Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5634–5638, October 1980
Biochemistry

Rate-limiting steps in RNA chain initiation

(RNA polymerase mechanism/abortive initiation)

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Communicated by Konrad E. Bloch, April 21, 1980

ABSTRACT **Promoter-specific lags in the approach to the steady-state rate of abortive initiation were observed when *Escherichia coli* RNA polymerase was added to initiate the reaction. The lag times were related to the time required for free enzyme and free promoter to combine and isomerize into a functionally active complex. The lag times measured for several bacteriophage and bacterial promoters differed widely (10 sec to several minutes) and in most cases corresponded to the rate-limiting step in the initiation process. The unique advantage in using the abortive initiation reaction to measure the lags was that the binding and isomerization steps in a simple two-state model could be quantitated separately. The separation of the contributions of both steps was effected by deriving an equation to describe the rate of formation of the active binary complex. Results from experiments based on the theory showed a linear relationship between the observed lag times and the reciprocal enzyme concentration. The slope and intercept of the equation yielded quantitative estimates of the binding and isomerization steps in initiation. The analysis was applied to the bacteriophage T7 A2 and D promoters to show the bases for the differences in *in vitro* initiation frequency that have been observed for these promoters.**

Transcription in *Escherichia coli* is catalyzed by DNA-dependent RNA polymerase (nucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). The initiation of RNA synthesis from bacterial and bacteriophage promoters is an important control point for gene expression. The frequencies of initiation vary considerably both *in vivo* and *in vitro*. Although activator proteins and repressor proteins provide important on/off switches for some operons, many initiation frequencies are determined solely by the interaction of RNA polymerase with the DNA in a promoter region. The sequences of about 50 such promoters have been determined (1, 2). Although striking sequence homologies are found in the DNA, general rules for initiation frequency based on DNA sequence have not emerged. The reason is that until recently there was no quantitative assay for *in vitro* chain initiation frequency that was also generally applicable to many promoters.

A minimal model for RNA chain initiation has existed for many years (3). The enzyme is thought to bind the DNA in the first step and to unwind the DNA in the second step. Subsequent triphosphate binding and phosphodiester bond formation would then lead to an elongating ternary complex. Chamberlin has focused attention on the two binary complexes and has referred to them as "closed" and "open" (4). For many promoters the formation of the open complex is likely to be rate determining in initiation because triphosphate binding and elongation have been shown to be very rapid (5, 6). Results obtained with different promoters and with various techniques have been interpreted as demonstrating that the binding step (7, 8) or the isomerization step (9, 10) was uniquely rate limiting. These results are not necessarily contradictory. Because promoters differ in sequence, initiation frequency may well be determined differently in the various promoters under study.

It is the purpose of this paper to demonstrate that an assay based on the abortive initiation reaction of promoter-bound RNA polymerase can be used to separate the contributions of binding and isomerization to overall rate determination in the RNA initiation mechanism. The key feature of the abortive initiation reaction that allows this advance is that abortive initiation is a promoter-specific steady-state reaction (11, 12). The presence of RNA polymerase in the open promoter complex can be monitored by quantitating the rate of formation of abortive initiation oligonucleotides. The observation relating this assay to the rate-limiting steps in initiation was that a lag was observed in the approach to the steady-state rate of abortive initiation when promoter and enzyme were mixed to initiate a reaction. Because the lag was found to be promoter-specific, the time required to reach the catalytically active open complex is interpreted as including the time required for the formation and subsequent isomerization of an intermediate (i.e., the closed complex). Finally, from a derivation of the kinetics predicted by the two-state model it is shown that the dependence of the lag on enzyme concentration allowed quantitative separation of the binding and isomerization steps in the initiation reaction.

MATERIALS AND METHODS

DNA Templates and Enzyme. Bacteriophage λ, T7 C5, and T7 D111 DNA were prepared from purified phage as described (12). Promoter-containing DNA fragments were obtained from *Hae* III digestions of the DNA as described (12). The *lac* P^+ and *lac* P^r L8 UV5 promoter-containing fragments were prepared from *Eco*RI digests of pMB-9 DNAs containing the *lac* operator-promoter region as a *Hae* 203 fragment (13). Poly[d(A-T)] and poly[d(I-C)] were synthesized with *E. coli* DNA polymerase I (Klenow fragment). *E. coli* RNA polymerase was isolated according to Burgess and Jendrisak (14); holoenzyme was separated from core according to Lowe *et al.* (15).

Abortive Initiation Assay. The steady-state properties and technical aspects of the abortive initiation assay have been reported (12). In this paper, standard assay conditions are 0.04 M Tris·HCl at pH 8, 100 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The RNA polymerase and template concentrations used are indicated in the figure and table legends. The substrates for the abortive initiation reactions used were: for the T7 D promoter, 2 mM GMP and 0.05 mM UTP to form pGpUpU; for the T7 A2 promoter, 2 mM GMP and 0.05 mM CTP to form pGpC (16). The appropriate pyrimidine[α-^{32}P]triphosphate was added to a specific activity of 300 cpm/pmol. The reaction mixtures were combined in a final volume of 0.25 ml at 37°C. Samples were taken at appropriate times (e.g., 0.5-min intervals) and applied to the origin of a

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Abbreviations: RP_c, closed RNA polymerase–promoter complex; RP_o, open RNA polymerase–promoter complex.

Whatman 3 MM paper chromatogram that had previousy been spotted with 0.1 M EDTA. The chromatogram was developed in the ascending direction (16 cm) with H_2O/saturated ammonium sulfate/isopropanol, 18:80:2 (vol/vol).

Evaluation of τ_{obs}. The rates of abortive initiation in control experiments initiated with nucleotides were determined by using a linear least squares analysis. The curves that resulted from experiments in which the reactions were initiated with enzyme were analyzed to yield τ_{obs} in two ways. A least squares line was calculated from those data points corresponding to times greater than 3 times the initial estimate of τ_{obs}. Second, a best fit line was drawn through the experimental curve parallel to the control rate. In most cases, the final steady-state rate after the lag was within 15% of the control rate and both methods yielded comparable results (± 15% τ_{obs}).

THEORY

The reaction scheme for the formation of an RNA polymerase-open promoter complex is:

$$R + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o \quad [1]$$

in which R and P correspond to free enzyme and promoter, respectively; RP_c is the closed complex; and RP_o is the open complex. The overall binding constant, K_o, is the product of the bimolecular association constant, K_I, and the isomerization equilibrium constant, K_{II}.

For the *in vitro* experiments, the following simplifying assumptions were made. (*i*) $[R] \gg [P_t]$ (i.e., the pseudo-first-order approximation, in which $[P_t]$ is the total promoter concentration). (*ii*) Steady state is assumed for $[RP_c]$, the closed complex. The rate equation to be solved is

$$\frac{d[RP_o]}{dt} = k_2[RP_c] - k_{-2}[RP_o]. \quad [2]$$

The general solution to this equation has been described (17).

Based on the above assumptions and the fact that the equilibria above lie far to the right

$$[RP_o] = [P_t](1 - e^{-k_{obs}t}) \quad [3]$$

$$k_{obs} = \frac{k_1[R](k_2 + k_{-2}) + k_{-1}k_{-2}}{k_1[R] + k_{-1} + k_2}. \quad [4]$$

In the work reported here, an additional simplifying assumption can be justified—namely, $k_{-2} \ll k_2$. In this case,

$$k_{obs} = \frac{k_1[R]k_2}{k_1[R] + k_{-1} + k_2}. \quad [5]$$

The reciprocal of Eq. **5** corresponds to the average time required for open complex formation, τ_{obs}:

$$\tau_{obs} = \frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{k_{-1} + k_2}{k_1[R]k_2}. \quad [6]$$

Thus, a plot of τ_{obs} versus $[R]^{-1}$ will yield $1/k_2$ on the ordinate; the slope is $1/k_{on}$ as discussed below. The experimental task is to evaluate k_{obs} at various concentrations of enzyme.

The detection of RP_o is based on the assumption that $[RP_o]$ is directly proportional to the rate of the RNA polymerase abortive initiation reaction. I consider this assumption in some detail in the *Discussion*. To evaluate k_{obs}, two reactions are run differing only in their initiation protocol. In the first reaction, enzyme and promoter are preincubated for a time sufficient to allow complete RP_o formation. Addition of the promoter-specific nucleotides required for the abortive initiation reaction at time zero initiates a steady-state rate of synthesis. The latter reaction serves as a control rate. If RNA polymerase is added to a solution of promoter and nucleotides at zero time, a lag in the approach to the control steady-state rate will be observed. Extrapolation of the steady-state rate observed after the lag in the polymerase-initiated reaction yields τ_{obs} as the intercept on the time axis.

There are two limiting expressions for Eq. **6** that can describe the RNA polymerase–promoter interaction. Depending on the relative magnitude of k_{-1} and k_2, I have called these limiting mechanisms I and II. The intercept on the ordinate of a tau plot (τ_{obs} vs. $[R]^{-1}$) is always $1/k_2$ (Table 1). The slope is the reciprocal of k_{on}, the apparent bimolecular association rate constant. Limiting mechanism I, in which $k_{-1} \gg k_2$, corresponds to the rapid equilibrium case, and the slope of a tau plot divided by the intercept is the dissociation constant for RP_c, K_I. In the case in which $k_{-1} \ll k_2$, limiting mechanism II, the kinetics actually correspond to two irreversible (pseudo) first-order reactions, and two exponentials describe the formation of RP_o. However, when the data are plotted according to the procedure used in this work, the slopes and intercepts will correspond to the expressions shown in Table 1.

Expressions for k_{on}, the bimolecular association rate constant, have been derived for the scheme shown in Eq. **1**. These equations correspond to the reciprocal of a tau plot slope and differ, as shown in Table 1, depending on the mechanism that obtains. Similarly, expressions for k_{off} can be derived for each mechanism, and $k_{on}/k_{off} = K_o$ in each case. The exceedingly long lifetimes of RP_o complexes correspond to $k_{off} = 10^{-3}$ to 10^{-5} sec^{-1} (18). Thus, reversal of RP_o formation can be ignored in the analysis presented here.

RESULTS

The time required for a promoter and RNA polymerase to combine and isomerize into an RP_o under a particular set of experimental conditions can be measured by initiating the promoter-specific abortive initiation reaction with RNA polymerase. Such a measurement is shown in Fig. 1 for the T7 D promoter. The reaction initiated with either GMP or UTP after preincubation of RNA polymerase and DNA proceeded

Table 1. Evaluation of rate constants corresponding to open complex formation

Mechanism	Additional assumption	tau plot parameters: Intercept	tau plot parameters: Slope	k_{off}
General case	—	$\frac{1}{k_2}$	$\frac{k_{-1} + k_2}{k_1k_2}$	$\frac{k_{-1}k_{-2}}{k_{-1} + k_2}$
Limiting mechanism I	$k_{-1} \gg k_2$	$\frac{1}{k_2}$	$\frac{k_{-1}}{k_1k_2}$	k_{-2}
Limiting mechanism II	$k_{-1} \ll k_2$	$\frac{1}{k_2}$	$\frac{1}{k_1}$	$\frac{k_{-1}k_{-2}}{k_2}$

The evaluation of rate constants is based on Eq. **6** and the information obtained from the slope and intercept when τ_{obs} is plotted versus $[R]^{-1}$, the tau plot. The general mechanism makes no assumptions beyond those identified in the derivation of Eq. **6**. The two limiting mechanisms correspond to cases in which k_{-1} is either greater than k_2 (mechanism I) or smaller than k_2 (mechanism II). The slope of a tau plot is $1/k_{on}$; the expression for k_{off} in each mechanism is also listed and must be evaluated independently as discussed in the text.

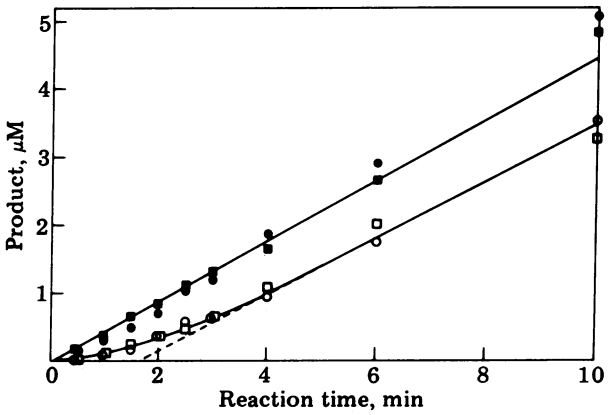

FIG. 1. Time required for RP_o formation on the bacteriophage T7 D promoter. The product is pGpUpU. T7 DNA was 2.4 nM genome. Two protocols were used to initiate the reaction. (*i*) RNA polymerase, at 90 nM, was preincubated for 10 min at 37°C with DNA template and either GMP (■) or UTP (●); the reactions were initiated at zero time with the other nucleotide. (*ii*) RNA polymerase was preincubated alone (□) or with GMP + UTP (○); the reactions were initiated at zero time with the addition of enzyme to the DNA solution. The second protocol resulted in a lag that preceded the final steady-state rate observed in all four reactions.

linearly over the time of the experiment. The steady-state rate extrapolated to zero product at zero time. However, when RNA polymerase was added to initiate the reaction a lag was observed in the approach to the steady-state rate. The same lag was obtained when RNA polymerase was preincubated with the nucleotides and then added to initiate the reaction. In all four reactions the final steady-state rate was the same within experimental error (± 15%).

To rule out the possibility that the lag occurred primarily as a result of the time required for an interconversion of RNA polymerase from a putative inactive form to an active form (e.g., dimer-to-monomer conversion), the enzyme was preincubated under standard assay conditions except that the KCl concentration was 0, 80, 300, or 800 mM. The enzyme was then added to reaction solutions prepared to yield a final KCl concentration of 80 mM in all cases. The observed lag was 80 ± 10 (mean ± SD) sec with no systematic trend in τ_{obs} values. I have also found that RNA polymerase can be added directly from a solution corresponding to standard assay conditions at 0°C, 23°C, or 37°C without a significant effect on τ_{obs}. These experiments do not rule out important conformational changes in RNA polymerase at extremes of salt concentration or temperature but such changes must occur rapidly compared to the times measured for RP_o formation on the T7 D promoter.

If the lag observed for the T7 D promoter is related to the rate-limiting step in initiation, the expectation is that the time required for RP_o formation, τ_{obs}, would be different for different promoters. Table 2 shows values of τ_{obs} obtained with RNA polymerase and several templates under comparable assay conditions. The frequently initiating major promoters of T7, A1 and A2, showed short lag times. The major promoters of bacteriophage λ, P_R and P_L, showed lag times that were somewhat longer than with the T7 promoters, in agreement with the *in vitro* transcription properties of this template. The τ_{obs} values for *E. coli lac* P^r L8 UV-5 and *lac* P^+ are also consistent with *in vitro* transcription results; *lac* UV-5 initiates frequently and *lac* wild type initiates infrequently (10, 19). Finally, the alternating copolymers poly[d(A-T)] and poly[d(I-C)] also displayed lags. Poly[d(A-T)] is a good template for *in vitro* RNA synthesis; τ_{obs} for this synthetic DNA was as short as found on the best natural promoters. The striking characteristic of all these promoters is that the times required for RP_o formation are long compared to the times required for elementary steps in most enzyme-catalyzed reactions.

Table 2. τ_{obs} on selected templates

Template	Product	τ_{obs}, sec
Bacteriophage promoters		
T7 A1	pA*pU	20
T7 A2	pG*pC	20
λ P_R	pA*pU	≤30
λ P_L	pA*pU	60
Bacterial promoters		
lac UV-5	ApA*pU*pU	30
lac wild type	ApA*pU*pU	400
Synthetic copolymers		
Poly[d(A-T)]	pA*pU	10
Poly[d(I-C)]	pG*pC	90

The lag times (τ_{obs}) were determined for the promoters listed with a protocol similar to that shown in Fig. 1 except that promoter-containing restriction enzyme fragments were used as templates (1–2 nM fragment). The synthetic templates were present at 100 μM DNA phosphate. The RNA polymerase concentrations were 50–100 nM. The products formed correspond to the starting sequence of each transcript. Purine monophosphates (or ApA for the *lac* promoters) and a pyrimidine [α-^{32}P] triphosphate were used to monitor the abortive initiation reactions.

The results obtained for several promoters under a specific set of conditions suggest that τ_{obs} is related to slow steps in the initiation process that precede any catalytic activity of the enzyme. As shown in the theory section, separation of the binding and isomerization contributions to overall rate limitation in RP_o formation is effected by measuring τ_{obs} at different concentrations of RNA polymerase.

In Fig. 2 the T7 D and A2 promoters are compared on a plot of τ_{obs} vs. reciprocal RNA polymerase concentration (tau plot). In both cases a linear relationship was found in conformity with Eq. 6. The values for the slope and intercept are shown in the figure legend. In both cases the intercept (which corresponds to $1/k_2$ in Eq. 1) is rather well determined. The slope is easily

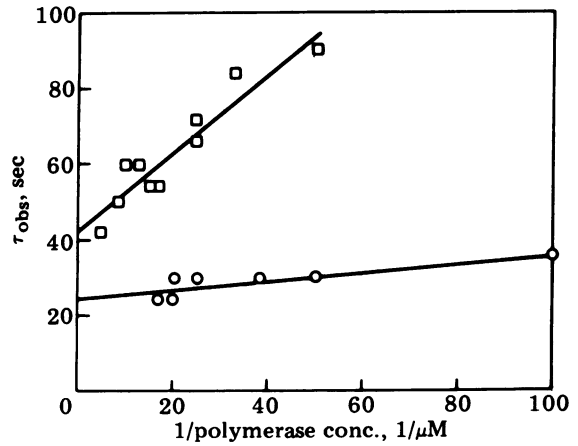

FIG. 2. tau plot for the bacteriophage T7 D and A2 promoters. The lag times observed (τ_{obs}) for pGpUpU synthesis from the D promoter (□) and pGpC synthesis from the A2 promoter (○) are plotted versus the reciprocal of the RNA polymerase concentrations used. The template was 1 nM T7 genome. Each determination of τ_{obs} was based on a control reaction initiated with nucleotides run in parallel with a reaction initiated with enzyme. In the latter case the evaluation of the resulting lags was carried out as described in *Materials and Methods*. The slopes and intercepts (determined by least squares) were: D promoter, 1.04 μM·sec and 42 sec; A2 promoter, 0.12 μM·sec and 25 sec.

measured for the D promoter at accessible enzyme concentrations. The low concentrations of enzyme required to show an effect on τ_{obs} for the A2 promoter allow only an upper estimate to be made for the slope.

Inspection of the tau plot in Fig. 2 is sufficient to decide which of the two promoters will initiate most frequently. At all RNA polymerase concentrations, RP_o is formed more rapidly on the A2 promoter. At saturating concentrations of enzyme the rates are limited only by k_2; the selectivity is about 2-fold. At typical concentrations of RNA polymerase used *in vitro* (e.g., 5 μg/ml or 10 nM) the overall rate of RP_o formation favors the A2 promoter by a factor of 6. At lower concentrations of enzyme, the selectivity for A2 becomes even more pronounced. Frequently initiating promoters as a class will have low intercepts and low slopes on a tau plot; less-efficient promoters are characterized by high intercepts or high slopes on a tau plot. This qualitative generalization follows directly from the fact that the times required for RP_o formation, τ_{obs}, are nearly always longer than the time required for any other step in initiation.

I emphasize that the measurement of τ_{obs} has been made without addition of drugs or other inhibitors to "trap" various states of promoter-bound polymerase. Instead, the measurement relies on an intrinsic catalytic property of RNA polymerase in the RP_o.

A quantitative comparison between the T7 A2 and D promoters is shown in Table 3. The analysis is based on conformity to limiting mechanism I (i.e., $k_{-1} \gg k_2$). I favor this mechanism because, if $k_{-1} \leq k_2$, the k_{on} values determined from the tau plots yield values of k_1 that are too small to correspond to an elementary bimolecular step (i.e., diffusional collision). Although it seems likely that the initial association of enzyme and promoter to yield RP_c is near equilibrium, this point has not been proven. Therefore, I have listed the values in Table 3 as apparent (i.e., model dependent) equilibrium constants.

The A2 and D promoters both have overall binding constants for RNA polymerase $> 10^{10}$ M^{-1}. The combined kinetic data are thus consistent with the tight binding behavior seen when these promoters were titrated with RNA polymerase in the nanomolar concentration range (18). It is also seen that a major portion of the free energy for RP_o formation accompanies the initial complexation to RP_c. The conversion from RP_c to RP_o is favorable by only 3–4 kcal/mol in each case.

DISCUSSION

The key finding reported here is that promoter-specific lags in the approach to the steady-state rate of abortive initiation can be related to the times required for RNA polymerase and a promoter to combine and isomerize into the functionally important RP_o. Moreover, the unique advantage found in using the abortive initiation assay to measure τ_{obs} was that the apparent binding and isomerization steps could be quantitated separately as a result of the dependence of τ_{obs} on enzyme concentration.

The functional comparison between the strong and weak T7 promoters detailed above in terms of relative initiation frequency can be applied to any set of promoters. Indeed, measurement of τ_{obs} under a given set of reaction conditions provides practical information about the length of time required during a preincubation period for RP_o formation to proceed to completion (e.g., $3 \times \tau_{obs}$ = 95% complete). But the real value in determining the slope and intercept of a tau plot for a given promoter lies in the mechanistic information obtained. The intercept corresponds to $1/k_2$; the slope corresponds to $1/k_{on}$. With additional information on the RP_o lifetime, it is also possible to determine the two equilibrium constants for RP_o formation. The separation and quantitation of these two steps that participate in overall rate limitation in RNA chain initiation provides an essential tool for quantitative comparisons of promoter function. It should now be possible to make measurements with selected promoters under different incubation conditions to determine the effect of solution conditions such as ionic strength, $[Mg^{2+}]$, etc. and to determine the effect of nonspecific DNA, supercoiling, promoter mutations, and other variables related to DNA template participation.

Table 3. *In vitro* comparison between a strong and a weak T7 promoter

	A2	D
Kinetic constants:		
$k_{on}\left(\frac{k_1k_2}{k_{-1}}\right)$, M^{-1} sec^{-1}	$>9 \times 10^6$	9.6×10^5
k_{off} (k_{-2}), sec^{-1}	1.7×10^{-4}	3.3×10^{-5}
k_2, sec^{-1}	4×10^{-2}	2.4×10^{-2}
Apparent equilibrium constants:		
K_I, M^{-1}	$>2 \times 10^8$	4.1×10^7
K_{II}	2.4×10^2	7.2×10^2
K_o, M^{-1}	$>5 \times 10^{10}$	3.0×10^{10}

The kinetic constants k_{on} and k_2 were evaluated from the slopes and intercepts of Fig. 2. k_{off} was determined by Cech and McClure (18). The equilibrium constants were calculated from the ratios k_{on}/k_2 for K_I, k_2/k_{off} for K_{II}, and k_{on}/k_{off} for K_o.

Validity of the Abortive Initiation Assay. The crucial assumption in this analysis is that the observed rate of abortive initiation is proportional to promoter occupancy in the RP_o. The evidence supporting this assumption is as follows. (*i*) The abortive initiation reaction is dependent on an intact promoter. Cleavage of λP_R with *Hin*cII destroys the promoter and also reduces the abortive initiation reaction to >95% (11). (*ii*) The abortive initiation reaction on promoters is absolutely dependent on the presence of sigma subunit (11); indeed, Hansen and McClure have devised a noncycling activity assay for sigma subunit based on the abortive initiation reaction (20). (*iii*) The abortive initiation reaction is promoter-specific. On an isolated restriction fragment containing only one promoter of known starting sequence the rate of abortive initiation corresponding to incorrect initiation is less than 5% (unpublished results). (*iv*) The abortive initiation reaction has been used to titrate active promoters on whole phage DNA and restriction endonuclease fragments (12, 18).

The finding that slow steps are involved in the formation of RP_os is not new. Relatively slow rates have been observed with various assay techniques *in vitro* with *lac* (10, 19), T7 (9), and phage fd (7) promoters. In the studies cited, only one or two RNA polymerase concentrations were used. Thus, even if the times observed corresponded to the τ_{obs} defined here, important information in the form of the intercept and slope of the tau plot was not obtained.

Rate-Limiting Steps in Initiation and Promoter Strength. Determination of τ_{obs} by using the abortive initiation assay measures the contribution of those steps preceding RP_o formation to the overall rate of RNA chain initiation. Initiation frequency could also be determined in part by the time required for an initiated RNA polymerase to elongate an RNA chain long enough to regenerate the free promoter—i.e., the promoter clearance time. The rate of formation of the first phosphodiester bond may be $\frac{1}{20}$th to $\frac{1}{10}$th the rate of subsequent catalytic steps (12), but if we conservatively assume a chain elongation rate of 20 sec^{-1} and further assume that the incorporation of about 75 nucleotides is necessary before the promoter is again accessible for RNA polymerase binding, then

the promoter clearance time is roughly 5 sec. The promoter clearance time may vary with transcription sequence if pauses (21, 22) or abortive starts (12) contribute to the time required to regenerate a free promoter. Both of these contributions to the promoter clearance time can be artificially increased by using low concentrations of one or more triphosphates, but intrinsic contributions of the transcribed sequence may occur even at saturating triphosphate concentrations (23, 24).

Promoter strength can also be quantitatively compared in terms of tau plot parameters. The best definition of promoter strength is RNA chain initiation frequency (initiation means the synthesis of a chain that with high probability will be elongated to the end of the transcription unit under study). The strongest promoters would be expected to have $1/k_2$ values <10 sec (i.e., approaching the promoter clearance time). In addition, the tau plot slope would be expected to be low, corresponding to a favorable binding step. These very strong *in vitro* promoters might approach an elongation-limited rate of initiation. In other words, as soon as an enzyme elongates beyond the promoter region, another enzyme would immediately bind and initiate another chain, etc. Good *in vivo* evidence for this maximum level of promoter strength exists for the rRNA operons (25) and for the activated, fully induced *lac* operon (26).

Having defined a maximum for promoter strength *in vitro* and *in vivo* in terms of an optimal RNA polymerase–promoter interaction, it is clear that an alteration in any of these individual steps could characterize a weak promoter. For example, if $k_2 \leq 0.01$ sec^{-1} then at any concentration of RNA polymerase, initiation frequency will be limited by the isomerization step. If $k_2 \leq 0.1$ sec^{-1} but the tau plot slope is high (e.g., $> 10^{-6}$ M·sec) initiation frequency will be limited by the binding of RNA polymerase at the typical concentrations of enzyme used *in vitro*. Combinations of a high intercept and a high slope on a tau plot would indicate an even more drastic reduction of initiation frequency. Thus, it is straightforward to understand the optimal characteristics of a strong promoter; weak promoters, however, will have to be characterized individually to determine the cause of a lower initiation frequency.

Limitations in the Model. Although the experiments reported here conform to the simple two-step model for RP_o formation, these data do not of course exclude more complicated pathways with more than one RP_c or RP_o state. If nonproductive binding, branching pathways, or several isomerizations occur, they would not be observed with this simple analysis because the steady-state assumption made at the outset combines these putative species into one intermediate, RP_c. In return for the loss in detail, we obtain a rather simple and pleasing view of overall RP_o formation. For most promoters the DNA sequence alone determines the magnitude of the binding and isomerization steps identified in this paper. If these steps ordinarily contribute to overall rate limitation in RNA chain initiation, the assay described herein should prove useful in dissecting the relationship between DNA sequence and initiation frequency.

The expert technical assistance of Mary Erfle is gratefully acknowledged. The work was supported by Grant GM 21052 from the National Institutes of Health.

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