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Kinetics of Open Complex Formation between *Escherichia coli* RNA Polymerase and the *lac* UV5 Promoter. Evidence for a Sequential Mechanism Involving Three Steps†

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ABSTRACT: The forward and reverse kinetics of open complex formation between *Escherichia coli* RNA polymerase and the *lac* UV5 promoter have been studied in the temperature range of 15-42 °C. The standard two-step model, involving the formation of a closed intermediate, RP_c, followed by an isomerization that leads to the active complex RP_o, could not account for the present data. The promoter-enzyme lifetime measurements showed an inverse temperature dependence (apparent activation energy, −35 kcal/mol). A third step, which is very temperature dependent and which is very rapid at 37 °C, was postulated to involve the unstacking of DNA base pairs that immediately precedes open complex formation. Evidence for incorporating a new binary complex, RP_i, in the pathway was provided by experiments that distinguished between stably bound species and active promoter after temperature-jump perturbations. These experiments allowed measurement of the rate of reequilibration between the stably bound species and determination of the corresponding equilibrium constant. They indicated that the third step became rate limiting below 20 °C; this prediction was checked by an analysis of the forward kinetics. A quantitative evaluation of the parameters involved in this three-step model is provided. Similar experiments were performed on a negatively supercoiled template: in this case the third equilibrium was driven toward formation of the open complex even at low temperature, and the corresponding step was not rate limiting.

It is generally postulated that the formation of an active complex between *Escherichia coli* RNA polymerase and a bacterial promoter involves at least two steps before triphosphate binding and chain initiation. An inactive binary complex is first formed: this transient species, the closed complex, undergoes a slow and thermodynamically favored isomerization to the transcriptionally active complex [cf. Walter et al. (1967) and Chamberlin (1974)]. This two-step model can be summarized as shown in Scheme I.

Scheme I

$$R + P \underset{K_B}{\rightleftharpoons} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o$$

For a given promoter, it is now possible to determine the rate of formation of the open complex RP_o through a simple enzymatic assay. This rate depends on the initial concentration

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of RNA polymerase as expected if Scheme I applies. This allows measurement of K_B and k_2. The reverse process can also be followed by challenging the preformed open complex with a competing polynucleotide; k_{-2} can be measured in this manner (McClure, 1980; Hawley & McClure, 1980). However, Scheme I must be an extreme oversimplification. Association of the enzyme with nonspecific DNA could contribute to the rate of closed complex formation [for a review, see von Hippel et al. (1984)]. This contribution is expected to be a significant factor for those promoters that combine with RNA polymerase at rates exceeding 10^8 M^{-1} s^{-1} [cf. Chamberlin et al. (1982), Bujard et al. (1982), Rosenberg et al. (1982), and Kadesch et al. (1982)]. For the UV5 promoter the apparent association rate constant K_Bk_2 is only 10^6 M^{-1} s^{-1}. Thus the possible contribution of nonspecific binding would not be significant in our experiments. The second step of Scheme I is probably extremely intricate for the UV5 promoter as well as for other promoters. In the open complex, the DNA structure of the promoter region is known to be strongly and specifically perturbed [see, for example, Wang et al. (1977), Siebenlist (1979), and Siebenlist et al. (1980)]. Since a cooperative unwinding reaction takes place, we reasoned that temperature as well as the degree of superhelicity of the DNA template could alter the kinetic pathway. We have therefore investigated how these two variables affect the rate of open complex formation and the residence time of RNA polymerase at the *lac* UV5 promoter. We have found that Scheme I was insufficient for our data. The minimal scheme consistent with the experiments reported in this paper is shown in Scheme II.

Scheme II

$$R + P \underset{K_1}{\rightleftharpoons} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} RP_o$$

Our primary evidence for incorporating RP_i in the reaction scheme is that, on a linear DNA, the rate of inactivation of the catalytically competent species observed after lowering the temperature is at least 1 order of magnitude faster than the rate of dissociation of the binary complexes measured by poly(deoxyadenylate-thymidylate) [poly[d(A-T)]] challenge. In the reverse pathway, a fast and reversible isomerization step ($RP_o \rightleftarrows RP_i$) must precede the isomerization leading to species sensitive to poly[d(A-T)] challenge, RP_c and R. This formulation explains the unusual dependence on temperature of the promoter–enzyme lifetimes and accounts for the forward kinetics. We report here an extensive test of this more comprehensive model. Insertion of an additional isomerization step does not alter the formalism proposed by McClure but requires a redefinition of the phenomenological parameters obtained through analysis of the forward or reverse kinetic processes. We give estimates of the values K_1, k_2, k_{-2}, k_3, and k_{-3} as the temperature of the assay or the superhelical density of the template is changed. From these variations, we assign the third step to the unstacking of the DNA base pairs during formation of the open complex. We show that, for the UV5 *lac* promoter, this unwinding step is ordinarily not rate limiting.

Materials and Methods

DNA and Enzyme Preparations. The *lac* UV5 promoter was studied either on a 211-base-pair linear fragment or as an insert corresponding to the same fragment ligated at the *Eco*RI restriction site of the pBR322 vector. These two DNA templates were prepared as described in Malan et al. (1984). RNA polymerase was purified according to Burgess & Jendrisak (1975), as modified by Lowe et al. (1979). It was 50–60% active as judged by quantitative activity measurements (Chamberlin et al., 1979) and promoter titration (Cech & McClure, 1980). Preparation of plasmids of various degrees of supercoiling and measurement of their superhelical densities were performed as described by Keller (1975). Unless otherwise stated, the superhelical density of the *lac* UV5 plasmid and of its parent vector was equal to −0.05. Less than 10% of the preparation was relaxed (and/or nicked) in typical preparations as judged by 0.8% agarose electrophoresis. Nicking activity was assayed under standard reaction conditions for times comparable to the lag, burst, and promoter lifetime measurements. No detectable relaxation was observed after 20 min when 10 μg/mL plasmid DNA and 100 nM RNA polymerase were incubated at 37 °C. For promoter lifetime measurements, an RNA polymerase preparation particularly devoid of nicking activity was used. With this preparation, more than 90% of the plasmid DNA remained superhelical for 3 h.

Abortive Initiation Assays. In the absence of a complete complement of nucleoside triphosphates, RNA polymerase synthesizes the tetranucleotide ApApUpU at the UV5 promoter. This reaction was assayed in the following standard buffer: 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 8.0, 100 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 100 μg/mL bovine serum albumin. The assay solution also contained [α-^{32}P]UTP (10 μCi/mL final concentration), 40 μM UTP, 450 μM ApA, 0.5–4 nM DNA, and 5–200 nM RNA polymerase. Temperature control of the thermostated bath (Lauda) was accurate to within 0.3 °C. For the activity assays, the number of moles to ApApUpU produced per mole of promoter was determined as a function of the reaction time t under three different conditions:

(1) The reaction was initiated by addition of UTP to the assay mixture already containing RNA polymerase and UV5 promoter; the preincubation of those solutions was 10 min. This procedure results in a linear accumulation of product $z(t)$ from which the steady-state activity s of the promoter–enzyme complex is deduced.

(2) The reaction was started by addition of RNA polymerase to the assay mixture containing the DNA, after both solutions had been incubated at the appropriate temperature. In this case, product accumulation $y(t)$ exhibits a lag that reflects the kinetics of approach to the steady-state rate. Analysis of this lag yielded a measure of the time τ required for the formation of an active complex between RNA polymerase and promoter (lag experiments).

(3) Only the substrates were preincubated at the final temperature. A concentrated mixture of RNA polymerase and UV5 DNA was preincubated at 40 or 37 °C so that the active complex was formed. The reaction was started by the dilution of the active complex into the assay mixture at T_f. In this case the product accumulation $y(t)$ follows another characteristic response, called a burst. The corresponding change in steady-state rate is interpreted here as reflecting the decay of the concentration of active species due to the change in temperature.

Kinetics of Approach to the Steady-State Rate: Lag Experiments. The forward reaction kinetic experiments correspond to the second experimental protocol described above. They were performed as described in Hawley & McClure (1980) adapted to the case of the UV5 promoter [cf. Malan et al. (1984)]. Portions (20 μL) were removed from the assay mixture described above at times ranging from 30 s to 20 min after the addition of RNA polymerase and spotted on Whatman 3MM paper chromatograms. Chromatography was performed as indicated in McClure et al. (1978). Radioactivity

migrating as ApApUpU and as UTP was measured on each of the chromatograms. Appropriate controls (without enzyme or without DNA) were run. Given the specific radioactivity of UTP, these values allowed the calculation of $y(t)$, the number of nanomoles of UTP incorporated into product per nanomole of *lac* UV5 promoter as a function of time. Care was taken to correct those values from the small fraction of the initial radioactivity (from 5×10^{-4} to 2×10^{-3}) that comigrated with the product. When this assay was used to characterize the behavior of the *lac* UV5 insert, subtraction of the small background due to abortive initiation at other sites on the pBR322 vector was performed, as described in Malan et al. (1984). Purity of radioactive UTP was checked as explained in Hansen & McClure (1979).

The progress curve, $y(t)$, was compared to a parallel experiment initiated by UTP addition. In this case, the synthesis of product, $z(t)$, was a linear function of time. A linear least-squares fit of $z(t)$ extrapolated at zero time to a value equal to zero within experimental error and was parallel to the final slope of $y(t)$. By definition, this slope s represents the steady-state activity of the enzyme–promoter complex under the conditions tested. At each temperature we compared the values of s observed on the linear DNA and on the insert. The ratio s(fragment)/s(insert) is called f^+.

The experimental ratio $q = y(t)/z(t)$ was then fitted to the theoretical equation that yields the characteristic time τ required for the formation of an active complex [cf. McClure (1980)]:

$$q = 1 - (1 - e^{-t/\tau})/(t/\tau)$$

The fit was done manually by comparing the set of experimental points $q = f(\log t)$ with the theoretical curve $q = f[\log (t/\tau)]$. A translation between the two curves was used to determine the best fit and therefore τ. When experimental errors made this approach uncertain, the final steady-state rate, s, and τ were independently determined by using a nonlinear least-squares analysis (M. E. Mulligan and W. R. McClure, unpublished method). These values of τ were plotted against the reciprocal of the RNA polymerase concentration as described in Hawley & McClure (1980) to yield the apparent constants K_B and k_f (cf. below).

Temperature Perturbation of the Steady-State Rate: Burst Experiments. In this case the third experimental approach outlined above was used. The reaction mixture (usually 300–400 μL), without RNA polymerase and DNA, was equilibrated for at least 15 min in a glass tube at temperature T_f. RNA polymerase and promoter fragments were incubated together in another bath regulated at the upper temperature, usually 37 °C. At time zero the RNA polymerase–promoter solution was mixed with the assay solution (20-fold dilution) and in less than 8 s placed back at temperature T_f. Portions (20 μL) were removed from the assay mixture, spotted on 3MM chromatograms, and developed and counted as described above. These data were analyzed by assuming that the active complex approaches its equilibrium concentration via a single slow isomerization step after the temperature perturbation. At each time t following the perturbation the fraction f of promoter that is present in the active complex is given by

$$f - f_e = (1 - f_e)e^{-\beta t} \quad (1)$$

where β is the relaxation rate constant and f_e the fraction of active complex at equilibrium. One does not follow f directly; rather, one follows the amount of product, y, released as a function of time by the complex still active at time t. Integration of the preceding expression yields the quantity that is experimentally measured:

$$y = s\left[t + \left(\frac{1 - f_e}{f_e}\right)\left(\frac{1 - e^{-\beta t}}{\beta}\right)\right] \quad (2)$$

The final rate is the steady-state rate of synthesis s corresponding to the fraction f_e of enzyme–promoter complexes active at this temperature. The initial rate is larger and represents the rate of synthesis at this temperature when all the complexes are active, s/f_e. A least-squares fit of the later time points was used to determine the final slope s; the intercept of this asymptote $y(\infty)$ with the ordinate gives the quantity $(s/\beta)[(1 - f_e)/f_e]$. The difference $y(\infty) - y$ was then plotted semilogarithmically vs. time. Hence one can estimate both β and f_e and eliminate the strong effect of temperature on the catalytic activity, which, in this formulation, is entirely taken into account in the term s/f_e.

RNA Polymerase–UV5 Promoter Dissociation Rates. In these experiments, preformed complexes between RNA polymerase and promoter were challenged at a given temperature by a competing polynucleotide [cf. Cech & McClure (1980)]. At various times after addition of poly[d(A-T)] the remaining fraction of active complexes was determined with abortive initiation assays. The enzyme (generally 40 nM) and promoter (1–2 nM) were preincubated under standard reaction conditions but in the absence of substrates for a time sufficient to form open complexes and to allow temperature equilibration (typically 10 min). Poly[d(A-T)] was added to a final concentration of 100 μM at times between 2 and 140 min; fractions of this solution (15 μL) were removed and mixed with 25 μL of prewarmed standard assay solution that also contained 2.5 mM AMP. After 10 min of reaction at 37 °C, 25-μL portions of each assay were sampled on 3MM paper. Chromatography and quantitation of the enzyme activity remaining after a given time of exposure to poly[d(A-T)] were performed as described above.

The inclusion of AMP in these assays resulted in the synthesis of pApU by RNA polymerase bound to poly[d(A-T)]. The UV5-specific product (ApApUpU) was well resolved from pApU by chromatography. The additional data provided by pApU synthetic activity provided an internal control for enzyme stability over the course of the (sometimes long) poly[d(A-T)] challenge and were also used to correct for the small differences in sampling volumes at subsequent reaction times in the abortive initiation assay. All of the activity assays were performed at 37 °C. Therefore, the measured activities could be related to the fraction of promoters bound by RNA polymerase in a poly[d(A-T)]-resistant form at any challenge temperature. As shown under Results the decrease in fractional occupancy was strictly first order down to at least 0.05.

An experiment was performed to test the efficiency of this method as well as possible interference introduced by the poly[d(A-T)] challenge protocol. Solutions containing UV5 promoter DNA (1.7 nM) or UV5 promoter DNA and poly[d(A-T)] (106 μM) were incubated at 10, 20, 30, and 40 °C. RNA polymerase (10 nM) was added, and each solution was incubated 10 min before assaying at 37 °C with a solution of ApA, AMP, and [α-^{32}P]UTP. In the reactions to which enzyme was added to promoter and poly[d(A-T)], essentially all of the assayable activity was associated with poly[d(A-T)] (pApU synthesis was 22 min^{-1} per enzyme). A small amount of UTP incorporated into product migrated as ApApU on the chromatogram. It corresponded to 0.42 UTP min^{-1} per enzyme. If all of this product had been synthesized at the UV5 promoter, we estimate an upper limit of promoter occupancy of 5%. We have treated this UTP incorporation as a part of the background in the ApApUpU product determinations.

Table I: Dissociation Rate Constants for RNA Polymerase Bound to *lac* UV5 Promoter at Different Temperatures[a]

temp (°C)	k_r ($\times 10^4$ s^{-1})	$t_{1/2}$ (min)	temp (°C)	k_r ($\times 10^4$ s^{-1})	$t_{1/2}$ (min)
14	5.7	20	33	0.90	129
17	4.9	24	37	0.40	290
22	2.6	45	19	2.7[b]	44
27	1.9	60		3.0[c]	38

[a] The poly[d(A-T)] challenge technique as described under Materials and Methods was employed in all cases except that the values listed for 19 °C correspond to different concentrations of poly[d(A-T)]. On the basis of linear least-squares analyses the errors in k_r were ±10% or less. [b] 37 μM poly[d(A-T)]. [c] 185 μM poly[d(A-T)].

The reactions run exclusively with promoter provided controls for the reactions containing poly[d(A-T)] and also showed insignificant pApU synthesis from the UV5 promoter. Moreover, we have found that the RNA polymerase–UV5 promoter dissociation rates were the same within experimental error when poly[d(A-T)] was employed in the concentration range of 35–266 μM (see Table I). We conclude that poly[d(A-T)] was an effective and nonperturbing challenge reagent over the entire temperature range reported in this paper.

Fraction F of UV5 Promoters Bound by RNA Polymerase As Assayed by Poly[d(A-T)] Challenge. The poly[d(A-T)] challenge protocol was employed except that activities were determined only 5 and 10 min after the addition of poly[d(A-T)]. A linear extrapolation to zero time was used to estimate the fraction of UV5 promoter productively bound. As this measurement was used to calculate the equilibrium distribution of bound and free promoter at each temperature, we compared the results of assays in which enzyme and promoter were preincubated at 23 °C before poly[d(A-T)] challenge with assays performed on an enzyme–promoter solution that was first preincubated at 37 °C for 10 min and then shifted to 23 °C for 10 min prior to poly[d(A-T)] challenge. The results were indistinguishable within experimental error. Thus, the poly[d(A-T)] challenge method is not sensitive to the prior history of the RNA polymerase–promoter complex as long as the preincubation times are long enough.

Results

In the introduction we have proposed to replace the classical two-step formalism for open complex formation (Scheme I), in the case of the *lac* UV5 promoter, by a sequential scheme involving three steps (Scheme II). The rationale for this proposal is more easily understood if one examines first the data obtained from perturbations of preformed RNA polymerase–promoter complexes [dissociation kinetics due to challenge by poly[d(A-T)] or transient inactivation caused by temperature perturbations].

Dissociation Rates. Challenge by poly[d(A-T)] monitors the decay of the kinetically competent complexes. This type of experiment yields the fraction of promoters engaged in such complexes F and the corresponding first-order dissociation rate k_r. The striking result shown in Figure 1 is that k_r increased with decreasing temperature. This observation is inconsistent with the simple two-state mechanism described in the introduction, because the rate of an elementary step (k_{-2} in the two-state model) must decrease with decreasing temperature. Therefore, a more realistic model for open complex formation must include at least one additional step.

The dissociation rate constants determined from the data shown in Figure 1 and from experiments performed at four other temperatures are compiled in Table I. The uncertainties in k_r estimated from the residual error in the least-squares

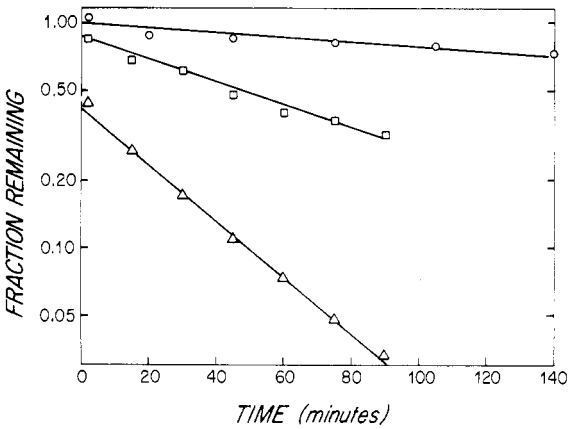

FIGURE 1: Dissociation rates for RNA polymerase bound to the *lac* UV5 promoter fragment increase with decreasing temperature. The fraction of the RNA polymerase–UV5 promoter complex remaining following poly[d(A-T)] challenge (see Materials and Methods) is plotted logarithmically vs. time. The reaction temperatures were (○) 37, (□) 27, and (△) 17 °C. The standard reaction conditions employed during poly[d(A-T)] challenge and the subsequent activity assay were 25 mM Hepes, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/mL bovine serum albumin, 1 nM *lac* UV5 fragment, and 40 nM RNA polymerase. Following preincubation of the enzyme and UV5 template for 10 min at the temperature indicated, poly[d(A-T)] was added to 100 μM at zero time. At the times indicated, aliquots were assayed for promoter occupancy with the abortive initiation assay described under Materials and Methods. Dissociation rate constants were evaluated by a linear least-squares analysis of the data and are compiled for these and other temperatures in Table I.

analyses for dissociation rates at temperatures below 30 °C were less than 10%. At the lower temperatures, the decay curves could be followed for 2–3 half-lives. The results obtained above 30 °C were less precise because dissociation becomes very slow. The initial rates reflected the true dissociation process because poly[d(A-T)] was an effective and nonperturbing challenging reagent over the entire temperature range of 10–40 °C, as detailed under Materials and Methods.

Temperature also affected F, the fraction of promoter–RNA polymerase complexes that was initially resistant to poly[d(A-T)] challenge. This effect was seen only at temperatures below 20 °C and was not pursued further.

The dissociation rates are shown in an Arrhenius plot in Figure 2. The apparent activation energy for dissociation from the linear UV5 promoter was −35 kcal/mol in the temperature range 25–37 °C. Data for the UV5 promoter (insert) corresponded to −15 kcal/mol in the same temperature range. Both of these values suggest a major change in the enzyme–promoter complex that precedes dissociation and that is accompanied by a large positive enthalpy in the forward direction.

Temperature Perturbations of Equilibria: Burst Experiment. In order to explain the dependence of dissociation rates on temperature, we assumed that two binary complexes, both resistant to poly[d(A-T)] challenge, coexisted at equilibrium. Besides the active (open) complex, we assumed that the other complex was inactive and that it was favored at low temperature. Dissociation proceeded from this complex. This simple reasoning led therefore to the inclusion of another intermediate, RP_i, in the mechanistic pathway:

$$R + P \rightleftharpoons RP_c \rightleftharpoons RP_i \rightleftharpoons RP_o$$

poly [d(A-T)] sensitive — poly [d(A-T)] resistant

We wanted first to show that the two postulated species were able to reequilibrate before any significant dissociation into poly[d(A-T)]-sensitive species took place. A complex between RNA polymerase and the UV5 promoter fragment was pre-

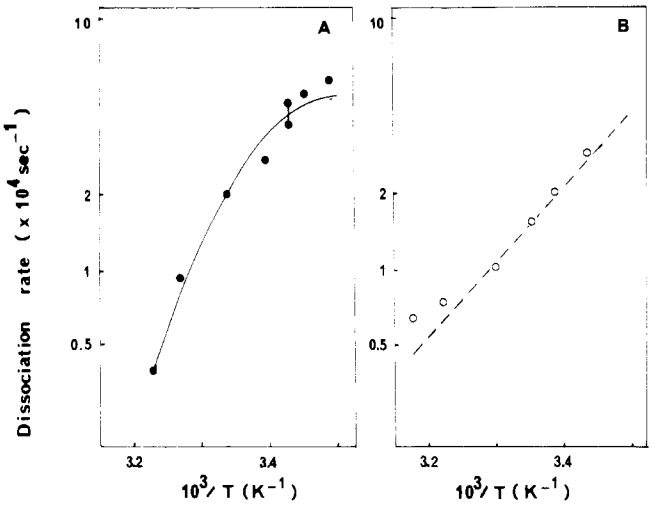

FIGURE 2: Temperature dependence of dissociation rates of RNA polymerase bound to the UV5 promoter. The dissociation rate constants are plotted logarithmically vs. the reciprocal temperature for the UV5 promoter fragment (●) and for the UV5 promoter insert (○). The dissociation rate constants, k_r, were determined with the poly[d(A-T)] challenge method described under Materials and Methods as shown in Figure 1. For the fragment, the solid line is a fit according to eq 6 with the values of K_3, $\Delta H(K_3)$, and E_{-2} given in Table III. For the insert, the dashed line corresponds to an apparent activation energy of −15 kcal/mol.

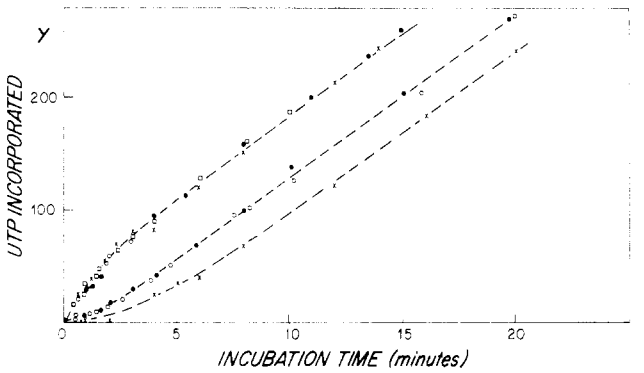

FIGURE 3: Burst and lag experiments for the UV5 promoter at 19 °C. The upper (burst) curve is a reaction progress curve for RNA polymerase and the UV5 promoter that occurred following preincubation at 37 °C for 10 min and 20-fold dilution into the standard assay solution equilibrated at 19 °C. The final enzyme concentrations were (●) 100, (□) 30, and (×) 15 nM. The final promoter concentration was 3 nM DNA fragment. The curve was calculated according to eq 2 in the text with $\beta^{-1} = 60$ s. The lower (lag) curves are reaction progress curves for RNA polymerase that was preincubated at 37 °C (●), or at 19 °C (○), and then diluted 20-fold into a solution containing the UV5 promoter (3 nM DNA fragment) in standard assay solution at 19 °C. In both reactions the final enzyme concentration was 100 nM. The curve was fitted as described under Materials and Methods for $\tau = 95$ s. A similar lag experiment corresponding to a final enzyme concentration at 15 nM is shown (×); $\tau = 210$ s.

formed at 37 °C and then rapidly cooled to 19 °C, in an assay medium where the abortive initiation reaction assay could immediately take place (see Materials and Methods). We reasoned that, if the active complex formed at 37 °C was transformed rather slowly into an inactive species, this should be reflected by a nonlinear accumulation of the product of the abortive initiation assay after the temperature shift, $y(t)$. The results of such experiments are shown in Figure 3 together with the corresponding controls. In the first 10 min the slope of $y(t)$, which represents the activity of the complex at time t, steadily decayed and reached a final value close to the one observed when the complex had not been preincubated at 37 °C. The kinetics and the amplitude of this process (called here a burst) did not depend on the RNA polymerase concentration. In contrast, the parallel experiments performed at 19 °C, in which the reaction was initiated by the addition of RNA polymerase at time zero, exhibited a lag before the steady state was reached. This lag, which reflects the time required for the formation of an active complex from the isolated components, did show, as expected, a marked dependence on RNA polymerase concentration. Incubation of one of the two components of the RNA polymerase–promoter complex at the upper temperature did not affect the lag. Therefore, the burst phenomenon revealed an intrinsic property of the preformed RNA polymerase–promoter complex. Burst kinetics were independent of enzyme concentration. They were faster than the dissociation kinetics (the characteristic relaxation time was 60 s instead of 3500 s). We conclude that the burst revealed an isomerization of the enzyme–DNA complex leading to an inactive (or to a less active) species.

We also performed the converse experiment. The protocol was similar to the previous one except for the inversion of the two thermostats: 20 nM UV5 fragment and 2 μM RNA polymerase were preincubated at 19 °C. This mixture was diluted 20 times into an assay buffer prewarmed at 37 or 30 °C. The formation of product proceeded then at a constant rate, characteristic of the abortive reaction at the final temperature (data not shown). Addition of poly[d(A-T)] to the assay mixture did not affect the course of the reaction. The absence of a burst or of a lag implied that the inactive, poly[d(A-T)]-resistant species present at the lower temperature was quickly converted to the active species during the mixing time.

Perturbation of the steady-state rate of product formation was then extended to other temperatures. In all cases the initial temperature was 37 °C. The results of such experiments, obtained with the UV5 fragment, are shown in Figure 4A. As the temperature of the assay increased, deviation from a linear accumulation of product was less and less marked. The reversibility of the process was checked. The complex, incubated for various times at the lower temperature, was brought back to 30 °C and assayed as described above. Below 17 °C incubation times longer than 5 min led to a partial and slow inactivation of the binary complex.

A similar set of experiments was performed with the UV5 insert. In this case, no burst was observed even when the temperature was shifted from 37 to 9 °C (Figure 4B). Conversely, if the RNA polymerase–UV5 insert complex was brought from 9 to 30 °C, the reaction took place immediately at a constant rate, the process being fully reversible. These findings were consistent with the idea that, on a negatively supercoiled template, the RP_i species was not populated.

Experiments shown in Figures 3 and 4A were then quantitatively interpreted, as explained under Materials and Methods. Each profile was characterized by a rate of transient inactivation β and by the ratio between the final and initial rates of synthesis of the abortive transcript, f_e. These data are given in Table II (cf. also Figures 5 and 6). It is clear that β is always much larger than k_r, the dissociation rate constant. Hence, dissociation must occur in at least two steps, and Scheme I cannot apply. The formalism corresponding to Scheme II was adopted. As shown in the Appendix, $f_e/(1 - f_e)$ yields the equilibrium constant K_3 between RP_o and RP_i, and β is the relaxation rate constant $k_3 + k_{-3}$. The values for f_e and β were used in combination to compute k_3 and k_{-3} (cf. Table III).

As shown in Figure 5, f_e increased sharply with temperature in the range 15–25 °C. Above 25 °C, direct estimates of f_e

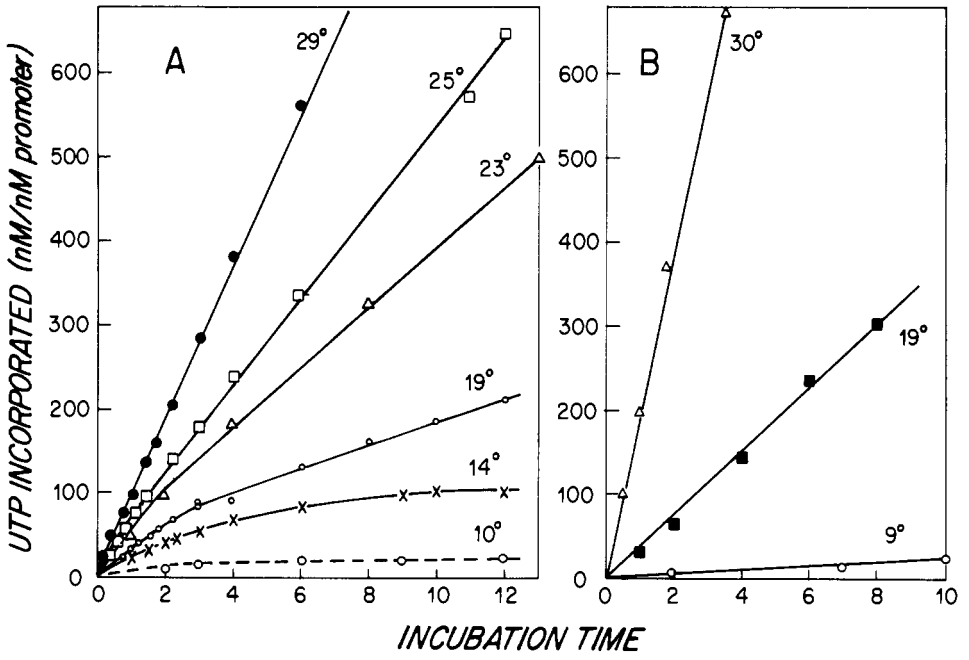

FIGURE 4: Temperature shift experiments performed with the RNA polymerase–*lac* UV5 promoter complex. The burst experiment described in Figure 3 was repeated at the final temperatures indicated in the figure. (A) The UV5 promoter (1–3 nM DNA fragment) was employed. (B) The UV5 promoter (insert; 1–4 nM) supercoiled DNA plasmid was used. The final RNA polymerase concentrations were 100 nM. The superhelical density of the plasmid was −0.035.

Table II: Parameters Defining Rate of Open Complex Formation and Rate of Transient Inactivation at *lac* UV5 (Linear) Promoter at Different Temperatures[a]

temp (°C)	K_B (μM^{-1})	k_f (s^{-1})	β (s^{-1})	f
42	>250	0.11		
37	160	0.09		
30	60	0.07		0.86*
25	45	0.035	0.07	0.65–0.70*
19	35	0.012	0.017	0.28
15			0.008	0.10
14			0.008	0.12*, 0.15
10			0.005	

[a] K_B and k_f were obtained from TAU plots (cf. Figure 7); kinetics of the burst yield β and f_e are explained under Materials and Methods. In the last column, f refers either to measures of f_e, obtained in the burst assay, or to the ratio f^+ between specific activities measured on linear vs. supercoiled templates (these last determinations are labeled with an asterisk). Other measurements of f are given in Figure 5.

Table III: Temperature Dependence on Various Rate and Equilibrium Constants for Formation of an Open Complex between RNA Polymerase and Linear *lac* UV5 Promoter[a]

	K_1 (μM^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	k_3 (s^{-1})	k_{-3} (s^{-1})
value at 37 °C	160	0.95×10^{-1}	0.95×10^{-3}	(2)	(0.08)
ΔH or E_i (kcal/mol)	30 ± 7	5.5 ± 1.5	4 ± 2	60 ± 7	19 ± 5

[a] Data are interpreted according to Scheme II through the procedure described in the Appendix. Values given in parentheses are extrapolated from the low-temperature range. Estimates for ΔH and E_i correspond to the best overall fit performed as described in the Appendix. Errors on these values are estimated from the iterative procedure used during the fit. Note that these errors are correlated: An increase in E_{-2} implies an increase in E_3 and E_2 and a decrease in ΔH_1.

were inaccurate. They were replaced by another estimate of the same quantity, f^+ (f^+ is the ratio between the specific rate of synthesis of ApApUpU found respectively on the linear fragment and on the insert at each temperature); f^+ is also a measure of the fraction of binary complexes that is still active, if one assumes (as suggested by the data shown in

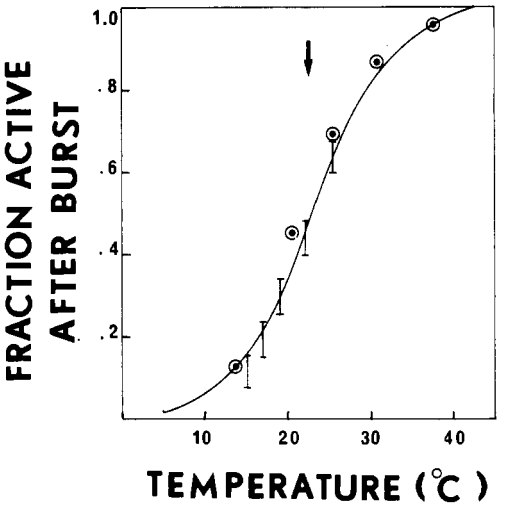

FIGURE 5: The fraction of RNA polymerase–UV5 promoter complexes that remain in open complexes after the burst phase shown in Figure 4 is plotted vs. the final temperature. The fraction of open complexes remaining after temperature downshift, f_e, was calculated according to eq 1 and 2 in the text (I). f^+, the ratio of the specific activity of the linear fragment to that observed for the insert, is plotted on the same figure (⊙). The arrow indicates the midpoint of the transition.

Figure 4B) that on a superhelical template the species RP_i is not populated. Indeed, when determinations of both f_e and f^+ could be made, they agreed reasonably well (cf. Figure 5). At the midpoint of the transition, 22–23 °C, K_3 is equal to 1. The equilibrium constant, K_3, is very temperature dependent (ΔH = 41 kcal/mol). Large activation energies were also calculated for k_3 and k_{-3} (cf. Table III).

Once k_3 and k_{-3} are known, a measurement of k_r can be used to determine the value of k_{-2} at each temperature since k_r is equal to $k_{-2}/(1 + K_3)$ (cf. eq 6 in the Appendix). A linear least-squares regression program yielded the fit given in Figure 2 and in Table III for the UV5 fragment. The decrease of k_r as the temperature increased was entirely accounted for by the large positive enthalpy associated with the third step in Scheme II. In addition, k_{-2} was found to be small compared

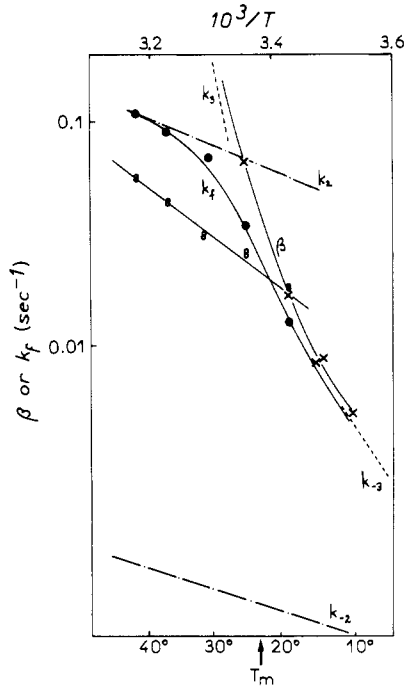

FIGURE 6: Temperature dependence of various kinetic parameters that characterize the burst phenomenon and the rate of open complex formation from lag experiments. The logarithms of the rate constants β and k_f are plotted vs. T^{-1}: (×) relaxation rate β corresponding to the burst experiment performed with the fragment; (●) k_f values for the fragment; (8) k_f values for the insert. k_f values were determined from TAU plots (cf. Figure 7). The dashed lines represent the change with temperature of the various rate constants k_2, k_{-2}, k_3, and k_{-3} when the data corresponding to the *lac* UV5 fragment are analyzed according to Scheme II. The solid lines correspond to the fit of β and k_f with the theory (eq 9a, 9b, and 13).

to k_{-3} and k_3 over the entire temperature range as required for eq 6. The apparent activation energy for k_{-2} was small, in contrast to the values found for k_3 and k_{-3}.

Association Kinetics Determined from TAU Plot Analysis. For the *lac* fragment, the equilibrium between RP_o and RP_i and the individual rate constants were strongly temperature dependent. On the *lac* UV5 insert, the same equilibrium appeared to be driven toward formation of RP_o over the entire temperature range examined. We tried to see how these differences affected the kinetics of open complex formation on these two templates. We used the abortive initiation assay and the methodology described in McClure (1980).

We assume that a first binary complex R·P is formed by the rapid association between RNA polymerase (free concentration [R]) and promoter (free concentration [P], [R] ≫ [P]). This intermediate then interconverts slowly to the active conformation. The identity of the postulated intermediate may change with temperature (i.e., from RP_c to RP_i in Scheme II). None of the constants are therefore assumed to correspond to an elementary step.

$$R + P \underset{K_B}{\rightleftharpoons} R{\cdot}P \overset{k_f}{\underset{k_R}{\rightleftharpoons}} RP_o$$

In this formalism, the rate of open complex formation is given by the equation:

$$k_{obsd} = \frac{k_f K_B[R]}{1 + K_B[R]} + k_R \qquad (3)$$

Whenever k_R is small compared to k_f:

$$1/k_{obsd} = \tau = (1/k_f)(1 + 1/K_B[R]) \qquad (4)$$

τ values were measured as indicated under Materials and Methods. k_f and K_B were determined by using a linear regression analysis of τ vs. $[R]^{-1}$. The results are compiled in Table II and shown in Figures 6–8.

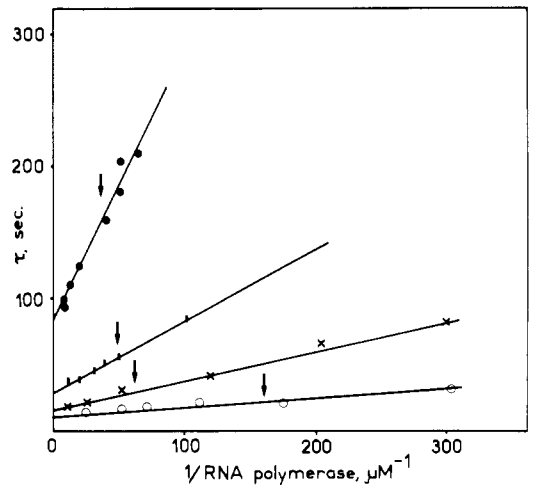

FIGURE 7: Effect of temperature on kinetics of the *lac* UV5 open complex formation. The time required for open complex formation, τ, is plotted vs. reciprocal RNA polymerase concentration. Assays were performed as described under Materials and Methods. Data correspond to a linear DNA and to four different temperatures: (●) 19, (‖) 25, (×) 30, and (○) 37 °C. K_B and k_f^{-1} values were obtained from the intercepts of these plots with the abscissa and the ordinate, respectively.

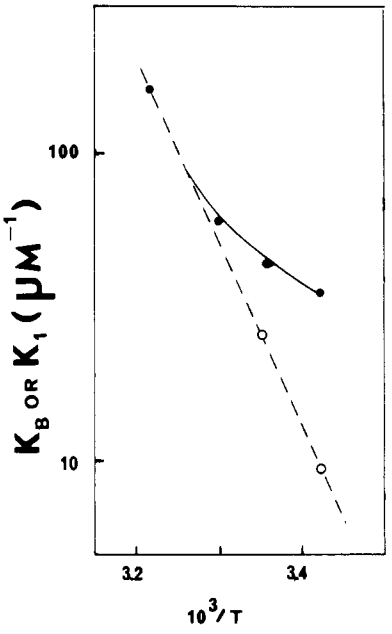

FIGURE 8: Dependence of K_B (●) and of K_1 (○) on temperature for the *lac* UV5 linear fragment. K_B values were obtained from TAU plots (cf. Figure 7), and K_1 values were computed from K_B, k_f, and β according to eq 16.

For the UV5 (insert) k_f was always very much larger than k_r (compare Figures 2 and 6). log k_f decreased linearly vs. $1/T$. The corresponding activation energy was small (9 kcal/mol). It was not possible to measure K_B accurately in this case because negative supercoiling greatly increased K_B [cf. Malan et al. (1984)] and because this first association step was also favored when the buffer was changed from tris(hydroxymethyl)aminomethane (Tris) to Hepes. K_B decreased, however, as the temperature was lowered.

In the case of the *lac* UV5 fragment, TAU plots were obtained at five temperatures from 42 to 19 °C. TAU plots did not show any significant downward curvature as would be the case if eq 3 could not be simplified into eq 4 (cf. Figure 7).

k_f was indeed found to be much larger than k_r (cf. Table II and Figures 2A and 6). The overall reaction was driven over the entire temperature range examined toward the formation of complexes that are far more stable than the initial ones defined by K_B. The dependence of k_f on temperature suggested to us a change in the rate-limiting step. As shown in Figure 6 the Arrhenius plot was not linear. In the high-temperature range, the apparent activation energy was close to the value observed for the UV5 insert. Below 30 °C, k_f decreased abruptly and approached the value of β, the rate constant that characterized the burst phenomenon. These results can be explained by the mechanism in Scheme II. Above 25 °C, the experiments performed after a temperature shift indicated that the isomerization $RP_i \rightarrow RP_o$ was too fast to be measured. The corresponding equilibrium was also driven toward formation of the active species. The formation of an open complex, monitored by the lag experiment, was expected to be limited by the rate of conversion between RP_c and RP_i. Thus, k_f should be approximately equal to k_2. In this temperature range, the values of k_f found for the fragment and for the insert are indeed of the same order of magnitude and follow approximately the same temperature dependence. With decreasing temperature the rate constants k_3 and k_{-3} fall abruptly, for the UV5 fragment only. In this case, the third step is expected to become rate limiting, and indeed, between 25 and 19 °C, we observe that k_f and β become equivalent. The two-step mechanism corresponding to Scheme I obviously fails to account for these observations.

We have therefore derived expressions relating K_B and k_f to the kinetic constants that define Scheme II, assuming this change in rate-limiting step as temperature decreased (cf. Appendix). Variations of K_B, k_f, and $\beta = (k_3 + k_{-3})^{-1}$ with temperature were used to compute estimates of K_1 and k_2 in the temperature range 42–25 °C (cf. Table III and Figures 6 and 8). A very reasonable fit was obtained that yielded estimates of k_2 and K_1 having a linear dependence on $1/T$ (cf. Table III and Figure 6). In the whole temperature range k_2 remained 50–100-fold larger than k_{-2}. The corresponding apparent activation energy was about 5.5 kcal/mol. This finding justifies the two assumptions made through the whole fitting procedure ($|RP_c| \ll |RP_i| + |RP_o|$; k_{-2} negligible with respect to all the other rate constants). log K_1 was linearly dependent on $1/T$ while log K_B manifested a curvature, attributed to the change in rate-limiting step [$\Delta H(K_1) = 30$ kcal/mol]. Thus, the three sets of experiments appeared to be mutually consistent, and the different rate and equilibrium constants could be estimated. Though errors made in the various determinations were quite large (up to 20%), these estimates, given in Table III, could not be changed very much without violating the model. K_1 and k_2 were more accurately defined above 25 °C, by the TAU plots, while k_{-2}, k_{-3}, and K_3 were better computed in the low-temperature range, where k_f was found to be a good approximation of k_{-2} and where the burst experiment was more accurate. Three independent constraints specified the range of possible variation of these parameters: first, the very narrow temperature interval for the transition between RP_i and RP_o with a midpoint between 21 and 24 °C; second, the observation that the rate-limiting step for the forward reaction changed between 19 and 29 °C; third, the very peculiar dependence of k_r on temperature. These limitations were very stringent for all the parameters because any change in one parameter strongly affected the others.

Estimates of the errors made on the various parameters are given in Table III. They rely on the above considerations as well as on the examination of the iterative process that yielded the final fit. The only significant discrepancy appeared when the experimental value of F, the fraction of promoter bound in a form resistant to poly[d(A-T)], was compared to the value expected from the theory (cf. eq 5, Appendix). For temperatures below 23 °C, F, computed through this equation, was consistently higher than its experimental determination. For example, F was found equal to 0.5 at 18 °C while computation predicted a midpoint around 8 °C. The overall observed enthalpy was however consistent with the one that could be calculated from $\Delta H(K_1) = 30$ kcal/mol, $\Delta H(K_2) = 2$ kcal/mol, and $\Delta H(K_3) = 41$ kcal/mol. We have not investigated this discrepancy further. Instead we note that Spassky et al. (1985) report in the following paper that a physical assay for open complexes yields an estimate of the K_3 midpoint in good agreement with the value of 22 or 23 °C suggested here.

Discussion

The reaction that leads to the formation of an active complex between RNA polymerase and a promoter is a slow and probably very intricate process. Three external variables have been used to perturb this pathway: ionic strength, since electrostatic interactions play a dominant role [cf. de Haseth et al. (1978)]; temperature, because the overall reaction is strongly endothermic [cf., for example, Hinkle & Chamberlin (1972), Mangel & Chamberlin (1974), and Seeburg & Schaller (1975)]; and the degree of supercoiling of the DNA template, since the promoter is unwound in the final state and since unwinding is strongly favored on a negatively supercoiled template [cf. Davidson (1972) and Richardson (1975)]. Using abortive initiation assays, it has been possible to characterize those steps of the pathway that are most sensitive to these variables. [For a similar approach, see Bertrand-Burgraaf et al. (1984).]

The simple two-step model for open complex formation originally proposed by Zillig and by Chamberlin cannot account for the temperature dependence of the reactions measured on the linear *lac* UV5 promoter. In particular, the lifetime of complexes resistant to poly[d(A-T)] challenge increased as temperature increased. A transient and reversible inactivation occurred when the open complex was brought from 37 to 19 °C. This inactivation took place at a rate much faster than the dissociation rate k_r. Also, when the complex was brought back to 30 °C, reactivation occurred faster than the overall rate of open complex formation observed by mixing RNA polymerase and DNA at this final temperature. Hence we were led to postulate an inactive intermediate, RP_i, stable at low temperature and resistant to poly[d(A-T)] challenge, which interconverts with the open complex RP_o faster than does the closed (poly[d(A-T)]-sensitive) intermediate, RP_c. Transition between the low-temperature and the high-temperature forms proceeded at a rate that was concentration independent as expected for an isomerization. Hence the second scheme proposed in the introduction:

$$R + P \underset{K_1}{\rightleftharpoons} RP_c \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} RP_i \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} RP_o$$

Scheme II accounts for the unusual temperature dependence of the lifetimes of the final complexes. The overall forward rate constant for open complex formation varies as temperature is lowered as if the rate-limiting step were changing from the second to the third step. It has been possible to obtain a quantitative fit of our data with this scheme. For the linear UV5 promoter, in Hepes buffer and 100 mM KCl, pH 8.0, K_1 is equal to 160 μM^{-1} at 37 °C. This first reaction is strongly endothermic in the range 42–19 °C ($\Delta H = 30$ kcal/mol) and is entropy driven ($\Delta S = 0.16$ eu at 37 °C). The rates and the

equilibrium constant associated with the second step are only slightly affected by temperature [K_2 changes from 100 to 85 between 40 and 10 °C; $E(k_2)$ = 5.4 kcal/mol; $E(k_{-2})$ = 4.1 kcal/mol]. In contrast, the last step is strikingly temperature dependent. The midpoint of the transition is 22–23 °C, the activation energies associated with k_3 and k_{-3} being equal to 60 and 19 kcal/mol, respectively.

The values assigned to each of the steps are limited somewhat because of the simplifications introduced in the data. Determination of those kinetic and equilibrium constants derives from various perturbations affecting the enzymatic activity of RNA polymerase during the abortive initiation assay. They are assumed to reflect solely changes in the concentration of the active species. Large errors affect the determination of the various constants (cf. Table III).

With those reservations in mind, one can, however, describe the effect of temperature on the pathway leading to active complex formation. Over the entire temperature range, K_2 is large and RP_c is therefore not significantly populated. Above 28 °C, the last step is very fast and driven toward RP_o; the steady-state level of RP_i is negligible [$k_f = k_2$; $k_r = k_{-2}/(1 + K_3)$]. Between 28 and 22 °C the rate-limiting step changes gradually from step 2 to step 3; the overall equilibrium is still driven toward RP_o. Below 22 °C, RP_i is more populated than RP_o, and the rate-limiting step in the formation of the active complex is governed by k_{-3}. k_3 and k_{-3} have such a steep dependence on temperature that one can easily imagine a hysteretic behavior. For example, if an active complex is formed at 37 °C and quenched at 4 °C, it would take hours before this complex isomerizes back into RP_i and then RP_c. Hysteresis of this type has previously been observed (Wang et al., 1977).

The steep temperature dependence of overall polymerase binding has been noted previously by several groups, using various assay techniques for different promoters (Chamberlin, 1974; Richardson, 1975; Dausse et al., 1976; Surzycki et al., 1976; Majors, 1977). A pathway involving three steps has been proposed for the T7 A1 promoter (Kadesch et al., 1982; Rosenberg et al., 1982) and for the λP_R promoter (Roe et al., 1984). The general characteristics of the pathway appear to be similar for all three promoters; however, a detailed comparison with UV5 is not possible because a variety of indirect techniques have been employed for all three promoters.

In the case of the *lac* UV5 promoter, we stress that temperature affects mainly the first and the third step of the pathway but not the second one. Negative supercoiling affects not only the same steps but also the rate constant k_2. In a previous publication (Malan et al., 1984) we reported that at 37 °C, in Tris buffer, the insertion of the *lac* UV5 fragment into a negatively supercoiled template resulted in a large increase in K_1 and a significant decrease in k_2. We show here that this is also true in Hepes buffer over the entire temperature range investigated. We interpreted these effects as meaning that the formation of the closed complex, RP_c, involves a significant unwinding of the DNA followed by a slight rewinding during the isomerization to RP_o. [Note that "unwinding" and "rewinding" have to be understood only in their topological meaning; cf. Davidson (1972).] We have shown here that, for the insert, k_r shows a negative dependence on T, that no transient inactivation can be observed, and that k_f behaves as if the rate-limiting step were not affected by temperature. In other words, over the entire temperature range, the insert behaves as the fragment above 30 °C. Moreover, if the RP_i species is still present as a transient intermediate, it is always unstable as compared to RP_o. This strong effect of negative supercoiling together with the observation that k_3 and k_{-3} are highly temperature dependent on the linear template led us to postulate that base-pair unstacking (Hsieh & Wang, 1978) and formation of single-stranded regions downstream of the Pribnow box (Siebenlist, 1979; Siebenlist et al., 1980) take place during the conversion of RP_i in RP_o. It has been shown that opening of base pairs by RNA polymerase on this promoter has a very steep temperature dependence; the midpoint of that transition is the same as that reported here for the equilibrium between RP_i and RP_o [cf. Kirkegaard et al. (1983) and Spassky et al. (1985)].

It follows that topological unwinding takes place gradually since it mainly affects steps 1 and 3 in the present scheme. Base-pair unstacking occurs during the last step. The step that is rate limiting for open complex formation on the supercoiled template (and above 25 °C for the linear DNA), step 2, is precisely the one that is less affected by temperature or by negative supercoiling. In these cases, open complex formation is therefore not limited by the rate at which specific base pairs between positions −10 and +2 become single stranded.

We also believe that DNA unstacking is not ordinarily rate limiting in vivo. The rate of initiation of transcription for the UV5 promoter is not very sensitive to drugs that change the degree of DNA supercoiling within the cell [cf. Sanzey (1979)]. The slight effect that is observed corresponds to an inhibition as negative supercoiling is increased, an effect that is consistent with the change of k_2 with superhelical density found in vitro. Hence for the *lac* UV5 promoter, temperature and negative supercoiling appear to be useful variables for investigating the structural changes arising during open complex formation in vitro. Except perhaps under extreme conditions (low temperature and low supercoiling) they probably do not affect significantly the rate of initiation of the *lac* UV5 message in vivo.

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APPENDIX

Under Materials and Methods we have shown how we determined several phenomenological equilibrium and rate constants. Poly[d(A-T)] challenge experiments are characterized by two values, F and k_r. Lag experiments, when analyzed according to McClure (1980), yield the constants K_B and k_f. The ratio f^* of the steady-state rate of synthesis on linear and supercoiled DNA can also be obtained from these measurements. Burst profiles, when observed, are defined by two other values, a rate constant β and the ratio between the final and the initial rates of synthesis, f_e.

Here, we derive expressions relating these experimental constants to the microscopic constants that characterize Scheme II; we show how we calculate from these relations K_1, k_2, k_{-2}, k_3, and k_{-3} and how we check the self-consistency of these determinations in the temperature range 14–42 °C.

In Scheme II we call P_T the total promoter concentration, A the concentration of RP_c, B the concentration of RP_i, and C the concentration of RP_o. At equilibrium, $K_2 = B_e/A_e$ and $K_3 = C_e/B_e$.

It is assumed that at each time t the rate of product formation is directly proportional to the concentration of active complex RP_o and that the proportionality factor a^* does not depend on the nature of the template. The rate of decay of activity at 37 °C after poly[d(A-T)] challenge reflects the

amount of stably bound species (RP_o in Scheme I, $RP_o + RP_i$ in Scheme II). The experiments are then directly related to the change in concentrations of the species postulated in the two schemes. This problem has been extensively treated in the literature [cf., for example, Pohl (1976) and Hagerman & Baldwin (1976)]. Here, we follow rather closely the approach given by Bernasconi (1976) and the treatment given in Frost & Pearson (1953) quoting Lowry & John (1910).

(*a*) *Dissociation Rates.* RP_c has been shown to be in rapid equilibrium with free RNA polymerase and DNA. Upon poly[d(A-T)] addition, Scheme II is reduced thereby to

$$RP_c \underset{k_{-2}}{\longleftarrow} RP_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} RP_o$$

RP_i and RP_o decay exponentially from their equilibrium concentrations, B_e and C_e, to zero. The total amplitude of the slow phase is expected to be

$$F = \frac{B_e + C_e}{P_T} = \frac{K_1K_2[R](1 + K_3)}{1 + K_1[R] + K_1K_2[R](1 + K_3)} \quad (5)$$

The corresponding rate constant derived for the slowest relaxation yielded the following expressions:

$$\frac{B}{k_{-3}} = \frac{C}{k_{-2} + k_3} = \frac{B + C}{k_{-2} + k_{-3} + k_3}$$

$$\frac{d(B + C)}{dt} = -k_{-2}B = -\frac{k_{-2}k_{-3}}{k_{-2} + k_3 + k_{-3}}(B + C)$$

Hence the rate constant of the exponential decay is given by

$$k_r = \frac{k_{-2}k_{-3}}{k_{-2} + k_3 + k_{-3}} \sim \frac{k_{-2}}{1 + K_3} \quad (6)$$

for $k_{-3} \gg k_{-2}$.

(*b*) *Approach to Equilibrium When the Free Species Are Negligible.* The burst experiments and the lag experiments represent two different ways of approaching a final equilibrium. In the first case, the active species RP_o is the only complex that is initially populated whereas in the lag experiments the closed complex, RP_c, is the only species initially present. For the coupled equilibria involved in Scheme II, i.e.

$$RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} RP_o$$

the rates of the two consecutive relaxation steps that describe these processes do not depend on the initial conditions, but their amplitudes differ [cf. Bernasconi (1976)]. The rates λ_1 and λ_2 are the roots of the secular equation

$$\lambda^2 - (k_2 + k_{-2} + k_3 + k_{-3})\lambda + k_2(k_3 + k_{-3}) + k_{-2}k_{-3} = 0 \quad (7)$$

If k_{-2} can be neglected, λ_1 and λ_2 are equal respectively to $k_3 + k_{-3}$ (the rate constants for isomerization between RP_i and RP_o) and k_2 (the rate constant for conversion from RP_c to RP_i).

Initial Condition $C = P_T$ (Burst Experiment). Under this condition the relaxation rate λ_2 cannot be detected because RP_o is not appreciably depopulated during the corresponding step. One follows the isomerization between RP_o and RP_i. The change in the concentration of the active species during this step is given by

$$C(t) - C_e = (P_T - C_e)e^{-(k_3 + k_{-3})t} \quad (8)$$

This expression can be identified with eq 1, the phenomenological equation used to characterize the burst phenomenon, provided that

$$\beta = k_3 + k_{-3} \quad (9a)$$

and

$$f_e = C_e/P_T \simeq K_3/(1 + K_3) \quad (9b)$$

Therefore, the burst experiment directly yields the rate constants corresponding to the third step. In conjunction with the measurement of k_r (eq 6), we can calculate the value of k_{-2}.

Initial Condition $A = P_T$. This case corresponds to lag experiments performed at saturating concentrations of RNA polymerase (R, in vast excess and not changing in concentration; P is therefore negligible). One follows the approach of RP_o to equilibrium through its activity in the abortive initiation assay. Here $C(t)$ is given by

$$\frac{C(t)}{P_T} = \frac{k_2k_3}{\lambda_2(\lambda_1 - \lambda_2)}(1 - e^{-\lambda_2 t}) - \frac{k_2k_3}{\lambda_1(\lambda_1 - \lambda_2)}(1 - e^{-\lambda_1 t}) \quad (10)$$

As above, $\lambda_1 = k_3 + k_{-3}$ and $\lambda_2 = k_2$. Integration of eq 10 over time yields the expression for product accumulation:

$$y(t) = (a^*/P_T)\int_0^t C(t)\,dt \quad (11a)$$

a^* being the steady-state rate of ApApUpU synthesis when $C_e = P_T$. It follows that

$$a^*C_e/P_T = s$$

After integration:

$$y(t) = s\left[t + \frac{\lambda_2}{(\lambda_1 - \lambda_2)\lambda_1}(1 - e^{-\lambda_1 t}) - \frac{\lambda_1}{(\lambda_1 - \lambda_2)\lambda_2}(1 - e^{-\lambda_2 t})\right] \quad (11b)$$

These complex equations can be simplified in the case of the UV5 promoter as follows: First, we assume that on a negatively supercoiled template, at equilibrium, $C_e = P_T$. Then

$$a^* = s(\text{insert})$$

Hence

$$s(\text{fragment})/s(\text{insert}) = f^* = (C_e/P_T)_{\text{fragment}} \quad (12)$$

Second, λ_1 and λ_2 have a very different temperature dependence for the fragment. Whenever one relaxation rate is small with respect to the other, the corresponding amplitude is large (cf. eq 10). In this case a single relaxation characterizes the appearance of the open complex, and by integration, the product accumulation takes place through a "lag" that measures the relaxation time corresponding to this rate-limiting step: k_2^{-1} for $RP_c \rightarrow RP_i$ and $(k_3 + k_{-3})^{-1}$ for $RP_i \rightleftarrows RP_o$. Third, in the small temperature interval where $\lambda_1 \simeq \lambda_2$, the experimental determination of the duration of the lag, τ, yields a weighted average of λ_1 and λ_2. The asymptotic behavior of $y(t)$ can be obtained from eq 11b. It can be shown that the asymptote $y(\infty)$ intersects the time axis at

$$\tau(\infty) = k_f^{-1} = k_2^{-1} + (k_3 + k_{-3})^{-1} = \frac{\lambda_1 + \lambda_2}{\lambda_1\lambda_2} \quad (13)$$

It can be also shown that this value closely approximates the single exponential that best fits our data in the intermediate temperature range. Hence the analyses of lag experiments performed at high RNA polymerase concentrations (intercept on τ plots) were used to determine either λ_1 or λ_2 or the average given by eq 13.

Approach to Equilibrium When the Free Species Are Not Negligible. The considerations developed above still apply.

For the temperature-shift experiments, provided that k_{-2} remains negligible and that, at $t = 0$, $C = P_T$, the burst experiment still reflects the isomerization between RP_i and RP_o. For the lag experiments, one has to take into account the fact that RP_c is in equilibrium with the free species. This equilibrium is assumed to be fast relative to k_2. Under these circumstances, one can write a steady-state condition for RP_c; this is equivalent to replacing k_2 in eq 10–13 given above by

$$k_2/(1 + [R]^{-1}K_1^{-1}) \quad (14)$$

[For a discussion of this point see Bernasconi (1976), Chapter 4.] Hence, τ will be given by

$$\tau = \frac{1}{k_3 + k_{-3}} + \frac{1}{k_2}(1 + [R]^{-1}K_1^{-1}) \quad (15)$$

Our experimental results have been analyzed according to a simpler two-step phenomenological model:

$$R + P \underset{K_B}{\rightleftharpoons} R{\cdot}P \xrightarrow{k_f} RP_o$$

It has been shown earlier that, in this model, the relaxation time τ depends on RNA polymerase concentration according to eq 4:

$$\tau = (1/k_f)(1 + [R]^{-1}K_B^{-1})$$

We have used this equation to analyze the lag experiments. In particular, we have derived from the TAU plot analysis (τ plotted vs. $[R]^{-1}$) values of k_f and K_B at each temperature [cf. McClure (1980)]. Comparison of eq 4 with eq 15 shows that the phenomenological constants are related to the microscopic constants involved in Scheme II as follows:

$$K_B^{-1} = K_1^{-1}\left(\frac{k_3 + k_{-3}}{k_2 + k_3 + k_{-3}}\right) \quad (16)$$

$$\frac{1}{k_f} = \frac{1}{k_2} + \frac{1}{k_3 + k_{-3}}$$

$k_3 + k_{-3}$ is known and equal to β. Hence K_1 and k_2 can be obtained from K_B and k_f.

In summary, the parameters defining Scheme II can be computed from the experiments described here. The burst experiment yields k_3 and k_{-3}. In conjunction with the measurements of the rates of open complex decay, we obtain k_{-2}. The rate of formation of open complexes, monitored by the abortive initiation assay, yields values of k_f and K_B and, in turn, k_2 (eq 13) and K_1 (eq 16). Assumptions made in the derivation (i.e., $k_{-2} \ll k_2$) can be examined at this point. The fraction of complexes present in the active form (f or f^*) or as species resistant to poly[d(A-T)] challenge, F, is used to check the internal consistency of those determinations.

Enthalpies, ΔH, and apparent activation energies, E_i, are obtained through the change of the corresponding constants vs. $1/T$ by linear least-squares regression. Each constant is assumed to be of the form

$$k_i = Ae^{-E_i/RT}$$

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

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Changes in the DNA Structure of the *lac* UV5 Promoter during Formation of an Open Complex with *Escherichia coli* RNA Polymerase†

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ABSTRACT: By chemical and enzymatic methods, two stable complexes between *Escherichia coli* RNA polymerase and a linear DNA fragment carrying the *lac* UV5 promoter have been identified. In these binary complexes, DNA can adopt two alternate conformations as a function of temperature. Contacts between RNA polymerase and the DNA phosphate backbone are indistinguishable in these two forms, as revealed by probing with pancreatic DNase I. Protection of enhancement of the reactivity of the bases toward $(CH_3)_2SO_4$ occurs, however, only in the form that predominates above 22 °C, RP_o. The form stable at low temperature, RP_i, is a "closed" complex since no single-stranded region is detectable in the DNA. The strong temperature dependence of the equilibrium constant, the midpoint value of the transition, and the rate of conversion between these two forms are in close agreement with a series of measurements performed by using a transcriptional assay and reported in the preceding paper [Buc, H., & McClure, W. R. (1985) *Biochemistry* (preceding paper in this issue)]. These data further support the postulated mechanism of open complex formation involving three sequential steps: $R + P \rightleftarrows RP_c \rightleftarrows RP_i \rightleftarrows RP_o$. The binary complex RP_c, which accumulates transiently at 37 °C before the isomerization leading to open complex formation, is not significantly protected against enzymatic cleavage or chemical modification and is therefore distinct from RP_i and RP_o. When considered in conjunction with the results of the kinetic studies, these characteristics of the three binary complexes, RP_c, RP_i, and RP_o, imply that the second step ($RP_c \rightarrow RP_i$) corresponds to the acquisition of a stable footprint and the last isomerization to the formation of single-stranded regions. The DNA structure of the UV5 promoter is further modified when substrates are added, following formation of the open complex: although the DNase I footprint is only slightly modified during the synthesis of the 5′-terminal tetranucleotide ApApUpU, the single-stranded region revealed by dimethyl sulfate reactivity moves downstream, and the methylation of a distal guanine at position −32 is affected.

The rate of formation of an active complex between *Escherichia coli* RNA polymerase and a DNA fragment containing a promoter sequence governs the frequency of initiation of synthesis of the corresponding messenger. This frequency defines the strength of the promoter (Seeburg et al., 1977; von Gabain & Bujard, 1977, 1979). Extensive studies have been performed to characterize the structure of the DNA in the active complex. One of the best known examples is the *lac* UV5 promoter [cf. Galas & Schmitz (1978), Johnsrud (1978), Simpson (1979), Siebenlist et al. (1980), and Chenchick et al. (1981)]. In this case, we know that the active complex RP_o is "open" because a well-defined region downstream of the Pribnow box is single stranded (Siebenlist et al., 1980; Kirkegaard et al., 1983) and because the promoter is topologically unwound.

In the preceding paper (Buc & McClure, 1985), we have explored the use of the abortive initiation assay devised by Johnston & McClure (1976) to investigate the kinetics of open complex formation. These studies led us to propose this multistep scheme:

$$R + P \rightleftarrows RP_c \rightleftarrows RP_i \rightleftarrows RP_o$$

The existence of a new species, RP_i, was postulated because the kinetically competent species can be rapidly and reversibly inactivated by lowering the temperature; this new binary complex, unlike RP_c, remains resistant to poly(deoxyadenylate-thymidylate) [poly[d(A-T)]] challenge at these lower temperatures. The location of RP_i in the pathway accounts well for the observed temperature dependence of all the kinetic constants.

In the work presented here, we have tried to characterize the reactivity of the promoter DNA in these different binary complexes as well as in ternary complexes formed after addition of the two substrates ApA and uridine 5′-triphosphate (UTP). First, independent support for the existence of the additional complex RP_i was obtained by monitoring the change in DNA reactivity in the temperature range where the two conformations RP_i and RP_o are postulated to occur. Second, we adapted our methods to kinetic studies and looked for structural changes occurring before the establishment of the

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