Dynamic and structural characterisation of multiple steps during complex formation between *E. coli* RNA polymerase and the *tetR* promoter from pSC101

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Received September 10, 1986; Revised December 15, 1986; Accepted December 22, 1986

ABSTRACT

Kinetic, functional and structural studies of the recognition of the *tetR* promoter from pSC101 by *E. coli* RNA polymerase allowed the characterization of several steps in the specific complex formation and transcription initiation process. First, enzyme and DNA enter in a short life-time complex. An isomerization will convert this unstable complex into a closed stable one where RNA polymerase is tightly attached without establishing stable chemical contacts with the bases. In the next step, stable close contacts appear between both macromolecules involving mainly the downstream part of the promoter. A further isomerization will lead to an open complex where DNA is locally melted and the system is able to initiate transcription. This latter process is accompanied by changes in the upstream part of the promoter. Finally, *in vitro* transcription assays showed that the position of the major transcription start sites depends on temperature. From the reported results, it appears that the recognition event is a sequential process where different structural elements of the promoter, that can be located apart in the sequence, are involved in a concerted manner in each stage.

1. INTRODUCTION

Initiation of transcription plays a major role in the regulation of *E. coli* gene expression. Interaction of RNA polymerase (RNAP) with constitutive promoters may provide the simplest control mechanism, involving only the intrinsic structure of the promoter and the polymerase itself. The molecular bases of the recognition process between *E. coli* RNAP and promoters and the mechanism leading to an active complex formation have been the subject of many structural and kinetic studies.

A two-step model for the specific complex formation process was first proposed, where a transient species in rapid equilibrium with free molecules, named "closed" complex, isomerizes into a stable transcriptionally active one (1). Results from kinetic studies (2,3) supported this basic model, the rate-limiting step was characterized as the conversion of the "closed" complex (RP_c) into the "open" one (RP_o) able to initiate transcription:

Evidence for additional steps were further reported, from binding studies (5,6), abortive (7) and productive (8) initiation kinetics, and their temperature dependence.

Results obtained with those approaches could not be interpreted in terms of a simple two-step model and led to the characterization of two types of stable RNAP-promoter complexes following (RP_C):

The existence of a new species RP_i was postulated in the lac UV5 system because kinetically competent species can be rapidly and reversibly inactivated by lowering the temperature; this new complex, unlike RP_C , remains resistant to poly(d(A-T)) challenge at the lower temperature (7). From temperature (A1 promoter from phage T7 (4)), and temperature and salt (λ p_R promoter (5,6)) dependence of association rate constants, it appeared that the minimum reaction mechanism required also an intermediary between the "closed" and the "open" complexes. For lac UV5 and λ p_R , the rate limiting step above 20° was identified as being associated with the interconversion of the transient intermediaries, corresponding to (conformationally) distinct forms of closed RNAP-promoter complexes.

Structural studies of the lac UV5 promoter DNA during complex formation at different temperatures by chemical and enzymatic methods (9) have further characterized the different aforementioned intermediaries.

We have already reported a static structural study of complex formation between E.coli RNAP and the tetR promoter from pSC101, which controls the expression of the tetracycline repressor. This work was carried out by DNA protection and premodification experiments using an alkylating reagent and by DNAase I footprinting (10). It was shown that the location, nature and intensities of the close contacts of the polymerase on the tetR promoter DNA conform to a common pattern shared by all other promoter systems studied so far. On the contrary, regions of enhanced cleavage in the DNAase I footprints appear to be characteristic of tetR suggesting that DNA adopts a particular conformation in the complex which differs from those occurring in other promoters. This latter fact could be related to the unusual length of the spacer domain in tetR (21 bp). From the comparison of temperature dependence of local unwinding around the transcription start site (detected by the appearance of single stranded cytosines) and DNAase I footprinting, it appeared that the process leading to stable complex formation can be achieved without disruption of base pairing as was reported for lac UV5 (9). A dynamic view of the various steps involved in complex formation studied by structural approaches was further required in order to understand the molecular mechanisms of transcription initiation in the tetR system, thus allowing a better identification of the nature of the promoter signal.

We present here results from kinetic, structural and functional studies, performed in the temperature range 10°-37°C. Stable complex formation was studied by the filter-binding assay. The kinetics of open complex formation was followed directly (for

reasons detailed in the next section) by a new approach, detecting the appearance of the single stranded DNA domain around the transcription start site. The transcription ability of the RNAP-promoter complex was studied by an *in vitro* transcription assay using a DNA fragment bearing the *tetR* promoter. Finally, structural analysis on intermediary and final complexes were performed by chemical and enzymatic protection experiments.

The results presented below show that the mechanism of active complex formation between *tetR* and RNAP conforms to those described for *lac UV5* and λ_{PR} systems and that they agree with a multi-step model. The temperature dependence of the interaction between both molecules reveals that above 20°C the rate-limiting step corresponded to the formation of the stable closed intermediary; below 20°C, the rate-limiting step shifts to the conversion of the stable closed intermediary into the open complex. Furthermore, temperature perturbation experiments allowed the characterization of a new transient species preceeding the open complex. The structural analysis of the different complexes show that distinct promoters domains are involved, probably sequentially, in each step of complex formation. Finally, the *in vitro* transcription studies reveal that the major transcription start site from *tetR* promoter moves upstream when the temperature decreases.

2. MATERIALS AND METHODS

2.1. Promoter fragment.

We have isolated and labelled at the EcoRI 3' end a 118 bp HphI-EcoRI fragment containing the *tetR* promoter from pAT153 (see 10).

2.2. RNA polymerase.

E. coli RNA polymerase holoenzyme was prepared according to (11). The preparation contains a minimum of 80-90% σ subunit and is at least 30% active according to the test described in (12). From titration experiments using the abortive initiation assay, we found that our enzyme preparation contains a minimum of 44% active molecules (13). Similar results were found by the nitrocellulose filter-binding assay (this paper).

2.3. Nitrocellulose filter binding assays.

General conditions were according to (14).

Association kinetics. Association kinetics using the filter-binding assay were performed under pseudo-first order conditions in the presence of at least a four-fold excess of active RNAP with respect to *tetR* promoter sites (118 bp fragment). Concentrations of DNA fragment employed in this work were between 0.5 to 3.0 nM. Complexes were formed in buffer N: 40 mM Tris-HCl pH 7.9, 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA and 100 µg/ml bovine serum albumin. Samples were between 50-100 µl. Promoter-RNAP complexes were revealed by a 20 second heparin challenge, performed after different incubation times (50 µg/ml heparin, final concentration), in order to eliminate filter retention of non-specific weak complexes. The heparin-quenched solutions were filtered (filters BA85, 0.45 µm, Schleicher and Schüll) and the filters were rinsed with 0.5 ml buffer W (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 100 mM KCl). Filters are dried, dissolved in a scintillation mixture and counted. Backgrounds were determined in the absence of RNAP. Controls with preformed stable complexes show a retention of the 118 bp fragment close to 100% in the above conditions.

Dissociation kinetics. Dissociation kinetic measurements were performed by forming stable complexes as described in the preceding section (preincubation times varied for the different temperatures, see Results section); heparin was added (50 $\mu\text{g}/\text{ml}$, final concentration) and 50-100 μl aliquots were withdrawn at different times and filtered. Subsequent rinse, dissolution and counting steps are as for the association assay. It is well known (5) and ref. therein) that heparin does not affect measurements of the promoter RNAP association constants. On the contrary, it was shown for many promoters that heparin destabilizes binary complexes; The dissociation rate constants would therefore be overestimated (25). We have measured the life-time of complexes using poly d(A-T), poly d(A-T) as competitor at 100 $\mu\text{g}/\text{ml}$ final concentration as in (7,25).

Determination of the activity of RNAP in binding the tetR promoter. Titration of the 118 bp fragment was performed under solution conditions where the fraction of stable complexes was found to be irreversible in the time course of the experiment at 37°C. After 30 minutes of preincubation of both macromolecules in buffer N, a heparin challenge and filter assay were performed as above.

2.4. Association kinetics studied by the extent of unpaired cytosines.

As described for promoters *lacUV5* (15-16), *A3* from phage T7 (15), *tac* (16), and RNA 1 (17), we have found a melted region in the complexed tetR promoter revealed by the presence of four single stranded cytosines, located at positions -8, -5, -4, and -2, with respect to the transcription start site (+1) on the antisense strand (10). We have improved the standard method described in (15) in order to follow the kinetics of open complex formation by reducing the alkylation time and detecting the appearance of the single stranded cytosines. The tetR promoter fragment (0.1-0.2 pmol) is incubated with a given RNAP concentration in 50 μl buffer H (25 mM HEPES, pH 8.0, 100 mM KCl, 10 mM MgCl_2 , 1 mM dithiothreitol and 100 $\mu\text{g}/\text{ml}$ bovin serum albumin). After different times of incubation at a given temperature (see Results section), 1 μl of 10.7 M dimethyl sulfate (DMS) (Aldrich, Steinheim) is added. The time of alkylation varies between 40 seconds at 30°C and 90 seconds at 15°C (alkylation conditions were previously optimized at the different temperatures). Methylation is stopped with 200 μl of 3 M ammonium acetate, 1 M β -mercaptoethanol, 20 mM EDTA and 10 $\mu\text{g}/\text{ml}$ tRNA. After ethanol precipitation, the pellet is resuspended in 20 μl H_2O followed by the addition of 20 μl hydrazine (Aldrich, Steinheim) and allowed to react for 7 minutes at 4°C. The pellet is dissolved in 100 μl 1 M piperidine and treated as in the chemical sequencing method. Single stranded cytosines, that were methylated on N3 position and further submitted to a mild hydrazine and piperidine treatments appear in autoradiograms as extra-bands in a G-lane.

Quantitative evaluation of the reactivity of cytosines was performed by scanning the autoradiograms in a Shimadzu CS-930 densitometer. Areas under the peaks were integrated by a on-line computer. When normalization was required it was performed taking into account the area associated with unperturbed regions of DNA far away from the promoter region.

2.5. In vitro transcription directed by the 118 bp fragment.

Transcription assays were performed in buffer B with 1 nM of 118 bp fragment and 140 nM active RNAP. The final nucleotide concentrations were 300 μM for ATP, GTP and CTP, and 30 μM for UTP, including (^{32}P)-UTP at 15 Ci/mMol (Amersham, England). When a heparin challenge was performed, the final heparin concentration was 50 $\mu\text{g}/\text{ml}$. Different kinds of experiment have been carried out and are detailed in the legends of the figures in the Results section. Typical experiments were carried out as follows: after a given preincubation time of RNAP and the 118bp fragment, depending on the chosen temperature, a mixture of the four nucleosides triphosphate and heparin is added. Incubation is continued for 5-10 minutes to allow the production of full length run-off transcripts. Reactions were quenched with an equal volume of saturated urea containing 0.05% bromo-phenol blue and xylene cyanol, 1 mM EDTA, and 0.1% SDS. Transcripts were separated by electrophoresis on 12% polyacrylamide, 7 M urea gels. Quantitative evaluations were performed by analyzing the corresponding autoradiographies by densitometry (cf. below).

26. Determination of the close contact points of RNAP on the *tetR* promoter.

Close contact points of RNAP on the 118 bp fragment were determined by protection experiments against DMS attack as described in (10). Only the anti-sense strand was analyzed. Methylation conditions were optimized for the different temperatures.

27. Temperature perturbation experiments.

The kinetic, functional and structural studies described above were also carried out preforming the RNAP-118 bp fragment complex at a given temperature. Cytosine reactivity, close contact points and transcription ability were then assayed at a different temperature, following a rapid shift up or down in temperature. Temperature shifts were carried out by diluting 2-5 fold the preincubation mixture in a buffer at the required temperature; this latter containing the NTP's when transcription was assayed.

RESULTS

1. Determination of the activity of RNAP in binding the *tetR* promoter.

Figure 1 shows the titration by the filter binding assay, under heparine challenge conditions, of the 118 bp *tetR* fragment with RNAP. Saturation is reached at 2.0 to 2.5 RNAP per DNA molecule. This is in agreement with what we found titrating promoters in pAT153 by the abortive initiation assay (13). Similar results, i.e. roughly 44% of RNAP preparations are active either in binding or in transcription initiation, have been reported by other authors (5,21). In what follows, we will use the active instead the total RNAP concentration in order to compare our data on the *tetR* system with other work. Other interpretations of the presence of a half population of active RNAP molecules cannot be excluded, for instance binding of dimers or cooperative binding (see 5,22).

2. Kinetics of formation of specific *tetR*-RNAP complexes.

a) Association kinetics studied by the filter binding assay.

The forward kinetics of stable complex formation have been studied by the nitrocellulose filter binding assay in the temperature range 10°-37°C. We have assayed different buffers (Tris-HCl, cacodylate, HEPES) and no significative difference have

Figure 1. Titration of the 118 pb HphI-EcoRI fragment, bearing the *tetR* promoter, with RNAP. The fragment (1nM) was incubated with RNAP at the indicated RNAP/DNA mole ratios at 37°C for 30 minutes in buffer N. The bound fraction was determined by filtration after a heparin challenge. Fits for $K_D = 1$ nM, $n=2$ and $n=2.5$, are presented.

Figure 2. Association kinetics experiments for the *tetR* promoter-RNAP complex formation.

A) Kinetics of formation of filter-retainable complexes at 30°C between *tetR* promoter (3nM) and RNAP, under heparin challenge conditions in buffer N. Active RNAP concentrations were: $\blacklozenge$: 74 nM, $\bullet$: 52 nM, $\blacksquare$: 34 nM, $\blacktriangle$: 16 nM, $\blacktriangledown$: 10 nM.

B) Kinetics of formation of open complexes at 30°C between the *tetR* promoter and RNAP, in buffer H, followed by the reactivity of cytosines -2, -4, -5 and -8 (anti-sense strand)(see fig.5). *tetR* fragment 3 nM; active RNAP concentrations: Δ : 82 nM, $\circ$: 62 nM, $\square$: 42 nM, ∇ : 11 nM.

C) Kinetic analysis of *tetR*-RNAP complex formation at 25°C studied by the filter binding assay (filled symbols) and by cytosine reactivity (open symbols). *tetR* was 3 nM; active RNAP concentrations: $\bullet$: 130 nM, $\circ$: 70 nM, $\square$: 62 nM, $\blacksquare$: 42 nM, $\blacktriangle$: 26 nM.

D) and E) Association kinetic data from figures 2 A,B and C) represented on a semilog plot of $1-\Theta$ versus time. D) data from figures 2 A and B: experiments performed at 30°C; E) data from figure 2 C: experiments performed at 25°C.

F) τ -plots from data presented in figures 2 A, B, and C. The time constants τ_{obs} are the inverse of the pseudo first-order rate constants (k_2) calculated from figures 2 D and E. τ_{obs} values are plotted as a function of the inverse of active RNAP concentration. The second-order rate constants (k_a) at 30° and 25°C are the reciprocals of the respective slopes. k_a values obtained in the range 10° to 37°C are given in table I. Filled symbols: data from the filter binding assay; open symbols: data from the kinetics of cytosine reactivity.

Figure 3. Dissociation kinetics of *tetR*-RNAP complexes at different incubation temperatures in buffer N studied by the filter binding assay, using either a heparin (a) or poly [d(A-T)] poly [d(A-T)] challenge (b). *tetR* promoter: 3 nM; RNAP: 74 nM. Incubation times before polyanion challenge and filtration were: 25' at 37°, 30' at 30°, 40' at 25°, 45' at 20°, 75' at 15°, and 90' at 10°C. k_d values at each temperature are given in table I.

been found in kinetic behaviour, within the limits of confidence of our experiments (data not shown).

Kinetic data for formation of filter retained (heparin-resistant) complexes between RNAP and the 118 bp fragment, and their analysis, are shown in figure 2 for incubation temperatures of 25° and 30°C. The linearity of the semilogarithmic plots (see figure 2 D and E) where the time evolution of the free promoter fraction is represented, agrees with a first-order law. The pseudo-first order rate constants (k_2), calculated from the semilogarithmic plots, are represented in a double reciprocal plot ($\mathcal{T}$ -plot) as a function of RNAP concentration (see figure 3F). The experimental $\mathcal{T}_{\text{obs}}$ values ($\mathcal{T}_{\text{obs}}=1/k_2$) exhibit a linear dependence with the inverse of RNAP concentration; the slope of the curve corresponds to the inverse of the overall second-order rate constant (k_a). The $\mathcal{T}$ -intercept for infinite RNAP concentration is different from zero reflecting—as already described and discussed (2,3) the existence of an intermediary rate-limiting step in the process leading to stable complex formation.

Values of k_a were determined in the range 10°–37°C (see table I). The temperature dependence of k_a gives a non linear Arrhenius plot (figure 4) which suggests that the rate-limiting step in the process of stable complex formation is

Figure 4. Thermodynamic analysis of the kinetic data for the tetR-RNAP interaction. A) Arrhenius plot of the temperature dependence of k_d ; B) Arrhenius plot for the temperature dependence of k_a ; C) van't Hoff plot for the temperature dependence of the equilibrium constant K_{eq} . The activation energies $E_{a,ass}$ and $E_{a,diss}$, ΔH and ΔS values are summarized in table 1. (●): data obtained with heparin challenge experiments; (○): data obtained with poly [d(A-T)], poly [d(A-T)] challenge experiments.

shifted when temperature varies. The estimated overall energy of association (E_{ass}) is around 31 Kcal in the range 20°–37°C and it increases to approximately 48 Kcal in the range 10°–20°C.

In the present experimental conditions, when no competitor is added, the total input DNA is retained instantly on the filter. As already demonstrated (see (18) for a review) this phenomenon corresponds to the rapid equilibrium leading to the formation of a heparin-sensitive transient species formerly called "closed" complex, the first intermediary in the process of specific complex formation.

b) Association kinetics studied by the extent of unpaired cytosines. Figure 5 shows autoradiograms of typical experiments at 25° and 30°C where the formation of the open promoter-polymerase complex is followed by the extent in the appearance of single stranded cytosines at different RNAP concentrations. Autoradiograms were analysed quantitatively (see densitogram in figure 5) and when required normalization was performed as described in the Materials and Methods section.

Results are shown in figures 2B and 2C. The kinetic data obtained by the present method agree with a first order law, and as evident in τ -plots of figure 2F the results agree with those obtained with the filter binding assay. The second-order rate constants at 25° and 30°C obtained from cytosine modification and nitrocellulose filter binding experiments are similar within experimental error. When the cytosine modification assay is performed below 20°C, cytosines remain almost unreacted in the time course of the experiments (up to 90 minutes), as we have already described (10).

Figure 5. Association kinetics of open complex formation between RNAP and the *tetR* promoter fragment, studied by the reactivity of cytosine residues to alkylation at the N³ position. After a mild hydrazin treatment, the DNA chain is splitted at the level of modification with piperidin. Single stranded cytosines appear as extra-bands in a G-lane. In all cases the *tetR* fragment was 3 nM.

A) active RNAP 62 nM, the incubation temperature was 30°C; B) active RNAP 42 nM, 30°C; C) active RNAP 42 nM, the incubation temperature was 25°C. D) typical densitogram showing the time evolution of cytosine reactivity in an association kinetics experiment; in the present case it corresponds to figure 5C. The arrows indicate cytosines -2, -4, -5, and -8 (non template strand).

E) Kinetics for the isomerization of a stable complex formed at 20°C into an open one, after a temperature shift to 30°C. Lane 1: control at 20°C, after 45' of preincubation. Methylation was performed after the temperature shift at the indicated times. DNA fragment: 3 nM; active RNAP: 140 nM.

F) As in E), but the preincubation was performed at 15°C, during 60'.

In all cases -R: control without RNAP (G-sequence lane).

Similar results were obtained with or without a heparin challenge (not shown).

In order to compare results from both approaches, the filter binding and cytosine modification assays, it must be considered that: i) by the filter binding assay we measured the association rate constant to form a "stable" RNAP-promoter complex, whereas by cytosine modification we measure the association rate constant for the "open" complex formation; ii) in the cytosine modification experiments, the dead time during the methylation procedure may introduce a systematic error in the measurements.

Keeping in mind these considerations, comparison of results obtained by the two approaches lead to the following conclusions: Above 20°C no difference in the kinetic behaviour is detected, by either monitoring the stable or the open complex formation. This suggests that formation of the latter is not rate-limiting in this temperature range; the corresponding forwards kinetics is too fast compared to the dead time in the cytosine modification procedure and the uncertainty limits of both techniques. In these conditions, the rate-limiting step corresponds, then, to the formation of some intermediary stable (heparin-resistant) species.

Below 20°C, stable complexes cannot be converted to open ones: the rate-limiting step appeared to be shifted to the opening step, as we already reported for the tetR promoter (10) and as has been described for lac UV5 (7,9) and λ_{PR} (5,6) promoters.

Finally, the non-linearity of the Arrhenius plot of figure 5b reveals the presence of a multi-step process, the energy barriers between the different steps being affected in different ways when temperature changes.

3. Kinetics of dissociation of RNAP from the tetR promoter.

We have studied the dissociation of stable RNAP-tetR promoter complexes formed at different temperatures, in the range 10° to 37°C, by the filter binding assay. Results are shown in figure 3a and b, where the fraction of undissociated complex is plotted on semilogarithmic scales as function of time. Preincubation times were between 30 and 90 minutes (see legend to figure 3); they were selected from the association kinetics data at the corresponding temperatures. It was shown (5) that at the heparin and poly d(A-T) concentrations employed in this work (50 µg/ml and 100µg/ml respectively) the dissociation process is irreversible.

As described for lac UV5 (7) and λ_{PR} (5), the k_d decreases with increasing temperature; Arrhenius activation energies for the dissociation process will be therefore negative (see figure 4A). As for the association kinetics, non-linear Arrhenius plots are obtained, suggesting that at the different temperatures we studied the dissociation kinetics of different intermediary species. Between 37° and 15°C only small differences are found between the set of experiments using poly [d(A-T)], poly [d(A-T)] or heparin as competitor. Below 15°C, K_d values obtained using heparin are roughly 5 times greater than those obtained with the poly [d(A-T)] challenge. The estimated

TABLE I
Temperature dependence of kinetics parameters
for the *tetR*-RNAP interaction.

	10°	15°	20°	25°	30°	37°
$k_a \times 10^{-3}$ ($M^{-1}s^{-1}$)	2	9	36	62	180	600
$k_d \times 10^4$ (s^{-1})	39.0 <i>7.5</i>	6.8 <i>5.4</i>	4.0 <i>3.2</i>	3.3 <i>2.7</i>	2.5 <i>2.1</i>	1.8 <i>1.5</i>
$K_{eq} \times 10^{-7}$ (M^{-1})	0.05 <i>0.27</i>	1.3 <i>1.7</i>	9.0 <i>11.3</i>	19.0 <i>23.0</i>	72.0 <i>86.0</i>	330.0 <i>400.0</i>
t 1/2 (min.)	4.3 <i>22.0</i>	24.5 <i>31.0</i>	41.7 <i>52.0</i>	49.9 <i>61.2</i>	66.7 <i>81.3</i>	99.6 <i>114.3</i>

Thermodynamic parameters for the *tetR* promoter-RNAP complex formation.

	range 10°-20°C	range 20°-37°C
ΔH°_{obs} (Kcal)	39 <i>40</i>	78 <i>63</i>
ΔS°_{obs} (e.u.)	179 <i>174</i>	304 <i>252</i>
$E_{a,diss}$ (Kcal)	-9.0 <i>-8.5</i>	-52 <i>-38</i>
$E_{a,ass}$ (Kcal)	31	48

In italics: values calculated from dissociation kinetic experiments using poly [d(A-T)]poly [d(A-T)] as competitor; in bold: values from heparin challenge experiments.

values of the corresponding activation energies for dissociation ($E_{a,diss}$) are given in table I.

If complexes were preformed above 20° and then assayed for dissociation at lower temperatures a different behaviour should be expected, introducing the reverse kinetics of all intermediary steps since the open complex.

From k_a and k_d measurements at the different temperatures we obtain the overall equilibrium constant (K_{eq}) values (see table I). The temperature dependence of K_{eq} yields a non-linear van't Hoff plot. The van't Hoff enthalpy change ΔH° is positive and very temperature dependent, increasing from 40 Kcal/mol in the 20°-37°C range to 63 Kcal/mol in the 10°-20°C range. Kinetic results are summarized in table I.

4. Temperature perturbation experiments

In a attempt to characterize intermediary species in the process of "open" complex formation we have analyzed the reactivity of single stranded cytosines after shifting up or down the temperature of complex formation.

When the RNAP-*tetR* complex is preformed at 20°C in conditions where at least 80-90% of the total DNA enters into stable complexes, and then the temperature is rapidly shifted to 30°C, the stable closed complex isomerizes rapidly to an open one. A burst leading to 100% open cytosines is observed (see figure 5D), the time course of wich is comprised in the dead time of the experiment.

When the complex is preformed at 15°C in conditions where a minimum of 60% of stable complex is present, and then the temperature is shifted to 30°C, the appearance of open complexes is not instantaneous. In our conditions we cannot have 100% of stable complexes at 15°C, therefore when the temperature is shifted to 30°C part of the free DNA fraction will enter into stable complexes, following the kinetics characteristic for the given temperature and RNAP concentration. The kinetics observed when the temperature is shifted from 15°C to 30°C will be then the sum of two processes: the conversion of stable closed complex formed at low temperature to open ones, plus the formation of new stable complexes at 30°C, rapidly (instantaneously considering the dead time of the present method) converted to open complexes. Experiments performed at different RNAP concentrations (not shown) revealed that the kinetics of cytosine reactivity, after the 15° to 30°C shift, correspond mainly to a pseudo-monomolecular process. Furthermore, the 15°-30°C shift experiment was carried out with a heparin challenge before the temperature shift ; in these conditions we eliminate the contribution of new complexes formed after the temperature perturbation. The appearance of the open complex in these conditions follows a relatively slow kinetics (data not shown) similar to that obtained without heparin challenge.

We can then conclude that the complexes formed at 20° and 15°C are different. In the former, some kind of rearrangement of both macromolecules has been already achieved which allows rapid open complex formation once an energy barrier is passed by raising the temperature. On the contrary, the closed stable complex formed at 15°C has been frozen in some intermediary state before this rearrangement.

5. In vitro transcription.

In order to characterize functionally the different intermediary species revealed by the filter binding assay and by the kinetics of cytosine reactivity, it was necessary to study the transcriptional ability of the tetR-RNAP complexes at different temperatures with and without temperature shifts. We have not been able to study the kinetics of transcription initiation from the tetR promoter either by the abortive initiation assay (2,3,7,13), nor by stabilization of the complex via ternary complex formation by the addition of the NTP's needed to synthesize the initial oligonucleotide as in (5,6). In both cases, although different combinations of ribonucleosides triphosphate or mono or dinucleosides monophosphate and ribonucleosides triphosphate were assayed, the signal to noise ratio was too low to allow quantitative analysis. We have then assayed the total in vitro transcription directed by the 118 bp fragment.

The kinetics of the elongation process at different temperatures was determined previously (data not shown) in order to ensure comparison of plateau values (under

Figure 6. *In vitro* transcription studies directed by the 118 bp fragment. In all cases the DNA fragment was 1 nM and active RNAP was 140 nM.

A) RNAP and the 118 bp fragment were incubated for 30' at 30° and transcription assayed for 5' at the indicated temperatures by adding the NTPs-heparin mixture after the temperature shift. Autoradiograms from transcription assays at the different temperatures of incubation are not shown but results are presented in E). M: size marker (G>A lane for the 118 bp fragment). T: control without RNAP.

B) and C) Time evolution of the transcription ability of a RNAP-*tetR* complex preformed at 30°C, after a temperature shift down to 20°C, with (B) or without (C) a heparin challenge. Transcription was assayed at the indicated times after the temperature shift. **D)** As in B), but the temperature shift was from 30° to 15°C.

E) Comparison of the extent of open cytosines at different incubation temperatures (data from (10), 118 bp fragment was 6.4 nM, active RNAP 140 nM) with the transcription ability assayed at different temperatures with and without a temperature shift down. --○--: extent of single stranded cytosines; --▲--: extent of transcription assayed at the same temperature of incubation; --▼--: extent of transcription assayed at the indicated temperatures after a 15' preincubation at 30°.

F) Densitogram of the *tetR* transcripts synthesized at different temperatures, showing the shift of the major transcription start site. The present densitogram corresponds to the results presented in A) but the same upstream shift when temperature decreases, appear when transcription is assayed directly at the same temperature without preincubation at 30° (not shown).

heparin challenge conditions, only one RNA chain is synthesized by active RNAP-promoter complex).

As shown in figure 6A, at 30°C, and in the absence of a heparin challenge, different transcripts appear: a) 118 bases long, corresponding to the end-to-end transcript; b) 81 bases long, present in small amount, corresponding to the mRNA initiated at the tetA promoter from position +45 from the EcoR I site up to the Hph I extremity; c) 37-38-39-40 bases long, proceeding from the tetR promoter (positions +34 to +37 from the EcoR I site) up to the EcoR I extremity. It has been already shown by S1 mapping that the tetR promoter initiates at four contiguous sites (A35, U36, U37, A38, from the EcoRI site), the pyrimidines being the major ones in standard conditions (19); this result was recently confirmed by primer extension (20).

When a heparin challenge is performed before adding the NTP's or simultaneously with the NTP's mixture (figure 6 A and B), the end-to-end transcript disappears almost completely, reflecting the short life-time of complexes between RNAP and the ends of the fragments (for a detailed analysis of this kind of complexes, see (23,24)).

In these conditions, the tetA transcript represents at most 3% of the tetR transcript, showing as we have already reported (10) that the tetA promoter is almost completely inactivated by cutting at the EcoRI site, located 45 bp upstream of the transcription start site.

Experiments performed in the temperature range 10°C to 37°C showed that the dependence of transcriptional activity with temperature parallels the curve of cytosine reactivity in similar conditions (figure 6E). The shift that appears between both curves could be due to the enhanced complex stability following the formation of ternary complexes.

When complexes were preincubated at 30°C and transcription assayed after a temperature shift down (see figure 6C and D) a hysteresis appears as shown in figure 6E. This suggests that the transconformation of the active species present at higher temperatures to that found at lower temperatures is a rather slow process (this result was further confirmed by cytosines reactivity).

The transcription ability was also assayed by adding the heparin-NTP's mixture at different times after the temperature shift in order to obtain the kinetic values for the inactivation process. At 20°C the inactivation of the complex is rather slow and follows a first order decay ($k_1 = 4.3 \cdot 10^{-4} \text{ s}^{-1}$). The inactivation at 15°C is faster and follows a more complicated kinetics suggesting that several steps take place simultaneously (probably dissociation of the complex in the time course of the experiment as well as inactivation after the temperature shift), (results not shown).

Transcription assays performed at different temperatures reveal also a striking

D) RNAP polymerase contact on the tetR promoter (10). Base pair positions are numbered relative to the start of transcription at +1. **G** and **A** : purines that polymerase protects against DMS attack in the complex; **Λ** : purines showing enhanced DMS attack in the complex; **▲** : methylated purines that interfere with binding of the polymerase; ***** : single stranded cytosines in the open complex. No difference in the methylation pattern was found in control experiments performed in the range 15° - 37° C.

phenomenon: the major transcription start site is shifted upstream when temperature decreases (figure 6A and F). To our knowledge this phenomenon has not been previously described. We don't know yet if it is a general characteristic of *E. coli* promoters or typical for *tetR* exclusively. In any case, in promoters displaying such a behaviour, quantitative in vitro assays, by techniques selecting a single initiation site, must be analyzed under well characterized conditions.

6. Structural studies

In order to characterize structurally the RNAP-*tetR* promoter complexes occurring in the different conditions described above, we have determined the close contact points of the enzyme on the promoter DNA (anti-sense strand) by studying the reactivity of purine residues in alkylation protection experiments.

As we previously reported (see (10)), a reproducible DNAase I footprint with the same hypersensitive sites to nucleolytic attack is obtained for complexes formed at any temperature between 10° and 30°C. On the contrary, the pattern of purines reactivity appears to be characteristic for the incubation conditions. Results presented in figure 7 show that complexes at 15°, 20° and 30°C, are structurally different.

At 15°C, as already described (10), no difference in the methylation pattern appears between complexed and free promoter DNA. When complexes are formed at 20°C, a protection profile becomes apparent at the downstream part of the promoter (mainly around position -15 from the transcription start site: G's -14 and -16 on the anti sense strand). The characteristic enhancement of methylation of G-37 (anti sense strand), is only slightly present. At 30°C, we found the protection pattern already described (10).

These different complexes, characterized here from a structural point of view, could correspond to intermediary species in the process leading to the final open transcriptionally active complex or to metastable species unable to be interconverted sequentially. In this way, we have analyzed the contact profile of RNAP on *tetR* promoter DNA, after temperature perturbation experiments: We have preincubated the complexes at 30°C and assayed them for alkylation at lower temperatures, and at different times after the temperature shift down.

As shown in figure 7C, the typical methylation profile obtained at 30°C is still present when DMS treatment is performed just after the RNAP-promoter complexes were transferred to 15°C. The characteristic reactivity pattern is, nevertheless, not stable at 15°C: as shown in figure 7C the enhancement of G-37 methylation disappears with a faster kinetics than the protections of G-14 and G-16.

DISCUSSION

The study of the RNAP-tetR promoter complex at different temperatures, including temperature perturbation experiments, and corresponding kinetic, structural and functional studies allows the characterization of several discrete steps in the recognition and transcription initiation process.

First, RNAP and promoter DNA enter into a complex in rapid equilibrium with free species (complex not resistant to heparin challenge). This complex, formerly called "closed complex", has already been well characterized (18,23) and its properties have been extensively analyzed in the literature. The conversion of this latter into a stable open active complex is the rate limiting step of the whole process (we will restrict the terms "stable" for complexes resistant to polyanion challenge and "open" for those including a DNA melted domain around the transcription start site).

When complex formation is performed at temperatures higher than 20°C the kinetics of the appearance of the stable species and the formation of the open complexes are similar (the kinetics of stable complex formation followed by the filter binding assay is similar to that obtained monitoring the reactivity of single stranded cytosines). In these conditions, the rate limiting step appears to be the isomerization of the short life-time closed complex into a stable heparin-resistant one (γ -intercept in the τ -plots, for infinite RNAP concentration, is significantly different from zero). From a structural point of view, a characteristic pattern of close contact points of RNAP on tetR promoter, already described (10), is present.

When the incubation temperature decreases, the stable RNAP-promoter complex is already formed but with slower forward kinetics and a faster dissociation rate. The increase of k_d when temperature decreases, accompanied by an important negative enthalpy change, suggests a major change in the protein-nucleic acid complex preceding the dissociation. Non-linear Arrhenius and van't Hoff plots, obtained in kinetics experiments, suggests already that complex formation is a multi-step process. The estimated thermodynamic parameters (see table I) suggest that tetR should be a less efficient promoter than λ_{PR} and lacUV5, for which thermodynamic parameters have been evaluated in similar conditions (5-7). These results agree with recent estimations of tetR efficiency (24).

Below 20°C, the local melting of DNA around the transcription start site is hindered. In these conditions the rate-limiting step is shifted to the conversion of the stable closed species in the open one, and we are in the presence of some stable closed intermediary, similar to that described in the lac UV5 system (7). Structurally, the enzyme is always positioned on the promoter as revealed by an unchanged DNAase I footprinting (10). Nevertheless, important differences are observed in studying the reactivity of purine residues to alkylation. Complexes formed at 20°C

show a close contact points pattern similar to that found at 30°C but without a single stranded domain. When this complex is transferred to 30°C the promoter DNA melts instantly around the transcription start site.

When the complexes are formed below 20°C a new species appears. Indeed, when RNAP and the promoter DNA are incubated at 15°C, a stable closed complex is already present, exhibiting a DNAase footprinting pattern no different with respect to that of the already characterized complexes but devoid of modifications in the reactivity of the purines: as already described (10), no close contact point of RNAP on the tetR promoter are evident at 15°C. When this complex is transferred to 30°C, it is converted into an open one, following a relative slow kinetics, as shown by the methylation on N³ of single stranded cytosines.

This stable closed species found at 15°C is then different from the stable-closed species characterized at 20°C. In the latter case, some kind of conformational rearrangement of the complex had already taken place, allowing the rapid nucleation of the melting process at 30°C. From the structural analysis, this rearrangement may be correlated to the establishment of close contacts between both macromolecules.

The lack of modification in the alkylation pattern at 15°C can be interpreted as reflecting the absence of close contacts between both molecules or at least the absence of close contacts with a life-time capable of interfering with the methylation process. When complexes are alkylated at 15°C, after preincubation of RNAP and promoter at 30°C, they show the characteristic close contact point pattern of RNAP on the tetR promoter. This pattern disappears, with a kinetics faster than that for the dissociation process in the same conditions and is associated with the loss of transcriptional activity. The enhancement of G-37 (sense strand) methylation disappears faster than the protections around -15. This conversion of the complex formed at 30°C to the complex formed at 15°C suggests that species characterized at 15°C and 20°C are probably intermediaries in the specific complex formation process, capable of interconversion from one into the other.

The reactivity of G-37 seems to precede the triggering of the local melting and the transcriptional activity. The local melting probably implies or determines further conformational changes in both macromolecules once the RNAP is tightly attached to the DNA and chemical interactions between them have been established. The enhancement of methylation could correspond to the formation of a hydrophobic pocket in the protein in the neighbourhood of G-37, or to a local conformational change in the DNA.

The enhanced sensibility of complexes formed below 15°C to heparin challenge, relative to poly [d(A-T)]poly [i(A-T)], suggest an additional step in the stable complex formation process. This requires, however, further investigation.

Finally, in vitro transcription analysis showed that in the tetR system the major transcription start site is shifted upstream when temperature decreases. This suggests that the determination of the +1 site depends also on subtle conformational modifications of the protein and the nucleic acid. This particular phenomenon displayed by the tetR promoter could be related to the fact that in this system the major transcription start positions correspond to pyrimidines. In any case it provides a useful model to study the constraints in the choice of the transcription start site.

From the present results and those already published (5-10,28), the recognition of promoters by E.coli RNAP appears as a succession of different correlated events. The present knowledge, lead to conclude that in order to characterize and to analyze promoters, it is not sufficient to dissect them into different independent structural domains. To understand the promoter recognition process it appears necessary therefore to look for concerted mechanisms, in which different structural elements can participate simultaneously or sequentially.

Furthermore, one can conceive that in the earliest stages of the recognition process some properties of the promoter sequences are able to "stop" RNAP. In this situation the enzyme would be stably anchored on the promoter, despite the fact that both molecules have not yet established stable close contacts. Physical properties, characteristic of promoter DNA, could be involved in the establishment of the early promoter target. Once RNAP is tightly fixed on the promoter region, rearrangements of both molecules could enable chemical interactions leading to the local melting of the DNA and transcription initiation at a precise address. It is interesting to remember that the alkylation of a single particular purine or a phosphate, upstream of the -32 address, prevents the formation of a stable complex although the corresponding positions are not protected against chemical attack in the final complex (10,15). Further work, should rely on the assumption that a promoter must not be a simple succession of chemical groups on both grooves of the double helix harbouring a precise nucleotidic sequence. Physical properties (flexibility, bending, local stacking and DNA conformation...) of the promoter and probably also those of the neighbouring sequences could be involved in defining the specific target for RNAP. These properties do not correspond necessary with literal homologies, admitting probably different "synonymous" base-pair arrangements.

AKNOWLEDGEMENTS: The authors thank C.Reiss, J.Gabarro-Arpa, A. Larousse, and W.Dalton for extensive discussions and constant encouragement; R. Schwartzmann for photographic work, and P. Hughes for help with the manuscript. The referees provided thoughtful comments on the manuscript.

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