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Effect of the sequence-dependent structure of the 17 bp AT spacer on the strength of consensus-like *E.coli* promoters *in vivo*

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ABSTRACT

Three *E.coli* promoters with the consensus sequences in the -35 and -10 regions and the 17 bp spacer made of random, heteronomous, and of both these classes of AT DNA simultaneously were constructed and cloned into plasmid pDS3. Electrophoretic gel mobilities of restriction fragments containing these promoters indicated that bending of the latter was proportional to the number of heteronomous AT DNA tracts. The strength of these promoters *in vivo* measured in relation to an internal transcriptional standard was shown to correlate well with gel mobilities of the respective restriction fragments and to decrease with the number of potential DNA bending sites encoded in the promoter structure.

INTRODUCTION

Promoter sequence data as well as promoter strength determination *in vitro* and *in vivo* for deletion mutants and consensus like synthetic promoters indicate that in all good *Escherichia coli* promoters the two highly conserved -10 and -35 hexameric regions, involved in specific recognition and binding of RNA polymerase, are separated by 17($\pm$1) base pairs (1). Formation of the open, transcriptionally active complex between a promoter and RNA polymerase results in topological unwinding of the DNA (2,3), whereas negative supercoiling of the template DNA promotes formation of the open complex (4-6) and alters the strength of many promoters (4-8). All these facts are interpreted at present (9,10) in terms of the necessity of proper mutual orientation of the -10 and -35 regions for initial topological recognition by the polymerase, and their ultimate strict positioning in the multistep process of the open complex formation. It could thus be expected that the sequence dependent variation in the spacer structure may affect to some extent thermodynamic and kinetic parameters of the transcription initiation and hence also the promoter function. Indeed, it has been demonstrated recently (11) that substitution of the spacer DNA of P_{RM} lambda phage promoter by a stretch of 9 GC base pairs in either orientation brings about a 2-3 fold reduction of the promoter strength. However, similar substitutions with alternating and heteronomous AT DNA tracts were accompanied by but a small increase in the promoter strength, considered by the authors as insignificant.

With the aim to further investigate the sequential pathway to open complex formation involving two isomerization steps, elucidated so far only for the *lac* UV5 (9) and P_R (12)

promoters, and in particular, possible effects of the sequence-dependent spacer DNA structure thereon, we constructed three consensus-like promoters with 17 bp spacer made of random, heteronomous, and of both these classes of AT DNA fragments. In this paper, we describe their functional properties and large differences in the promoter strength determined *in vivo*, relative to an internal standard, according to the method of Deuschle *et al.* (13).

MATERIALS AND METHODS

Materials

E.coli C600 and DZ291 strains, pDS3 plasmid and derivatives of bacteriophage M13, containing fragments of *bla* and *dhfr* genes were kindly provided by H.Bujard. *E.coli* RNA polymerase was purified by the method of Burgess and Jendrisak (14), except that Bio-Gel A 5m was replaced by Sephacryl S300. Radioactive ^{32}P-, ^{3}H-isotope-labelled chemicals were from Amersham, while enzymes were from the following firms: Biolabs (T4 polynucleotide kinase, T4 DNA ligase), Boehringer-Mannheim (S1 nuclease, calf intestinal alkaline phosphatase, Asp700I, EcoRI, HindIII, XhoI), Pharmacia (HinfI, SalI) Sigma (ribonuclease A). All other chemicals were of reagent grade.

Synthesis and cloning of the promoters

Each of the three promoters synthesized (Fig.1) was planned to be made of four oligomers and to have at the 5' end and 3' end XhoI and EcoRI overhanging sites, respectively. The oligomers were synthesized manually by the solid phase phosphoramidite method (15,16). They were purified by polyacrylamide-urea gel electrophoresis followed by DEAE-Sephacel column chromatography. Oligomers with the 5' end occurring inside the planned promoter sequence were phosphorylated by polynucleotide kinase, annealled with complementary ones and ligated with the use of T4 DNA ligase. The duplex formed was again purified by polyacrylamide gel electrophoresis followed by DEAE-Sephacel column chromatography. The duplexes were cloned into the pDS3 plasmid (Fig.2) previously digested with XhoI and EcoRI enzymes, and then transferred into *E.coli* JM 101. Recombinants were selected by chloramphenicol resistance. Those with proper SalI/EcoRI restriction patterns were sequenced. Sequencing was done directly on denatured closed circular plasmid by the method of Sanger.

Run-off transcription

RNA polymerase (0.2 pmol) and DNA (0.2 pmol) were preincubated in 0.020 ml of the transcription buffer (120 mM KCl, 10 mM $MgCl_2$, 20mM Tris-HCl pH 8.0, 1 mM dithiothreitol, 5% glycerol) for 10 minutes at 37°C, then ribonucleoside triphosphates (final concentrations in the reaction solution: 0.3 mM ATP, 0.3 mM CTP, 0.3 mM GTP,

0.05 mM UTP + 0.5Ci/l [α-^{32}P]UTP) in 0.005 ml of the transcription buffer were added to start the reaction. After 10 minutes the reaction was stopped either by adding an equal volume of the electrophoresis loading buffer (95% formamide, 45 mM Tris-borate, 2mM EDTA, dyes) or by phenol extraction and ethanol precipitation. The transcripts were resolved on 6% polyacrylamide-urea gel.

Mapping of transcription startsites

In this experiment XhoI-Asp700I restriction fragments containing cloned promoters were labelled at the Asp700I 5' end with ^{32}P by kination. The DNA probe (0.1 pmol) and complementary RNA formed on plasmids *in vitro* (0.02 pmol plasmid) or *in vivo* (isolated from 0.3 ml culture at A_{600}=0.4) were hybridized in 0.020 ml buffer (400 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 80% formamide) at 45°C for 6 hours following incubation at 85°C for 10 minutes. The hybridization solution was mixed with 0.2 ml ice-cold buffer (400 mM NaCl, 50 mM AcONa pH 4.8, 2 mM $ZnSO_4$) containing 100 units of S1 nuclease, and then was transferred to 20°C water bath. The digestion was stopped by phenol extraction and ethanol precipitation. The DNA probe protected from digestion was run on 6% polyacrylamide-urea gel along with products of the Maxam-Gilbert sequence reactions.

Determination of promoter strength *in vivo*

The strength *in vivo* of the promoters studied was determined according to Deuschle *et al.*(13). In this method the promoter under investigation controls transcription of the coding sequence of the mouse dihydrofolate reductase gene (*dhfr*). The quantity of *dhfr*-specific RNA synthesized is compared with that of an internal standard - ß-lactamase (*bla*) specific RNA which is transcribed from the same plasmid under the control of its own P_{bla} promoter (cf Fig.2). In our experiments RNA was labelled *in vivo*, isolated and subjected to hybridization exactly as described by (13), except that 20 mg/l chloramphenicol was used instead of ampicillin, and [5,6-^{3}H]uridine was added to cultures at A_{600}=0.4. In determination of the strength of the promoters we assumed that the half-life times of the *dhfr* transcripts investigated by ourselves and of those dealt with by Deuschle *et al.* (13) are similar, as we have not introduced any long sequences, and only a very few bases at the beginning of the 5' end of *dhfr* transcript. To be able to compare the strength of our promoters with the published data (13) we labelled RNA *in vivo* for only 1 minute - a period shorter than the half-life time (~ 1.5 min.) of *bla* and *dhfr* transcripts. Certainly, the obtained order of the promoter strength should not be influenced at all by the stability of RNA *in vivo*, as transcripts from all these promoters are of identical sequence and length. The only problem in evaluating the strength that we have come across, is that noted in (13), i.e., that the promoter strength determined from independent cultures varied up to 15%. Variations in the case of independent RNA preparations from the same culture in our experiments did not exceed 3%.

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          XhoI    / -35 /     spacer     / -10 /     * EcoRI

(a)  CTCGAGTTATTGACAATTATTTATTTATTATTTATAATTATTTAATTGAATTC
(b)  CTCGAGTTTTTGACAATTTTTTATATATTTTTTATAATTTTTTAATTGAATTC
(c)  CTCGAGTTTTTGACAATTTTTTTTTTTTTTTTTATAATTTTTTAATTGAATTC
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Fig.1. Sequences of synthetic promoters. The /+1/ transcription startsite proved experimentally (cf Fig. 4 and 5) is indicated with an asterisk. Runs of T bases, long enough to cause DNA bending at their boundaries are underlined.

RESULTS AND DISCUSSION

Planning sequences of consensus-like *E.coli* promoters we intended to encode pronounced differences in their strength, while retaining RNA polymerase-promoter specific interactions defined by the -10 and -35 consensus hexamers and the consensus spacer length of 17 bp. In view of the proposed (9) involvement of the spacer DNA itself in isomerization of the closed RNA polymerase-promoter complex to the open transcriptionally active one, the spacer seemed to be the most logical candidate for sequence modification. The heteronomous poly(dA)·poly(dT) DNA having rather unusual structural features e.g. decreased helical repeat, pronounced propeller twist and inability to undergo B⟶ A transition (17) seemed very promising in this respect. Moreover, $(dA)_n \cdot (dT)_n$, $n \geq 4$ tracts were shown (17,18) to be the cause of DNA intrinsic bending beginning at the junctions between these tracts and adjacent orthodox sequences. The overall DNA bending is most pronounced in the cases when n is 5 or 6 and such tracts are repeated and phased with helical periodicity. Thus, we introduced a tract of $(dA)_{16} \cdot (dT)_{16}$ between the -12 and -29 position of the spacer with a dA in the -29 position (cf promoter **c** in Fig.1). Next we decided to alter it by inserting an ATATA stretch between the -17 and -23 positions to

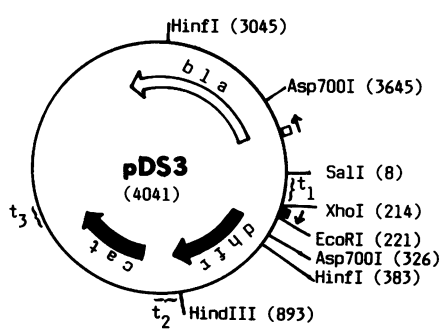

Fig.2. Schematic map of plasmid pDS3. Promoter cloning site and P_{bla} promoter are denoted as a black and a white square, respectively. The directions of transcription from both promoters are indicated by arrows; t_1 and t_2 and t_3 denote transcription terminators; structural regions of the genes used in transcription experiments are abbreviated: *bla*, *cat* and *dhfr*. Positions of restriction sites employed are given in parentheses.

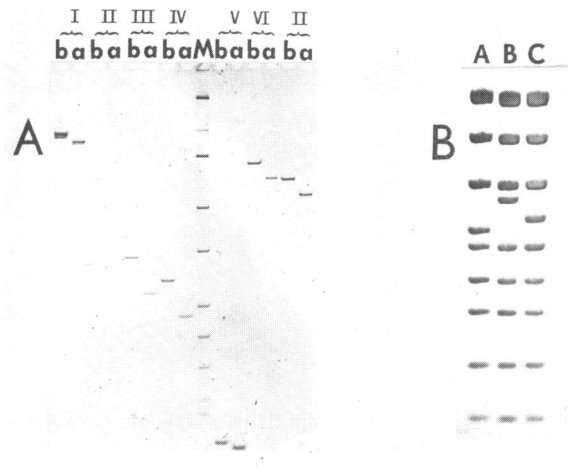

Fig. 3. Electrophoretic gel analysis of mobilities of DNA restriction fragments containing synthetic promoters.
<A> 6% polyacrylamide gel run at room temperature in 40 mM Tris-HCl, 20 mM AcONa, 2 mM EDTA, pH 7.8; stained with ethidium bromide. Lanes marked /a/ and /b/ refer to fragments containing promoters **a** and **b**, respectively. Pairs of lanes, with size of the fragments in bp in parentheses: /I/ HinfI-HinfI(1315), /II/ Asp700I-Asp700I(772), /III/ SalI-HinfI(415), /IV/ SalI-Asp700I(358), /V/ XhoI-Asp700I(152), /VI/ SalI-HindIII(925). Lane /M/ - DNA size marker (3611, 1166, 606, 517, 396, 318, 263, 222, 186, 141).
<B> 5% polyacrylamide gel run at room temperature in 40mM Tris-acetate, 2mM EDTA, pH 8.3; stained with ethidium bromide. Lanes marked A, B, C, show restriction pattern of SalI/HinfI digest of the pDS3 plasmid containing the promoter **a**, **b** or **c**, respectively. The fragments containing these promoters are positiond in lanes A-C at different height.

encode an additional potential bending site within the spacer (cf promoter **b** in Fig.1). It should be noted that these promoters contain two additional identical bending sites outside the spacer region (cf. legend to Fig.1) kept in phase with those inside the spacer, adding to the overall DNA bending, which may also contribute to the promoter-RNA polymerase interactions. For the control promoter we have chosen a random AT sequence DNA avoiding any additional TATAAT boxes and any bending sites (cf promoter **a** in Fig.1). All three promoters were synthesized and cloned into the pDS3 plasmid (Fig.2) as described under MATERIALS AND METHODS.

In the light of recent work (18), DNA bending can be easily assessed by measurements of its electrophoretic mobility in polyacrylamide gel. From our electrophoretic experiments (Fig.3A and 3B) conducted for various restriction fragments it can be seen that the fragments containing promoter **b** exhibit abnormally slow mobility in comparison to analogous fragments containing either promoter **a** or **c**. The presence of the latter imposes also slower mobility on respective restriction fragments when compared with

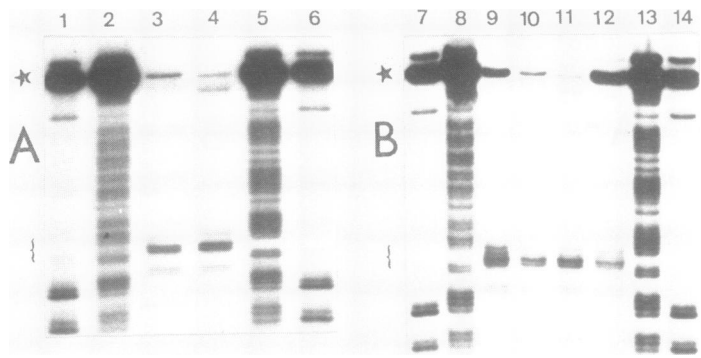

Fig.4. Mapping of the 5' ends of transcripts with S1 nuclease and radiolabelled DNA probes. Asterisks indicate the full-length DNA probes. Brackets indicate fragments of the DNA probes protected from S1 nuclease digestion for 45 minutes - panel <A> and for 30 minutes - panel <B> by transcripts formed *in vitro* - lanes: 3, 4, 9 and 12, and formed *in vivo* - lanes: 10 and 11. As a size marker for the protected fragments the same DNA probes were cleaved in sequence reaction for guanine - lanes: 1, 6, 7, 14; and for guanine and adenine - lanes: 2, 5, 8 and 13. The DNA probe containing promoter **a** was used in lanes: 1, 2, 3, 7, 8, 9 and 10, and containing promoter **b** - in lanes: 4, 5, 6, 11, 12, 13 and 14.

those containing the control promoter **a**. The lower electrophoretic mobility of promoter **b** and **c** bearing fragments, as well as the difference in their mobility, correlates well with the number of potential bending sites encoded in their sequence.

The transcription startsites of promoters **a** and **b** *in vitro* and *in vivo* were mapped with S1 nuclease (Fig.4A and 4B). *In vitro*, the transcription startsites of promoter **c** were found by comparing the length of run-off transcripts from promoters **c** and **a** (data not shown). It can be concluded from these experiments that, although we observed heterogeneity of startsites, transcription started at the same bases on all these three promoters both *in vitro* and *in vivo*; moreover, these bases were the startsites expected from the consensus promoter sequence. Additional information gained from run-off transcription experiments is that there is no up-stream transcription from any of the promoters investigated as shown for promoter **a** and **b** in Fig.5. Even if a small number of run-off transcripts were synthesized up-stream from promoter **a** or/and **b**, the transcripts should have been visible somewhere in the middle of the gel (Fig.5) as bands in lanes 1 and 2 at a common level or/and in lanes 3 and 4 analogously, because the DNA fragments used as the templates have common ends up-stream of the promoters.

The strength of promoters **a**-**c** determined *in vivo*, in two *E. coli* strains C600 and DZ291, expressed in *bla* units, is given in Table 1 along with that reported for some natural and synthetic promoters (13). Fortunately enough, the strength of promoters **a** and **b** happened to be similar to that found in the same DZ291 strain for both consensus

Fig.5. Electrophoretic 6% polyacrylamide-urea gel analysis of radioactive run-off transcripts formed on the ^{32}P end labelled DNA restriction fragments. On the right side of the photograph bands of radiolabelled DNA templets are marked with asterisks and radiolabelled transcripts are marked with arrows. Lanes 1 and 2 correspond, respectively, to SalI-Asp700I and SalI-HinfI fragments containing promoter **a**, while lanes 3 and 4 correspond to analogous fragments containing promoter **b**.

Table 1. The relative strength of promoters *in vivo* expressed in *bla* units. *E.coli* C600 and DZ291 strains were used as the hosts. Each value represents an average obtained from at least four independent determinations; the standard deviation did not exceed 10 %.

	C600	DZ291	
a	7.8	11.	this work
b	2.7	2.4	this work
c	5.6	--	this work
lac	--	5.7	ref. 13
lac UV5	--	3.3	ref. 13
tacI	--	17.	ref. 13
con	--	4.	ref. 13

synthetic promoters: *con* and *tac* I, and to some of the natural ones like *lac* and *lac* UV5. The life time of their complexes with RNA polymerase can thus be expected to be sufficiently long for planned steady-state kinetic studies on abortive transcription *in vitro*. The differential strain effect observed in activity ratio (cf. Tab.1) between promoter **a** and **b** is larger than the experimental errors involved. It seems to be specifically connected with somewhat different organization of plasmid transcription in the two strains.

Decrease of the strength of the promoters in the order: **a** > **c** > **b** goes along with their electrophoretic mobilities on polyacrylamide gels (cf Fig.3A and 3B). Hence, the several fold difference in the strength between these promoters can primarily be attributed to the differences in the extent of their bending. The insertion of the ATATA stretch between the -17 and -23 positions, of the spacer of the promoter **c**, resulting in an additional bending of the middle part of the spacer DNA, led to a two fold decrease in its strength *in vivo*. Since that region is lacking demonstrated RNA polymerase contacts the decrease in the promoter activity can be attributed to an indirect but sequence-dependent effect of the spacer structure connected with spatial positioning of the promoter regions involved in specific interactions with RNA polymerase.

Auble *at al.* reported recently (11) that the strength *in vivo* and *in vitro* of P_{RM} lambda phage promoter bearing a substituted heteronomous $(dA)_9 \cdot (dT)_9$ sequence within the spacer was not affected at all. In their experiments the relative promoter strength was measured by determination of enzymatic activity of ß-galactosidase, the structural gene of which remained under control of the cloned promoter. Provided that the sensitivity of this method is similar to that of the method used in our work, the result obtained by Auble *et al.* could be compared with our promoter strength data. Thus it could be concluded that the presence of only one heteronomous AT DNA tract, imposing promoter bending, possibly also because of its improper location within the spacer, does not affect measurably the promoter strength.

What is obvious at present is that the very bendings limited to a small region of the spacer (when the promoter **b** is compared to promoter **c**) or encompassing the whole promoter region (when promoters **b** or **c** are compared to **a**) neither do stop the promoter function nor change the startsites. This remains in agreement with the earlier observations (19,20) that small changes (1-2 bp) in the spacer length do not affect position of the startsite. Although we observed that the strength *in vivo* of the promoters studied is lowered by DNA bending we can not exclude that a bending differently phased or in another direction could enhance the strength *in vivo*. Such a possibility was suggested by Dripps *et al.* (21) on the basis of the observed strong correlation between the promoter strength and curvature scores due to the potential bending sites in the up-stream regions of *E.coli* promoters.

In order to be able to distinguish the effect of the bending on the promoter strength,

originating from the presence of heteronomous AT DNA within the spacer and in the up-stream and down-stream promoter regions, it would be desirable to complement the present work with experiments on appropriately constructed promoters from the **a-c** promoter family.

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