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DNA flexibility of the UP element is a major determinant for transcriptional activation at the *Escherichia coli* acetate promoter

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ABSTRACT

The specific interaction of the upstream element-containing promoter of the *Escherichia coli* acetate operon with either the RNA polymerase holoenzyme or its α subunit has been analyzed by the base removal method. Our results indicate that: (i) direct and specific base contacts can be detected in the acetate promoter-α subunit complex; (ii) base elimination in the upstream element of the acetate promoter enhances the binding of RNA polymerase. A similar effect is observed when studying the interactions between RNA polymerase and the *rrnB* ribosomal operon P1 promoter.

INTRODUCTION

It has recently been shown that, in addition to its two major determinants (the –10 and –35 hexamers), the promoter strength of the *Escherichia coli* RNA polymerase can be greatly increased by a third *cis*-acting recognition element: the upstream (UP) module. Thus, in the case of the *rrnB*P1 (promoter of the ribosomal RNA operon), Ross *et al*. (1) have demonstrated that interactions between the UP element, which spans an (A+T)-rich region (residues –40 to –60), and the α subunit of the RNA polymerase induce a 30-fold stimulation, compared to the promoter activity lacking the UP element. Studies performed on mutants of RNA polymerase holoenzyme (RNAP) have shown that the C-terminal domain of the α subunit (αCTD) is directly involved in such stimulation (1–3). The three-dimensional structure of the C-terminal domain of the α subunit of RNAP, recently determined by NMR analysis (4), contains a spatial arrangement of four helices and two arms enclosing a hydrophobic core. When incubated with a 25 bp DNA containing the *rrnB*P1 UP element, the region of the protein including in particular the amino acid residues of helix 1, and the N-terminal end of helix 4 have been shown to participate in the binding process. However, no detailed description of the structure of the αCTD–*rrnB*P1 UP complex has been provided so far, and the process of recognition between the bases of the DNA and the amino acid residues of the α subunit remains to be determined (5).

Therefore, in this work, the binding of either purified α subunit alone, or total RNAP, to the promoter of the *ace* operon (the operon encoding the enzymes for acetate utilization) of *E.coli* has been studied by using the 'missing contact' chemical approach described by Brunelle and Schleif (6). We have shown that while the removal of certain bases between positions –32 to –50 of the *ace*P (promoter region of the *ace* operon) *interferes* with the binding of the α subunit, the elimination of single bases from either strand of the UP-like element results, in contrast, in an *enhanced* binding affinity of the RNAP. This finding suggests that disruption of the helix in this particular region promotes local DNA flexibility that stabilizes the RNAP–promoter complex. Similar results have been obtained when incubating RNAP specifically with the well-characterized UP element of *rrnB*P1, which supports the concept that the interference footprinting method is an efficient tool for studying the binding of RNAP to different UP module-containing promoters.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Fluka except hydrazine which was from Aldrich Chemical Co. Restriction enzymes and DNA modification enzymes were obtained from Promega Corp. The *E.coli* RNAP (100% σ^{70}-saturated) was from Epicentre Technologies. [α-^{32}P]dATP (3000 Ci/mmol), [α-^{32}P]dGTP (3000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were obtained from Du Pont-New England Nuclear. Ni^{2+}-nitrilotriacetic acid–agarose resin and pQE30 plasmid were from Quiagen Inc. The Protein Pak Glass DEAE 5PW column was from Waters Co.

Construction of plasmid pJCD6

A *Bam*HI–*Dra*I DNA fragment containing the *rpoA* gene encoding the α subunit of RNA polymerase was amplified by

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polymerase chain reaction (PCR) using *E.coli* K12 chromosomal DNA as template and two oligonucleotides, one carrying a *Bam*HI site within the following sequence, 5′-TAT**GGATC-C**ATGCAGGGTTCTGTGACAGAGTTTC-3′, and the other with a *Dra*I site in a sequence complementary to the 3′-end of the gene, 5′-TAT**TTTAAA**TGCCAGACGACGATTAGCAAC-3′. Following PCR, the fragment was restricted with *Bam*HI+*Dra*I and cloned into the compatible sites *Bam*HI+*Sma*I of the expression vector pQE-30 to create plasmid pJCD6. This plasmid encoded the α subunit under control of the *E.coli* phage T5 promoter. The *rpoA* gene contained a 6× His-tag at its 5′-end to facilitate the purification of the corresponding protein by affinity chromatography.

Expression and purification of the recombinant α subunit

Plasmid pJCD6 was transformed into *E.coli* strain JM105 (*endA*1, *thi*, *rpsL*, *sbcB*15, *hsdR*4, Δ(*lac-proAB*), [F′, *traD*36, *proAB* , *lacI*^{q}ZΔM15] (7). Cells were grown at 37°C in 2× YT broth (8) in the presence of ampicillin (50 µg/ml) to an absorbance value of 0.7–0.8 at 600 nm, and treated with the inducer isopropyl-β-thiogalactopyranoside (IPTG) at a final concentration of 2 mM. After 2 h of induction, cells were harvested by centrifugation, disrupted in a French pressure cell at 10 000 p.s.i. in buffer A (10 mM Tris–HCl, pH 7.3, 10 mM β-mercaptoethanol, 10% glycerol) containing 150 mM NaCl, 30 mM imidazole and 1% Triton X-100. A high-speed supernatant (S30) was then prepared by centrifugation for 30 min at 30 000 *g* and applied, in a first step, onto a Ni^{2+}-nitrilotriacetic acid–agarose column equilibrated with buffer A. After elution of the His_6-tagged α subunit with buffer A containing 250 mM imidazole, column fractions enriched with the protein were pooled and dialyzed against buffer A containing 50 mM NaCl. In a second step, the corresponding fractions were loaded onto a DEAE anion exchange column, and elution was achieved with a 0–500 mM NaCl linear gradient in buffer A. Pure α subunit protein was concentrated and dialyzed against buffer A containing 50% glycerol by using a Vivaspin-50 (Vivasciences Co.) filtration device, and stored at –20°C. Protein concentration was estimated according to the method of Bradford (9). Size exclusion chromatography showed that, after purification, α subunit was present as a homodimer in solution (10,11).

Labelling of promoter DNA fragments

Two plasmids, pJCD7 and pJCD8, derived from pBluescript II KS vector (GenBank accession no: X52327, Stratagene) were used to generate radioactively labelled DNA fragments for gel retardation and base removal studies. Plasmid pJCD7 carried a 219 bp (base pairs) *Eco*RI–*Not*I fragment containing the *ace* promoter regulatory region (12), and plasmid pJCD8 had a 160 bp insert containing the *rrnB*P1 (13). In each case, the *Eco*RI+*Not*I fragment was end-labelled at the *Not*I site by [α-^{32}P]-dGTP (top strand) or at the *Eco*RI site by [α-^{32}P]dATP (bottom strand), using the Klenow fragment of DNA polymerase.

Interference experiments

Premodifications of the promoter-containing DNA fragments were carried out by using the base removal approach described in (6) and modified as follows: (i) promoter DNAs were chemically modified either by formic acid (G+A reaction) or by hydrazine (C+T reaction) in conditions that allowed an average of one modifiing event per DNA molecule (14) (Table 1); (ii) after ethanol precipitation, modified DNAs were loaded on a non-denaturing 4% polyacrylamide gel and electrophoresed by using Tris-borate buffer; (iii) following autoradiography, the fastest-moving DNA molecules were electroeluted from gels and assessed in subsequent binding experiments.

Retardation assays were performed essentially as already described (15). A typical assay mixture contained in 200 µl: 12 mM HEPES-NaOH, pH 7.9, 4 mM Tris–HCl, pH 7.9, 95 mM KCl, 1 mM EDTA, 1 mM DTT, 9% (v/v) glycerol, 0.02% (v/v) Nonidet P-40, 2 µg poly(dI–dC).poly(dI–dC) as bulk carrier DNA, 10 µg bovine serum albumin, chemically-modified DNA probe (1.7×10^6 c.p.m.), and either α subunit (2.4 µM) or RNAP (2 nM). After incubation for 10 min at 25°C, the mixtures were loaded on a 4% preparative polyacrylamide gel at high-ionic-strength and electrophoresed for 1 h at 30 V/cm. Bands corresponding to free and complexed DNA were visualized by autoradiography on the wet gel after overnight exposure at 4°C. Labelled DNA was excised from the gel, eluted for subsequent piperidine cleavage (14), and analyzed on a 6% sequencing gel to reveal the position of the interfering modifications. Autoradiograms were analyzed by densitometry using a Personal Densitometer SI from Molecular Dynamics.

Table 1. Protocol for generating partially depurinated or depyrimidated DNA used in interference studies

	G+A reaction	C+T reaction
Mix at 0°C	5×10^6 c.p.m. DNA in 200 µl H_2O	5×10^6 c.p.m. DNA in 200 µl H_2O
	20 µl formic acid 4%	300 µl hydrazine 98%
Incubate	30 min, 37°C	15 min, 25°C
Add and mix	200 µl NaOAc 300 mM, pH 7.0 and 3 vol ethanol	
Precipitate	5 min in liquid nitrogen	
Centrifuge	10 min at 20 000 *g*	
Dissolve in	200 µl loading buffer [10% glycerol, 0.1% (w/v) bromophenol blue,	
	and 0.1% (w/v) xylene cyanol]	
Electrophoresis in	4% non-denaturing polyacrylamide gel (80:1) in 0.5× TBE buffer	

Transcription assay

The plasmid pJCD9 used as a template in the *in vitro* transcription assay carried the *ace*P region followed by the transcription termination signal T1T2 of the *rrnB* operon (16), inserted between the *Eco*RI and *Afl*III sites of the pUC19 vector (GenBank accession no: X02514). Transcription experiments were performed at 37°C in a buffer containing 50 mM Tris-acetate (pH 8), 100 mM KOAc, 8% glycerol, 0.1 mM EDTA, 8 mM MgOAc, 0.1 mM DTT and 500 U/ml RNasin. In a 20 µl typical assay, 60 nM of supercoiled pJCD9 plasmid purified by centrifugation in CsCl/ethidium bromide were incubated for 20 min, with 67 nM of either wild-type RNAP or C-truncated α-256 RNAP to allow open complex formation. The mutant RNA polymerase containing α-256 was prepared as described in (17). Transcription was initiated by adding a nucleotide mix of ATP, GTP and CTP (200 µM each), UTP (10 µM), [α-^{32}P]UTP (2.5 µCi at 800 Ci/mmol) and 0.1 mg/ml of heparin. After 5 min of incubation, the reaction was stopped by adding 1 vol of formamide loading dye (98% deionized formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 10 mM EDTA). Following heat denaturation at 65°C, a 5 µl aliquot of the assay was electrophoresed on a 6% sequencing gel and visualized by autoradiography on a Kodak Biomax MR film at –70°C.

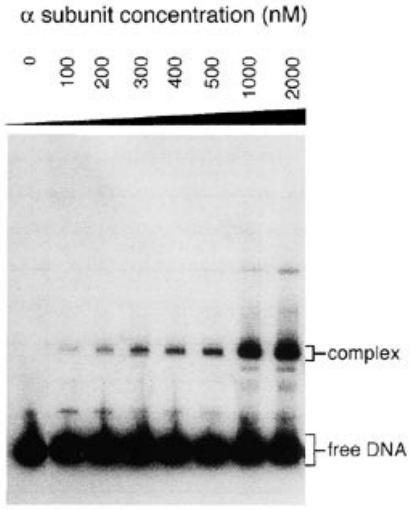

Figure 1. Electrophoretic mobility shift assay for interaction of the α subunit with the *ace*P region. The end-labelled 219 bp insert of plasmid pJCD7 containing the *ace*P was incubated with a varying amount of purified α subunit, and electrophoresed in a native polyacrylamide gel according to Garner and Revzin (15).

RESULTS AND DISCUSSION

Alpha subunit interaction with the *ace*P

In a number of prokaryotic promoters, the presence of a third recognition element interacting with RNAP, upstream from the –10 and –35 hexamers, remains to be demonstrated. For this purpose, the promoter region of the *ace* operon (12) containing a putative UP element, centered around base –50, was studied in order to analyze in detail its interaction with the α subunit.

The 219 bp *Eco*RI/*Not*I insert obtained after restriction of plasmid pJCD7 (see Materials and Methods) was labelled with [α-^{32}P]dATP, and then used in a gel retardation assay. After incubation of this DNA with a varying amount of α subunit followed by electrophoresis in a 4% native polyacrylamide gel, a single DNA–protein complex was detected (Fig. 1). This result indicates that α alone can form a *stable* and *specific* complex with the *ace*P DNA fragment, with a binding affinity in the range of that observed for the *rrnB*P1 (see fig. 3 in ref. 18).

Identification of the α-binding site in *ace*P

The precise location of the α-binding site within the *ace*P DNA was determined by the base removal method (6). In this approach, the 219 bp end-labelled DNA fragment containing *ace*P was depurinated or depyrimidated at a level of slightly less than one base removed per DNA fragment. This partially modified DNA was incubated with the α subunit, then free and complexed DNA were separated by electrophoresis on a non-denaturing gel. After cleavage at the positions of missing bases, DNA molecules were separated on a sequencing gel to reveal the positions either irrelevant or, instead, crucial to binding. Experiments were performed by removing either G+A residues by treatment with formic acid, or C+T residues by treatment with hydrazine (6,14). The corresponding electrophoretic patterns for both top and bottom DNA strands are shown in Figure 2A, and a summary of the effects of DNA modifications on α binding is presented in Figure 3A. The comparison of band intensity in the lanes of *bound* and *free* DNA in Figure 2A, indicates that missing bases at positions –41 to –50 in the *ace*P fragment significantly affect α binding to its DNA target. A weaker effect was observed for bases spanning positions –31 to –37. All together these results show that (i) isolated α subunit of RNAP actually behaves as a DNA-binding module (19) capable of interacting specifically, by direct contacts, with a limited number of bases of the UP element in the *ace* operon; (ii) recombinant α subunit which was purified as a homodimer, partly contacts a region of the *ace*P that exhibits palindromy (Fig. 3A). This specific mode of interaction with DNA might be related to the fact that the α subunit does not contain the well-characterized helix–turn–helix motif detected in many other bacterial DNA-binding proteins (20); (iii) most of the contacted bases detected by base removal experiments span a region that overlaps the *ace* operator of the IclR repressor previously characterized by chemical and enzymatic footprinting (21,22). Since these two sequences are, in this case, essentially coincident, one can consider that the negative regulator IclR could partially inhibit transcription by steric hindrance, i.e., by preventing access of RNAP to the extended promoter of the *ace* operon.

RNAP interaction with *ace*P

The binding of RNAP to *ace*P was studied by using the same chemical interference approach as that described above. Figure 2B shows the autoradiograms of the depurination (G+A) and the depyrimidation (C+T) footprints obtained for both the top strand and the bottom strand of *ace*P complexed with RNAP. Comparison of the band intensity of bound and free fractions reveals two distinct regions where a missing base enhances the affinity of RNAP for its promoter. The first region, between positions –5 and –14 (Fig. 3B), including the *ace*P –10 hexamer, likely corresponds to the 'melting domain' of the initiation complex. This result is in agreement with the footprinting

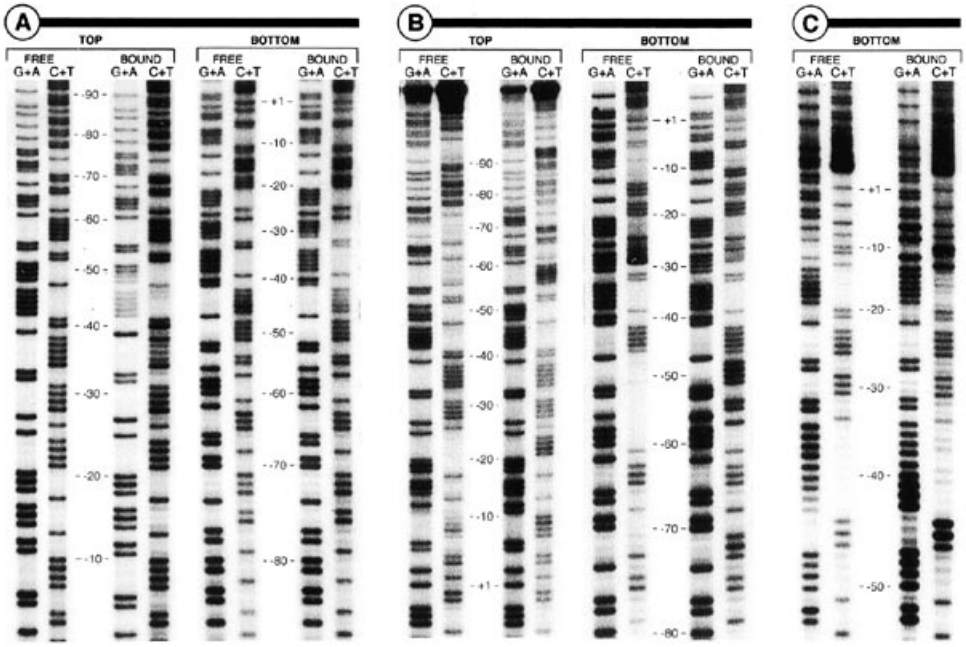

Figure 2. Base removal experiments with *ace*P and *rrnB*P1. The 219 bp DNA fragment containing *ace*P was ^{32}P-labelled at either the *Not*I site referred to as 'TOP', or at the *Eco*RI referred to as 'BOTTOM' (see Materials and Methods). DNA molecules were treated with either formic acid to remove G+A, or hydrazine to remove C+T, and incubated with α subunit (**A**) or RNAP (**B**) before electrophoresis and autoradiography. BOUND, gel lanes with the modified DNA isolated from complexes; FREE, gel lanes with the DNA that had dissociated or was free of complexes. (**C**) Experiments carried out in the same conditions as described above, between RNAP and the *Eco*RI–*Not*I insert of plasmid pJCD8 (bottom strand) containing *rrnB*P1.

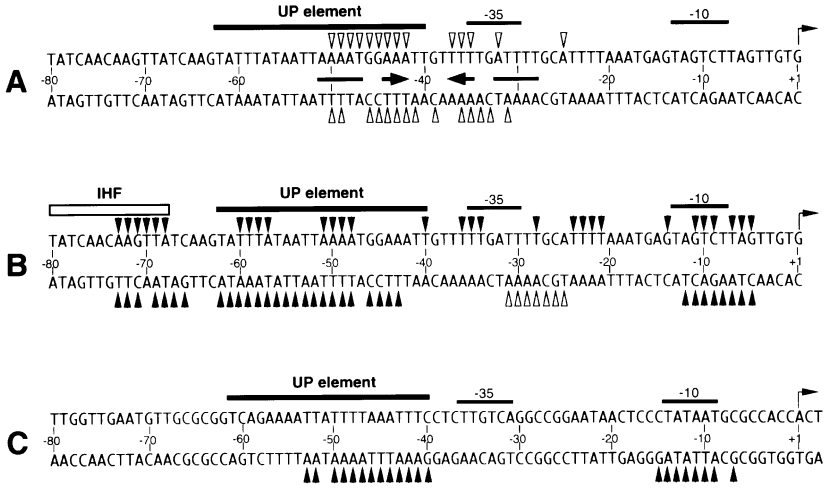

Figure 3. Summary of base removal experiments. (**A** and **B**) Data obtained for the *ace* promoter complexed with α subunit and RNAP, respectively. (**C**) Data obtained for the *rrnB*P1 promoter complexed with RNAP. In each case, Δ indicates the position where a missing base results in binding interference; ▲, indicates the position where base removal leads to enhanced binding. The numbering gives the position of nucleotides relative to the first nucleotide of the transcribed region, referred to as +1. Palindromy is underlined by two converging horizontal arrows.

experiments previously performed with the promoter A1 of the *E.coli* RNAP and with the promoter sites of the RNA polymerases of bacteriophages T3, T7, and SP6 (23,24). Of particular interest was the detection of an increased affinity of RNAP for *ace*P when the bases in the region –43 to –73 on either strand of DNA were removed (Figs 2B and 3B). The DNA between positions –43 and –62 in this region of the promoter shares common features with the UP element of the ribosomal *rrnB*P1 (1).

These data provide evidence, on the one hand, that the mode of interaction of RNAP with the UP element of the *ace*P is essentially dependent on the structure of DNA. On the other hand, base removal interference experiments carried out with isolated

α subunit have shown that only a few direct base contacts occur with a region of DNA that partially overlaps the UP module. This quite different behavior suggests that, when it is part of RNAP, the α subunit can recognize a higher order structure of the UP element rather than its primary nucleotide sequence. This finding could be connected with the previous observation that the purified α subunit of RNAP retains the ability to bind the UP element of the *ada* and *rrnB*P1 promoters (1,25).

Moreover, the DNA encompassing residues –66 to –73 partially overlaps the 3′-end of a proximal IHF-binding site recently characterized in the *ace* promoter/operator regulatory region (Fig. 2B) (26). In several operons the IHF factor is known to facilitate the loop formation responsible for the contacts between RNAP and DNA sequences located further upstream from the *ihf* site. In our case, such a structural role for IHF could be mimicked by the functional replacement of the IHF-binding site by a DNA of enhanced flexibility. The detection of both an IHF-binding site and an UP element has been already reported in the case of the *ilvGMEDA* operon of *E.coli* (27). Mutagenesis experiments recently performed on the promoter area have shown that transcriptional activation of these two regions are functionally independent (28).

Finally, it can be noted that, when they are missing, the four thymines (–92 to –95) in the top strand of the *ace*P DNA also increase RNAP binding (Fig. 2B). This long-distance stimulation of the promoter strength was previously detected in several natural and hybrid promoters, and could be related to the presence of A+T-rich tracts in the DNA (29–32).

In vitro transcription from *ace*P with wild-type and mutant α-256 RNAPs

Specific recognition between the α subunit of RNAP and the UP-element of *ace*P has been analyzed by *in vitro* transcription. In this assay, the supercoiled plasmid pJCD9 containing the *ace*P region upstream from the *rrnB* transcription termination signal T1T2, was incubated with either wild-type RNAP or mutant RNAP containing the C-truncated α-256 subunit (17). RNA transcripts generated in both reactions were then separated on a 6% sequencing gel and revealed by autoradiography.

Comparison between tracks in Figure 4 shows that while, on the one hand, an equivalent amount of the control transcript RNA-1 was synthetized in both cases, on the other hand, the *ace* RNA was only present at a level ~8-fold higher when transcription was initiated with wild-type RNAP. These results therefore demonstrate that the specific recognition between RNAP and the UP-element is mediated by the C-terminus of the α subunit which stimulates the *ace* promoter expression.

Binding of RNAP to *rrnB*P1

In order to check the results presented above, base removal experiments were performed on the *rrnB*P1–RNAP complex. Figure 2C shows that, in this case again, two sites of increased affinity for RNAP can be detected when the bases from positions –7 to –15, and from positions –40 to –53, were removed by chemical treatment of the bottom DNA strand. These regions of functional importance could be related with the 'melting domain' and with the UP module of the *rrnB*P1 promoter, respectively. Indeed, this latter region coincides with the (A+T)-rich α-binding site which was characterized by DNase I and hydroxyl radical protection assays (1,2).

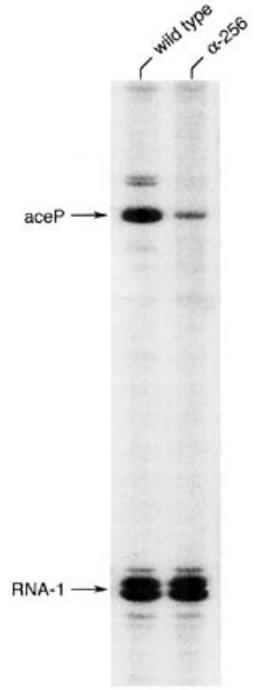

Figure 4. *In vitro* transcription from *ace* promoter with wild type RNAP and a mutant polymerase containing a C-terminally truncated α subunit (α-256). Transcription assays were performed according to the procedure described in Materials and Methods with the pJCD9 plasmid. The respective positions of the RNA I (107–108 nt) and *ace*P (179 nt) transcripts are indicated by arrows.

Our results concerning two different UP module-containing promoters suggest that local DNA flexibility generated in the UP element facilitates its recognition by RNAP. This observation is in agreement with previous data that demonstrate the ability of intrinsically curved DNA, upstream the –35 region, to increase the interaction of *E.coli* RNAP with its promoter (33,34). Moreover, by studying both *in vivo* and *in vitro* a series of mutations within the –10 to –35 region of the *proU* promoter of *Salmonella typhimurium*, it has been suggested that increased DNA flexibility is required for the activation of these mutant promoters (35).

Furthermore, recent work carried out on the spacer DNA, a region that has been proposed to contact specifically the α subunit, between the CRP-binding site and the *lac* promoter, has shown that it contains no specific sequence determinant for *lac* transcription activation (36).

Taken together, these results could explain the previous failure to define a precise consensus α-binding site from all the UP elements detected so far.

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