Effect of A_n tracts within the UP element proximal subsite of a model promoter on kinetics of open complex formation by *Escherichia coli* RNA polymerase✪

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In the open transcription complex (RPo), *Escherichia coli* RNA polymerase σ^{70} and α subunits are known to be in contact with each other and with the promoter region overlapping the −35 hexamer and the proximal part of the UP element. To probe the effect of A_n DNA bending tracts in this region on initiation of transcription, kinetics of the formation of RPo by *Escherichia coli* RNA polymerase at two groups of synthetic consensus-like promoters bearing single DNA bending tracts (i) A_5 within the proximal subsite region of the UP element (promoters Pk and Pl) and (ii) A_5 (Pg) or A_8 (Pm) in the region including the downstream end of the proximal UP subsite and the −35 consensus hexamer was studied *in vitro* using the fluorescence-detected abortive initiation assay. The kinetic data obtained demonstrate that the overall second-order rate constant k_a of RPo formation is: (i) by almost one order of magnitude larger at Pk and Pl, relative to that at a control unbent promoter, and mainly due to a higher value of the equilibrium constant, K_1, of the initial closed complex; and (ii) several-fold smaller at Pg and Pm owing to a strongly decreased value of K_1. For Pm, the latter parameter was found to be dependent exponentially on four Mg^{2+} ions, as compared with the seven ions remaining in equilibrium with the initial closed complex at the parent Pa promoter. This indicates that promoter region bearing a stiff $A_8 \cdot T_8$ fragment of B′-DNA forms a smaller number of ionic contacts with the α subunit. These findings provide a new insight to and support the present model of interactions between RNA polymerase α and σ^{70} subunits with the proximal UP subsite and the −35 region of promoters.

Abbreviations: $E\sigma^{70}$ or R, *Escherichia coli* RNA polymerase; RPo, open transcription complex; P, promoter; UP, upstream promoter region recognized specifically by RNA polymerase; αCTD, C-terminal domain of RNA polymerase α subunit.

Discovery of the UP elements rich in $A_n \cdot T_n$ tracts in bacterial promoters led to numerous studies on their structure and interaction with cognate RNA polymerases (cf. Ross *et al.*, 1998; Helmann & deHaseth, 1999). Our earlier investigations (Łoziński *et al.* 1989; 1991; Łoziński & Wierzchowski, 1996) on the effect of location of $A_n \cdot T_n$ (n = 5, 6) DNA bending tracts within various domains of a model Pa promoter on the gross structure of open complexes with *Escherichia coli* RNA polymerase ($E\sigma^{70}$) have shown that promoters bearing these tracts immediately upstream of the –35 consensus hexamer exhibit lower mobility indicative of DNA compaction due to a larger DNA/$E\sigma^{70}$ interface. According to the present model (Ross *et al.*, 2001), UP promoter elements consist of one or two subsites, proximal and distal, each interacting specifically with the C-terminal domain of one of the two α subunits of RNA polymerase (αCTD). Thus, the macroscopic curvature imposed by A_n tracts does not seem to be essential for UP element function (Ross *et al.*, 1998). UP elements containing only a proximal subsite are the most frequent class of UP elements in the *E. coli* genome (Estrem *et al.*,1999). Some of the promoters studied by us previously belong to this group as they contain a single $A_5 \cdot T_5$ sequence separated by one nucleotide upstream of the –35 consensus hexamer. In others, this sequence overlaps partially the –35 region, recognized specifically by a helix-turn-helix motif of σ^{70} region 4.2 (Siegele *et al.,* 1989; Dombroski *et al.,* 1992; Mekler *et al.,* 2002). These two groups of promoters offer thus a unique possibility to study the effects of $A_n \cdot T_n$ sequences on promoter interaction with αCTD and region 4 of σ^{70} at a junction between these two functional polymerase domains. It seemed thus worth to extend our previous studies by investigating the effect of these sequences on kinetics of open transcription complex formation at these promoters (Kolasa, 2001). We have shown recently (Kolasa *et al.*, 2001) that formation of the open transcription complex at the parent Pa promoter conforms to the commonly accepted three-step mechanism involving the initial fast binding, slow isomerization of binary complex and DNA melting steps (Rosenberg *et al.*, 1982; Buc & McClure, 1985; Roe *et al.*, 1984; 1985; Tsodikov & Record, 1999). Here we present further results of these studies and their interpretation in connection with present views on the molecular mechanism of interaction of proximal subsite of the UP element with αCTD domain of $E\sigma^{70}$.

MATERIALS AND METHODS

RNA polymerase. RNA polymerase was prepared from *E. coli* C600 strain according to Burgess & Jendrisak (1975) except that Sephacryl S300 was used instead of Bio-Gel A5m, and was kept in a storage buffer (50% glycerol, 100 mM NaCl, 10 mM Tris/HCl, pH 7.9, 0.1 mM dithiothreitol). Quantitation of its activity according to Chamberlin *et al.* (1983) has shown that 50% of the holoenzyme form was active. The enzyme concentrations reported here refer to the active form of $E\sigma^{70}$.

Promoters. *E. coli* control promoter Pa, made of the consensus –35 and –10 hexamers separated by a 17 bp spacer, and its four analogues: Pg, Pm, Pk and Pl, each containing a single $A_n \cdot T_n$ bending sequence, located as shown in Fig. 1, were synthesized and cloned into pDS3 plasmid as described earlier (Łoziński *et al.*, 1989; 1991; Łoziński & Wierzchowski, 1996). Control promoter Pac bearing A–36C mutation was constructed and cloned similarly. For studies on the open complex formation, 226 bp pDS3 DNA fragments containing these promoters were obtained by PCR amplification with the use of appropriately designed primers and an Ampligene thermocycler. Concentrations of PAGE purified fragments were determined spectrophotometrically.

Reagents and chemicals. γ-ANS-UTP (γ-aminonaphthalene-sulfonate-UTP) was prepared and purified according to Yarbrough *et*

al. (1979). ANS was from Fluka. UTP, ApA, heparin and stock solution of 1.0 M magnesium chloride were purchased from Sigma. All other chemicals were also of reagent grade.

Fluorescence-detected abortive initiation (FDAI) assay of association kinetics. In this assay, γ-ANS-UTP was used as an elongating NTP (Bertrand-Burgraff *et al.*, 1984; Suh *et al.*, 1992) and ApA as the initiating nucleotide, so that ApApUpU was the only abortive transcription product at all the promoters studied (cf. Fig. 1). The amount of fluorescent ANS-pyrophosphate liberated in the course of the reaction was measured spectrofluorimetrically. A laboratory-made double-monochromator ratio-recording and computer controlled spectrofluorimeter equipped with a thermostated cell compartment was used to monitor at 500 nm the fluorescence intensity of ANS excited at 360 nm. Reactions were initiated by addition of $E\sigma^{70}$ in solution at 35 ± 0.1°C to the reaction mixture held at the same temperature in the fluorimetric cuvette and fast mixing for about 15 s with a Pasteur capillary pipette. Final concentration of components in the reaction mixture of 0.8 ml was as follow: 60–125 mM $MgCl_2$, 25 mM Hepes buffer, pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 0.45 mM ApA, 0.1 mM γ-ANS-UTP, 5 nM promoter DNA, 25–200 nM $E\sigma^{70}$, as well as 10 mM NaCl and 5% glycerol from the RNA polymerase storage buffer. Fluorescence intensity was measured every 1–10 s with a maximum of 500 points per sample for a period corresponding to at least 7 time constants (τ_{obs}) for the reaction, and stored on an IBM PC. Data from 3–6 independent reactions at every $E\sigma^{70}$ concentration were analyzed simultaneously by a nonlinear least-squares weighted (fluorescence intensity fluctuations as weighting factors) fit to the function:

$$N = N_0 + Vt - V\tau_{obs} (1 - \exp(-t/\tau_{obs})) \quad (1),$$

where N and N_0 are fluorescence intensities at time t and $t = 0$, respectively, V the product [M] per promoter [M] per second, t the time

(s), and $\tau_{obs} = 1/k_{obs}$, where k_{obs} (s^{-1}) is the observed second-order rate constant (Suh *et al.*, 1992). Standard errors of τ_{obs} were calculated using the Marquardt algorithm for minimization of χ^2. Steady-state rates (V) obtained in lag assays at different enzyme concentrations for the same promoter under the same set of solution conditions agreed within ±10% with those determined in control reactions initiated by addition of ApA and γ-ANS-UTP to preformed open complexes.

RESULTS

Sequences of non-template strand of the promoters studied, the parent one, Pa, and its derivatives Pac, Pg, Pk, Pl and Pm, are shown in Fig. 1. Promoter Pg bears a single –34TT-TTT–38 tract, partially overlapping the –35 hexamer, formed by the A(–36)T point mutation in Pa. In Pm the length of this tract is extended to eight bases. Pk and Pl bear –37AA-AAA–41 and –37TTTTT-41 tracts, respectively, separated from the –35 hexamer by cytosine in –36 position. Promoter Pac differs from Pa by a single A(–36)C mutation. This promoter was used as a proper control in experiments with Pk and Pl. Note that bending sequences of Pk, Pl and Pm promoters are located within the proximal subsite of the consensus UP element (cf. Fig. 1).

Kinetics of the open complex formation was studied under assumption of the minimal three-step mechanism (Scheme 1), expected to be independent of the promoter sequence (Tsodikov & Record, 1999) and shown to be fully applicable to the parent Pa promoter (Kolasa *et al.*, 2001). According to this model, the first intermediate closed complex (I_1), remaining in rapid-equilibrium with RNA polymerase (R) and promoter DNA (P), undergoes isomerization to a long-lived intermediate (I_2) followed by DNA melting in the –10 domain and formation of the open complex (Rpo),

$$R + P \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} I_1 \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} I_2 \underset{k_{-3}}{\overset{k_3}{\longleftrightarrow}} RPo \qquad \text{(Scheme 1).}$$

(a)

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                  -35                                              -10              +1
Pa 5'-CTCGAGTTATTGACAATTATTTATTTATTATTTATAATTATTTAATTGAATTC-3'
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(b)

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                   -45 -44 -43 -42 -41 -40 -39 -38 -37 -36 -35 -34
Pa:                          C   T   C   G   A   G   T   T   A   T   T   G   A   C   A
Pac:                         C   T   C   G   A   G   T   T   C   T   T   G   A   C   A
Pg:                          C   T   C   G   A   G   T   T   T   T   T   G   A   C   A
Pm:                  C   T   C   G   A   G   T   T   T   T   T   T   T   T   G   A   C   A
Pk:                  C   T   C   G   A   G   A   A   A   A   A   C   T   T   G   A   C   A
Pl:                  C   T   C   G   A   G   T   T   T   T   T   C   T   T   G   A   C   A
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(c)

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                    -45 -44 -43 -42 -41 -40 -39 -38 -37 -36
Proximal UP subsite:  A   A   A   A   A   A   R   N   R   N
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Figure 1. Sequences (non-template DNA strand) of the synthetic *Escherichia coli* promoters Pa and Pac and their derivatives Pg, Pk, Pl and Pm bearing single T_n or A_n tracts upstream of the −35 element.

(a) Full sequence of the parent promoter Pa with restriction sites at the ends used for cloning into pDS3 plasmid **(b)** sequences of Pa and its derivatives upstream of the −35 hexamer region in which they differ from each other **(c)** consensus proximal UP subsite according to Estrem *et al.* (1999); the bending tracts are underlined −10 and −35 hexamers in bold font.

The observed rate, $k_{obs} \equiv 1/\tau_{obs}$, of the second-order transcription reaction is related to the composite overall second-order association rate constant k_a and the composite first-order isomerization rate constant k_i by Eqn. 2:

$$\tau_{obs} \equiv (k_{obs})^{-1} = (k_a)^{-1} [R]_T + (k_i)^{-1} \qquad (2),$$

where $[R]_T$ is the total concentration of $E\sigma^{70}$ and τ_{obs} — a lag-time necessary to reach the steady-state by the second-order transcription reaction. Provided that the association reaction exhibits at $[R]_T \geq 0.3 \, k_i/k_a$ single-exponentiality, and the fraction of long-lived complexes approaches unity, then $k_i \approx k_2 \ll k_{-1}$ and $k_a = K_1 k_2$ (Tsodikov & Record, 1999). These rate constants were determined by measuring τ_{obs} in function of the enzyme concentration. Fluorescence-detected abortive initiation assay (FDAI) with γ-ANS-UTP as a substrate were used to follow continuously the progress of the reaction and to extract the parameter τ_{obs} there from, as described under Methods. Linear weighted least-squares fit of eqn. 2 to the experimental τ_{obs} ($[R]_T$) data, plotted in Figs. 2a and 2b, yielded k_a and k_i parameters, collected in Table 1. Using the k_a and k_i values obtained, the corresponding K_1 equilibrium constants were calculated (Table 1). As can be judged from the experimental data, the formulated conditions of single exponentiality were satisfactorily fulfilled.

Since abortive reactions were initiated by manual mixing of the reactants for approximately 15 s, values of τ_{obs} should be higher than 50 s to be measured with a sufficient accuracy. Since k_a is known to depend exponentially on 12 mono- (Roe *et al.*, 1985) or 7 divalent (Mg^{2+}) cations (Kolasa *et al.*, 2001), the appropriate experimental conditions were sought by variation of salt concentration in the transcription buffer. Unfortunately, it proved impossible to find common salt conditions for all the promoters investigated, so that the association reactions were conducted either at 100 or 125 mM $MgCl_2$, and in some cases in function of $MgCl_2$ concentration, to obtain the sought k_a values by linear extrapolation of double logarithmic plots to a desired salt concentration. $MgCl_2$ was chosen as the sole salt in order to avoid complicated kinetics observed in mixed salt transcription buffers (Record *et al.*, 1977).

Changes in the rate constant of RPo formation upon modification of promoters were ex-

Table 1. Kinetic parameters of open complex formation at control promoters Pa and Pac and their bent derivatives in the transcription buffer (25 mM Hepes, pH 8, 35°C) containing 100 or 125 mM $MgCl_2$ (in brackets standard errors at 0.95 confidence)

Promoter	k_a 10^5 M^{-1} s^{-1}		k_i 10^{-2} s^{-1}		K_1 10^7 M^{-1}	
	100 mM $MgCl_2$	125 mM $MgCl_2$	100 mM $MgCl_2$	125 mM $MgCl_2$	100 mM $MgCl_2$	125 mM $MgCl_2$
Pa	6.3 (0.3)	1.3 (0.1)	2.7 (0.2)	2.3 (0.5)	2.3 (0.1)	0.6 (0.1)
Pac	2.0 (0.04)	0.4 (0.03)[*]	1.4 (0.4)	–	1.45 (0.05)	0.3[*$]
Pk	–	3.1 (0.5)	–	3.8 (1.6)	–	0.8 (0.4)
Pl	–	2.6 (0.2)	–	1.9 (0.2)	–	1.4 (0.2)
Pm	0.5 (0.06)	0.21 (0.05)[#]	3.6 (3.4)	–	0.15 (0.02)	0.06[#$]
Pg	1.1 (0.2)	–	1.7 (0.8)	–	0.65 (0.3)	–

[*]from extrapolation of $\log k_a$ vs. $\log[MgCl_2]$ plot using slope: –7.2 (0.6); [#] ditto using slope: –4.3 (0.3); [*$] and [#$] estimated from extrapolated values of k_a under assumption that k_i is independend of $MgCl_2$ concentration between 100 and 125 mM.

pressed as the ratio k_a/k_a(con), i.e. between the rate constant for a given promoter and that for the control one (Pa or Pac), and analyzed according to Scheme 2, which shows the relationships between the control promoter and its derivatives.

$$Pa \xrightarrow{0.18} Pg \xrightarrow{0.45\ (0.08)} Pm$$

$$\downarrow 0.32$$

$$Pl \xleftarrow{6.5} Pac$$

$$\downarrow 7.8$$

$$Pk \qquad \text{(Scheme 2)}$$
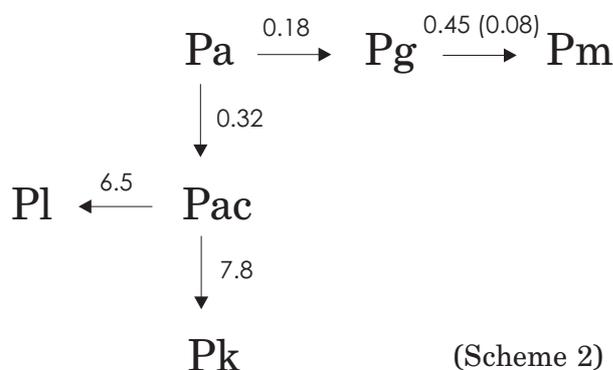
Insertion to Pac of the $-41(A_5 \cdot T_5)-37$ sequence in two orientations, resulting in promoters Pk (5′AAAAA3′) and Pl (5′TTTTT3′), caused a similar about 7–8-fold increase in k_a. The magnitude of this effect is similar to that brought about by the presence of UP proximal subsite on strong promoters with the consensus –35 and –10 elements (Strainic *et al.*, 1998). However, the presence of the same sequence in Pg, with the T_5 run in the non-template strand between positions –38 and –34, led to a several-fold decrease in k_a. Upstream extension of the T_5 sequence by 3

bases, forming $-41(T_8)-34$ run of promoter Pm, brought about a further about twofold drop in this rate constant. Since A(–36)C and A(–36)T mutations in Pa reduced k_a about 3- and 6-fold, respectively, the nature of the base in this position seems to be very important for promoter strength. The A(–36)T mutation in Pa, leading to Pg, formed simultaneously a $-38(T_5)-34$ run, so that the observed effect on k_a would be due both to that mutation and distorted conformation of bent DNA in this region.

The values of k_a were determined quite accurately from the slopes of the tau-plots (Figs. 2a, b). The corresponding k_i isomerization rate constants (cf. Table 1) bear a much larger error, however, connected with a low accuracy of determination of the intersection of these plots with the tau-axis (cf. Eqn. 2). At a rate of the isomerization $I_1 \to I_2$ step in the range of 10^{-1}–10^{-2} s^{-1}, characteristic for most promoters (Leirmo & Record, 1990), values of $1/k_i$ were very small as compared with those of τ_{obs}. The average k_i values: 2.5 (2.5) $\times 10^{-2}$ s for Pa and 5.9 (6.9) $\times 10^{-2}$ s for P_m were found independent of $MgCl_2$ concentration (between 60 and 125 mM) within the standard errors indicated, in agreement with earlier documentation (Leirmo & Record, 1990) that the isomerization step was little influ-
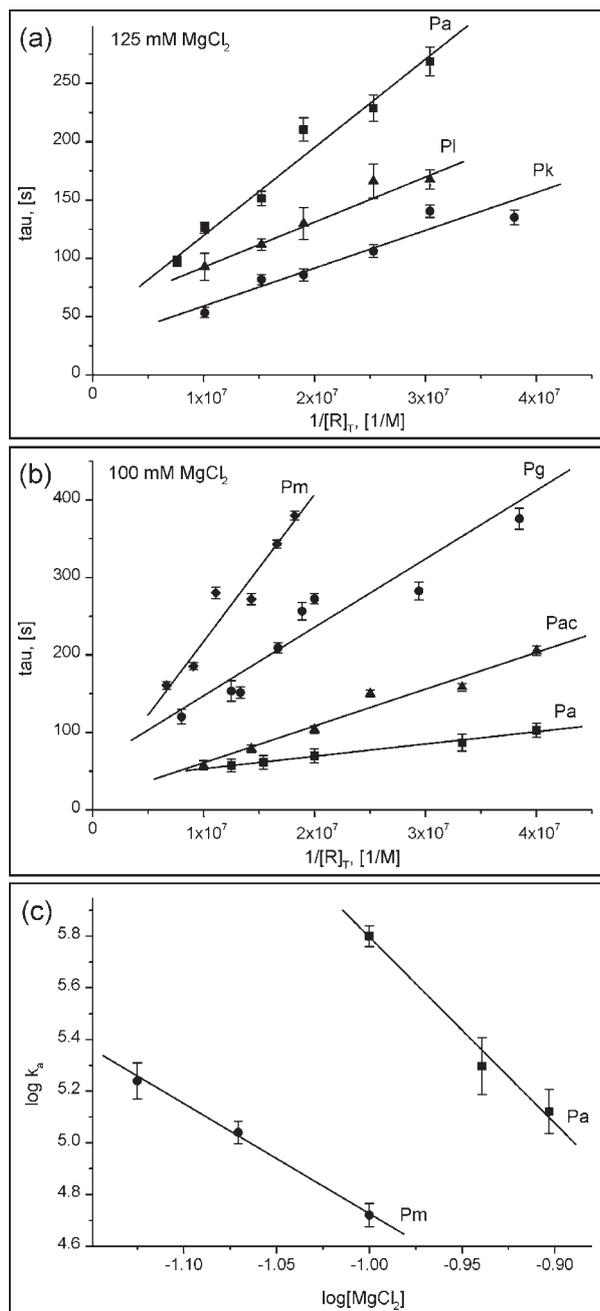
Figure 2. Kinetics of the open complex formation.

Plots according to Eqn. 2 of experimental τ_{obs} data at 35°C (a) for Pa, Pk and Pl promoters at 125 mM $MgCl_2$ and (b) for Pa, Pac, Pg and Pm at 100 mM $MgCl_2$ (c) double-logarithmic plot of k_a vs $MgCl_2$ concentration for Pm with the slope of –4.3 (0.3); for comparison purposes a similar plot for Pa with the slope of –7.2 (0.6) (Kolasa et al., 2001) is shown.

enced by ionic exchange equilibria. Bearing this in mind, inspection of the data in Table 1 allows to conclude that changes in k_a are for the most part due to changes in the binding

equilibrium constant K_1. An increase in K_1 has been also observed for promoters having the natural UP element and was accompanied by a two-fold faster rate of closed complex isomerization (Strainic et al., 1998). Our k_i data for Pa, Pk and Pl (Table 1) do not allow to postulate similar effect of the proximal UP subsite on this process.

The strong dependence of k_a on salt concentration has been shown to be due to mono- or divalent cations acting as non-specific competitors for RNA polymerase at the binding step (Roe et al., 1985). For Pa, the number of Mg^{2+} ions released from the protein/DNA interface upon formation of I_1 closed complex was found equal to 7 (Kolasa et al., 2001). This number is equivalent to 12 Na^+ ions found to remain in equilibrium with the closed complex at λ_{PR} promoter (Roe et al., 1985), since for every Mg^{2+} ion bound 1.8 Na^+ ions are displaced from DNA solvation sphere (Misra & Draper, 1999). However, for Pm the slope of the log k_a vs. log[$MgCl_2$] plot (cf. Fig. 2c) indicates that only 4 Mg^{2+} ions are released. This finding is consistent with the large drop in K_1 value on going from Pa to Pm, indicative of loss of a number of specific ionic contacts between the promoter DNA upstream of the –35 hexamer and the α subunits of RNA polymerase.

DISCUSSION

Analysis of the kinetic data on the open complex formation at the studied group of promoters has demonstrated that (i) presence of $-41(A_5 \cdot T_5)-37$ sequence in either orientation, i.e. $5'$-T_5-$3'$ or $5'$-A_5-$3'$, at the downstream region of the proximal UP consensus subsite caused a significant rise in the promoter strength (k_a) of respective derivatives of the control Pac promoter; (ii) the same sequence with $5'$-T_5-$3'$ run in the non-template strand between –38 and –34 positions, partially overlapping both the –35 hexamer and proximal UP subsite, had an opposite effect on k_a; (iii) upstream extension of the latter to

T_8 caused a further decrease in the promoter strength, accompanied by significant reduction in the number of Mg^{2+} ions released from the promoter/protein interface upon the closed complex formation; and (iv) the nature of the base at -36 position adjacent to the -35 hexamer affects also the overall rate of RPo formation.

How can these findings be interpreted in view of the present model of molecular interactions between the UP promoter element and the α subunits of $E\sigma^{70}$?

A preliminary model of how α might interact with its binding site in the UP element proximal subsite has been recently proposed (Ross *et al.*, 2001) on the basis of extensive experimental data (including hydroxyl radical footprinting, effects of missing single bases, U for T and 7-deaza-7-nitro-A for A substitutions, protection from various chemical modifications, etc.) for promoter complexes with purified αCTD, wild and αCTD lacking RNA polymerases. According to this model, each of the two helix-hairpin-helix motifs in the αCTD (Jeon *et al.*, 1995; Shao & Grishin, 2000) makes sequence specific backbone contacts with one of DNA strands from the minor groove. The sequence specificity derives probably from the narrow width of the minor groove in an A_n (n = 5, 6) tract, shown to decrease from $5'$ to $3'$ direction along the DNA strand (MacDonald *et al.*, 2001), and from contacts of the exposed in that groove functional groups: N3 of adenine and O2 of thymine, through hydrogen bonds with the basic side chains of R265 and K298 of αCTD. NMR and molecular modeling studies on interaction between αCTD and UP element have indicated (Yasuno *et al.*, 2001), however, that (i) αCTD is not inserted in the minor DNA groove deeply, being in contact with DNA backbone alone, and (ii) the guanidine group of arginine 265 can interact most strongly with both sides of the negatively charged phosphate backbone when within the narrowest part of the minor groove at the $3'$ end of an A_n tract.

Our kinetic data for Pk and Pl promoters seem to be in general agreement with, and add

support to this model. First of all, the $-41(A_5 \cdot T_5)-37$ sequence in these promoters is located within the downstream part of the proximal UP subsite, so that αCTD may recognize it specifically. The similar direction and magnitude of the up-effect exerted on k_a and K_1 by this sequence in either orientation is fully understandable if one considers where the width of the minor groove of $-41(A_5)-37$ tract in either of these two promoters should be the smallest, as compared with the consensus proximal subsite. In Pl, this tract lies in the template DNA strand, so it has $3'$ to $5'$ orientation and thus the width of its minor groove is expected to be the smallest close to $-41A$. By a similar token, in Pk the narrowest part of the minor groove of this tract, located in the non-template strand, lies at its other end close to $-37A$. The A_6 tract in non-template strand of the consensus UP proximal subsite, in promoters with 17 bp spacer, would have the narrowest minor groove near $-40A$, that is barely by one nucleotide downstream or two nucleotides upstream of its location in Pk and Pl, respectively. Recent studies on positional requirements of the cognate UP element in *rrn*P1 promoter and the role of the RNA polymerase α subunit interdomain linker length (Meng *et al.*, 2001) have shown that displacement of the UP element by one helical turn from the wild type location caused an over 20-fold decrease in transcriptional activity, whereas displacement of this element by any other distance, including displacement by only 5 nucleotides, abolished the UP element dependent transcription. Thus, αCTD may interact with promoter sequences located on the same face of DNA helix as the rest of $E\sigma^{70}$ and the optimal UP element function at some promoters may require contact between αCTD and σ^{70} bound at the -35 region. Hence perturbation in DNA structure by bending tracts in the very region where these two $E\sigma^{70}$ subunits are in contact with each other and with promoter DNA may profoundly affect promoter function. This seems to be the case for Pg and Pm promot-

ers. In Pg, the $-38(A_5 \cdot T_5)-34$ sequence contains a highly conserved $-35T_2-34$ dinucleotide motif of the consensus -35 hexamer. In the open complex at natural promoters, the -35 element lies on the DNA face in a specific contact *via* its major groove with the postulated helix-turn-helix motif of the σ^{70} region 4 (Siegele *et al.*, 1989; Dombroski *et al.*, 1992; Mekler *et al.*, 2002). Bending of DNA in the -35 region by the $-38 A_5 -34$ tract in the template strand towards the minor groove directs the upstream part of DNA to the outside of the promoter/σ^{70} interface (Łoziński & Wierzchowski, 1996; Kolasa, 2001). This can be expected to perturb severely specific contacts of the promoter region with σ^{70}. The strongest perturbation of the B-DNA structure by this tract would occur near its $3'$ end at $-38A$, where the width of minor groove attains a minimum value. Thus, specific interactions between $E\sigma^{70}$ and some nucleotides upstream of the -35 hexamer can be also affected. The most vulnerable site seems to be that immediately adjacent to the -35 element, since site-specific protein–DNA phosphate photo-crosslinking studies on the open complex at *lac*UV5 promoter (Naryshkin *et al.*, 2000) have shown that at -37 non-template position (equivalent to -36 in Pg and Pm) crosslinking involves besides αCTD also σ^{70}, β and β' polymerase subunits. Furthermore, the identity of C residue at this position is known to be very important for transcription from the *rrnB* P1, λ_{PR} and *lac* promoters (Ross *et al.*, 1998). Also values of statistical weight matrix for the four bases at this position differ greatly from each other (Herz & Stormo, 1996), while the occurrence of T is the least probable. These facts and considerations well explain the large drop in k_a and K_1 values brought about by formation of the $-38(A_5 \cdot T_5)-34$ sequence in Pg as a result of the single A(-36)T mutation in Pa. A further decrease in the values of these two parameters for Pm seems to be due, in addition to the A(-36)T mutation, also to a more compact and stiff B$'$-DNA structure of the $-41A_8 \cdot T_8$

-34 sequence (Nelson *et al.*, 1987) which apparently prevents its effective interaction with αCTD. In spite of the fact that it is located in the $-41...-37$ region, in which the $A_5 \cdot T_5$ sequence, in Pl and Pk promoters, was shown to stimulate greatly the transcription. This conclusion is strongly supported by the smaller number of 4 Mg^{2+} ions found to be involved in control of K_1 for Pm, as compared with that of 7, determined for Pa (Kolasa *et al.*, 2001). The smaller number of these ions released upon closed complex formation at Pm would indicate a loss of some specific ionic contacts between the minor groove DNA phosphates and basic amino acids of αCTD, postulated in the model (Ross *et al.*, 2001) referred to above. As it has been shown recently (Heyduk *et al.*, 2001), enthalpy driven formation of 1:1 complex between isolated α subunit and UP element of *rrnB* P1 promoter is also accompanied by a net release of $1-2$ ions. In this context it would be of great interest to study the influence of the UP proximal subsite on the rate of open complex formation at a model promoter in function of a salt concentration. For Pk and Pl promoters this proved not feasible by the method used in this work owing to too low efficiency of abortive transcription at high concentrations of $MgCl_2$.

The effect of A(-36)C mutation in Pa on kinetics of RPo formation at its Pac derivative deserves an additional comment. On the basis of the largest statistical weight for C in this position (Herz & Stormo, 1996), and importance of this base for a number of natural promoters reported by Ross *et al.* (1998), we expected that this mutation should improve contacts of Pac with $E\sigma^{70}$. On the contrary, the mutation caused a two-fold decrease in k_a. Apparently, selection of a base at this position for optimal contacts of a promoter with $E\sigma^{70}$ depends on the sequence context.

Finally, we would like to comment on our earlier findings (Łoziński *et al.* 1989; 1991; Łoziński & Wierzchowski, 1996) that open complexes formed at Pk, Pl, Pg and Pm, promoters exhibited a similar decrease in electro-

phoretic mobility on acrylamide gel (PAGE) as compared with that at the parent Pa promoter, as well as a somewhat decreased transcriptional activity *in vivo*. Photo-cross-linking studies on the open complex formed by $E\sigma^{70}$ at *lac*UV5 promoter (Naryshkin *et al.*, 2000) have indicated that, in the absence of upstream sequence determinants, αCTD interacts nonspecifically with the upstream part of promoter DNA, making alternative transient contacts with any one of the first five to six minor grooves upstream of the core promoter and causing compaction of DNA. This may apply also to Pa positioned in the central part of a *Sal*I (–250)... *Hinf*I (+160) DNA restriction fragment (Łoziński *et al.*, 1991). Insertion to Pa in either of the two upstream regions, –41...–37 or   –38...–34, of a single $A_5 \cdot T_5$ tract, shown to cause bending of DNA axis towards the $E\sigma^{70}$/promoter interface (Łoziński & Wierzchowski, 1996; Kolasa, 2001), leads apparently to an additional compaction of promoter DNA by facilitating its wrapping around the enzyme. Macroscopically, this manifests itself in PAGE retardation of the open complexes. At the molecular level, however, the small difference in location of this sequence affects in a different way the interaction of promoter DNA with $E\sigma^{70}$ and thus also kinetics of the open complex formation *in vitro,* as discussed earlier in the text. In our investigations on promoters' strength *in vivo* by quantification of the amount of full-length RNA transcript formed, referred to above, the reaction involved an additional kinetic control point, *viz.* transition from initiation to elongation stage, absent in the *in vitro* experiments by the abortive transcription assay. Since all the promoters studied contain consensus –35, –10 and 17 bp spacer regions, the escape of RNA polymerase from the open complex can be expected to be the rate controlling step of transcription *in vivo*. Moreover, it was shown recently (Strainic *et al.*, 1998) that synthetic promoters bearing both these consensus elements and the UP one were cleared slower than those without the latter. This would explain why the stimulatory effect of $A_5 \cdot T_5$ sequences on transcription initiation from Pk and Pl promoters documented in this work has not been observed under conditions *in vivo*.

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