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Dedicated to Professor David Shugar on the occasion of his 80th birthday

Effect of reversed orientation and length of $A_n \cdot T_n$ DNA bending sequences in the –35 and spacer domains of a consensus-like *Escherichia coli* promoter on its strength *in vivo* and gross structure of the open complex *in vitro**

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In continuation of an earlier study (Łoziński *et al.*, 1991 *Nucleic Acids Res.* 19, 2947–2953) a series of consensus-like *E. coli* promoters with bending $A_n \cdot T_n$ sequences of different length (n = 3–8) and orientation in the –35 and spacer domains was constructed, cloned into the plasmid pDS3 and their strength *in vivo* measured in relation to an internal transcriptional standard. Gel mobilities of free DNA restriction fragments carrying these promoters and of open transcriptional complexes with cognate RNA polymerase were determined by polyacrylamide gel electrophoresis and the gross structure of the complexes interpreted in terms of the theoretically predicted superstructure of DNA restriction fragments. The results obtained together with those reported earlier show that bending of the DNA helix axis immediately upstream of the –35 domain generally lowers the promoter strength *in vivo* and brings about shortening of the mean square end-to-end distance between free DNA ends in the open complex *in vitro*. T_4(–34...–37) and T_5(–34...–38) tracts located in the nontemplate DNA strand had the largest and comparable effect on the promoter strength, while the $A_5 \cdot T_5$ (–37...–41) sequence in either orientation (A_5 tract in the template or nontemplate strand) exerted a much smaller effect. Promoters with the spacer bent by about 40° but in different directions, by two A_n (n = 5 or 6) tracts aligned in phase with the B-DNA repeat and located either in the template or nontemplate strands, had somewhat lower strength *in vivo* but the gross geometry of the respective open complexes was the same as that of a control promoter with straight spacer. Implications of these findings are discussed in connection with the existing model of *E. coli* transcriptional open complex.

At present, the structure of the open transcription complex formed by *E. coli* RNA polymerase (RNAP, $E\sigma^{70}$) on any of the cognate promoters and the structure of RNAP itself are not known in sufficient detail to explain properly the mechanistic role of various domains of a consensus-like promoter involved in the multistep process of protein-DNA recognition [1–9]. The role of these domains has been probed by various techniques including mutation of promoter DNA (see ref. [2] for a review) and σ^{70} subunit of RNA polymerase [10–14]

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Abbreviations: CAP, catabolite activator protein; HS, homology scores; RNAP, RNA polymerase; RS, relative strength of promoters; UP element, upstream element.

involved in the promoter recognition, insertion into the spacer domain of DNA sequences of different conformation and flexibility [15–19], by footprinting of various transcriptional complexes with $OH^{\cdot}$ radicals [20–22], etc.

We probed the mechanistic role of the 17 bp spacer and two –35 and –10 recognition hexamers [23, 24] by insertion into the nontemplate chain of a consensus-like *E. coli* promoter of appropriately located DNA bending $T_n \cdot$ (n = 5, 6) tracts and determination of the effect of these sequences on promoter strength *in vivo* and gross structure of the open complex *in vitro* by polyacrylamide gel electrophoresis (PAGE). It has been found that insertion into the spacer domain of two tracts T_6(–12...–17) and T_6(–23...–28), expected to bend the helical axis of B-DNA form by about 40° to the outside of the DNA-RNAP interface [25–27], lowered the promoter strength by about 20% but had no effect on the electrophoretic mobility of the open complex, and thus on its gross structure either. However, replacement of the whole spacer domain by a stiff $T_{17} \cdot A_{17}$ B'-DNA fragment [28] or presence of T_6(–7...–2) bending tract in the –10 domain had no effect whatsoever on these two parameters. On the other hand, all the promoters bearing the T_5(–34...–38) sequence in the nontemplate strand of the –35 recognition domain exhibited much lower strength *in vivo* and a change in the gross structure of the open complex *in vitro*, irrespective of the presence or absence of other T_n bending sequences in the spacer or in the –10 domain. In the present work, we report the results of further experiments along the same line with synthetic promoters bearing (i) $A_n \cdot T_n$ bending sequences in the spacer region and upstream of the –35 domain in reversed orientation, or (ii) T_n sequences of different length overlapping the T_2(–34,–35) doublet of the –35 canonical hexamer. The results obtained in this work and in earlier studies [23, 24] are interpreted in the light of theoretically calculated promoter DNA curvature [29] and the existing model of the transcriptional open complex [9, 10].

MATERIALS AND METHODS

Materials. *E. coli* C600 strain, plasmid pDS3 and the derivatives of bacteriophage M13 containing fragments of *bla* and *dhfr* genes, were kindly provided by Dr H. Bujard. *E. coli* RNA polymerase was purified by the method of Burgess & Jendrisak [30], except that Bio-Gel A 5 m was replaced by Sephacryl S300. [γ-^{32}P]ATP was made in the Institute of Biochemistry and Biophysics, enzymes and [5,6-^{3}H]uridine were from Amersham, and all other chemicals were of reagent grade.

Synthesis and cloning of the promoters. Promoters were obtained as described earlier [23]. Oligomers for the promoters specified under "Results" were synthesized by the solid phosphoramidite method and purified by PAGE; promoter DNA's obtained subsequently by annealing of the complementary oligomers were then cloned into pDS3. The recombinants obtained were selected by chloramphenicol resistance and the promoter region was sequenced.

Calculation of the promoter DNA curvature. The path in the three-dimensionl space and parameters of curvature for promoter DNA restriction fragments studied were calculated with help of the computer program SuperDNA, based on a theoretical model of DNA curvature of De Santis *et al.* [29], kindly made available to us by P. De Santis.

Mapping of transcription start sites. In this experiment with the use of *Sal*I-*Hin*fI restriction fragments carrying the promoters specified under "Results", ^{32}P-labelled run-off transcripts were obtained *in vitro*, and their length analyzed by PAGE and autoradiography as described earlier [23].

Electrophoretic gel analysis of free DNA and the open complexes. Restriction fragments carrying the studied promoters were 5'-end labelled with ^{32}P, resolved by electrophoresis on 6% polyacrylamide gel in TBE buffer (90 mM Tris/borate, pH 8.3; 2 mM EDTA, pH 8.0) at 4°C and visualized by autoradiography. The RNA polymerase-promoter open complexes were formed exactly as described earlier [24] on the ^{32}P-end-labelled DNA fragments and resolved on 3.8% polyacrylamide gel in TB-$MgCl_2$ buffer (90 mM Tris/borate, pH 8.3; 5 mM $MgCl_2$) at 30°C, followed by autoradiography.

Determination of promoter strength in vivo. The strength *in vivo* of the promoters studied was determined according to Deuschle *et al.* [31], exactly as described earlier [23]. In that method the promoter under investigation controls transcription of the coding sequence of the

mouse dihydrofolate reductase (*dhfr*) gene. The quantity of *dhfr* specific RNA synthesized is compared with that of the internal standard, which is the β-lactamase (*bla*) specific RNA transcribed from the same plasmid under control of its own constitutive P_{bla} promoter.

RESULTS AND DISCUSSION

Structure of promoters

The synthetic promoters **i** and **k–o** (shown in Fig. 1) were constructed as variants of the promoters **a**, **e**, and **g** (cf. Fig. 1) of the previously studied series **a–h** [24], with the aim of investigating the effect of (i) reversed orientation of the DNA-bending $A_n \cdot T_n$ sequences in the spacer region and upstream of the –35 hexamer, that is A_n instead of T_n tracts in the nontemplate DNA strand, and (ii) the length of the T_n tract upstream of, and partially overlapping the –35 hexamer, on the promoter strength *in vivo* and gross structure of the open complex *in vitro*.

Relative strength of promoters *in vivo*

All the newly constructed promoters proved to be fully functional. When cloned into the plasmid pDS3, they were able to direct transcription of the *cat* and *dhfr* genes both *in vitro* and *in vivo*, as it was shown to be also the case with all the previously constructed promoters **a–h** [24]. This indicated proper utilization of their main promoter features, viz. the –10 and –35 canonical hexamers separated by 17 bp, and purines at the start site. To confirm this conclusion directly we have determined the actual start sites for all the new promoters by comparative PAGE analysis of the length of the run-off transcripts obtained *in vitro*. All the transcripts appeared to be of the same length as that for the control promoter **a** (not shown). The start sites *in vitro* and *in vivo* for the latter pro-

A

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          XhoI        -35                        -10        +1    EcoRI
a:        CTCGAGTTATTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
b:        CTCGAGTTTTTGACAATTTTTTATATATTTTTTTATAATTTTTTA*A*TTGAATTC
e:        CTCGAGTTATTGACAATTTTTTATATATTTTTTTATAATTATTTA*A*TTGAATTC
g:        CTCGAGTTTTTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
i:        CTCGAGTTATTGACAATAAAAATATATAAAAATATAATTATTTA*A*TTGAATTC
k:   CTCGAGAAAAACTTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
l:   CTCGAGTTTTTCTTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
m:   CTCGAGTTTTTTTTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
n:        CTCGAGATTTTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
o:        CTCGAGAATTTGACAATTATTTATTTATTATTTATAATTATTTA*A*TTGAATTC
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B

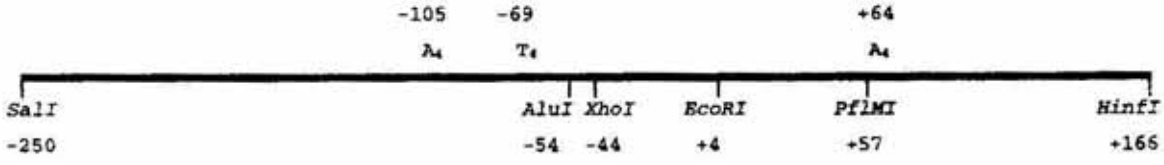


Fig. 1. **(A)** *Sequences of consensus-like promoters; nontemplate DNA strand.*
Transcription start sites occurring at –1 and +1 positions are indicated by asterisks (*); consensus hexamers in the –10 and –35 regions in **bold font**; tracts expected to bend DNA underlined; the promoters **a**, **b**, **e** and **g** are included for comparison from ref. [24]. **(B)** DNA restriction fragments of pDS3 plasmid carrying promoters used in PAGE experiments; numbers relative to the start site of promoter **a** indicate positions of respective restriction enzymes, and centers of A_4 and T_4 tracts, outside of the promoter region.

moter were mapped previously [23] by the S1 nuclease assay, and shown to occur at the –1 and +1 positions occupied by an AA dinucleotide (cf. Fig. 1). These are thus also the start sites on all the promoters **i** and **k–o** presently studied.

The relative strength (RS) *in vivo* of all the promoters: **i** and **k–o**, proved somewhat lower than that of the control promoter **a** (Table 1). The largest drop in RS by about 50% was found for promoter **n** bearing T_4(–34...–37) tract in the nontemplate DNA strand, a drop similar to that observed previously [24] for the promoter **g** with the T_5(–34...–38) tract, longer by only one T residue. Extension of the T_2(–34,–35) dinucleotide sequence upstream of the –35 canonical hexamer by just one T residue (promoter **o**) or by as many as six T residues (promoter **m**) had a similar but much smaller effect on RS. Also insertion of $A_5 \cdot T_5$ sequence in either orientation at –36...–40 location, promoters **k** and **l**, brought about a much smaller decrease in RS compared with that found for promoters **g** and **n**. Note that in two former promoters the $A_5 \cdot T_5$ sequence is separated from the –35 hexamer by one GC pair. However, the corresponding A→C point mutation leads to only a small drop in the homology score of the canonical *E. coli* promoter [32], and thus it can be expected to result also in a small change in RS. Comparison of the RS values for promoters **k** and **l** shows that reversed orientation of the $A_5 \cdot T_5$(–36...–40]) sequence brought about a change in RS by only some 20%.

Effects of insertion of phased $A_5 \cdot T_5$ sequences upstream the –35 domain of a core promoter were recently subject of two independent investigations in plasmid *in vivo* systems [33, 34]. In the first study [33] on two synthetic promoters, rate-limited at a step following open complex formation (accumulation of stalled initial transcribing complexes observed by permanganate probing), insertion of three phased A_5 tracts in the nontemplate strand upstream of the –35 domain, with the first tract centered around position –40, brought about a six-fold decrease in the promoter strength. No such stalling was observed, however, for any of the promoters here studied with $A_n \cdot T_n$ sequences upstream the –35 region (Łoziński, T., unpublished). So that their decreased expression can not be ascribed to a reduced ability of the polymerase to escape to form an elongation complex *in vivo*. In the second study [34], a set of the β-lactamase promoter derivatives was constructed by substitution into a region of naturally occurring right-handed superhelical curvature immediately upstream the –35 position of double-stranded oligonucleotides, each with unique DNA writhe imposed by 3–4 variously spaced T_5 sequences in the nontemplate strand. In all these promoters the first T_5 tract spans from –34 to –40, like in the case of our promoter **g**. The strength of all the mutant β-lactamase promoters was reduced 5–30 fold with respect to the wild-type one. Since the smallest drop in strength exhibited promoters with right-handed superhelical writhe upstream the promoter core region, the authors hypothesize that it is this superhelical sense which may partially compensate for a decrease in promoter strength brought about by lack of sequence homology upstream the –35 region between the mutant promoters and the wild-type promoter. These observations are in general agreement with present and earlier findings (cf. ref. [24] and reference [4] cited therein) that T_n or A_n tracts (in the nontemplate strand) partially overlaping the –35 consensus hexamer or immediately upstream thereof, cause a drop in promoter strength.

The reversed orientation of the two phased $A_n \cdot T_n$ bending sequences within the spacer domain in promoters **e** (n = 6) and **i** (n = 5) had

Table 1

The relative strength of the studied promoters in vivo (in bla units); pDS3 plasmid and E. coli C600 were used as the vector and host, respectively; each value is an average from four independent determinations with standard deviation corresponding to less than 10%; in parenthesis values from ref. [24].

Promoters forming open complex of perturbed gross geometry								
a	**e**	**i**	**g**	**k**	**l**	**m**	**n**	**o**
(11.4)	(9.1)	8.2	(6.2)	10.7	8.6	8.4	5.5	8.1

also a little effect on the promoter strength, as evidenced by their similar RS values. As it will be shown in the next section, only the promoters bearing a bending $A_n \cdot T_n$ sequence in the –35 region formed open complexes of perturbed gross structure.

To check whether the observed variation of RS among members of the **a**, **o**, **n**, **g**, and **m** group of promoters is not simply due to deviation of the promoter sequence in the (–34...(–34-n)) region from that of the consensus promoter, we calculated homology scores (HS) [32] for these promoters. The relative HS values, normalized to the HS = 84 for the control promoter **a**, show very small variation in the whole series: 1.0, 0.95, 0.96, 0.99 and 1.01, while the corresponding RS *in vivo* values differ to a much larger extent: 1.0, 0.71, 0.48, 0.54 and 0.74, respectively.

PAGE analysis of the open complexes

To estimate the effects of the various $A_n \cdot T_n$ tracts encoded in a promoter sequence on the extent of promoter DNA bending elicited by their presence, and on the gross structure of the open promoter complexes formed with RNAP, we measured PAGE mobilities of these complexes and of the respective free DNA restriction fragments under non-denaturing conditions (Tables 2 and 3).

According to theories on DNA mobility in gel electrophoresis [35, 36], mobility of DNA fragments of the same length, measured in base pairs (bp), is proportional to their mean square end-to-end distance. The latter is determined by the DNA superstructure, which can be represented as a writhing of the helix axis resulting from local sequence-dependent deviations from the uniform standard B-DNA structure [29]. Mobility of an intrinsically bent DNA fragment is thus expected to be retarded relative to that of a linear fragment of the same size. Gel electrophoresis has been shown to be a very sensitive method for vizualization of even small differences in the DNA superstructure induced by the presence of $A_n \cdot T_n$ bending sequences or specific binding of regulatory proteins [37–41].

The PAGE data for free *SalI-HinfI* DNA fragments carrying the studied group of promoters with inserted DNA bending $A_n \cdot T_n$ sequences (Table 2) indicate that their relative gel retardation, R_{exp} (in respect to a linear fragment of the same size), varies in the range of 1.17 – 1.40. The experimental R_{exp} values proved to correlate well with those predicted (Table 4) by the theoretical cyclic permutation analysis, R_{th}, [42], discussed in the next chapter. Note that the R_{exp} values for the fragments with promoters **k**, **l** and **m** correspond to a similar (promoter **m**) or even higher gel mobilities (promoters **k** and **l**) in comparison to promoter **a**, in spite of the fact that they all contain DNA bending $A_n \cdot T_n$ sequence. This is due to the somewhat different construction of these promoters, which required insertion of additional 3 bp between the *XhoI* restriction site and the –35 hexamer (cf. Fig. 1).

The mobility of RNAP-promoter open complexes formed on *SalI-HinfI* DNA fragments was always lower than that of the corresponding free DNA's (cf. Fig. 2) and strongly dependent on the concentration of polyacrylamide gel used in PAGE analysis. For instance, the ratio of (free DNA mobility)/(RNAP-DNA mobility) differed by a factor of about 2 between 3.2% and 4.4% gels. It would be, of course, desirable to relate always PAGE retardation of the complexes to that of the respective DNA fragment determined under the same experimental conditions. Unfortunately, this proved impossible because of very different gel concentrations required for good resolution of small differences in gel mobility between the free DNA's (at least 6% gel), on the one hand, and between their complexes with RNAP (about 4% gel), on the other. At higher than 4% gel concentrations, the

Table 2

PAGE retardation, R_{exp}, of the SalI-HinfI free DNA fragments carrying the promoters studied.

PAGE mobilities of the fragments were measured relative to that carrying the promoter **a**, for which we have assumed $R_{exp} = R_{th}$; R_{th} was calculated with use of De Santis SuperDNA program (cf. Fig. 3 and Table 4); R_{exp} values for promoters **a**–**h** were calculated from mobilities reported in ref. [24].

a	b	c	d	e	f	g	h	i	k	l	m	n	o
1.19	1.40	1.26	1.19	1.27	1.25	1.23	1.20	1.25	1.18	1.17	1.19	1.23	1.21

Table 3

PAGE retardation of open complexes formed on three types of restriction fragments (cf. Fig. 1), measured as the ratio of mobility of the open complex of promoter **a** *to the mobility of that of the indicated promoter for the same fragment type; n.d., not determined.*

Promoter	*SalI-HinfI*	*SalI-PflMI*	*AluI-HinfI*
a	**1.00**	**1.00**	**1.00**
e	1.00	n.d.	n.d.
g	1.22	1.08	1.00
i	1.00	n.d.	n.d.
k	1.22	1.02	1.00
l	1.15	1.07	1.00
m	1.30	1.14	1.00
n	1.22	n.d.	n.d.
o	1.22	n.d.	n.d.

complexes did not move well through the gel phase. All this indicates that PAGE mobility of the complexes was strongly influenced also by the presence of bound RNAP. Thus, retardation of the complexes with bent promoters was calculated relative to the mobility of the complex with promoter **a**, assumed as equal to 1.0. For smaller transcription protein factors like CAP (catabolite activator protein) [43] such a significant retardation of DNA-protein complexes by protein itself was not observed.

We found previously [24] that mobility of the open complexes formed on the analogous group of synthetic promoters **a**–**h** (located within the same *SalI-HinfI* restriction fragment as used in the present work) depended on the presence or absence of the T_5(–34...–38) tract in the nontemplate DNA strand. Complexes formed on promoters (**b**, **c**, **f**, **g**) bearing the latter sequence, called β, exhibited lower mobility (higher retardation) than those, called α, of the control promoter **a** and its derivatives lacking this tract.

Inspection of the PAGE data obtained (Table 3, Fig. 2) indicates that complexes formed on the promoters **k**–**o** exhibited a retardation similar to that of the promoter **g**, bearing the T_5 (–34...–38) tract, and higher than that of the complex formed on the control promoter **a**. They could be thus assigned to the β class. In particular, the same value of PAGE retardation, 1.22, was obtained for the complexes of promoters **o**, **n** and **g**, in spite of the fact that the latter differ in length, and thus also in bendability, of the T_n tract (n = 3, 4 and 5, respectively) overlapping the T_2(–34,–35) sequence of the –35 hexamer. The complexes with promoter **m** were the most retarded. The difference in PAGE retardation between the complexes of the promoters **k** and **l** was found to be rather small (ΔR = 0.07) in spite of the reversed orientation of the $A_5 \cdot T_5$(–37...–41) sequence, that is with A_5 or T_5 tract in the nontemplate strand, respectively.

The open complex formed on promoter **i** exhibited the same PAGE mobility as did the α complexes of the promoters **a** and **e** (Table 3), so that it could be classified accordingly. Since the promoters **e** and **i** carry in the spacer domain two phased $A_n \cdot T_n$ sequences of reversed orientation (n = 6 or 5, respectively), we conclude that this structural difference does not affect measurably the gross structure of the open complex characterized by the mean square distance between the ends of the template DNA. This confirms our earlier observation [24] that variation of the sequence dependent DNA bendability in the spacer region does not influence the gross structure of the open complex.

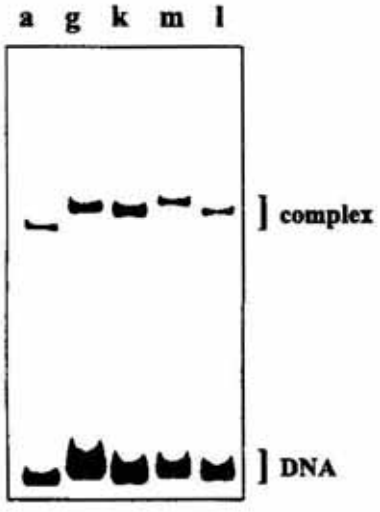


Fig. 2. Electrophoretic gel analysis of the open complexes formed by E. coli RNA polymerase on the **a**, **g**, **k**, **l**, *and* **m** *promoters located within the* ^{32}*P-labelled SalI-HinfI restriction fragments.*

The letters at the tracts denote the respective promoters studied; 3.8% polyacrylamide gel, TB-$MgCl_2$ buffer (90 mM Tris/borate, pH 8.3; 5 mM $MgCl_2$) at 30°C.

Table 4

Retardation parameters from the theoretical cyclic permutation analysis, according to De Santis SuperDNA program, for three types of restriction fragments carrying the studied promoters **a - o** *(cf. Fig.1; for sequences of promoters* **c, d, f** *and* **h** *see ref. [24]); R_{th}, R_{max} and R_{min} are the actual, maximal and minimal retardation values obtained from the permutation diagrams (cf. Fig. 3), respectively; $\Delta R = (R_{max} - R_{min})$.*

DNA fragment	Promoter	R_{th}	R_{max}	R_{min}	ΔR
SalI-HinfI	**a**	1.190	1.200	1.050	0.150
	b	1.494	1.513	1.066	0.447
	o	1.218	1.227	1.053	0.174
	n	1.218	1.230	1.053	0.177
	g	1.230	1.240	1.053	0.187
	e	1.344	1.360	1.056	0.304
	i	1.367	1.383	1.058	0.325
	k	1.170	1.170	1.053	0.117
	l	1.140	1.140	1.052	0.088
	m	1.187	1.190	1.055	0.135
	c	1.330	1.350	1.064	0.286
	d	1.227	1.240	1.058	0.182
	f	1.282	1.293	1.060	0.233
	h	1.225	1.244	1.058	0.186
*SalI-Pf1*MI	**a**	1.127	1.150	1.050	0.100
	g	1.157	1.187	1.060	0.127
	k	1.157	1.163	1.047	0.116
	l	1.135	1.140	1.042	0.098
	m	1.173	1.180	1.050	0.130
AluI-HinfI	**a**	1.053	1.061	1.038	0.024
	g	1.060	1.087	1.042	0.045
	k	1.047	1.049	1.034	0.015
	l	1.044	1.044	1.027	0.017
	m	1.051	1.056	1.037	0.019

Location of the downstream lying free DNA end in the open complex is determined by the RNAP-induced promoter bending, which in the case of the A1T7 phage promoter has been shown to be centered close to the start site [20, 44]. We have found recently by $KMnO_4$ footprinting of the open complexes of the **a–o** group of promoters formed in the presence of Mg^{2+} ions (Łoziński, T., Smagowicz, W.J. & Wierzchowski, K.L., unpublished) that T residues in this region of the template DNA strand are most susceptible to permanganate oxidation, what may indicate that also in this case the center of DNA bending is similarly located. The presence of DNA bending tracts in the adjacent spacer region apparently does not affect the RNAP-induced DNA bending and, thus, the location of the downstream DNA trajectory outside the complex. It seems thus very likely that more distantly located DNA-bending tracts in the –35 domain do not influence spatial disposition of the downstream DNA end, either. Therefore, the reason for lower mobility of the β complexes with promoters bearing the $A_n \cdot T_n$ runs in the –35 recognition domain could be sought in such a change in the DNA superstructure of the upstream lying free DNA end, which leads to lowering of the mean

end-to-end distance between the two free ends of the *Sal*I-*Hin*fI (–250 ...+166) DNA fragment. To verify this supposition we studied the effect of shortening of each of the two flanking free DNA strands of *Sal*I-*Hin*fI fragments on gel mobility of the open complexes. For this purpose we prepared *Alu*I-*Hin*fI(–54...+166) and *Sal*I-*Pfl*MI(–250...+57) restriction fragments carrying selected promoters (**a**, **g**, **k**, **l**, **m**, **n**, **o**). PAGE mobility of the complexes formed on the DNA fragment ending at the *Alu*I site (–54), adjacent to the promoter sequence covered by RNAP [20], proved identical for all the promoters investigated (Table 3), irrespective of the actual length and location of the DNA bending $A_n \cdot T_n$ sequence. The complexes formed on the same promoters contained within the *Sal*I-*Pfl*MI(–250 ...+57) fragment still exhibited gel retardation relative to the mobility of the control promoter **a**. This finding seems to support the notion that the observed retardation of the β complexes formed on the *Sal*I-*Hin*fI DNA fragments was due primarily to a changed disposition of the upstream lying free DNA arm, enforced by the presence of $A_n \cdot T_n$ sequences in the –35 domain.

The β form of complexes of all the promoters carrying the DNA-bending tracts in the –35 region was always accompanied by a small amount of a complex of PAGE mobility similar to that of the α complex formed at the control promoter **a**, as it has been also observed by us previously for other promoters of this class [24]. Simultaneous appearance of the two complexes was tentatively interpreted as originating from fast dynamic equilibrium between the unbent and bent DNA species in solution during formation of the RNAP-promoter complex or from thermal isomerization of the perturbed β complexes to their regular α counterparts. In order to verify this hypothesis we studied the temperature dependence of formation of the open complexes and thermal stability of the preformed complexes for a number of promoters of the studied series (**g**, **m**–**o**). We found that the relative amount of the β and α complexes was independent of the incubation temperature in the range of 10°C–56°C (not shown). Also incubation of the complexes preformed at 37°C, at 42°, 56° and 65°C for a prolonged period of time (from 10 to 120 min) led only to slow disappearance of both complexes without any change in their relative amounts (not shown). This indicated that the isomerization hypothesis should be discarded. We have then measured the relative content of the two complexes formed with different RNAP preparations (commercial from Boeringer and two obtained in this laboratory) and found substantial differences. It seems thus that the small admixture of the so called α complexes, of similar PAGE mobility as that of the open complex formed at the control promoter **a**, results probably from a heparin-resistant RNAP-promoter complex containing an additional protein component present in varying amounts in the RNAP preparations used.

Theoretical evaluation of promoter DNA bending and its relevance to the experimental PAGE data

To rationalize the observed variation in PAGE mobility among *Sal*I-*Hin*fI DNA fragments carrying different promoters of the **a**–**o** group we calculated the intrinsic DNA superstructures and related parameters corresponding to the experimental PAGE data with help of the De Santis *et al.* [29] theoretical model of DNA curvature and SuperDNA software. The validity and predictive power of this model have been amply demonstrated [45]. In particular, theoretical prediction of permutation gel electrophoresis results, based on this model, has been shown [42] to be a valid alternative to the experimental method [43, 46] used to locate the curved regions of DNA fragments. We applied the former method to evaluate DNA curvature induced by the studied group of promoters on the three free DNA restriction fragments: *Sal*I-*Hin*fI, *Sal*I-*Pfl*MI and *Alu*I-*Hin*fI. Illustrative permutation assays for each of these fragments are shown in Fig. 3 for the control promoter **a**, while the numerical data for retardation factors: R_{th}, R_{min}, R_{max}, and ΔR, defined in Fig. 3, for all the promoter fragments are compiled in Table 4. We calculated also curvature vectors, which represent in the complex plane (in modulus and phase) the directional change of the double helix axis [29] for respective DNA sequences. The thus derived writhings of the DNA double helix axis, visualized as pairs of stereoprojections of a given promoter DNA fragment, are depicted in Fig. 4 for selected promoters.

The theoretically predicted retardation factors, R_{th}, for the *Sal*I-*Hin*fI free DNA fragments

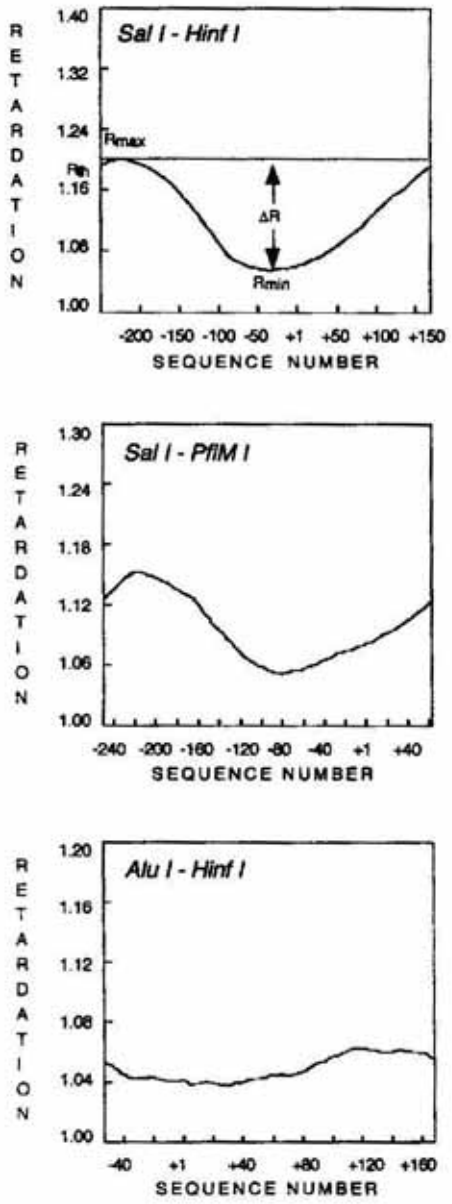


Fig. 3. Cyclic permutation diagrams obtained with help of De Santis SuperDNA program for the three types of restriction fragments carrying promoter **a**; *sequence numbers relative to the transcription start site.*

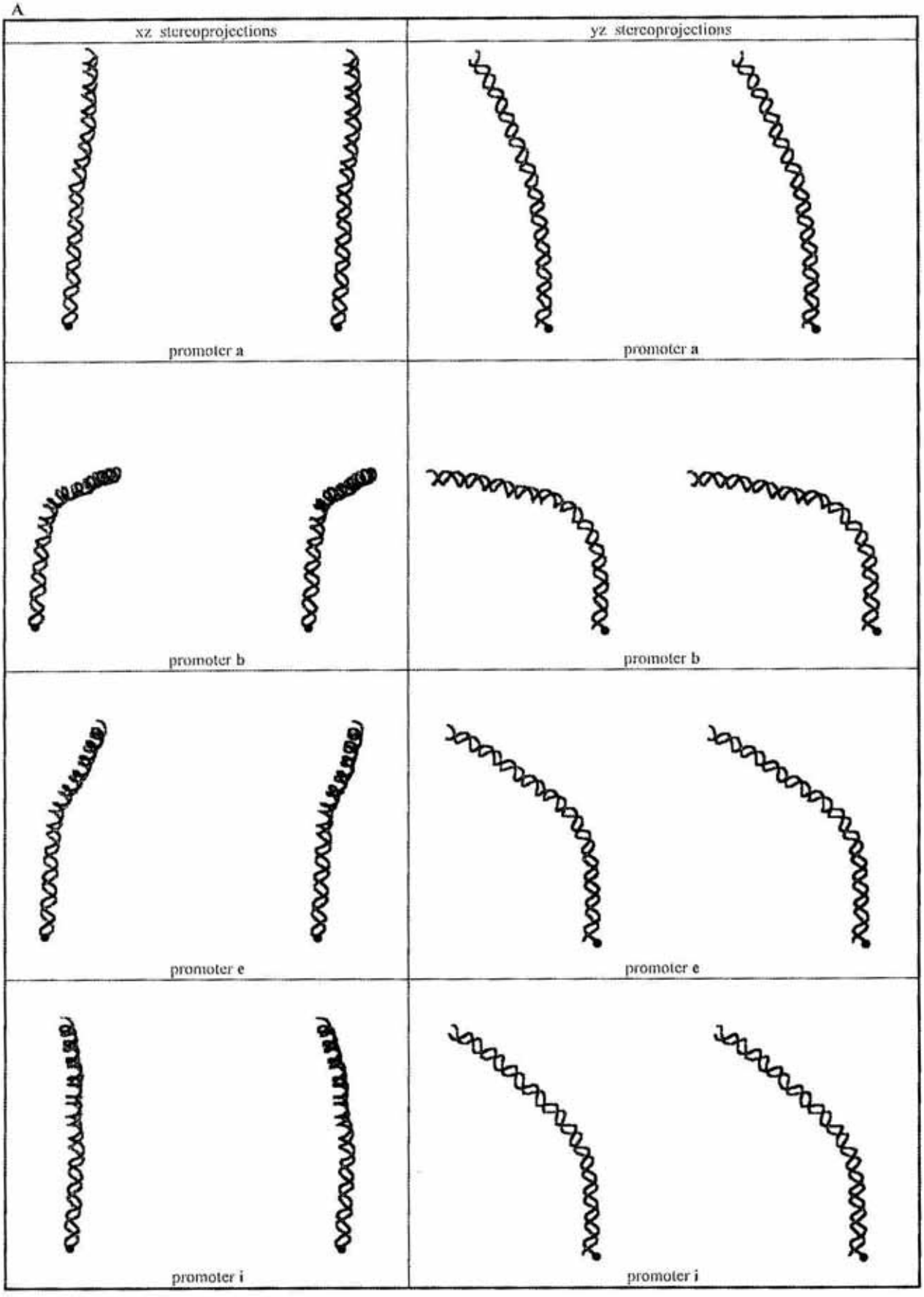
The minimum, R_{min}, and maximum, R_{max}, values of retardation correspond to molecules having the locus of curvature nearest their end and center, respectively; R_{th} is the retardation corresponding to location of a promoter as depicted in Fig. 1.

containing all the so far studied promoters (**a**–**h**, and **i**–**o**) proved to correlate linearly with a reasonable accuracy (correlation coefficient 0.95) with those measured by PAGE (Table 2). In view of this agreement between the experimental and theoretical R factors for the *Sal*I-*Hin*fI fragments, the theoretically predicted values of R_{th} for the two groups of shorter restriction DNA fragments, as well as the other calculated curvature parameters can be regarded as closely reproducing the behaviour of the studied DNA's.

The parameter ΔR, i.e. the difference between the maximum and minimum values of R on the R *v.* (sequence number) plot (Fig. 3), corresponding to molecules having the locus of curvature nearest their center and end, respectively, measures the magnitude of the intrinsic curvature of a whole DNA fragment [46]. Inspection of the calculated ΔR and R_{th} data for *Sal*I-*Hin*fI DNA fragments (Table 4) shows that (i) extension of T_2(–35, –34) tract in promoter **a** upstream of the –35 recognition domain is accompanied by a steady small increase in the intrinsic DNA curvature (promoters **o**, **n**, **g**); (ii) two $A_n \cdot T_n$ sequences in the spacer region located in phase with the helical symmetry double the curvature of promoter **a**, irrespective of their orientation (promoters **e** and **i**); and (iii) four such bending sequences (promoter **b**) give rise to about three times higher curvature. All these changes in DNA curvature show up more vividly in steroprojections of the writhing of the double helix axis within promoter sequences (Fig. 4).

The *Sal*I-*Hin*fI fragment carrying only sligthly bent control promoter **a** (cf. Fig. 4A), exhibits also a curved superstructure (cf. R_{th} and ΔR parameters in Table 4, what could be expected because of the presence of two A_4 and T_4 tracts upstream of the promoter *Xho*I site and of an A_4 one located downstream of the promoter *Eco*RI site (cf. Fig. 1). This was fully confirmed by the results of theoretical permutation analysis of *Sal*I-*Hin*fI fragments with deleted two bending sequences upstream of the *Xho*I site: 64 and 74 bp long deletions led to a large decrease in the magnitude of R_{th} and ΔR parameters (data not shown). Somewhat lower overall curvature for the *Sal*I-*Hin*fI fragments with promoters **k**, **l**, or **m** (Table 4), cloned in the place of promoter **a**, indicated also by corresponding experimental gel retardations (Table 2), is caused by the presence of a 3 bp long insertion between the *Xho*I restriction site and the –35 hexamer (cf. Fig. 1). This insertion changes the phasing between the bending tracts located in the promoter –35 domain and the sequence upstream of *Xho*I site by about one quarter of the helical

A


xz stereoprojections
yz stereoprojections
promoter a
promoter a
promoter b
promoter b
promoter e
promoter e
promoter i
promoter i

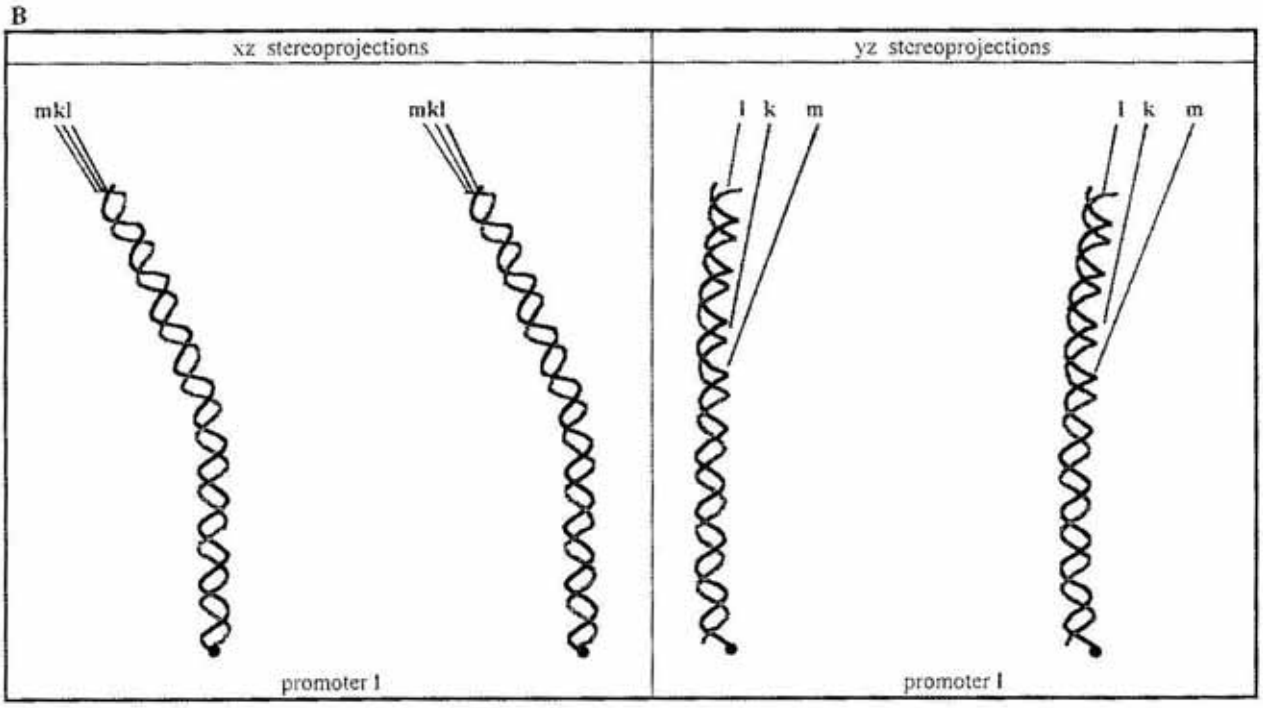


Fig. 4. Stereoprojections of the writhing of the double helix axis for selected bend promoters in two planes (xz and yz), calculated by using De Santis SuperDNA program; the filled circle indicates the 5′ end of the template DNA strand; for better visualization of differences in promoter DNA bending, (+21...–90) and (+5...–85) DNA fragments are shown for promoters **a**, **b**, **e** *and* **i** *(A), and the promoter* **l** *(B), respectively; solid lines in stereoprojections of promoter* **l** *indicate directions of the double helix axis upstream of the –35 hexamer for promoters* **k**, **l** *and* **m**.

turn thus increasing the mean square end-to-end distance between the two DNA ends.

To evaluate the expected effect of opposite orientation of $A_n \cdot T_n$ sequences on the direction of DNA bending in (**e**, **i**) and (**k**, **l**) promoter pairs, relative inclination of the double helix axes in stereoprojections of corresponding promoter fragments was compared. As it can be seen from Fig. 4A, in the xz plane the helix axis upstream of the –35 hexamer in promoters **e** and **i** is bent in somewhat different directions, while in the yz plane the inclination of this axis in both promoters is the same. In promoters **k** and **l**, contrary to expectations, inclinations of this axis upstream of the –35 region in both planes differ only slightly one from the other (Fig. 4B). This indicates that direction of DNA bending by a $A_5 \cdot T_5$ tract may depend also on its sequential context. Also contrary to expectations based on the higher rigidity of $A_n \cdot T_n$ sequences in the B′-DNA form [28], the $A_8 \cdot T_8$ sequence in promoter **m** proved to induce stronger bending of the helix axis than do $A_5 \cdot T_5$ sequences in related **k** and **l** promoters.

The theoretically predicted parameters of DNA curvature for *Alu*I-*Hin*fI (–54...+166) fragments (Table 4): very small ΔR and similar values of R_{th} and R_{min}, indicate very low writhing of the double axis consistent with localization of the $A_n \cdot T_n$ bending promoter sequences near the *Alu*I end of DNA's, so that all these fragments can be regarded as almost linear. The calculated data (cf. Table 4) clearly show that the *Sal*I-*Pfl*MI(–250...+57) fragments retain most of the curvature patterns of the parent *Sal*I-*Hin*fI DNA's, largely due to the highly curved and long arm upstream the –35 promoter domain, as discussed above.

In the light of the curvature characteristics of the studied DNA restriction fragments, the experimentally observed similar magnitude of PAGE retardation for all the β class open promoter complexes on *Sal*I-*Hin*fI fragments points to their similar gross structure, viz. comparable mean square end-to-end distance between DNA's termini. The latter is primarily governed by promoter curvature in the –35 domain induced by a $A_n \cdot T_n$ sequence and helical phasing between this sequence and the two $A_4 \cdot T_4$ sequences upstream of the –64 locus. The observed small differences in gel retardation between complexes formed on promoters **k**, **l**

and **m** reflect thus corresponding differences in local curvature of promoters in the –35 domain (cf. Fig. 4). The complexes formed on the *Sal*I-*Pfl*MI(–250...+57) fragments exhibited generally lower retardation, compared with their *Sal*I-*Hin*fI counterparts, owing to considerably shorter free DNA arm extending downstream out of the promoter region covered by bound RNAP. Provided that the total footprint of the RNAP molecule in the open complex spans promoter DNA from about –54 to +22 position, like in the case of A1T7 promoter [20], this arm would be about 3 helical turns long, compared with 14 turns of the arm ending at the *Hin*fI site. The lower experimental gel mobility of the complexes with promoters **g**, **k–m**, bearing $A_n \cdot T_n$ bending sequence in the –35 domain, is governed by the very same curvature vector properties of the upstream free DNA arm as in their *Sal*I-*Hin*fI counterparts. In the case of complexes formed on the *Alu*I-*Hin*fI fragments, the lack of any reporter arm extending upstream out of the promoter region covered by bound RNAP, and almost linear DNA located downstream of the promoter, fully explain similar gel mobility of the complexes with bent and not bent promoters.

Similar gross geometry of the α class complexes of promoters **e** and **i** with the spacer bent in different planes at approximately 40° [27], and that of the control promoter **a**, is most probably due to removal of the DNA bend in the spacer region upon promoter binding to σ^{70} subunit of RNAP. Hence, the trajectories of both free DNA ends outside the RNAP-promoter complex are for the most part determined by the specific interactions of σ^{70} subunit of the RNAP holoenzyme with the canonical –35 and –10 hexamers [1, 2, 20], and by RNAP induced bending of promoter DNA around the transcription start site [20].

CONCLUDING REMARKS

The results obtained in this work and in our earlier studies [23, 24] show that bending of a consensus-like synthetic *E. coli* promoter **a** by $A_n \cdot T_n$ sequences exerts a measurable effect both on its strength *in vivo* and gross structure of the open transcription complex *in vitro*, the magnitude of which depends on the length and location of the bending sequence. DNA bending in the –35 recognition domain brings about a decrease in the promoter strength *in vivo* and in the mean end-to-end distance between the free DNA ends of the transcription open complex *in vitro*. The largest effect on the promoter strength was observed when the bending T_n tract in the nontemplate strand was 4 or 5 bp long and included the T_2 doublet of the consensus TTGACA hexamer. No or very little concomitant variation in the gross geometry of the transcription open complex *in vitro* was observed, as judged from similar gel retardation of the complexes with respective promoters. DNA bending in the spacer region resulted also in a decrease in the promoter strength *in vivo*, but irrespective of its direction, it had no effect whatsoever on the gross geometry of the open complex *in vitro*.

According to the present model of *E. coli* RNA polymerase open transcription complex [9], deduced from the low resolution (23 Å) three-dimensional structure of the holo and core RNAP forms [8, 9], and DNase I and hydroxy radical footprinting results [1, 2, 20], an about 250 Å long promoter DNA (–54...+22) is wrapped around the polymerase and from about –10 to +20 goes through a closed channel (25 Å in diameter and 45 Å in length) within a thumb-like domain on the protein surface. Unfortunately, this model does not show location of the polymerase α, β, β′ and σ^{70} subunits. Nonetheless, knowledge of their amino-acid sequences and results of mutational studies allowed to postulate specific recognition of the promoter –35 hexamer by a helix-turn-helix motif of the σ^{70} subunit [10–13]. Furthermore, it has been shown recently [47] that the UP (upstream) element of the *E. coli* ribosomal RNA promoter *rrnaB* P1, rich in AT pairs and located in the –40...–60 region, comprises a third promoter recognition region interacting with the α subunit of RNAP. In the light of these models, the observed effects of promoter DNA bending on the gross geometry of the open complex and of promoter strength can be rationalized in the following way. The largest decrease in relative strength found for promoters bearing the T_4(–34...–37) and T_5(–34...–38) tracts suggests that these sequences may perturb subtle specific interactions between the –35 recognition promoter domain and the hypothetical helix-turn-helix motif of the σ^{70} subunit by enforcing repositioning of the DNA

helix on the RNAP surface. The crucial role of two adjacent T residues in the TTGACA -35 canonical hexamer is well documented by strong downmutations, which map preferentially in this region [2], and by chemical T→U replacements leading to substantial reduction of promoter activity *in vitro* [48]. Thus, promoter bending in the -35 domain may result in sub-optimal σ^{70}-promoter contacts. Provided that in our system the α subunit is to some extent also involved in promoter recognition, the latter can be simultanously perturbed by DNA bending in the -35 domain.

Formation of the open complex with promoters bearing a bend in the spacer region would require adjustment of the DNA writhing inherent to free DNA to that determined by promoter DNA wrapping around RNAP. The activation energy necessary for removal at room temperature of a 40° bend from the spacer, like in the case of promoters **e** and **i**, can be estimated [49] to be about 2 kT, that is of the order of magnitude of the thermal energy. Moreover, DNA bending by asymmetric phosphate neutralization [50] upon one-sided promoter binding by RNAP [49] may help to reduce this unfavourable energy, so that adjustment of a bent spacer to the RNAP surface during formation of the open complex could be expected fully feasible energetically. This should, however, affect thermodynamics of complex formation. Indeed, we have shown earlier [24] for the most strongly bent promoter **b** (cf. Table 4 and Fig. 4) that its affinity to RNAP is considerably lower and the rate of isomerization of the closed complex to its open form is much faster than for the linear promoter **a**.

Recent computer simulations, based on an elastic DNA model, have clearly demonstrated that sequence-directed and protein-induced bending may change dramatically the overall folding and twisting of circular or looped DNA supercoils (see for a review [51]), much in agreement with earlier electron microscopic observations [52]. Such changes suggest structural mechanisms whereby molecular interactions occurring in transcription can be regulated in the cell by specific base-pair sequences. They should be thus taken into consideration in any interpretation of effects of DNA bending on promoter strength *in vivo*.

In the light of the above discussion, it is obvious that a deeper explanation of the observed effects of DNA bending in the -35 recognition and spacer domains of the consensus-like promoter **a** would require a more detailed information concerning the extent and nature of protein-DNA contacts in the open complex of the parent promoter and its selected sequence variants, the influence of DNA supercoiling on these contacts, and knowledge of thermodynamic parameters of the open complex formation for the promoters with bends located in particular functional domains. Further studies in these directions are under way in this and in other laboratories.

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