Characterization of Ribonucleic Acid Polymerase–T7 Promoter Binary Complexes†

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ABSTRACT: Several experimental approaches have been employed to independently determine rates of dissociation for *Escherichia coli* RNA polymerase from the T7 bacteriophage A1, A2, A3, and D promoters. Heparin challenge measurements employing abortive initiation turnover rates as an index of promoter occupancy were carried out over a range of heparin concentrations in order to separate intrinsic dissociation rates from dissociation due to direct heparin attack of the polymerase–promoter complex. Dissociation rates were found to vary widely even among major promoters considered to have equivalent strength in vivo. Direct heparin attack was found to occur slowly with respect to intrinsic dissociation of polymerase from most promoters. Results were verified by gel analysis of full-length transcripts after heparin challenge and by measuring abortive initiation rates after poly[d(A-T)]·poly[d(A-T)] challenge. In the latter case, it was discovered that the equilibrium distribution of polymerase between a promoter and poly[d(A-T)]·poly[d(A-T)] could be measured.

Escherichia coli RNA polymerase selectively initiates RNA chains at specific sites on a DNA template (major promoter sites) much more frequently than at other sites (minor promoter sites). [See Losick & Chamberlin (1976) for a review of RNA polymerase properties.] Early in vivo transcription from bacteriophage T7 DNA initiates and proceeds rightward from three closely spaced promoters near the left end of the genome on the standard genetic and physical map. These sites have been termed promoters A1, A2, and A3 and are located approximately 470, 570, and 700 base pairs from the left end, respectively (Hsieh & Wang, 1976). In vitro transcription with excess polymerase has also been observed to proceed rightward from minor promoter sites B, C, and E, located further to the right on the T7 template (Minkley & Pribnow, 1973; Stahl & Chamberlin, 1977). Transcription also occurs leftward in vitro from the D promoter located 200 base pairs from the left. The in vivo function of these minor promoters has not been demonstrated.

The mechanism by which such selection of promoters occurs remains largely undetermined. It is generally believed that RNA polymerase associates rapidly with DNA to form a closed complex, one in which DNA strands remain base-paired or in which some other conformational feature of the binary complex is maintained that prohibits initiation. The complex is then thought to undergo a first-order conversion to a form capable of rapid initiation of RNA synthesis. Stahl & Chamberlin (1977) have studied the A1 promoter in comparison with the minor promoters on intact T7D111 DNA, a deletion mutant missing the A2 and A3 promoters. Under "prebinding conditions", i.e., incubation of low concentrations of RNA polymerase and promoter before the addition of nucleoside triphosphates, the A1 promoter was found to bind polymerase preferentially; increasing concentrations of polymerase resulted in RNA transcription from all promoters. If nucleoside triphosphates were present before addition of polymerase, however, transcription began preferentially at A1 regardless of the RNA polymerase concentration. These results were interpreted as an indication that important rate differences between major and minor promoters must exist in the slow conversion step. A more detailed mechanism has not been proposed. The contribution of binary complex dissociation rates to promoter utilization has not been studied in detail. Measurements have been made on fd promoters (Seeburg et al., 1977) and T7 promoters (Pfeffer et al., 1977; Miller & Burgess, 1978) using filter binding after excess cold single-stranded DNA chase or transcription after heparin challenge, respectively. Differences in dissociation rates were found in all cases but with no apparent correlation to promoter strength. However, the observed dissociation rates determined by these techniques may have been perturbed by promoter-dependent response to the filter binding procedure or by the direct attack of heparin on polymerase–promoter complexes (Pfeffer et al., 1977).

In this paper we show that dissociation rates of the binary complexes can be obtained with methods that do not significantly perturb the RNA polymerase–promoter complex. Moreover, we show that although heparin does perturb the observed rate of dissociation, the pattern of heparin inactivation can be determined in a manner that allows a measurement of intrinsic dissociation rates. To ensure clear interpretation of our results, we employed HaeIII digestion of the appropriate T7 or T7 deletion templates to generate fragments containing only the four promoters at the far left end of T7.

Polymerase occupancy at individual promoters was selectively quantitated by employing the abortive initiation assay (Cech et al., 1980). Key features of our results were also verified by gel analysis of full-length RNA transcripts produced on restriction fragment templates. Finally, poly[d(A-T)]·poly[d(A-T)] challenge experiments led to the discovery that equilibrium distributions of RNA polymerase between two of the T7 promoters and the poly[d(A-T)]·poly[d(A-T)] template could be measured.

Materials and Methods

Reagents. Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals, and [α-^{32}P]UTP and -CTP (100 mCi/μmol) were obtained from New England Nuclear. The unlabeled nucleoside triphosphates were further purified as described by McClure et al. (1978). Heparin was purchased

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from Sigma Chemical Co.; poly[d(A-T)][1] was synthesized with *E. coli* DNA polymerase I (Klenow fragment). Abortive initiation products were chromatographed on Whatman 3MM paper in WASP solvent.

RNA Polymerase. *E. coli* B RNA polymerase was purified by using the method of Burgess & Jendrisak (1975). The σ content was at least 60% based on densitometry of sodium dodecyl sulfate–polyacrylamide gels.

DNA Template. T7C5 and T7D111 (from D. McConnell) were grown by infection on *E. coli* B and *E. coli* C, respectively. The lysate was precipitated with Fisher carbowax 6000. The precipitate was resuspended in buffer, purified on a CsCl step gradient, and then sedimented to equilibrium in CsCl at 33 000 rpm for 24 h. The purified phage preparation was treated with sodium dodecyl sulfate for 15 min at 65 °C; protein was precipitated with KCl addition. The DNA was dialyzed into a final storage solution containing 0.1 M KCl, 0.01 M Tris, pH 8, and 1 mM EDTA. To prepare restriction fragments, we digested 2 mg of DNA with 500 units of HaeIII endonuclease (New England Biolabs) for 3 h in 70 mM KCl, 6 mM $MgCl_2$, 10 mM Tris (pH 8), and 1 mM DTT. The DNA was ethanol-precipitated, redissolved in TBE buffer (90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA), and electrophoresed on a 3.5% polyacrylamide gel. The T7 early promoter-containing left-end fragments were located by UV shadowing, cut out in gel strips, and placed in large dialysis bags containing 5 mM Tris and 2.5 mM acetic acid, pH 8.3. Voltage was applied across the short axis of the bags 20 min–1 h. The buffer contained in each dialysis bag was ethanol-precipitated and resuspended in 0.1 M KCl. The DNA fragments were then separated from ultraviolet-absorbing contaminants by adsorbing the DNA to a 0.5-mL Sephadex A-25 column equilibrated in 0.1 M KCl. The DNA was eluted with 1.0 M KCl and was then dialyzed against 0.1 M KCl, 10 mM Tris, pH 8, and 1 mM EDTA for storage.

Transcription Assays. Abortive initiation experiments were performed as described in Johnston & McClure (1976) and in Cech et al. (1980). Standard reaction conditions were 80 mM KCl, 40 mM Tris (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM initiating nucleotide, and 40 μM elongating nucleotide with the corresponding ^{32}P labels added to a final specific activity of 100–300 cpm/pmol. Unless specified, the DNA template was 2 nM genome, and RNA polymerase was 40 nM. Full-length RNA was transcribed under identical reaction conditions except that all four nucleoside triphosphates were present at 0.2 mM except UTP which was 5 μM with [α-^{32}P]UTP added to 10 000–15 000 cpm/pmol. Transcription reactions were terminated with EDTA; urea was added to 7 M, and the samples were boiled 3 min before loading onto gels. Gels were 5% acrylamide [29:1 acrylamide–bis(acrylamide)] and 7 M urea, run in TBE. After electrophoresis, an autoradiogram was made from the gel. The gel was then cut into 2–2.5-mm strips and counted in H_2O by employing the Cerenkov effect.

Heparin Challenge Assays. Under standard assay conditions, in the absence of heparin, the abortive initiation rate is constant for at least 2 h or until substrate is depleted. Similarly, if RNA polymerase is incubated with a DNA template in the absence of triphosphates, aliquots taken at any time up to at least 2 h will yield a constant abortive initiation rate when assayed with an appropriate combination of nucleoside triphosphates.

We reasoned that by measuring the perturbation of these two control rates by heparin we could determine a lifetime of the binary RNA polymerase–promoter complexes. The first, or synthesis mixture, challenge method was performed by preincubating RNA polymerase and the promoter-containing DNA fragment for 10 min at 37 °C. At zero time, the promoter-specific abortive initiation reaction was begun with the addition of nucleotides followed 30 s later with the addition of heparin to the concentrations indicated. The result was a progress curve that was linear with time in the absence of heparin; in the presence of heparin, the rate between subsequent time points decreased exponentially with time. The second, or binary complex, challenge method was performed by preincubating RNA polymerase and the promoter-containing DNA fragment for 10 min at 37 °C. At zero time, heparin was added at the concentrations indicated. Samples were then withdrawn at selected times and added to reaction buffer containing the nucleotides corresponding to the promoter-specific abortive initiation assay under study. The ensuing fixed-time assay (typically 5–10 min) yielded rates that decreased exponentially with time of incubation in the presence of heparin. The concentrations of heparin employed are given in micrograms per milliliter. The calculation of the second-order attack constant for heparin (M^{-1} min^{-1}) assumed an average molecular weight of 12 500.

Poly[d(A-T)] Challenge Assays. The protocol employed for poly[d(A-T)] challenge was identical with that of the binary complex challenge described above for heparin except that poly[d(A-T)] at the concentrations indicated was used in place of heparin. At the lowest poly[d(A-T)] concentrations employed, the dissociation of RNA polymerase did not proceed to completion at long times. Instead, a plateau in promoter occupancy was achieved that we interpreted as an equilibration of RNA polymerase between the promoter and the poly[d(A-T)] (see Results). In order to measure the true equilibrium distribution more conveniently, we preincubated RNA polymerase with poly[d(A-T)] for 10 min at 37 °C and then added a promoter-containing DNA fragment. Subsequent abortive initiation assays were performed to determine the relative promoter occupancy.

Calculation of Relative Binding Constants for RNA Polymerase Association with a Promoter or Poly[d(A-T)]. We define an overall equilibrium constant between RNA polymerase and promoter as

$$R + P \rightleftharpoons RP \qquad K_{promoter} = [RP]/([R][P])$$

We define an overall equilibrium constant between RNA polymerase and poly[d(A-T)] as

$$R + dAT \rightleftharpoons R{\cdot}dAT \qquad K_{dAT} = [R{\cdot}dAT]/([R][dAT])$$

where [R] is the free RNA polymerase concentration, [P] is the free promoter concentration, [dAT], the free concentration of poly[d(A-T)], is by convention calculated as moles of DNA phosphate per liter, and [R·dAT] is the concentration of polymerase complexed to poly[d(A-T)]. The relative binding constant for RNA polymerase binding to a promoter is calculated as the ratio

$$R = K_{promoter}/K_{dAT} = \frac{[RP][dAT]}{[P][R{\cdot}dAT]} \qquad (1)$$

At the lowest concentration ($\leq$10 μM) of total poly[d(A-T)] employed, it was necessary to calculate [dAT] by correcting

[1] Abbreviations used: poly[d(A-T)], poly[d(A-T)]·poly[d(A-T)]; WASP chromatography, Whatman 3MM paper developed in ascending chromatography for 16 cm in water–saturated $(NH_4)_2SO_4$–2-propanol (18:80:2) that was also 1 mM in EDTA; TBE, 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA.

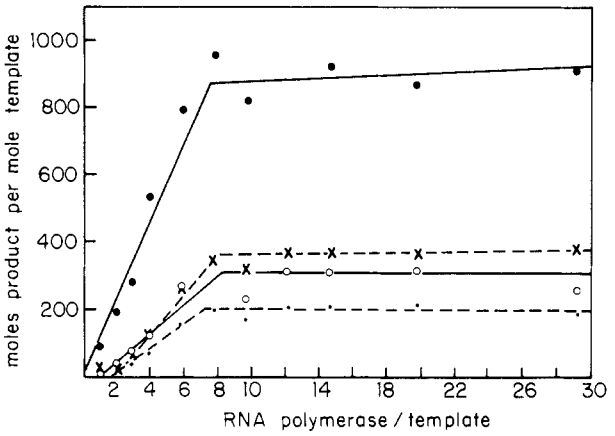

FIGURE 1: Rate of abortive initiation product synthesis from the T7C5 HaeIII$_{1200}$ fragment as a function of RNA polymerase concentration. In all reactions, the indicated amount of polymerase was incubated with 1 nM fragment at 37 °C for 10 min in 40 mM Tris (pH 8), 80 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT. Reactions were initiated by the addition of 0.5 mM initiating nucleotide and 0.04 mM elongating nucleotide in the following combinations: (●) ATP plus UTP, producing pppApU from the A1 and A3 promoters; (○) ATP plus UTP plus GTP (5 μM), producing pppApU from the A1 promoter only; (×) GMP plus CTP, producing pGpC from the A2 promoter; (–·–) GMP plus UTP, producing pGpUpU from the D promoter. Synthesis was terminated after 20 min, and reaction aliquots were chromatographed as described under Materials and Methods.

for the amount of [R·dAT] present by employing an effective site size for RNA polymerase of 70 base pairs (Hansen & McClure, 1979). In all cases we assumed that at equilibrium free [R] was negligible.

Results

Titration of T7 Promoters on T7C5 HaeIII$_{1200}$ Fragments. Saturating RNA polymerase concentrations were determined for the A1, A2, A3, and D promoters by titrating the T7C5 HaeIII$_{1200}$ fragment with increasing concentrations of RNA polymerase. The occupancy of each promoter was determined by measuring the rate of abortive initiation as a function of RNA polymerase concentration (Figure 1). The start sequences for the four promoters are as follows: A1, pppApUpC (Kramer et al., 1974; U. Siebenlist, personal communication); A2, pppGpCpU (Pribnow, 1975a); A3, pppApUpGpApApA (Pribnow, 1975b); D, pppGpUpUpG (Cech et al., 1980). Therefore, incubation of RNA polymerase with the T7C5 fragment in the presence of only ATP plus UTP resulted in pppApU synthesis from the A1 and A3 promoters. Addition of GTP at low concentration to this reaction allowed transcripts originating at A3 to elongate to a hexanucleotide. Formation of this product prevented further abortive initiation from A3. We have therefore assumed that the synthesis of pppApU in the presence of GTP measured abortive initiation at the A1 promoter only. Abortive initiation in the presence of GMP plus CTP resulted in pGpC synthesis from A2, and the GMP plus UTP combination produced pGpUpU from the D promoter. GMP can initiate an RNA chain but cannot serve in elongation; it was employed because the pGpC product was better resolved from the labeled CTP substrate than was pppGpC (Cech et al., 1980). For all four promoters, the rate of product synthesis increased linearly with the increasing RNA polymerase concentration to a ratio of approximately eight polymerases per DNA fragment; the rate then plateaued sharply and remained essentially constant following subsequent polymerase addition. At very low concentrations of polymerase, only the A3 promoter appeared to be occupied. The other three promoters exhibited an apparent lag such that the linear increase in rate of product synthesis did not begin until about two polymerases per fragment were added.

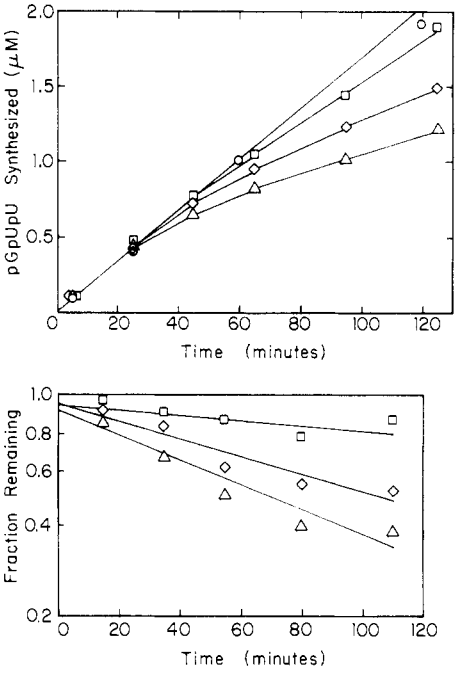

FIGURE 2: Synthesis mixture heparin challenge determination of the dissociation rate constant for RNA polymerase bound to the D promoter, as a function of heparin concentration. RNA polymerase was incubated with the T7C5 HaeIII$_{1200}$ fragment at 37 °C for 10 min, and then GMP and UTP were added to begin abortive initiation of pGpUpU. Heparin was immediately added to the following concentrations: (○) no heparin; (□) 38 μg/mL; (◇) 250 μg/mL; (△) 375 μg/mL. Aliquots were taken at the times indicated, after addition of heparin, and the accumulated amount of abortive initiation product was determined as described under Materials and Methods. The slopes of the curves shown in the upper figure were compared, as discussed under Results, to the slope of the curve representing no addition of heparin, in order to estimate the fraction of active complex remaining at each time point. This fractional occupancy is plotted logarithmically vs. time in the lower figure. First-order decay rates are 2.0×10^{-3} min^{-1} at 38 μg/mL heparin, 6.8×10^{-3} min^{-1} at 250 μg/mL heparin, and 10.4×10^{-3} min^{-1} at 375 μg/mL heparin.

Determination of Polymerase–Promoter Complex Lifetime by Heparin Challenge. The synthesis mixture challenge method applied to the D promoter on the T7C5 HaeIII$_{1200}$ fragment is shown in Figure 2, upper curve. As described under Materials and Methods, pGpUpU synthesis was initiated at zero time, and samples taken at subsequent time points were run on chromatograms to determine the accumulated amount of pGpUpU. The rate of synthesis during each time interval was estimated by calculating the increment in product accumulated between two time points and dividing by the respective time span. A semilogarithmic plot of rate of synthesis (converted to fraction of complex remaining) vs. time resulted in linear curves, indicating first-order decay (Figure 2, lower curve).

The observed dissociation rate, k_{obsd}, for the D promoter determined by the synthesis mixture challenge was measured at three heparin concentrations. A plot of k_{obsd} vs. heparin concentration is shown in Figure 3A. A linear relationship was obtained that extrapolated to $k_d = 0.89 \times 10^{-3}$ min^{-1} at zero heparin, corresponding to a half-life of 780 min. If the effect of heparin is assumed to be a direct bimolecular attack on the polymerase–promoter complex, the observed rate should be a sum of the true first-order rate of dissociation plus the rate of heparin attack, i.e.

$$k_{obsd} = k_d + k_{Hep}[Hep] \quad (2)$$

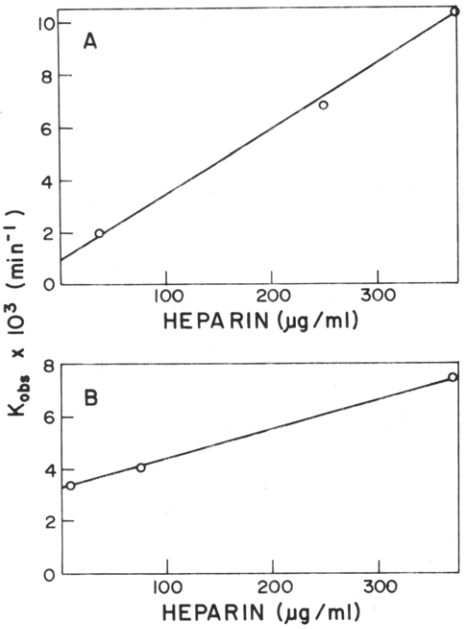

FIGURE 3: (A) The observed rate constant for dissociation of RNA polymerase from the D promoter is plotted as a function of the heparin concentration according to eq 2 in the text. Data are those determined in Figure 2, measured by the synthesis mixture challenge method. A value of k_{Hep} = 340 M^{-1} min^{-1} was obtained from the slope. The intercept corresponded to $k_d = 0.89 \times 10^{-3}$ min^{-1}. (B) The observed rate constant for dissociation of RNA polymerase as determined by the binary complex challenge method is plotted as a function of heparin concentration; k_{Hep} = 140 M^{-1} min^{-1} and $k_d = 3.3 \times 10^{-3}$ min^{-1}.

The slope in Figure 3A corresponded to $k_{Hep} = 3.4 \times 10^2$ M^{-1} min^{-1}. An attempt was made to determine the rate of direct heparin attack at the A2 promoter, but no measurable effect of heparin concentration was detected. This is as expected for the A2 promoter if $k_d = 0.023$ min^{-1} and $k_{Hep} = 340$ M^{-1} min^{-1}. For example, at 50 μg/mL Hep, $k_{obsd} = 0.023$ min^{-1} + (340 M^{-1} min^{-1})(4 × 10^{-6} M) = 0.024 min^{-1}, not detectably different from k_d. The A3 promoter could not be assayed (independent of A1) and therefore was not measured for rate of heparin attack.

Figure 3B shows the results of a binary complex challenge experiment applied to the D promoter of the T7C5 $HaeIII_{1200}$ fragment. The abortive initiation product pGpUpU produced in a 10-min reaction period was measured by assaying aliquots of the polymerase–DNA mixture by addition of GMP plus UTP as described under Materials and Methods. At 9 μg/mL heparin, $t_{1/2}$ for the polymerase–D promoter complex was ~205 min, corresponding to an observed dissociation rate k_{obsd} = 3.4 × 10^{-3} min^{-1}. We found that the D promoter also exhibited apparent first-order decay properties when analyzed by this method. Since the half-life was long relative to the 10-min abortive initiation assay, dissociation during the assay period was neglected. This was not the case with the other three promoters on the T7C5 $HaeIII_{1200}$ fragment, but lifetime estimates were nevertheless made for A1 and A3 using the same method (Table I). A biphasic curve was obtained for pppApU production in the presence of ATP and UTP, with one observed half-life of 5 ● 2 min and the other of 60 ± 20 min. To assign these dissociation rates, we did an identical experiment on the T7D111 $HaeIII_{1500}$ fragment, carrying A1 but not A3. At 100 μg/mL heparin the observed half-life for A1 was 7 ± 3 min.

The D promoter binary complex challenge experiment was repeated at two additional heparin concentrations. Analysis of k_{obsd} as a function of heparin concentration (Figure 3B) proceeded by the same arguments presented above for synthesis

Table I: Dissociation Rate Constants for RNA Polymerase Bound to Promoters on the T7C5 $HaeIII_{1200}$ Fragment

	rate constants (min^{-1}) for the promoters			
	A_1	A_2	A_3	D
binary complex challenge				
heparin	0.14[a]		0.014[a]	3.3×10^{-3}[c]
poly[d(A-T)]		0.010		2.0×10^{-3}
synthesis mixture challenge				
heparin		0.023[b]		0.89×10^{-3}[c]

[a] Dissociation rates were measured at 80 μg/mL heparin. [b] Dissociation rate was measured at 50 μg/mL heparin. [c] Dissociation rates were measured at several heparin concentrations and then extrapolated to zero heparin concentration as shown in Figure 3.

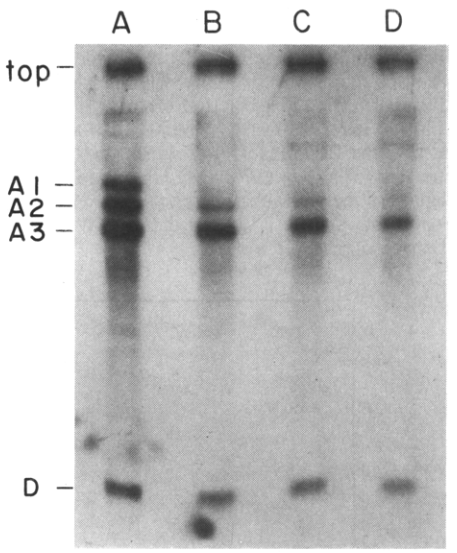

FIGURE 4: RNA transcripts from the T7C5 $HaeIII_{1200}$ fragment initiated (A) 1, (B) 15, (C) 30, and (D) 60 min after addition of heparin. Polymerase (21 nM) was incubated with the T7C5 $HaeIII_{1200}$ fragment (2 nM) in standard assay buffer at 37 °C for 10 min before addition of 57 μg/mL heparin. At the times indicated, RNA synthesis was initiated by the addition of ATP (0.2 mM), GTP (0.2 mM), CTP (0.2 mM), UTP (0.005 mM), and [α-^{32}P]UTP to 10 000 cpm/pmol of UTP. After 15 min, the reaction products were separated on a 5% acrylamide–7 M urea gel, as described under Materials and Methods. The bands are labeled to correspond to the transcripts from the A1, A2, A3, and D promoters. In slot A, the A1 transcript accounts for 10% of all transcripts, A2 accounts for 21%, A3 accounts for 41%, and D accounts for 27%.

mixture challenge experiments. We found, in this case, an extrapolated first-order dissociation rate $k_d = 3.3 \times 10^{-3}$ min^{-1} and a rate of direct heparin attack k_{Hep} = 140 M^{-1} min^{-1}.

Information obtained through binary complex challenge experiments can be verified qualitatively by separating the promoter-specific RNA transcripts on polyacrylamide gels. The gel approach also unambiguously resolved the question of assigning A1 and A3 promoters to the components of the abortive initiation bimodal decay curve for pppApU synthesis. Aliquots of preincubated RNA polymerase–DNA binary complexes plus heparin were added at fixed time points to tubes containing all four nucleoside triphosphates. The resulting RNA transcripts were treated as discussed under Materials and Methods. The pattern of transcription 1 min after addition of heparin was essentially the same as that reported previously from a $Hinf_{1100}$ fragment (Hsieh & Wang, 1976). The differences in transcript size between RNA produced on the HaeIII fragment as opposed to the Hinf fragment corresponded to the differences in DNA fragment lengths. Analysis of the radioactivity in each full-length band (Figure

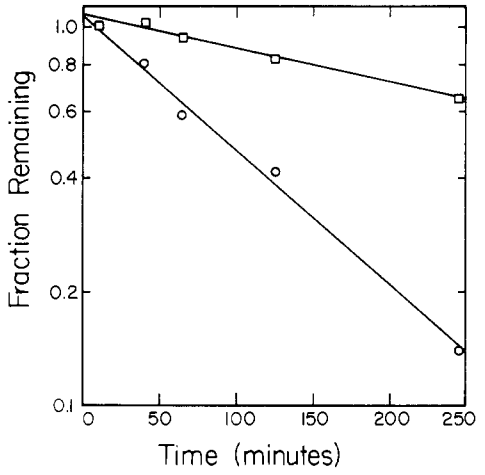

FIGURE 5: Binary complex poly[d(A-T)] challenge determination of the dissociation rate constant for RNA polymerase bound to the A2 and D promoters of T7. The fraction of each binary complex remaining is plotted logarithmically vs. time. RNA polymerase was preincubated with the T7C5 $HaeIII_{1200}$ fragment at 37 °C for 10 min. At zero time poly[d(A-T)] (170 μM) was added. At the times indicated, aliquots of the preincubation solution were assayed separately (10 min) for pGpC synthesis [A2 promoter (○)] and pGpUpU synthesis [D promoter (□)].

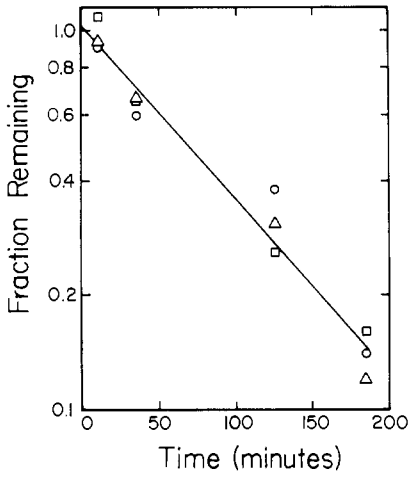

FIGURE 6: Binary complex poly[d(A-T)] challenge yields dissociation rates that are independent of poly[d(A-T)] concentration. The fraction of the RNA polymerase–A2 promoter binary complex remaining following poly[d(A-T)] challenge is plotted logarithmically vs. time. The protocol described in Figure 5 was employed to determine the dissociation rate except that the poly[d(A-T)] concentration was varied as follows: (○) 67; (△) 133; (□) 267 μM.

4) as a function of time after addition of heparin agreed quantitatively with the decay rates determined by both abortive initiation methods. Values of $t_{1/2}$ were 20 ± 10 min for A2, 40 ± 10 min for A3, and 300 ± 100 min for the D promoter. The A1 promoter decayed with a lifetime less than or equal to 5 min and is clearly the more rapidly dissociable of the A1 and A3 promoters.

The final approach employed to measure dissociation rates was to perform the binary complex challenge experiments with double-stranded poly[d(A-T)] in place of heparin. As shown in Figure 5, the rate of synthesis of pGpC from the A2 promoter decayed exponentially as a function of time after addition of poly[d(A-T)]. A decay half-life of 69 min was determined. Similarly, pGpUpU synthesis from the D promoter decayed with a half-life of 345 min at all poly[d(A-T)] concentrations tested. Since A1 and A3 synthesize pppApU, abortive initiation could not be used for these promoters in

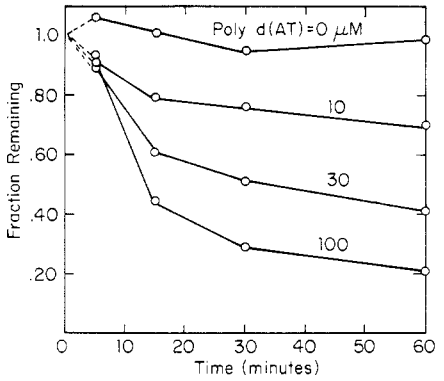

FIGURE 7: The extent of binary complex dissociation from the A2 promoter depends on poly[d(A-T)] concentration. The fraction of binary complex remaining following poly[d(A-T)] challenge is plotted vs. time. The protocol described in Figure 5 was employed except that the poly[d(A-T)] concentration was varied as indicated.

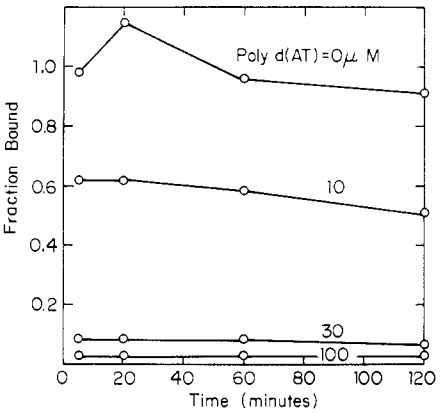

FIGURE 8: Equilibration of RNA polymerase between the A2 promoter and poly[d(A-T)]. The fraction A2 promoter occupied by RNA polymerase is plotted vs. time. RNA polymerase was first preincubated with poly[d(A-T)] at the concentrations indicated for 10 min at 37 °C. At zero time, the T7C5 $HaeIII_{1200}$ fragment was added. At the times indicated, aliquots were assayed for pGpC synthesis. The average values over the 2-h period were used to calculate a relative binding constant for the A2 promoter as described in the text.

the presence of poly[d(A-T)]. The half-life was independent of poly[d(A-T)] concentration within the range tested (70–270 μM) (Figure 6).

Relative Binding Constants Determined by Poly[d(A-T)] Competition. The observation that poly[d(A-T)] at high concentrations could bind free enzyme after it had dissociated from a binary promoter complex suggested that the same reagent could be employed to probe the reversibility of the binding interaction. In Figure 7 we show that at lower poly[d(A-T)] concentrations, the dissociation of a preformed polymerase–promoter complex follows about the same kinetics shown in Figure 6, but here the extent of reaction is clearly dependent on the poly[d(A-T)] concentration. Indeed, the final promoter occupancy at long times as monitored by abortive initiation rates can be used to calculate $R = K_{promoter}/K_{dAT}$ as discussed under Materials and Methods. The calculation can be made more directly, however, from the results of an experiment in which RNA polymerase is first preincubated with varying concentrations of poly[d(A-T)], followed by the addition of the promoter-containing restriction fragment. As shown in Figure 8, the rate of approach to equilibrium is dominated by the dissociation rate from poly[d(A-T)] which we estimate to occur in less than 5 min (i.e., the first time point). The final promoter occupancy was the same employing both protocols. This result proves that we

are in fact observing an equilibration of polymerase between the promoter and poly[d(A-T)]. The second protocol is more convenient for estimating R because of the rapid approach to equilibrium in that method. Calculating average values for R over the entire time course shown in Figure 8 yielded for the A2 promoter $R = 400$. The identical protocol applied to the D promoter yielded a value of $R = 20$ (data not shown).

Discussion

We have previously reported (McClure et al., 1978) abortive initiation rates determined as a function of RNA polymerase concentration from the bacteriophage λ $HaeIII_{890}$ fragment containing the λ P_R promoter. In that experiment, little or no product was synthesized until a ratio of two polymerases per fragment was reached, at which point synthesis began to increase linearly with polymerase concentration to a ratio of eight polymerases per fragment. An almost identical pattern was observed for the titration of the four promoters on the T7C5 $HaeIII_{1200}$ fragment. On the basis of our estimate of 50% enzyme activity, the 8:1 titration ratio for all T7 promoters suggests that the fragment contains only four binding sites, each of which competes nearly equally for RNA polymerase until saturation is achieved. The sharpness of the titrations is in contrast to the titration of intact phage DNA (McClure et al., 1978) and is consistent with a binding constant greater than 10^{10} M^{-1} for all binding sites. The primary result, however, is that a ratio of eight polymerases or more per T7 fragment clearly ensured maximum synthesis from all four promoters in subsequent experiments. Binding of polymerase to the A3 promoter may occur preferentially at very low polymerase concentration, but this preference may reflect kinetic differences as well as overall binding affinity. We have also not pursued the fact that pppApU synthesis from A1 alone was only one-third the combined synthesis rate of A1 and A3 together. The A1 promoter for unknown reasons may not turn over product as quickly as the A3 promoter. Alternatively, the presence of GTP may have had a slight inhibitory effect on A1 abortive initiation. Behavior of these promoters at varying polymerase concentration is currently being studied more thoroughly.

RNA polymerase–promoter dissociation rates as measured by both heparin challenge techniques clearly vary considerably between promoters, even between those classed as major promoters. All approaches employing heparin yielded substantially the same results. Measurements can be made reproducibly to within better than ±50% for the more rapidly dissociable complexes. The major difficulty in these cases is that the synthesis mixture challenge method requires estimates of the slope between each time point while the binary complex challenge involves continuing dissociation of the complex during each fixed-time assay. Measured half-lives for the more stable complexes vary over factors of 2–3, but here the uncertainty is increased by the additional contribution of the direct heparin attack rate. We expected that the synthesis mixture challenge method would result in longer half-times for dissociation because a fraction of the enzyme–DNA complex would be involved in the catalytic cycle of abortive initiation and be stabilized thereby. This is seen in the case of the D promoter where the $t_{1/2}$ extrapolated to zero heparin concentration was 770 min for the synthesis mixture challenge whereas the extrapolated $t_{1/2}$ for the binary complex challenge was only 209 min. The half-life obtained with the nonperturbing poly[d(A-T)] binary complex challenge was 350 min. The stabilizing effect is significant but rather small. The explanation is likely to lie in the fact that the nucleotide concentrations employed to measure abortive initiation result in observed velocities that are only about 20% of the maximal velocity. In other words, most of the RNA polymerase–promoter complex is uncomplexed with nucleotides and dissociates from the promoter at a rate close to the intrinsic rate observed without nucleotides present.

Analysis of full-length RNA transcripts after heparin challenge corroborates data obtained with the abortive initiation assays, further supporting our assertion that abortive initiation can be used interchangeably with other techniques for characterizing and measuring promoter occupancy. Larger amounts of template were necessary for the full length transcript experiments than with abortive initiation assays in part because less than stoichiometric amounts of full-length transcript were synthesized per promoter. Smaller discrete sized transcripts were always observed on gels, so a certain percentage of initiated chains must end prematurely at specific sites. The A1 and A2 transcripts usually appeared at full length in fewer number than the A3 and D transcripts. It is possible that this observation does not reflect differences in promoter occupancy but rather selective premature termination of the A1 and A2 transcripts shortly after initiation. The total number of full-length transcripts in these experiments was generally 20% or less the maximum expected starts based on template concentration.

Heparin concentration dependence of dissociation rates studied by two methods on two promoters consistently showed a second-order attack rate between 150 and 340 M^{-1} min^{-1}. Previously published work on dissociation from the A1 promoter at different heparin concentrations and under different reaction conditions than those employed here (Pfeffner et al., 1977) can be replotted and analyzed in a similar manner. We have estimated the second-order attack rate in those experiments to be 400 M^{-1} min^{-1}, i.e., not significantly different from our measurement. Furthermore, Majors (1977) has reported heparin challenge lifetimes of RNA polymerase bound to the *lac* UV-5 promoter at various heparin concentrations that can be calculated to yield $k_{Hep} = 170$ M^{-1} min^{-1}. We agree with Pfeffer et al. (1977) that heparin directly attacks promoter-bound polymerase; we argue that the rate is slow compared to the normal dissociation rate at many promoters. Even when the normal dissociation rate is on the order of several hours, a pseudo-first-order rate is obtained when low concentrations of heparin are employed that does not significantly differ from the true first-order rate.

The mechanism of heparin inactivation of promoter-bound polymerase remains unclear. Our data are consistent with a bimolecular combination of heparin and promoter-bound polymerase according to eq 2. There are two considerations that suggest that this cannot be a complete description of the inactivation process. First, any reasonable mechanism would consider an initial bimolecular step leading to complex formation in analogy to a Michaelis complex in an enzyme–substrate reaction. The prediction from such a model is that the data in Figure 3 would be a hyperbola with a plateauing of the observed rate of dissociation with increasing heparin concentrations. If this is the case, our results would be explained by arguing that we have employed heparin concentrations in the first-order region of the heparin saturation curve. Second, the observed bimolecular rate of heparin attack is only ~300 M^{-1} min^{-1}. This value is clearly too low for an elementary bimolecular step. Thus, we suggest that the observed rate is actually the product of (at least) one equilibrium constant times the intrinsic bimolecular step for heparin binding. We do not know whether the suggested equilibrium is a property of the polymerase–promoter complex, the heparin

molecule itself, or a combination of the two. The measured rate of direct attack is twofold lower for the D promoter under conditions of binary complex challenge than with synthesis mixture challenge. It is possible that this difference reflects an additional susceptibility of a reiteratively initiating complex to heparin attack. Finally, we note that heparin is a complex mixture of several molecular species ranging in molecular weight from 10000 to 25000. Because of these mechanistic ambiguities, we have not attempted to measure dissociation rates under a variety of solution conditions with either heparin challenge technique. Ensurance of accuracy would seem to require an extrapolation to zero heparin concentration in each case for each promoter studied.

Heparin is frequently added simultaneously with triphosphates to ensure one round of synthesis from preincubated RNA polymerase–promoter complexes. Our results show that if heparin is employed in the range of 5–10 μg/mL, the destruction of preformed complexes would be negligible in most cases. On the other hand, for mechanistic experiments to be performed conveniently (e.g., determination of k_{on} and k_{off}), an RNA polymerase sequestering reagent is required that binds only to free enzyme. The search for this mechanistic probe showed that poly[d(A-T)] fulfills many of the criteria demanded of such an ideal reagent.

The use of double-stranded poly[d(A-T)] as a binding reagent for uncomplexed polymerase yielded dissociation rates that were independent of poly[d(A-T)] concentration. At the concentrations employed, poly[d(A-T)] binding site concentration was at least 10^3-fold in excess of polymerase concentration. Several other properties of poly[d(A-T)] recommend it as a sequestering agent for RNA polymerase. The functional (Hansen & McClure, 1979) and physical (Williams & Chamberlin, 1977) site sizes for the enzyme are known. We have shown here that the binding is reversible with known relative affinities for two of the T7 promoters. These data in combination allow a choice of the poly[d(A-T)] concentration for specific RNA polymerase–promoter challenge experiments. Dissociation rates determined by poly[d(A-T)] challenge are the same for the D promoter as determined by heparin challenge and a factor of 2 slower for the A2 promoter. The absence of a poly[d(A-T)] concentration dependence in the determination of intrinsic dissociation rates is the single most important criterion for favoring this reagent over heparin. The binding stoichiometry and binding constant of heparin for RNA polymerase are unknown. As discussed above, the mechanistic basis for the concentration dependence of k_{obsd} on heparin concentration is also not well understood. For all of these reasons, we favor the use of poly[d(A-T)] for routine determination of dissociation rates. However, we still do not know whether poly[d(A-T)] will perform ideally under all reaction conditions or for all RNA polymerase–promoter complexes. In particular, promoters with the starting sequence pppApU cannot be studied with the poly[d(A-T)] challenge.

In comparison to existing estimates of promoter–polymerase complex dissociation rates, those reported here are generally faster. Miller & Burgess (1978) reported $t_{1/2}$ values of 1 and 1.4 h, respectively, for the A1 and A3 promoters measured at 160 mM KCl after heparin challenge. Since their procedure involved quantitation of γ-^{32}P-labeled oligonucleotides on PEI-cellulose two-dimensional chromatograms after RNase T1 digestion of full-length RNA transcripts, they were not able to distinguish initiation products from promoters producing GTP starts. Our reaction conditions differed only in the use of 80 mM KCl rather than 160 mM, a factor which would be expected to stabilize rather than destabilize the polymerase–promoter complex. The discrepancy is unresolved, except for the possibility that the use of intact T7 template by Miller & Burgess (1978) might result in contamination by oligonucleotides from highly stable minor promoter complexes not present on the restriction fragments employed here. The work by Pfeffer et al. (1977) cited above was performed by monitoring production of full-length transcripts from intact T7-D111 DNA after heparin challenge in a reaction buffer containing bovine serum albumin but no monovalent cation. The A1 promoter–polymerase complex dissociated with a $t_{1/2}$ of $\sim$5 min at 100 μg/mL heparin, but the lifetime increased as heparin concentration was decreased to the extent that with no addition of heparin, initiation of transcription remained the same after many hours of incubation. However, data obtained without heparin or any other polymerase binding reagent are not interpretable because, once the enzyme dissociated, it or another in solution is expected to rebind the promoter. When the latter case is ignored, therefore, the published data replotted as k_{obsd} vs. heparin concentration extrapolate linearly to a true $k_d = 3.3 \times 10^{-3}$ min^{-1} ($t_{1/2}$ = 210 min). This value is still not in agreement with that obtained from the work reported here; differences in reaction conditions may possibly be responsible. The apparent second-order rate of heparin attack does agree with our estimate, however.

In conclusion, a variety of experimental approaches have shown that *E. coli* RNA polymerase bound to the four promoters located at the left end of T7 exhibits widely varying promoter-dependent dissociation rates. Measurements of these rates are within at most a factor of 2–3 in the most variant cases whether determined by heparin challenge (at low heparin concentration) or poly[d(A-T)] challenge. Examination of direct heparin attack in detail indicated that such attack occurs at approximately the same rate or slower than true dissociation from the promoters studied and is slow enough not to seriously distort measured dissociation rates at concentrations in the range of 5 μg/mL heparin.

Equilibrium distributions of polymerase between T7 promoters and poly[d(A-T)] binding sites showed that the A2 promoters must have an overall equilibrium binding affinity for polymerase 20 times larger than the D promoter binding constant. On the basis of relative dissociation rates, A2 must form an initiation complex at least 80 times as fast as the D promoter.

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Purification and Characterization of the Amino-Terminal Propeptide of Pro α1(I) Chains from Embryonic Chick Tendon Procollagen†

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ABSTRACT: A peptide with an apparent molecular weight of 23000 was isolated from the medium of cultured chick embryo tendons. Comparison of tryptic peptides derived from the medium peptide and from the amino-terminal, bacterial collagenase resistant portion of Type I procollagen indicated that the medium peptide represented the amino-terminal precursor-specific region of the pro α1(I) chain of procollagen. This conclusion was supported by the demonstration that antibodies against the medium peptide reacted with Type I procollagen in a radioimmune assay but did not react with a peptide derived from the carboxy-terminal propeptide of Type I procollagen. In addition, the reaction with Type I procollagen was inhibited with the purified amino-terminal, collagenase-resistant portion of pro α1(I) chains. Finally, amino acid sequencing demonstrated that the amino propeptide of dermatosparactic calf pN α1(I) chains and the medium peptide have similar amino-terminal sequences. Carbohydrate analysis established the presence of one residue of *N*-acetylglucosamine and a trace of mannose and galactose.

Collagen comprises a family of molecules with similar structural features. Each molecule consists of three helical polypeptide chains (α chains) held together by hydrogen bonds to form a triple helical structure. Collagens are synthesized as procollagens that are larger than collagens because of additional amino acid sequences at both the amino and carboxyl ends of the α chains of collagen molecules [for a review, see Fessler & Fessler (1978)]. During the extracellular conversion of procollagen to collagen, these propeptides are removed by procollagen proteases to yield the collagen molecule [see Fessler & Fessler (1978)].

A truncated form of procollagen with an amino propeptide but lacking the carboxyl propeptide has been found to accumulate in the skin of animals with dermatosparaxis (Lenaers et al., 1971; Furthmayr et al., 1972; Becker et al., 1976, 1977). This pN-collagen[1] has provided material for chemical, immunological, and physical studies on the amino propeptide of Type I procollagen (Becker et al., 1976; Rohde et al., 1976; Engel et al., 1977; Bruckner et al., 1978; Hörlein et al., 1978, 1979; Rohde & Timpl, 1979). These studies show that the amino-terminal propeptide of dermatosparactic calf and sheep pN α1(I) chains contains about 140 amino acid residues arranged in three structural domains. The amino-terminal portion of the peptide (~90 residues) contains a region which is resistant to bacterial collagenase. The central portion (~30 residues) is sensitive to bacterial collagenase, and this region is linked to a short, nonhelical sequence (~10 residues) which connects the propeptide to the collagen α1(I) chains (Becker et al., 1976). The complete amino acid sequence of the amino-terminal propeptide from dermatosparactic calf skin has been established (Hörlein et al., 1979). Also, the complete sequence of the collagenase-resistant domain of the dermatosparactic sheep peptide has been determined (Rohde et al., 1979). The sequence data for the calf and sheep dermatosparactic peptide indicate a highly conserved structure with only a few amino acid substitutions.

It has been assumed but not proven chemically that amino propeptides of Type I pN-collagen isolated from dermatosparactic skin are identical with amino propeptides of Type I procollagen secreted by cells in culture. Although it is clear that dermatosparactic pN-collagen is the product of normal processing at the carboxyl end of Type I procollagen, it is also possible that the amino-terminal propeptide is partially processed, so that truncated amino propeptides are produced. Here, we compare the structure of the pro α1(I) amino propeptide of Type I procollagen secreted by chick embryo fibroblast in suspension culture to that of calf and sheep dermatosparactic propeptides. Our data demonstrate that the propeptides of the dermatosparactic calf and sheep pN α1(I)

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[1] Abbreviations used: BAPN, β-aminopropionitrile; NEM, *N*-ethylmaleimide; CM, carboxymethyl; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; Tos-Phe-CH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; pN, precursor molecule with an amino-terminal extension; $NaDodSO_4$, sodium dodecyl sulfate.

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