Synthetic curved DNA sequences can act as transcriptional activators in *Escherichia coli*

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Can a transcriptional activator known to bend DNA be functionally replaced by a sequence-directed bend in *Escherichia coli*? To investigate this question, a partially truncated promoter was used, deleted of its −35 region and of its CRP binding site, leaving only two Pribnow boxes as functional elements. Synthetic and naturally occurring curved DNA sequences introduced upstream from these elements could restore transcription at either one of the two natural starts. Some of these hybrid promoters turned out to be more efficient than the CRP activated wild-type *gal* promoter *in vivo*. Control experiments performed with very similar sequences devoid of any curvature produced weak promoters only. Minimal changes in the location of the centre of curvature or perturbation in the amount of curvature strongly affected the level of expression. No significant stimulation of transcription could be detected *in vitro*. Furthermore, both *gal* P1 and P2 starts could be activated *in vivo* but also *in vitro* via a properly positioned CRP binding site. This partial analogy suggests that bending induced by the cAMP−CRP complex upon binding to its site may be biologically relevant to the mechanism of transcriptional activation.

Key words: bent DNA/transcription activation

Introduction

The sequence determinants of a prokaryotic promoter, i.e. the −10 and −35 regions, and, to a lesser degree, other sequences located further upstream or downstream, dictate its kinetics of association with RNA polymerase and the efficiency of mRNA initiation (Hawley and McClure, 1983; Deutschle *et al.*, 1986). A transcriptional activator is sometimes required to increase the synthesis of a given gene product. The catabolite activator protein (CRP) is a classical example. Complexed with cAMP, this protein binds to an upstream site in many *Escherichia coli* promoters to activate transcription (de Crombrugghe *et al.*, 1984). This binding has been shown to induce a bend in the DNA (Kolb *et al.*, 1983; Wu and Crothers, 1984). Does this bend have any functional role or is it just a non-significant consequence of CRP binding? In other words, could bent DNA *per se* have any effect on transcription? In the case of CRP activation, this hypothesis has already been formulated on the basis of rather indirect arguments (Wu and Crothers, 1984; Busby and Buc, 1987; Busby *et al.*, 1987). More generally,
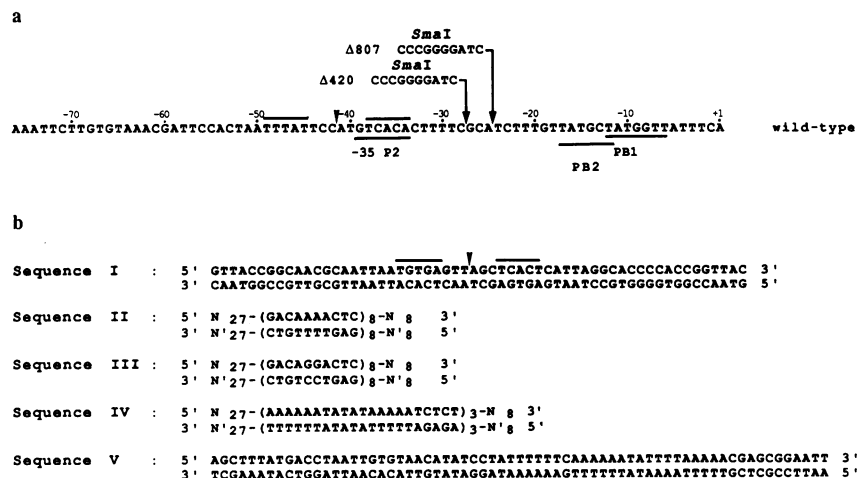
stretches of poly(dA), known to cause DNA bending, have been reported upstream of strong promoters (Galas *et al.*, 1985; Plaskon and Wartell, 1987) and in some cases mutation analysis or genetic selection indicated that they were important for promoter strength (Banner *et al.*, 1983; Lamond and Travers, 1983; Bossi and Smith, 1984; Gourse *et al.*, 1986; Bauer *et al.*, 1988; McAllister and Achberger, 1988, 1989). To address a similar issue, we decided to carry out more drastic experiments. The *E.coli gal* control region was deleted of its −35 region and of its CRP binding site located at −40. It was first shown that the whole upstream part of this sequence could be efficiently replaced by inserts containing the lactose CRP binding site. Reactivation of one of the two overlapping promoters occurred in a cAMP dependent manner and was optimal when the centre of the site was positioned with respect to the transcription start as in the original *lac* promoter (at −60, i.e. two helix turns upstream from the *gal* CRP binding site). In a second step, similar constructs were made using various synthetic and natural DNA sequences known to be curved. Our results indicate that some of these sequences could promote transcription better than the original CRP activated wild-type promoter *in vivo*. This enhancement of transcription may represent an important biological role played by curved DNA sequences.
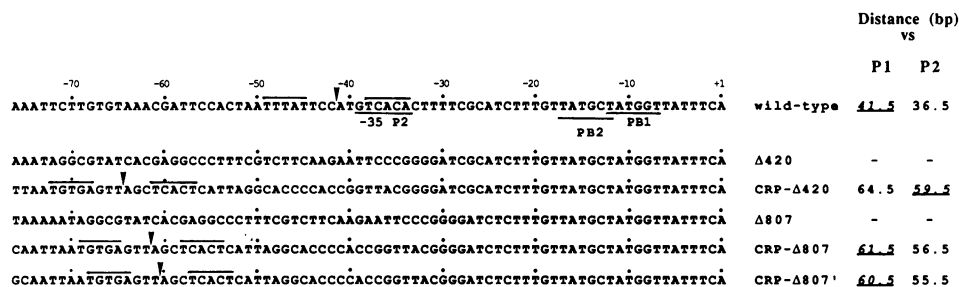
Results

We used the *gal* promoter of *E.coli* for our study. The organization of this promoter is shown in Figure 1a. It is actually composed of two overlapping promoters P1 and P2, both active *in vivo*. The −35 sequence at P2 is weak and the corresponding region for P1 strongly deviates from the consensus. The CRP protein, upon binding to a site centred at −41.5 bp from the P1 start, stimulates transcription *in vivo*, inhibiting P2 and activating P1. The presence of two Pribnow boxes, separated by 5 bp, increased our initial chances of correctly positioning potentially crucial elements with respect to these two promoters.

Construction of the synthetic promoters

In the plasmid pAA187, the *gal* promoters control the expression of the structural genes of the lactose operon. We used deletions which created a *Sma*I site and removed upstream sequences either from the −24 (Δ807) or from the −27 (Δ420) position (+1 being the P1 start of transcription, see Figure 1a) (Busby *et al.*, 1983). Sequences inserted in the *Sma*I site of Δ420 and Δ807 are listed in Figure 1b. We chose the *lac*−CRP binding site (sequence I) to check whether both truncated *gal* promoters, P1 and P2, could be activated by another CRP binding site. Sequences II and III consist of tandem repeats (× 8) of a 10mer, flanked by residual pBR322 sequences. This 10mer is either 4A10: 5′-GACAAAACTC-3′ (which has been designed to phase at 10 bp intervals consecutive dA stretches in the polymer)

a


```
                                          SmaI
                              Δ807  CCCGGGGATC┐
                                          SmaI
                              Δ420  CCCGGGGATC┐
                                                    ▼
     -70        -60        -50         -40        -30    ▼  -20        -10        +1
AAATTCTTGTGTAAACGATTCCACTAATTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCTATGGTTATTTCA   wild-type
                          -35 P2                              ‾‾‾‾‾‾‾‾PB1
                                                           PB2
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b

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Sequence  I    :  5'  GTTACCGGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCACCGGTTAC  3'
                  3'  CAATGGCCGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTGGCCAATG  5'

Sequence  II   :  5'  N 27-(GACAAAACTC)8-N 8    3'
                  3'  N'27-(CTGTTTTGAG)8-N'8    5'

Sequence  III  :  5'  N 27-(GACAGGACTC)8-N 8    3'
                  3'  N'27-(CTGTCCTGAG)8-N'8    5'

Sequence  IV   :  5'  N 27-(AAAAAATATATAAAAATCTCT)3-N 8    3'
                  3'  N'27-(TTTTTTATATATTTTTAGAGA)3-N'8    5'

Sequence  V    :  5'  AGCTTTATGACCTAATTGTGTAACATATCCTATTTTTTCAAAAAATATTTTAAAAACGAGCGGAATT  3'
                  3'  TCGAAATACTGGATTAACACATTGTATAGGATAAAAAAGTTTTTTATAAAATTTTTGCTCGCCTTAA  5'
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Fig. 1. (a) Sequence of the wild-type galactose promoter. The +1 position corresponds to the P1 start. The two Pribnow boxes PB1 and PB2 and the '−35 region' are underlined. The conserved motifs of the CRP site are overlined and the pseudo-axis of symmetry symbolized by a caret. The positions of the Δ420 and Δ807 deletions are also indicated. (b) Sequences inserted in the *SmaI* site of pAA187-Δ420 and pAA187-Δ807. Sequence I, the CRP binding site. The conserved motifs of the CRP site are overlined and the pseudo-axis of symmetry indicated by a caret. Sequence II, (4A10)$_8$ polymer flanked by residual pBR322 sequences (N$_{27}$ and N$_8$). Sequence III, (1A10)$_8$ polymer flanked by residual pBR322 sequences (N$_{27}$ and N$_8$). Sequence IV, the 5A/6A Ulanovsky sequence flanked by residual pBR322 sequences (N$_{27}$ and N$_8$). Sequence V, the upstream *Spo*VG element.


```
                                                                          Distance (bp)
                                                                               vs

                                                                          P1     P2
     -70        -60        -50        -40        -30        -20        -10        +1
AAATTCTTGTGTAAACGATTCCACTAATTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCTATGGTTATTTCA  wild-type  41.5  36.5
                          -35 P2                              PB1
                                                           PB2

AAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCCCGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA  Δ420       -     -

TTAATGTGAGTTAGCTCACTCATTAGGCACCCCACCGGTTACGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA  CRP-Δ420  64.5  59.5

TAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCCCGGGATCTCTTTGTTATGCTATGGTTATTTCA  Δ807       -     -

CAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCACCGGTTACGGGGATCTCTTTGTTATGCTATGGTTATTTCA  CRP-Δ807  61.5  56.5

GCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCACCGGTTACGGGGATCTCTTTGTTATGCTATGGTTATTTCA  CRP-Δ807'  60.5  55.5
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Fig. 2. Sequence of the starting deletion promoters and of the *lac*−CRP hybrid promoters. The conserved motifs of the CRP site are overlined and the pseudo-axis of symmetry indicated by a caret. The distance (bp) corresponds to the interval between the centre of the CRP binding site to the P1 and P2 starts.

or 1A10: 5'-GACAGGACTC-3' (which serves as a negative control). Sequence II is strongly curved as detected by its abnormal migration within a polyacrylamide gel matrix contrary to sequence III which showed no sign of curvature (Diekmann, 1987). Sequence IV is a tandem repeat (× 3) of 21 bp studied by Ulanovsky and colleagues which contains alternating stretches of 5 and 6 dAs at 10.5 bp intervals (Ulanovsky *et al.*, 1986). Measurements of k factors (ratio of the apparent length of a DNA fragment as deduced from its mobility to its actual length) indicated a more intense curvature for this sequence than for sequence II (Diekmann, 1987). Sequence V represents an AT rich region found upstream of the *Spo*VG promoter in *Bacillus subtilis*, which has been shown to be an important determinant of this promoter and to migrate abnormally in electrophoresis (Banner *et al.*, 1983). This sequence was only inserted in Δ420. The blunt end cloning procedure was used to generate plasmids with sequences inserted in the two possible orientations.

In vivo strength of the hybrid gal promoters

The lac−CRP constructs. Three synthetic promoters were characterized for this study. The corresponding sequences from +1 to −76 are shown in Figure 2. Two of these promoters, CRP-Δ807, CRP-Δ420 were expected from our

Table I. β-Galactosidase activities associated with the *lac*−CRP hybrid promoters

	β-Galactosidase (−cAMP)	β-Galactosidase (+2 mM cAMP)	Activation[a]	Distance[b]
gal wild-type	1240	4700	3.8	41.5 (P1)
Δ420	520	310	0.6	−
Δ807	820	490	0.6	−
CRP-Δ807	1120	4340	4.0	61.5 (P1)
CRP-Δ807'	1035	5520	5.3	60.5 (P1)
CRP-Δ420	420	2530	6.0	59.5 (P2)

[a]Defined as the ratio of the β-galactosidase units in the presence and the absence of cAMP.

[b]Defined as the interval between the centre of the CRP binding site and the transcription start of the promoter indicated in parenthesis.

original design. The third promoter, CRP-Δ807' is a mutant of CRP-Δ807 having a 1 bp deletion at position −30 (part of the *SmaI* cloning site). These plasmids were used to transform a Δ*lac cya* strain. β-Galactosidase assays were performed in the absence or presence of cAMP in the growth medium to measure the *in vivo* strength of these promoters as well as the extent of activation by the cAMP−CRP complex (Table I). These constructs could be activated by the cAMP−CRP complex by roughly the same factor (4−6
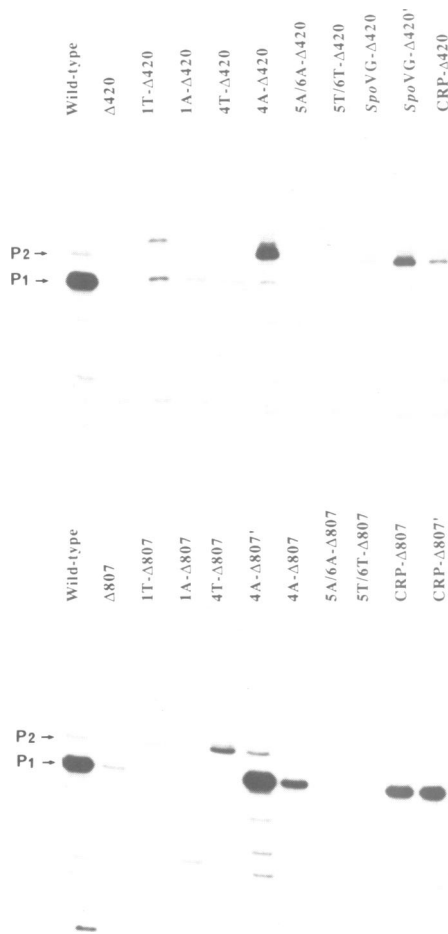
Fig. 3. Analysis of the *in vivo* transcripts: origins and quantitation of the mRNA products. The bands corresponding to a P1 or P2 start of transcription are indicated. See Materials and methods for the reaction conditions.

times). The start and the amount of the mRNA transcripts originating from these hybrid promoters were then determined by a primer extension method using total RNA synthesized *in vivo* in a parent *cya*⁺ strain, M182*recA* (see Materials and methods for genotype) (Figure 3). For each construct, one or two main bands are observed corresponding either to a P1 or to a P2 start. CRP-Δ807 and CRP-Δ807′ corresponded excusively to a P1 start whereas P2 was utilized in CRP-Δ420. Thus, both truncated promoters could be activated by the cAMP−CRP complex through a properly positioned binding site (59.5−61.5 bp instead of 41.5 in the wild-type P1 *gal* promoter).

The bent DNA sequences. The sequences of the hybrid promoters constructed with these curved DNA fragments are listed in Figure 4. The corresponding plasmids were used to transform M182 *recA*. Phenotypes and β-galactosidase activities associated with each construct are indicated in Table II. The plasmids carrying the deleted promoters, Δ420 and Δ807, gave a Lac⁻ phenotype of MacConkey lactose indicator plates, whereas the wild-type *gal* promoter corresponded to a Lac⁺ phenotype. 4A-Δ807 and 4A-Δ420 strongly promoted β-galactosidase expression (91 and 142% respectively of the wild-type activity). Their corresponding controls 1A-Δ807 and 1A-Δ420 produced weak promoters. The two other promoters originating from the 4A10 motifs,

4T-Δ420 and 4T-Δ807, gave intermediate levels of β-galactosidase (42 and 29%). Nevertheless, 4T-Δ807 was five times more active than its control 1T-Δ807, whereas 4T-Δ420 and 1T-Δ420 gave identical results. Interestingly, the Ulanovsky sequence in each of the four plasmids, 5A/6A-Δ420, 5T/6T-Δ420, 5A/6A-Δ807 and 5T/6T-Δ807 were totally inefficient as upstream promoter elements (∼ 10% of the wild-type activity). Similarly, *Spo*VG-Δ420 could not restore a Lac⁺ phenotype (20% of the wild-type activity). Thus, a subset of the curved DNA sequences appeared to be responsible for a strong promoter activity. The origin and the amount of the transcripts were again determined by the primer extension method (Figure 3). The relative intensities of the extended chains were in qualitative agreement with the results of the β-galactosidase assay considering the three times better translational efficiency of the mRNA originating at P2 versus P1 (Queen and Rosenberg, 1981; Dreyfus *et al.*, 1985). Since the plasmid copy number did not appreciably change among all the constructs, we attributed the observed stimulation by some of the curved sequences to a direct transcriptional effect. Both promoters could then also be specifically and strongly activated by curved DNA sequences: P1 in 4A-Δ807, P2 in 4A-Δ420 and to a lesser extent in 4T-Δ807.

The proper location of the bent DNA sequences is a highly sensitive parameter for this upstream activation

We were able to isolate two mutants, 4A-Δ807′ and *Spo*VG-Δ420′, in which a single base pair deletion was fortuitously created at the *Sma*I junction (see Figure 4). The effect of this deletion on the *in vivo* strength of these two promoters was remarkable, increasing their efficiency by a factor of 3−4.5 (Table III). 4A-Δ807′ now corresponded to a promoter almost three times as active as the CRP induced wild-type *gal* promoter and *Spo*VG-Δ420′ was nearly as efficient as wild-type. The mRNA analysis revealed that 4A-Δ807′ was still utilizing *gal* P1 and that *Spo*VG-Δ420′ originated transcription at P2 (Figure 3).

Effect of the DNA superhelicity in vivo on the activation by curved sequences

The *in vivo* strengths of the wild-type and some hybrid promoters were tested in a top⁻ and a gyr⁻ genetic background by performing β-galactosidase assays (Table IV).

Activation by the curved sequences consisting of the 4A10 polymer (in 4A-Δ807 and 4A-Δ807′) and of the *Spo*VG upstream element (in *Spo*VG-Δ420 and *Spo*VG-Δ420′) was observed in the three different genetic backgrounds. In the gyr⁻ allele, no significant difference in the amount of activation was observed. With the top⁻ allele, the extent of the stimulation was significantly modulated by the degree of superhelicity only in the case of *Spo*VG-Δ420 and *Spo*VG-Δ420′, where an increase in negative supercoiling weakened the activation by 60%.

The activation of transcription by the (4A10)₈ motif could not be reproduced in vitro

We attempted to reproduce our *in vivo* data, using linear DNA templates *in vitro*. Significant activation of run-off transcription starting at the expected origin was observed for CRP-Δ420 and CRP-Δ807. However, no stimulation of transcription could be detected with 4A-Δ420 and 4A-Δ807′

Distance (bp)
vs

P1 P2

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           -70        -60        -50      -40        -30        -20        -10      +1
      AAATTCTTGTGTGTAAACGATTCCACTAATTTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCTATGGTTATTTCA  wild-type    -  -
                                              -35  P2                      PB1
                                                              PB2

      AAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCCCGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA   Δ420         -  -

      ACTCGACAAAACTCGACAAAACTCGACAAAACTCGATAAGCTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    4A-Δ420     46 41

      ACTCGACAGGACTCGACAGGACTCGACAGGACTCGATAAGCTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    1A-Δ420      -  -

      TTGTCGAGTTTTGTCGATGATAAGCTGTCAAACATGAGAATTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    4T-Δ420     65 60

      CTGTCGAGTCCTGTCGATGATAAGCTGTCAAACATGAGAATTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    1T-Δ420      -  -

      TATCCTATTTTTTCAAAAAATATTTTAAAAACGAGCGGAATTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    SpoVG-Δ420  46 41

      ATATCCTATTTTTTCAAAAAATATTTTAAAAACGAGCGGAATTGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    SpoVG-Δ420' 45 40

      TATAAAAATCTCTAAAAAATATATAAAAATCTCTGATAAGCTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    5A/6A-Δ420  48 43

      ATATTTTTTAGAGACGATGATAAGCTGTCAAACATGAGAATTGGGGATCGCATCTTTGTTATGCTATGGTTATTTCA    5T/6T-Δ420  68 63

      TAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCCCGGGGATCTCTTTGTTATGCTATGGTTATTTCA    Δ807         -  -

      CAAAACTCGACAAAACTCGACAAAACTCGACAAAACTCGATAAGCTGGGGATCTCTTTGTTATGCTATGGTTATTTCA   4A-Δ807'    42 37

      AAAACTCGACAAAACTCGACAAAACTCGACAAAACTCGATAAGCTGGGGATCTCTTTGTTATGCTATGGTTATTTCA    4A-Δ807     43 38

      AGGACTCGACAGGACTCGACAGGACTCGACAGGACTCGATAAGCTGGGGATCTCTTTGTTATGCTATGGTTATTTCA    1A-Δ807      -  -

      GTTTTGTCGAGTTTTGTCGATGATAAGCTGTCAAACATGAGAATTGGGGATCTCTTTGTTATGCTATGGTTATTTCA    4T-Δ807     62 52

      GTCCTGTCGAGTCCTGTCGATGATAAGCTGTCAAACATGAGAATTGGGGATCTCTTTGTTATGCTATGGTTATTTCA    1T-Δ807      -  -

      ATATATAAAAATCTCTAAAAAATATATAAAAATCTCTGATAAGCTGGGGATCTCTTTGTTATGCTATGGTTATTTCA    5A/6A-Δ807  45 40

      TATATATTTTTTAGAGACGATGATAAGCTGTCAAACATGAGAATTGGGGATCTCTTTGTTATGCTATGGTTATTTCA    5T/6T-Δ807  65 60
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Fig. 4. Sequence of the bent DNA hybrid promoters and of their controls. The important elements (dA or dT stretches and homologous control sequences) are in bold face. The starts of transcription have been materialized by arrows of various sizes indicative of the strength of the promoter. The −41.5 and −46.5 positions are indicated by vertical arrows. The distance (bp) corresponds to the interval between the end of the proximal dA or dT stretch to the P1 and P2 starts. The relevant distances in the activated constructs in the last two columns have been italicized and underlined.

with respect to their controls, 1A-Δ420 and 1A-Δ807. In addition, all transcripts originated from P2 whereas 4A-Δ807' corresponded to P1 *in vivo* (data not shown). Similar results were obtained when purified negatively supercoiled plasmid DNA was used in place of linear DNA. 4A-Δ807' was chosen since two parameters, the amount and the origin of the transcripts were different *in vivo* and *in vitro*. 4A-Δ807' and 1A-Δ807 produced comparable amounts of transcripts (Figure 5, cf. lane 2 with lane 4 or lane 3 with lane 5). 4A-Δ807' appeared slightly stronger than 1A-Δ807 but this difference was minimal compared with the activation observed *in vivo*. In addition, the main *in vitro* start was still P2 in contrast to the *in vivo* results.

Is a DNA binding protein involved in the activation by curved sequences?

Activation could also have resulted from the action of a DNA binding protein facilitating the positioning of RNA polymerase on a promoter containing a curved upstream region. The first candidate was the cAMP−CRP complex itself which could have interacted directly with RNA polymerase at these loci. This hypothesis was discarded by comparing the level of activation observed for the 4A-Δ807' constructs (with respect to 1A-Δ807) in a crp⁻ or cya⁻ background (in the presence or absence of cAMP). In those strains 4A-Δ807' remained a strong promoter insensitive to the presence of a functional cAMP−CRP complex inside the cell. 1A-Δ807 remained uniformly weak (data not shown). We also considered the possible involvement of IHF in recognition of the bent motifs. The activation *in vivo* by the 4A10 sequences was maintained in *E.coli* strain 02014 (*hip*) and purified IHF did not influence the *in vitro* transcription either (data not shown).

Table II. Phenotype, β-galactosidase activities and *in vivo* origins of transcription associated with the bent DNA hybrid promoters and their controls

	Phenotype[a]	β-Galactosidase activity[b]	Origin of transcription[c]
Wild type	Lac⁺	100	P1
Δ420	Lac⁻	7	P1
4A-Δ420	Lac⁺	142	P2
1A-Δ420	Lac⁺ᐟ⁻	23	P1
4T-Δ420	Lac⁺ᐟ⁻	29	P1
1T-Δ420	Lac⁺ᐟ⁻	28	P1
5A/6A-Δ420	Lac⁻	12	P1
5T/6T-Δ420	Lac⁻	13	P1/P2
*Spo*VG-Δ420	Lac⁺ᐟ⁻	20	P2
Δ807	Lac⁻	12	P1
4A-Δ807	Lac⁺	91	P1
1A-Δ807	Lac⁻	14	P1
4T-Δ807	Lac⁺	42	P2
1T-Δ807	Lac⁻	9	P2
5A/6A-Δ807	Lac⁻	8	P2
5T/6TΔ807	Lac⁻	7	P2

[a]Observed on MacConkey lactose medium.
[b]Expressed as percentage of the β-galactosidase activity measured in the wild-type strain (2800 units).
[c]cf. Figure 3.

Other experiments were performed in parallel *in vitro* by comparing the relative affinity of several DNA binding proteins for curved versus straight sequences. Gel retardation assays showed that the *E.coli* DNA binding protein H1 (Spassky *et al.*, 1984) had a better affinity for the (4A10)₈ sequence than for the (1A10)₈ sequence (Figure 6). To a

Table III. Influence of a 1 bp deletion on the *in vivo* promoter strength of two bent DNA hybrid promoters

Promoter	Phenotype	β-Galactosidase activity[a]	Origin of transcription
Wild-type	Lac[+]	100	P1
4A-Δ807	Lac[+]	91	P1
4A-Δ807'	Lac[+]	270	P1
*Spo*VG-Δ420	Lac[-]	20	P2
*Spo*VG-Δ420'	Lac[+]	92	P2

[a]Expressed as percentage of the β-galactosidase activity measured in the wild-type strain (2800 units).

Table IV. Influence of the superhelicity on the *in vivo* activation by the 4A10 polymer

	T13*recA*	T23*recA* (*top*[-])	T33*recA* (*gyr*[-])
Wild-type	100[a]	100[b]	100[c]
4A-Δ807'	350	265	360
4A-Δ807	115	98	118
1A-Δ807	25	23	26
Δ807	24	19	18
*Spo*VG-Δ420	41	27	37
*Spo*VG-Δ420'	105	62	117

[a,b,c]Expressed as percentage of units for the wild-type promoter in a given strain.
[a]100 is equivalent to 3200 units for wild-type.
[b]100 is equivalent to 2400 units for wild-type.
[c]100 is equivalent to 3500 units for wild-type.

1 2 3 4 5 6 7

P2 →
P1 →
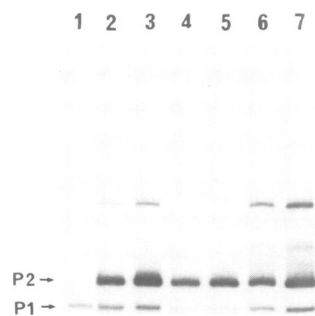
Fig. 5. *In vitro* transcription on purified plasmid DNA templates. The P1 and P2 starts of transcription are indicated. **1**, *in vivo* start of transcription from 4A-Δ807' (inserted to emphasize the difference between the *in vivo* and the *in vitro* starts for 4A-Δ807'. No comparison in band intensities should be made). **2**: 4A-Δ807', at 37°C for 10 min. **3**: 4A-Δ807', at 37°C for 30 min. **4**: 1A-Δ807, at 37°C for 10 min. **5**: 1A-Δ807, at 37°C for 30 min. **6**: 4A-Δ807', at 16°C for 10 min. **7**: 4A-Δ807', at 16°C for 30 min.

lesser extent, it also seemed to be true for the histone-like protein HU (Drlica and Rouvière-Yaniv, 1987, J.Rouvière-Yaniv, personal communication) and RNA polymerase.

H1   0   .4   .6   .8

4A →
1A →

POL   0   .003   .01   .03   .1

4A →
1A →
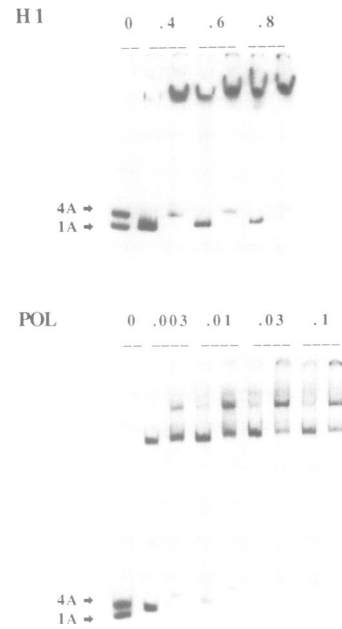
Fig. 6. Comparative gel retardation analysis showing the binding of H1 and RNA polymerase to 113 bp *Eco*RI–*Hind*III fragments (Diekmann, 1987), containing eight decamers of either the curved sequence (fragment 4A) or the control sequence (fragment 1A) as indicated in Materials and methods. In **lane 0**, an equimolar mixture of 4A and 1A fragments was loaded with no protein. The protein concentration is given in μM. At each protein concentration, the first lane corresponds to the 1A10 polymer and the second lane to the 4A10 polymer.

However, the addition of H1 or HU to the transcription reaction using plasmid DNA template neither activated the 4A-Δ807' promoter with respect to the 1A-Δ807 nor did it induce any shift towards a preferred utilization of the P1 promoter.

Discussion

The results obtained with the *lac*–CRP hybrid constructs indicate that both *gal* promoters when deleted upstream of position −24 or −27 can be reactivated in a cAMP dependent manner by the insertion, at a proper distance from the transcription start, of the lactose CRP binding site. The −35 regions show no significant homology with the consensus sequences, suggesting that no element other than a Pribnow box and a good binding site for the activating complex is required to make those promoters functional in the presence of cAMP.

In the wild-type *gal* control region the centre of the binding site for the activating complex is located at −41.5 from the P1 promoter which is activated. In CRP-Δ807, the same promoter is now turned on by a site inserted at the same distance as in the original *lac* background (61.5 bp). Hence, swapping between two CRP sites is possible in the *gal* P1 background, and activation proceeds as well, whether the distance is 41.5 or 61.5 bp. The results obtained with CRP-Δ420 (P2 activated) and CRP-Δ807' (P1 activated) indicate that deviation from the 61.5 bp spacing could be accommodated by the nucleoprotein assembly and still lead to promoter activation (the corresponding distances to the start

sites are 59.5 and 60.5 bp). In the case of the *lac* promoter, two reports (Mandecki and Caruthers, 1984; Straney *et al.*, 1989) indicated that the insertion of 5 bp, i.e. half a turn of B-DNA helix, between the CRP binding site and the -35 region prevented CRP activation. This activation was partially restored when 11 bp replaced the 5 bp insertion. Deletions and insertions of 1 or 2 bp also modulated the activation of CRP (Mandecki and Caruthers, 1984). A more extensive study of our hybrid constructs will be reported elsewhere.

We have demonstrated that naturally bent sequences could activate transcription *in vivo* in place of a CRP site when positioned upstream of the same *gal* Pribnow boxes. The most striking example, 4A-Δ807', was 20 times more efficient than its corresponding negative control, 1A-Δ807. This activation could affect both promoters, *gal* P1 and *gal* P2. The same sequence (II, see Figure 2) in one of the two possible orientations activated P1 in the promoter 4A-Δ807 and activated P2 when moved upstream by 3 bp in 4A-Δ420. In both cases, replacement of sequence II by sequence III (cf. results of 1A-Δ420 and 1A-Δ807 in Table II) led to low levels of β-galactosidase activities, comparable to the ones observed in the starting deletions, and originating from the same promoter, P1. Thus the shift from P1 to P2 observed in 4A-Δ420, as well as the increase in expression, are indeed due to the insertion of the phased 4A sequences. The results obtained with the 1A-Δ420 control rule out, in particular, the objection that perhaps the -35 region of P2, also shared by 4A-Δ420, could be more efficient than the corresponding -35 region of P1. None of these sequences show good homology with consensus hexamers, but in view of recent results indicating that such non-homologous -35 sequences could produce very efficient promoters (Horwitz and Loeb, 1988; Jacquet and Ehrlich, 1985), it was essential to show that no element present in the 1A sequences led to significant promoter activity. Finally, sequence II can be inserted in the opposite orientation placing the dA stretches on the other strand, and still lead to a significant activation (cf. 4T-Δ807). In general, the *gal* control region appears very suitable for such replacements, as also shown recently by Chan and Busby (1989).

It is possible to score the promoter strength of P1 and P2 in the various constructs as a function of the distance of the first curved motif to the two starts, taking into account not only the positive outcomes but also the negative results (no significant activity). As shown in Figure 4, there is a narrow window of spacings leading to activity when distances, taken from the 3' end of the dA stretch closest to the transcription starts are $<40-43$ bp$>$. In the most active promoter, 4A-Δ807', this interval is 42 bp. However, three constructs lie in the corresponding window and fail to yield marked stimulation, *Spo*VG-Δ420, 5A/6A-Δ420 and 5A/6A-Δ807 (all with respect to P2). None of the constructs derived from the Ulanovsky sequence yields an active promoter. The stronger curvature observed with these upstream elements may be detrimental to the activation. Another almost equivalent way to look at the problem is to position the centre of the *gal* P1 CRP site (at -41.5) or of the CRP site which would activate *gal* P2 (at -46.5) with respect to the bent sequences which stimulate a given promoter. This is done in Figure 4. One finds that in the activated constructs, the 3' end of the first A stretch, which has been associated with a more pronounced bend in the so-called junction model (Koo

and Crothers, 1988; Koo *et al.*, 1986) comes very close to this position. In their analysis of promoter sequences from *E.coli*, Galas *et al.* (1985) found a significant subset of promoters possessing an A_n sequence centred around -44 bp, which might fulfill a similar function. Plaskon and Wartell (1987) have independently noticed that such tracts were often phased. In *B.subtilis*, McAllister and Achberger (1989) have shown that the phasing between the -35 region and poly(dA) stretches known to act as positive upstream elements was essential to promoter activity: 11 and 21 bp insertions (i.e. one and two helical turns of B-DNA) between these two regions only slightly affected promoter efficiency, while a 15 bp insertion was very detrimental to promoter activity. RNA polymerase binding experiments suggested that the curvature associated with these upstream elements may allow additional polymerase−DNA contacts which would be responsible for the observed effects on promoter function. Some of our results could be interpreted in the same way. We note that the 3' proximal dT in the promoter 4T-Δ807 is located 57 bp away from the activated start, i.e. 15 bp upstream (or 1.5 helical turn of B-DNA) from the 3' position of the 4A sequences which activate the 4A-Δ807' promoter (cf. underlined numbers in the two last columns of Figure 4). When dA stretches have been replaced by dT stretches, we would have precisely expected such a correlation if the primary determinant for activation was the rotational orientation of the curved DNA sequence.

The proper positioning of bent upstream elements is of the upmost importance for promoter function. A 1 bp insertion completely destroyed the ability of the *Spo*VG sequence to activate transcription and reduced the ability of the 4A10 motif to act as an upstream promoter element (see Table III; Figure 3). In the same line of reasoning, we note that negative superhelicity sometimes modifies the relative activation *in vivo* by curved sequences suggesting that untwisting affects phasing. Thus, there is a similarity between this activation and the activation by the CRP protein: both P1 and P2 promoters can be stimulated *in vivo* in both instances and the location of the A_n stretch and of the CRP binding site effectively modulates this phenomenon. However, this analogy stopped when *in vitro* experiments were performed. With the CRP constructs, the amount and start of the transcripts were in total correspondence with the *in vivo* results whereas these were not matched with the hybrid promoters containing the bent DNA sequences.

Several laboratories have studied the influence of naturally occurring A or T stretches positioned upstream of -35 regions on promoter efficiency. None of the activation effects were as large as the ones reported here. In many reports, only *in vivo* experiments involving mutations and deletions have been performed (Lamond and Travers, 1983; Gourse *et al.*, 1986) but, in three instances, *in vitro* studies supported the *in vivo* data. Bossi and Smith (1984) reported a 2-fold activation of the *his* tRNA promoter by upstream bent sequences in *Salmonella typhimurium*. A 3 bp deletion destroyed the curvature and the activation as well. This result could be reproduced *in vitro* using *E.coli* RNA polymerase. Another study (Banner *et al.*, 1983) demonstrated the positive effect, *in vivo*, of bent upstream sequences on the two *Spo*VG promoters P1 and P2 in *B.subtilis*. Run-off experiments *in vitro* on linear templates using a mixture of the σ^{37} and σ^{32} factors associated to core RNA polymerase confirmed their *in vivo* data. As mentioned above, McAllister

Table V. Strains of *E.coli* K12

Name	Relevant genotype	Origin	Reference
M182	Δ*lacX74 galK galU rpsL*	S.Busby	Casadaban and Cohen (1979)
T13	M182 *pyrF*	this work	
T14	M182 *pyrF* Δ*cya854 ilv*::Tn*5*	this work	Ebright *et al.* (1984) for the Δ*cya854* character
T23	M182 *pyrF top10*	this work	Sternglanz *et al.* (1981)
T33	M182 *pyrF gyrB226 tnaA*::Tn*10*	this work	Drlica *et al.* (1986)
GC2226	Hfr KL16 *recA srl*::Tn*10*	C.Wandersman	Wandersman *et al.* (1980)
MCL30	Hfr PO45 *thi-1* Δ(*srl-recA*)306 :: Tn*10*	M.Lorence *via* A.Pugsley	Lorence and Rupert (1983)
02014	F-*araD139 lac strA supF trp*::Tn*10 hip*::*cat*	R.Weisberg	

and Achberger (1988) have investigated the influence of three phased poly(dA) stretches on two *B.subtilis* promoters. *In vivo*, such sequences activated one of the two promoters but weakened the second one. These effects could be somewhat qualitatively reproduced *in vitro* using the major σ^{43} *B.subtilis* RNA polymerase. In the case where the dA stretches acted as positive elements, the use of supercoiled templates *in vitro* partially compensated for the lack of these upstream sequences.

Contrary to these reports, and in spite of numerous attempts and changes in experimental conditions, we could not find an *in vitro* system where the *in vivo* results could be reproduced using *E.coli* σ^{70} RNA polymerase and linear or supercoiled templates. We can invoke four explanations for this negative result.

First, a very specific range of superhelical density of the template could be required to lead to promoter activation. Cases are indeed known where the strength of a given promoter is increased in a rather narrow range of negative supercoiling (cf. e.g. Brahms *et al.*, 1985). We are pursuing along these lines *in vitro* experiments.

Second, it is still possible but less likely that the enzyme operating on these templates *in vivo* is not the major form of RNA polymerase. Preliminary *in vivo* experiments disfavour a specific recognition of these sequences by σ^{32}, the heat shock sigma subunit, but other minor forms have not been investigated.

Third, auxiliary factors present in the *E.coli* chromoid could bind to these upstream sequences and be essential for the proper positioning of RNA polymerase (cf. better affinity shown above of factor H1 for the 4A10 sequence as compared with 1A10). They could act directly as activators, or release a prior inhibition due to improper RNA polymerase positioning. In the above mentioned studies by Bosssi, by Banner and by McAllister, activation could be

reproduced *in vitro* without additional factors. It is not unlikely that, in our case, both a change in negative superhelicity and the addition of at least one factor are required to observe a significant activation *in vitro*.

Fourth, the situation could be even more complex if the bent sequences prevented the formation of a chromoid structure within the cell which could facilitate the access to the promoter. In yeast, two reports (Russell *et al.*, 1983; Chen *et al.*, 1987) indicated that poly(dA-dT) could act as upstream transcriptional activator and that the probable reason for this effect was a displacement of the chromatin structure around the promoter. Indeed, this activation could not be reproduced *in vitro* either. More recently, A.P.Wolffe and H.Drew (unpublished data) have reconstituted chromatin on closely related DNA fragments containing curved sequences and found that the positioning of nucleosomes was strongly affecting transcription.

Whatever the explanation is, we want to emphasize that the effects reported here are very large and that they afford the possibility of a genetic analysis of the partners involved. Goodman and Nash (1989) have recently demonstrated that a sequence-directed bend could substitute for a protein induced bend (via IHF) in the integration process of λ. Similarly, our results show that the cAMP–CRP complex could be efficiently replaced by curved sequences in the activation of two overlapping promoters.

Materials and methods

Constructions of hybrid promoters and nucleic acid techniques

Sequences II, III and IV listed in Figure 1b are *Eco*RI–*Hind*III fragments extracted from plasmids pK1A108, pK4A108 and pK5/6A216 described by S.Diekmann (1987 and personal communication). The sequence V corresponds to the *Eco*RI–*Hind*III fragment extracted from plasmid pCB5162 (gift from Richard Losick) which contains the A/T rich upstream region of promoter *Spo*VG cloned in pBR322 (Banner *et al.*, 1983). The sequence I has been generated by joining a blunt-ended *Bst*EII linker to the *Hha*I–*Bst*NI fragment of the *lac* promoter sequence (Douc-Rasy *et al.*, 1989). It contains the quasi-palindromic CRP binding site. All five fragments were blunt-ended with the Klenow fragment.

The vector plasmids pAA187Δ807 and pAA187Δ420 are described in Busby *et al.* (1983). Each bears an *Eco*RI–*Hind*III fragment 72 and 75 bp long respectively, containing the deleted *gal* promoters which control the expression of the *lac* operon. Their unique *Sma*I site served for the blunt end ligations of the five fragments listed in Figure 1b. The ligation products were transfected in M182recA. Selective growths were performed on MacConkey lactose agar plates supplemented with 100 μg/ml ampicillin. On this medium the original plasmids give rise to Lac⁻ white colonies while plasmids having recovered a functional promoter give red Lac⁺ colonies. Minilysates from these colonies have been examined for the presence of insertions by measuring the apparent size of the *Eco*RI–*Hind*III fragments. Orientation of the insertions have been determined by end labelling the plasmids at the *Eco*RI site and looking for the length of the labelled fragments generated by a convenient secondary restriction (*Ban*I for sequence I, *Alu*I for inserts II, III and IV, and *Dra*I for the sequence V). Selected constructs were sequenced according to the dideoxy procedure using MMLV reverse transcriptase or the Sequenase kit provided by US Biochemicals Corporation.

Strains

Table V lists the strains which have been used in this study.

Strain M182 was a kind gift from S.Busby. P1 co-transduction of Δ*cya854* with *ilv*::Tn*5*, selecting for kanamycin resistance and cya⁻ character led to strain M182Δ*cya* (the donor strain is described in Ebright *et al.*, 1984).

The gene coding for topoisomerase I is located between *pyrF* and the *trp*EDCBA operon. M182 and M182Δ*cya* were then rendered *trp*⁻ and Tc^R by insertion of a Tn*10* transposon in the *trp* operon. P1 transduction from KZ43 (*pyrF*) and KZ44 (*pyrF top10*) (both gifts from J.Wang) and selection for the trp⁺–pyr⁻ characters in derivatives of strain M182 led

to strains T13, T14 and T23. The top⁻ character of strain T23 was checked by a published procedure (Sternglanz *et al.*, 1981).

P1 transduction from strain KD112 (JTT1 *gyrB226 tnaA*::Tn*10* (Tc^R), a gift from K.Drlica) into strain T13 and selection for the Tc^R character led to strain T33. The gyr⁻ character of this strain was verified by its enhanced sensitivity to 50 µg/ml novobiocin relative to strain T13. To prevent possible recombination events between the polymerized motifs (1A10, 4A10, 5A/6A), strain M182 and strains T13, T23, T33, T14 were all rendered *recA* by mating with strains GC2226 and MCL30 respectively. The *recA* character of the resulting strains was corroborated by their increased sensitivity to UV light.

Strain 02014 (*hip*) was generously donated by R.Weisberg.

β-Galactosidase assay

50 µl of an overnight culture in rich medium were used to inoculate 5 ml of M63B1 medium supplemented with fructose 0.5% (w/v), casamino acids 0.5% (w/v), uracil (50 µg/ml) (+50 µg/ml kanamycin for T14*recA*). Cells were grown to $OD_{600} = 0.3-0.4$ and the assay performed as in Miller (1972) using $CHCl_3$, 0.1% SDS to permeate the cells. The linearity of the assay with the cell density and the time of reaction with ONPG has been verified. The results indicated represent the average of at least three separate sets of experiments, each one with duplicate samples, the error on the assay being <15%.

mRNA analysis

Total RNA was extracted according to a published procedure (Aiba *et al.*, 1981) and quantified by its UV spectrum. A labelled probe (16mer, 1 pmol) corresponding to the beginning of the coding region was hybridized to 0.5 µg total RNA in 0.4 M KCl by slowly cooling down from 90 to 20°C. Following extension by 100 U of MMLV reverse transcriptase in 50 mM Tris pH 7.6, 3 mM $MgCl_2$, 130 mM KCl, 10 mM dithiothreitol (DDT), 0.5 mM of each dNTP for 1 h at 37°C, the products were resolved by PAGE (10% acrylamide, 7 M urea). Under these conditions, the intensity of the extended chain was found to be proportional to the amount of total RNA used in the hybridization. The major two bands observed were shown to correspond to a P1 or a P2 start by using dideoxy-sequencing reactions as markers.

In vitro transcription

Plasmids 4A-Δ807' and 1A-Δ807 were prepared on a large scale and purified using the protocol and the pZ523 columns from 5 Prime → 3 Prime, Inc.

Escherichia coli RNA polymerase was prepared as described (Lowe *et al.*, 1979). 5 nM plasmid DNA was preincubated with 100 nM RNA polymerase in 40 mM Tris−HCl pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA for 30 min at 37 or 16°C. Transcription was initiated by the addition of XTPs (0.5 mM each). The reaction was stopped at 90°C after 10 or 30 min. The origin and amount of transcripts were determined by the primer extension technique described in the preceding paragraph. IHF protein was kindly provided by H.Nash.

DNA binding assays

H1 and HU were generous gifts from Drs S.Rimsky and A.Spassky, and E.Bonnefoy and J.Rouvière-Yaniv respectively. Plasmids pK4A108 and pK1A108 were cut with *Eco*RI and 3' end-labelled with [α-³²P]dATP using Klenow fragment and then cut by *Hae*III restriction enzyme. The 256 bp *Eco*RI−*Hae*III fragments were purified on a 5% polyacrylamide gel and subsequently cut by *Hin*dIII restriction enzyme, to give the 113 bp *Eco*RI−*Hin*dIII fragments, named 4A and 1A respectively.

For binding experiments 4 µl of each 113 bp fragment (5 × 10⁻¹⁰M) were gently mixed with 4 µl of the diluted protein in 20 mM Tris−HCl pH 7.9, 0.1 mM EDTA, 50 mM NaCl and 100 µg/ml BSA. After 1 h incubation at room temperature, 1.5 µl of 50% glycerol with 0.01% xylenecyanol blue in the same buffer was added and the mixture directly loaded on a 7.5% polyacrylamide gel (75 × 75 × 0.8 mm), run at 12 V/cm in 45 mM Tris−borate, 1.25 mM EDTA until the dye migrated 4 cm. For RNA polymerase binding, the mixture was incubated for 30 min at 37°C, then 2 µl of the glycerol buffer dye containing 120 µM M13 single-stranded DNA was added to the sample before loading on a 4% polyacrylamide gel.

Acknowledgements

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