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Relevance of UP elements for three strong *Bacillus subtilis* phage ϕ29 promoters

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ABSTRACT

Various *Escherichia coli* promoters contain, in addition to the classical –35 and –10 hexamers, a third recognition element, named the UP element. Located upstream of the –35 box, UP elements stimulate promoter activity by forming a docking site for the C-terminal domain of the RNA polymerase α subunit (αCTD). Accumulating genetic, biochemical and structural information has provided a detailed picture on the molecular mechanism underlying UP element-dependent promoter stimulation in *E.coli*. However, far less is known about functional UP elements of *Bacillus subtilis* promoters. Here we analyse the strong early σ^A-RNA polymerase-dependent promoters C2, A2c and A2b of the lytic *B.subtilis* phage ϕ29. We demonstrate that the phage promoters contain functional UP elements although their contribution to promoter strength is very different. Moreover, we show that the UP element of the A2b promoter, being critical for its activity, is located further upstream of the –35 box than most *E.coli* UP elements. The importance of the UP elements for the phage promoters and how they relate to other UP elements are discussed.

INTRODUCTION

Bacteria have a multisubunit RNA polymerase (RNAP) with a conserved subunit composition. The core enzyme is composed of a β, β′ and two α subunits. Association of a σ subunit with the core enzyme forms the holoenzyme and determines the specificity of promoter utilization. The *Escherichia coli* and *Bacillus subtilis* RNAP holoenzymes present during logarithmical growth contain σ^{70} and σ^A factors, respectively. These σ factors recognize the canonical hexameric sequences 5′ TATAAT 3′ and 5′ TTGACA 3′, centred ~10 and 35 bp upstream of the transcription start site, respectively (1,2). The similarity of the –35 and –10 elements to the consensus recognition hexamers and the spacing between them determine to a great extent the strength of a promoter. In addition, the presence of a third recognition element, located upstream of the –35 hexamer of various promoters, can enhance promoter strength significantly. In particular, studies on the strong *E.coli rrnB* P1 promoter provided a major breakthrough in understanding the mechanism by which –35 upstream sequences, named UP elements, enhance promoter strength [for reviews see Busby and Ebright (3), Ebright and Busby (4) and Gourse *et al*. (5,6)]. UP elements were demonstrated to be a docking site for the C-terminal domain (CTD) of the α subunit of RNAP. They stimulate promoter activity primarily by increasing the initial equilibrium constant (K_B), although subsequent step(s) in the transcription initiation pathway (k_f) may also be affected in at least a subset of UP element-containing promoters (7,8). UP elements are independent promoter modules because they can stimulate transcription when fused to other promoters (9,10).

The *E.coli* α subunit contains two independently folded domains connected by a flexible linker (11). Whereas the N-terminal domain is responsible for dimerization and interaction with the β and β′ subunits (12), the CTD can interact with DNA and/or transcriptional regulators [for review see Ebright and Busby (4) and Hochschild and Dove (13)]. A full UP element, characterized by a high A + T content, actually consists of two subsites, each of which can bind one α subunit (14). Genetic studies have identified amino acid residues in the *E.coli* αCTD that are crucial for both DNA binding and UP element function (15,16). Moreover, functional, biochemical and structural studies, including the determination of the solution and crystal structures of αCTD and αCTD–DNA complex, showed that the residues crucial for DNA binding and UP element function reside in two helix–hairpin–helix (HhH) motifs that interact with UP element DNA in and across the minor groove (14,17–21).

Relatively little is known about UP elements present in *B.subtilis* promoters. Early reports have demonstrated that the *B.subtilis spoVG* promoter requires an AT-rich region between positions –40 and –70 for high activity *in vivo* and *in vitro* (22,23). Similarly, high expression of the autolysin gene *cwlB* requires a DNA region upstream of its –35-promoter box (24). In the case of the more thoroughly studied flagellin gene (*hag*), it was shown that the RNAP α subunit binds to the UP element, which is required for high expression of this promoter (25). Peculiarly, these *B.subtilis* promoters are recognized by RNAP containing the alternative σ factor σ^D (*hag* and *cwlB*) or σ^H (*spoVG*). Statistical analysis of 142 *B.subtilis* σ^A-dependent promoter sequences revealed that upstream promoter regions (–36 to –80) are enriched for short A and T tracts (26), suggesting that UP elements may be

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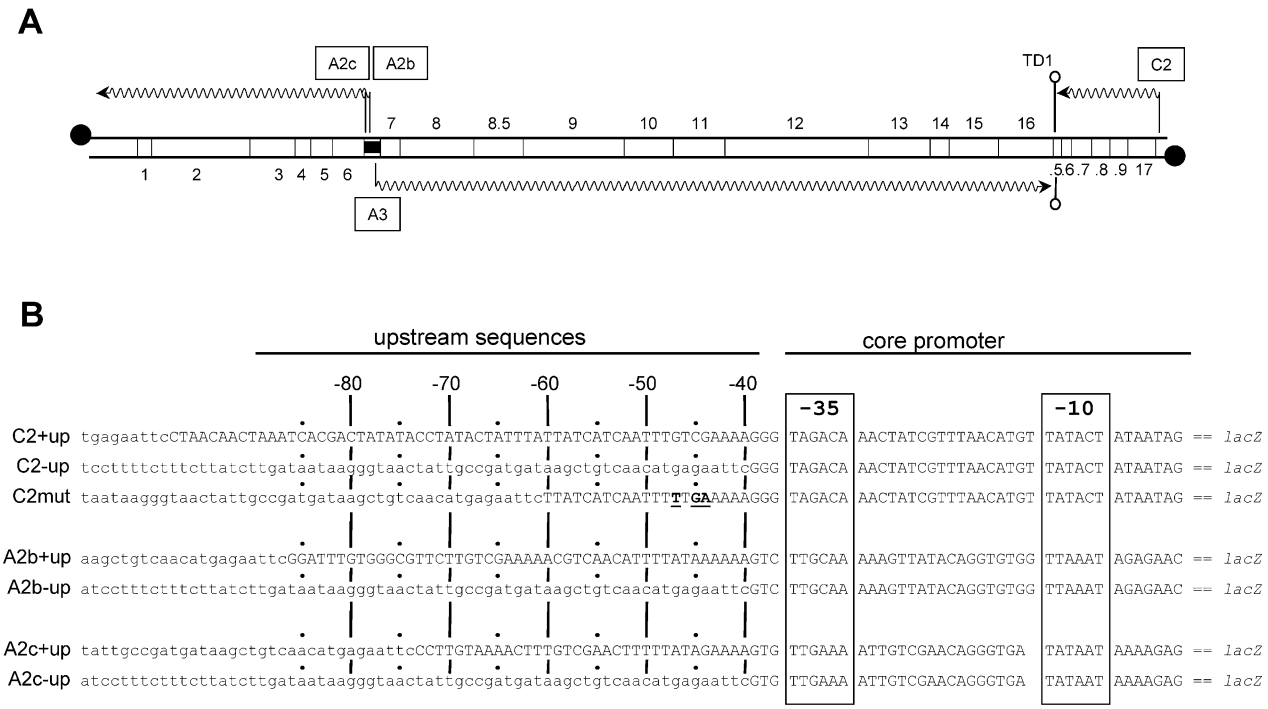

Figure 1. Position of the main ϕ29 promoters in the phage genome (**A**) and sequences of the early ϕ29 promoters C2, A2b and A2c used in this study (**B**). A genetic and transcriptional map of the ϕ29 genome is given in (A). The direction of transcription and length of the transcripts are indicated by wavy arrows. The transcripts of late and early expressed operons and the late and early promoters (boxed) are shown below and above the map, respectively. Some additional minor promoters [for review see Meijer *et al*. (28)] are omitted. The positions of the various genes, indicated with numbers, are indicated between the two DNA strands. The positions of gene 16.7 and ORFs 16.9, 16.8, 16.6 and 16.5, located at the right side of the genome, are indicated with the numbers .7, .9, .8, .6 and .5, respectively. The bidirectional transcriptional terminator TD1 between the convergently oriented late and early operon located at the right side of the genome is indicated with a hairpin structure. A black box indicates the region spanning the early promoters A2b and A2c, and the late A3 promoter. Black circles represent the terminal protein covalently attached to the 5′ DNA ends. Sequences of the phage ϕ29 early promoters C2, A2b and A2c containing either native or substituted upstream sequences are presented in (B). Native sequences are given in upper case letters, and substituted sequences, corresponding to the pRD62 integration vector, are in lower case. The various promoters are aligned according to their –10 and –35 hexamers (boxed). The three point mutations present in the upstream sequence of promoter variant C2mut are in bold and underlined. Numbering of the upstream sequences was according to that determined for promoter C2.

common for σ^A-dependent *B.subtilis* promoters. So far, experimental evidence for the presence of a UP element has been provided only for the σ^A-dependent *B.subtilis* promoters *tms* and *veg* (27).

Bacteriophage ϕ29 is a lytic *B.subtilis* phage with a rather simple life cycle [for a review see Meijer *et al*. (28)]. The genome of ϕ29 is a linear double-stranded DNA of 19 285 bp that contains a terminal protein linked at each of its 5′ ends. A genetic and transcriptional map of the ϕ29 genome is presented in Figure 1A. Shortly after infection, an early operon, which contains the gene encoding the ϕ29 transcriptional regulator protein p4 and the essential genes required for phage DNA replication, is expressed from two strong, tandemly organized promoters named A2b and A2c. Also, a second operon, located at the right side of the ϕ29 genome, is expressed soon after infection. This operon is under the control of the strong early promoter C2. As infection proceeds, the late A3 promoter is activated and the two early operons become repressed.

Several features made the three strong early ϕ29 promoters appealing for these studies. First, the presence of A + T rich regions upstream of their –35 hexamers suggested that they might contain UP elements and, secondly, it was intriguing to study whether known differences between the regulation of these strong early ϕ29 promoters would be related to the presence of possible UP elements.

In this study, we show that native DNA sequences located upstream of the –35 boxes of the ϕ29 promoters C2, A2b and A2c enhance promoter activity *in vivo* as well as *in vitro*. In addition, interaction of the αCTD of the *B.subtilis* RNAP with these upstream promoter sequences is required for stimulating promoter activity. Based on the results obtained, we conclude that the early ϕ29 promoters A2b, A2c and C2 contain functional UP elements. Moreover, we show that these UP elements affect differently the strength of their cognate promoter and that the UP element of the A2b promoter, which is critical for its activity, is positioned farther upstream of the –35 box than is commonly found.

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages

The bacterial strains and plasmids used in this study are listed in Table 1. All *B.subtilis* strains were isogenic with wild-type *B.subtilis* strain 168 (*trpC2*). Chloramphenicol and ampicillin were added to *E.coli* and *B.subtilis* cultures or plates to final concentrations of 1 and 100 μg/ml, respectively.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Source or reference
B.subtilis		
168	*trpC2*	BGSC[a]
M1_C2B	*trpC2*, *amyE::C2+up-lacZ*	This study
M1_C2C	*trpC2*, *amyE::C2-up-lacZ*	This study
M1_C2D	*trpC2*, *amyE::C2mut-lacZ*	This study
M1_A2cA	*trpC2*, *amyE::A2c+up-lacZ*	This study
M1_A2cC	*trpC2*, *amyE::A2c-up-lacZ*	This study
M1_A2bA	*trpC2*, *amyE::A2b+up-lacZ*	This study
M1_A2bC	*trpC2*, *amyE::A2b-up-lacZ*	This study
E.coli XL1-Blue	F′::Tn*10 proA+B+ lacI*^{q} Δ(*lacZ*)*M15*/*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_K-M_K+) *supE44 relA1 lac*	(55)
Plasmids		
pUC19Ω	pUC19 containing the Omega interposon in the NarI site	(33)
pRD62	*B. subtilis amyE* integration vector containing possible *yllC* promoter fragment	(30)
pDM1_C2B	EcoRI–BglII C2 promoter with upstream sequences inserted into pRD62	This study
pDM1_C2C	EcoRI–BglII C2 promoter without upstream sequences inserted into pRD62	This study
pDM1_C2D	EcoRI–BglII C2 promoter with upstream sequences containing three point mutations inserted into pRD62	This study
PDM1_A2bA	EcoRI–BglII A2b promoter with upstream sequences inserted into pRD62	This study
pDM1_A2bC	EcoRI–BglII A2b promoter without upstream sequences inserted into pRD62	This study
pDM1_A2cA	EcoRI–BglII A2c promoter with upstream sequences inserted into pRD62	This study
pDM1_A2cC	EcoRI–BglII A2c promoter without upstream sequences inserted into pRD62	This study
pOC2B	pUC19Ω derivative having XbaI fragment containing promoter C2 and upstream sequences	This study
pOC2C	pUC19Ω derivative having XbaI fragment containing promoter C2 without upstream sequences	This study
pOC2D	pUC19Ω derivative having XbaI fragment containing promoter C2 and upstream sequences with three pointmutations	This study
pOA2bA	pUC19Ω derivative having XbaI fragment containing promoter A2b and upstream sequences	This study
pOA2bC	pUC19Ω derivative having XbaI fragment containing promoter A2b without upstream sequences	This study
pOA2cA	pUC19Ω derivative having XbaI fragment containing promoter A2c and upstream sequences	This study
pOA2cC	pUC19Ω derivative having XbaI fragment containing promoter A2c without upstream sequences	This study

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DNA techniques

All DNA manipulations as well as transformation of $CaCl_2$-treated *E.coli* cells were carried out according to Sambrook *et al*. (29). *Bacillus subtilis* strains were transformed as described (30). Restriction enzymes were used as indicated by the suppliers. [α-^{32}P]dATP, [γ-^{32}P]ATP and [α-^{32}P]UTP (3000 Ci/mmol) were obtained from Amersham International plc. Plasmid DNA was isolated using a Wizard® Plus DNA purification kit (Promega, Madison, WI). DNA fragments were isolated from gels using the Qiaquick Gel Extraction Kit (Qiagen Inc., Chatsworth, USA). The dideoxynucleotide chain termination method (31) with Sequenase (United States Biochemicals sequencing kit) was used for DNA sequencing.

PCR techniques

PCRs were carried out essentially as described (32) using the proofreading-proficient Vent DNA polymerase (New England Biolabs, Beverly, MA). Template DNAs were denatured for 1 min at 94°C. Next, DNA fragments were amplified in 30 cycles of denaturation (30 s; 94°C), primer annealing (1 min; 55°C) and DNA synthesis (30 s; 73°C). Purified phage ϕ29 DNA served as template for amplification of the desired promoter-containing regions.

Oligonucleotides

The sequences of the synthetic oligonucleotides (Isogen Bioscience BV, The Netherlands) used are listed in Table 2. All oligonucleotides used for cloning of the ϕ29 promoters contain an XbaI restriction site near their 5′ termini. In addition, oligonucleotides corresponding to upstream (designated 'U') and/or downstream (designated 'L') promoter sequences contained an EcoRI or BglII restriction site, respectively, located 3′ with respect to the XbaI restriction site. The seq-series of oligonucleotides were used for sequencing or to provide fragments amplified in PCRs with a unique restriction site at their end to be used for labelling.

Construction of integration plasmids

DNA fragments containing promoter A2b, A2c or C2, either with or without upstream sequences, were amplified by PCR using genomic ϕ29 as template DNA together with the appropriate oligonucleotides (listed in Table 2). After purification, the PCR products were digested with XbaI and ligated

Table 2. Synthetic oligonucleotides used

Name	Sequence (5′–3′)[a]	Position
C2_L1	ttt ttc tag act agt aga tct GGT TGT CTT ATT ACC TTA CTT CTA TTA TAG	
C2_U2	ttt ttc tag aag ctt gaa ttc CTA ACA ACT AAA TCA CGA CTA TAT ACC	
C2_U3	ttt ttc tag aag ctt gaa ttc GGG TAG ACA AAC TAT CGT TTA ACA TG	
C2_U4	ttt ttc tag aat tcT TAT CAT CAA TTT **TTG A**AA AAG GGT AGA CAA ACT ATC GTT TAA	
A2c_L1	ttt ttc tag act agt aga tct GTA GAC TCT GTA TCT CTT CTA CTC	
A2c_U1	ttt ttc tag aag ctt gaa ttc CCT TGT AAA ACT TTG TCG AAC TTT TT	
A2c_U2	ttt ttc tag aat tcG TGT TGA AAA TTG TCG AAC AGG GTG ATA TAA T	
A2b_L1	ttt ttc tag atc tct ata aaa agt tcg aca aag ttt tac aag g	
A2b_U1	ttt ttc tag aag ctt gaa ttc GGA TTT GTG GGC GTT CTT GTC GAA	
A2B_U3	ttt ttc tag aat tcG TCT TGC AAA AAG TTA TAC AGG TGT GGT TAA AT	
seq1	ggg atc gcc aag ctt cat gtg	34[b]
seq2	ggg taa cta ttg ccg atg ata agc tgt c	36
seq3	gtg ttt ttt taa agg att tga gcg tag cg	97
seq4	aag gcg att aag ttg ggt aac gcc agg g	97
seq6	ttt ttg atc cta tct tga taa taa ggg taa cta ttg ccg atg	50
seq7	gtg ggg atc cgt gtt ttt tta aag gat ttg agc gta gcg aaa	97

[a]Nucleotides identical to the ϕ29 sequences are in upper case letters. Lower case letters in the oligonucleotides other than the seq-series represent 5′ extensions specifying restriction sites used for cloning. Mutations present in oligonucleotide C2_U4 are in bold. The seq-series of oligonucleotides were applied in PCR assays to generate substrates used in *in vitro* transcription or DNase I footprint assays.
[b]Numbers indicate the position of the 5′ end of the oligonucleotide with respect to the upstream EcoRI (seq2, seq3, seq6 and seq7) and downstream BglII (seq1 and seq4) cloning sites present in the pDM1-series of integration vectors.

to XbaI-linearized pUC19Ω (33) DNA. The ligation mixtures were used to transform competent *E.coli* XL1-Blue cells. Plasmid DNA from ampicillin-resistant colonies was isolated and DNA sequence analysis was used to verify the correctness of the cloned inserts. Next, the EcoRI–BglII ϕ29 promoter-containing fragments were isolated and used to replace the EcoRI–BglII fragment located in front of the *lacZ* gene present in the pRD62 integration vector, which contains the *B.subtilis yllC* promoter fragment (30). The *B.subtilis* integration vector pRD62 is based on the *E.coli* pBR322 replicon and contains sequences corresponding to the N- and C-terminal parts of the *B.subtilis amyE* gene, which are separated by the divergently oriented *cat* and *lacZ* genes. The resulting integration plasmids, the pDM1-series, are listed in Table 1.

Construction of chromosomal *lacZ* fusions and β-galactosidase assays

The pRD62-derived integration vectors were transformed into *B.subtilis* 168 cells, with selection for chloramphenicol resistance, and the loss of amylase activity was used to confirm that insertion had occurred by a double crossover recombination event. Levels of β-galactosidase activity during growth on liquid medium were determined as described by Daniel *et al.* (30).

Protein purification

The σ^A-RNAP, and the wild-type and mutant α subunits were purified as described before (34,35). Reconstituted RNAP containing either the wild-type or the mutant α subunit lacking its 15 C-terminal amino acids was obtained as described by Mencía *et al.* (35) with the modifications introduced by Calles *et al.* (36).

In vitro transcription reactions

Run-off transcription assays (25 µl) contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 200 µM ATP, CTP and GTP, 50 µM UTP, ~150 µM [α-^{32}P]UTP (1 µCi), 1 µg of poly[d(I–C)], 10 U of RNasin, 4 nM of DNA template, 50 nM *B.subtilis* σ^A-RNAP and KCl as indicated. The concentration of the DNA templates was determined by measurement of optical density at 260 nm and verified by quantitative analysis of ethidium bromide-stained 1.5% agarose gels. After 10 min at 37°C, reactions were stopped with EDTA and SDS (final concentrations of 20 mM and 0.15%, respectively). Non-incorporated NTPs were removed through Sephadex-G50 spin columns. Transcripts were ethanol precipitated in the presence of 10 µg of carrier tRNA. They were then resolved by denaturing PAGE (6%) and quantified with a BAS-IIIs Fuji imaging analyser. Promoter-containing DNA fragments were generated by PCR using the proper pDM1 integration vector as template DNA and the same sets of primers as described for the generation of DNase I footprinting templates.

DNase I footprinting

Binding reactions contained, in 20 µl, 1 nM end-labelled DNA, 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 2 µg of poly[d(I–C)], 2 µg of bovine serum albumin, the indicated amount of KCl and 70 nM of either wild-type or αΔ15-reconstituted σ^A-RNAP. Control experiments showed that the reconstitution procedure by itself (35) did not affect the binding characteristics of the RNAP as judged from the identical footprints generated by non-reconstituted or wild-type α reconstituted RNAP. Binding reactions were incubated for 10 min at 37°C before DNase I (0.05 U) was added. Digestion was allowed to proceed for 2 min at 37°C before being stopped with EDTA (20 mM final concentration). The DNA was then ethanol precipitated using linear polyacrylamide as carrier and subsequently analysed in denaturing 6% polyacrylamide gels.

In all cases, substrates were obtained by PCR using the appropriate pDM1 vector as template. The substrates were labelled at their 3′ end by digesting the PCR fragments with

Table 3. *In vivo* activity of promoters with or without native upstream sequences

Strain	Promoter	Upstream end point	β-Gal activity (Miller units)[a]	Relative activity[b]
M1_C2B	C2+up	–98	93.4	100
M1_C2C	C2-up	–39	46.6	49
M1_C2D	C2mut	–60	148.2	158
M1_A2bA	A2b+up	–86	49.1	100
M1_A2bC	A2b-up	–39	6.5	12
M1_A2cA	A2c+up	–73	52.8	100
M1_A2cC	A2c-up	–39	20.2	38

[a]Values are the average of at least three independent experiments, which among themselves differed <10%.
[b]Each *lacZ* fusion contains native sequences downstream of the –10 box up to at least 19 bp downstream of its respective transcription start point. The *lacZ* gene of this vector contains the RBS and initiation codon from the *B.subtilis sspB* gene (30). Note that the β-galactosidase activities of this *lacZ* construct are lower than those obtained by similar integration vectors in which the *lacZ* gene is translationally controlled by the *B.subtilis spoVG* gene signals (56).

the proper restriction enzyme (either EcoRI, HindIII or BamHI) and filling in the generated 3′-recessive ends with Klenow enzyme. The 5′-labelled C2 substrates were obtained by treating primer seq4 with polynucleotide kinase in the presence of [γ-^{32}P]ATP prior to PCR. To analyse fragments of similar size, PCR mixtures contained primers seq3 and seq4 in the case of amplification of DNA fragments encompassing promoters C2-up and C2mut, and seq2 and seq4 in the case of amplification of promoter C2+up. To label the 3′ end, the fragments were digested with HindIII, after which the purified fragments were treated with Klenow enzyme in the presence of [α-^{32}P]ATP. In the case of the A2b promoter, PCRs were carried out using the appropriate pDM1 vector as template DNA and the promoter-downstream primer seq1 combined with the promoter upstream primer seq6 (A2b+up) or seq7 (A2b-up). Next, the PCR products were digested with BamHI, after which the Klenow enzyme was used to label these fragments at their 3′ end. Primer seq7 hybridizes 47 bp upstream of primer seq6 in the backbone of the pDM1 vector. Consequently, the A2b+up and A2b-up core promoter sequences were located at exact equidistant positions with respect to their labelled DNA end. Similarly, primers seq7 and seq4 were used in PCRs with plasmid pDM1_A2cA as template DNA to generate a DNA fragment encompassing the A2cA promoter. The fragment was digested with BamHI, and Klenow enzyme was used to label the 3′ DNA end.

RESULTS

The transcription start sites of the ϕ29 promoters C2, A2b and A2c have been determined (37,38). For an easier interpretation, the promoter sequences shown in Figures 1B and 6 are aligned according to their –35 boxes, and numbering of the upstream sequences of these three promoters is according to that determined for promoter C2. As a consequence, the numbering of promoters A2b andA2c used in this work is –1 (A2c) or +1 bp (A2b) with respect to their published numbering. The numbering coincides with the numbering of the *E.coli rrnB* P1 promoter and derivatives (9).

Effect of upstream sequences on transcription *in vivo*

The effect of upstream sequences on promoter activity *in vivo* was determined for the ϕ29 early promoters C2, A2b and A2c in the absence of additional viral factors. For this, each promoter with or without native sequences upstream of the –35 box was fused to *lacZ* using the *B.subtilis* integration vector pRD62 (see Fig. 1B). Next, each transcriptional *lacZ* fusion was integrated into the wild-type *B.subtilis* strain 168 at the *amyE* locus. Likewise, a *B.subtilis* strain was constructed in which the upstream C2 promoter region contains three point mutations. Each strain containing a single copy of the transcriptional *lacZ* fusion was grown in liquid medium, and β-galactosidase activity was determined to measure promoter activity during mid-logarithmic growth. The results, summarized in Table 3, show that each promoter containing its native upstream sequences displayed higher activity compared with the corresponding derivative lacking these sequences. This demonstrates that the presence of native upstream sequences enhances the strength of these promoters *in vivo*. The effects were different, though, for each promoter. Whereas the lack of native upstream sequences led to a relatively moderate decrease in activity of promoters C2 and A2c (~50 and 60%, respectively) it caused a dramatic decrease in promoter A2b activity (~90%).

The point mutations introduced in the upstream region of promoter C2, variant C2mut, make the upstream region more similar to the consensus *E.coli* UP element sequence, as defined by Estrem *et al.* [(39); see Fig. 6 and Discussion]. Analysis of the C2mut promoter showed that its *in vivo* activity was higher than that of promoter C2+up containing native upstream sequences.

Effect of upstream sequences on transcription *in vitro*

The effect of upstream sequences on promoter strength was tested in *in vitro* transcription assays in the absence of any factor other than purified *B.subtilis* σ^A-RNAP. *In vitro* transcription of promoters C2+up, C2-up and C2mut was studied at low (0), intermediate (100 mM) and high (200 mM) salt concentration (Fig. 2). At low and intermediate salt concentrations, the relative activities of the C2 promoter variants *in vitro* were comparable with those observed *in vivo*; i.e. the activity of promoter C2+up is higher than that of C2-up but lower than that of C2mut. However, whereas promoter activity dropped only modestly at high salt concentrations in the case of promoters C2+up and C2mut, a dramatic decline in promoter activity was observed for promoter C2-up. Thus, the

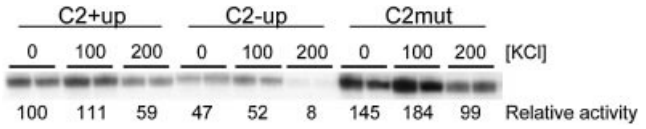

Figure 2. *In vitro* transcription of C2 promoter derivatives with native (C2+up), substituted (C2-up) or mutant upstream sequences (C2mut) at different ionic strengths. Run-off assays were performed in duplicate. Reaction mixtures contained 4 nM of the appropriate C2 promoter template, 40 nM purified σ^A-RNAP and the indicated amount of KCl. After exposure of the dried gel, the signals were quantified using a Bas-IIIs image analyser. The relative promoter activities are given at the bottom.

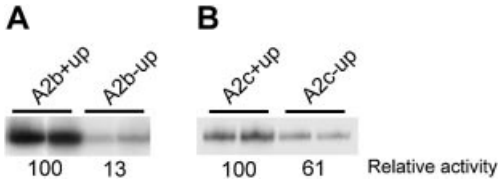

Figure 3. *In vitro* transcription of A2b (**A**) and A2c (**B**) promoter derivatives containing native or substituted upstream sequences. Run-off assays were performed in duplicate. Reaction mixtures contained 4 nM of the appropriate template DNA and 40 nM purified σ^A-RNAP. After exposure of the dried gel, the signals were quantified using a Bas-IIIs image analyser. The relative promoter activities are given at the bottom. *In vitro* transcription of A2b and A2c promoters with their native upstream sequences by RNAP containing a mutant α subunit lacking its 59 C-terminal amino acids was mildly and severely affected, respectively, when compared with transcription performed with wild-type RNAP (42).

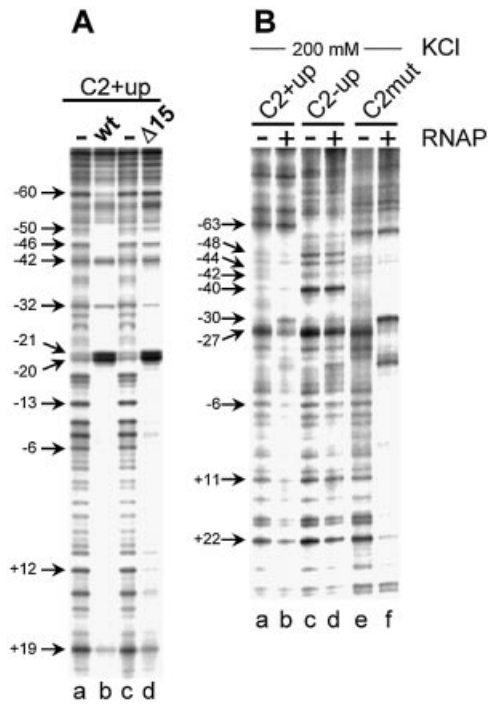

Figure 4. DNase I footprints of RNAP on promoter C2 of ϕ29. C2 promoter-containing DNA fragments were end-labelled at the non-template (A) or template (B) strand and analysed for the binding of σ^A-RNAP (70 nM). (**A**) DNA template containing promoter C2+up was incubated in a low salt buffer with RNAP containing wild-type (wt) or a truncated mutant α subunit lacking its C-terminal 15 residues (Δ15). (**B**) C2 promoters C2+up, C2-up and C2mut were tested for the binding of wild-type σ^A-RNAP under stringent conditions (200 mM KCl). Positions relative to the C2 transcription start site are indicated.

in vitro results demonstrate a direct effect of the promoter C2 upstream region on promoter strength that is most prominent in high salt conditions. Analysis of *in vitro* transcription of the A2b and A2c promoters (Fig. 3A and B, respectively) with or without their upstream sequences at low salt concentration showed that promoter activity was reduced in the promoter variants lacking their upstream sequences. Moreover, the absence of upstream sequences affected the *in vitro* activity of these promoters in a similar way to that observed *in vivo*; thus, the lack of upstream sequences had a drastic effect on the activity of promoter A2b but affected the A2c promoter activity only moderately.

Thus, the *in vitro* results demonstrate that the presence of upstream sequences enhances the activity of the three early ϕ29 promoters, although the degree of stimulation is different for each promoter. In addition, the stimulatory effect of upstream sequences on promoter strength observed *in vitro* at low salt concentration corresponds well to those observed *in vivo*.

RNA polymerase α subunit interacts with the promoter upstream sequences

Promoter C2. The *in vivo* and *in vitro* analyses described above indicate that the early ϕ29 promoters C2, A2c and A2b contain functional UP elements. Characteristically, RNAP forms extended footprints on UP element-containing promoters due to binding of the αCTD to the UP element. DNase I footprint analyses were therefore performed to study whether *B.subtilis* σ^A-RNAP forms extended footprints on the native ϕ29 promoters and whether this is due to the αCTD. For the latter objective, RNAP holoenzyme containing a mutant α subunit lacking its 15 C-terminal amino acids (αΔ15-RNAP) was used. Although this mutant α subunit contains the conserved residues that have been demonstrated for the *E.coli* αCTD to be crucial for UP element binding, it is unable to bind DNA (unpublished results) and it is dysfunctional in transcriptional regulation, most probably because the structure of the CTD is disrupted in the truncated protein (33,35,40).

Thus, a DNA fragment encompassing the C2+up promoter labelled at the non-template strand at its 3′ end was incubated with RNAP holoenzyme containing either wild-type or mutant α subunit and analysed by DNase I footprinting (Fig. 4A). In both cases, RNAP binds to promoter C2 forming an open complex that gives a DNase I footprint spanning positions –42 to +19 relative to the transcription start site and generated two hypersensitive bands at positions –20 and –21. However, only when the RNAP contained wild-type α subunit was the additional full protection of positions –45, –46, –49 and –50, and partial protection of –60 observed (compare lanes b and d). Control assays showed that these latter bands were also protected by wild-type RNAP that had undergone the α subunit reconstitution procedure (results not shown). These results demonstrate that the wild-type α subunit is responsible for protection of the native upstream region of promoter C2.

We also analysed binding of wild-type RNAP to promoters C2+up, C2-up and C2mut using templates that were labelled at the 5′ ends of the template strand (Fig. 4B). These DNase I assays were performed under stringent conditions (200 mM

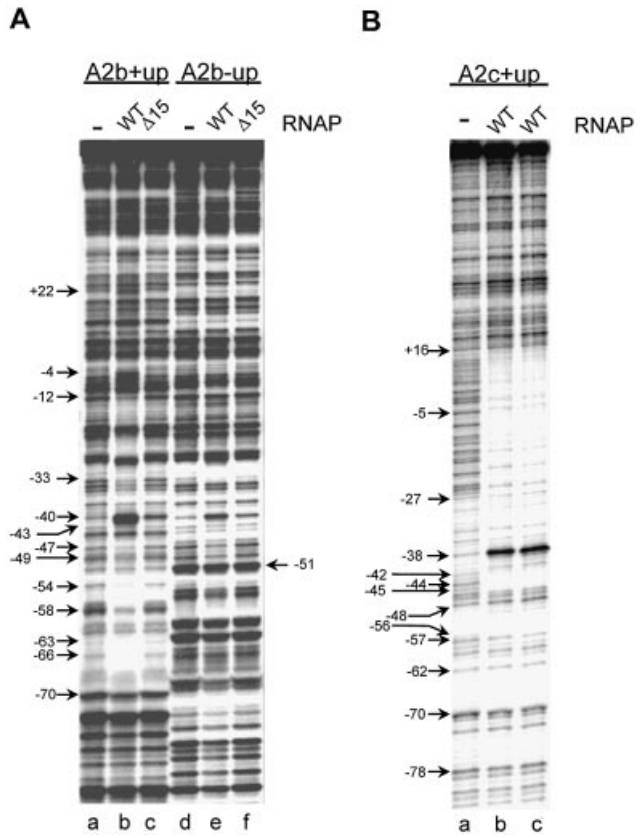

Figure 5. DNase I footprints of σ^A-RNAP on promoter A2b (**A**) and A2c (**B**). DNA templates encompassing promoter A2b+up, A2b-up or A2c+up were labelled at their 3′ end at the template strand and incubated with 70 nM of wild-type (WT) or αΔ15-RNAP (Δ15). Positions are indicated corresponding to the numbering of the A2b and A2c promoters used in Figures 1B and 6. DNA templates were amplified by PCR using the appropriate pDM1 variant with the promoter downstream primer seq1 and the promoter upstream primer seq6 or seq7 in the case of promoter A2b+up and A2b-up, respectively, and with primers seq7 and seq4 in the case of promoter A2c+up. Primer seq7 hybridizes 47 bp upstream of primer seq6 in the backbone of the pDM1 vector. Consequently, the A2b+up and A2b-up core promoter sequences were located at exact equidistant positions with respect to their labelled DNA end. Lanes b and c of the A2c promoter (B) are duplicate reactions.

KCl) for two reasons: (i) to prevent possible non-specific interactions of the α subunit with the promoter upstream region; and (ii) to correlate the footprints with the *in vitro* transcription activity of these promoters performed under stringent conditions. Although the high salt concentration affected the quality of the footprints, they provided the following information. The C2+up core promoter region was only partially protected, indicating that the high ionic strength conditions affected RNAP binding. Nevertheless, the promoter upstream positions –42, –44 and –48 were partially protected under these stringent conditions (lane b). These upstream region positions were also protected in the C2mut promoter (lane f). In this case, the core promoter region was almost completely protected and the hypersensitive band at position –30 was much more pronounced compared with that generated in promoter C2+up. Thus, the point mutations present in the upstream region of promoter C2mut clearly enhance RNAP binding. In contrast, hardly any footprint was observed in the case of the C2-up promoter (lane d). These results are consistent with the view that the C2 promoter upstream region is an important determinant for RNAP binding.

The DNase I footprint results obtained under high ionic strength conditions are in agreement with the *in vitro* transcription results of the three C2 promoter variants carried out at the same salt concentration. Thus, C2mut had the highest *in vitro* promoter activity and protected the core and upstream promoter region rather well in DNase I assays; C2+up had a lower *in vitro* promoter activity and its core and upstream promoter region was less well protected; and C2-up displayed very low *in vitro* promoter activity and gave a hardly detectable footprint.

Promoter A2b. Although A2b is a strong promoter, wild-type RNAP forms very unstable open complexes at this promoter (37,38,41). Most probably, the high activity of promoter A2b is due to efficient conversion of open complexes into elongation complexes (37). In addition, the A2b promoter is highly salt sensitive (37,41). Binding of wild-type RNAP to the A2b+up promoter was detected at low salt concentrations in DNase I footprint analysis (Fig. 5A, lane b). One of the most characteristic changes upon binding of the RNAP to the A2b+up promoter is the generation of a strong hypersensitive site at position –40 (position –41 related to the real A2b transcription start site) of the template strand [Fig. 5A, lane b (41)]; hypersensitive sites were also generated at positions –4 and +22 upon RNAP binding. In addition, the following positions in the native promoter upstream region were partially or fully protected by wild-type RNAP: –47, –49, –54, –57, –58, –63 and –66. Interestingly, whereas the upstream regions protected by RNAP on UP element-containing promoters are generally limited to approximately position –60, Figure 5A shows that positions between –60 and –70 are protected in the A2b+up promoter. None of the positions in the upstream promoter region was protected by αΔ15-RNAP (lane c). Actually, hardly any footprint was detected at the A2b+up promoter when RNAP containing αΔ15 instead of wild-type α was used (compare lanes b and c); only position –40 became moderately hypersensitive, indicating that the αCTD plays a crucial role in recognition of this promoter.

Binding of RNAP containing either wild-type or the mutant α subunit to the A2b-up promoter was also analysed by DNase I footprinting (Fig. 5A, lanes d–f). Most importantly, in sharp contrast to promoter A2b+up, the upstream region of the A2b-up promoter was hardly protected by wild-type RNAP (lane e). Moreover, position –40 became only moderately hypersensitive. In fact, the moderate hypersensitivity generated by wild-type RNAP on the A2b-up promoter is comparable with that generated by αΔ15-RNAP on the A2b+up promoter (compare lanes c and e). In addition, the hypersensitivity of position –40 was decreased even further when αΔ15-RNAP was used at the A2b-up promoter (lane f). These results, therefore, substantiate the conclusion that the αCTD is crucial for A2b promoter recognition through binding to its promoter upstream region.

Promoter A2c. A DNA fragment encompassing the A2c+up promoter containing its native upstream promoter sequences,

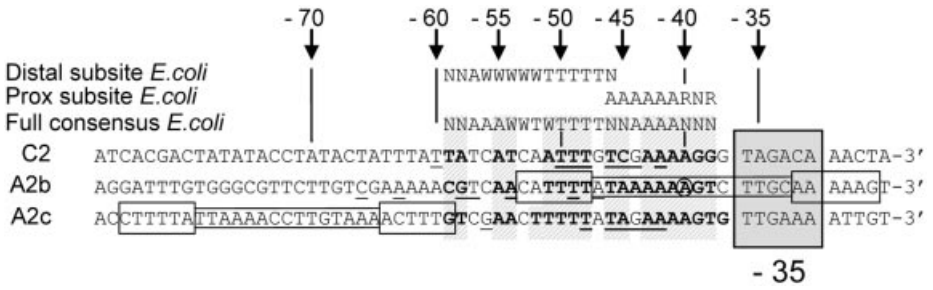

Figure 6. Sequences of the –35 boxes and upstream regions of the phage ϕ29 early promoters C2, A2b and A2c aligned with the consensus UP element sequence determined for *E.coli* promoters. The promoters are aligned according to their –35 hexamers (grey box). The full *E.coli* consensus UP element sequence was taken from Estrem *et al.* (39), and those of the proximal and distal subsites were taken from Estrem *et al.* (14). W = A or T; R = A or G; N = A, C, G or T. The non-template strand positions protected by RNAP in DNase I footprints are underlined. The p4-binding sites 2 (upstream of A2c promoter) and 3 (A2b promoter) are boxed. Nucleotides conserved with respect to the *E.coli* consensus UP element sequence are in bold and shaded. The characteristic RNAP binding-induced hypersensitive band upstream of the A2b promoter is circled. Note that the αCTD-binding site of the A2b promoter is located upstream of the p4-binding site (see text for details).

3′ end labelled at the template strand, was used in DNase I footprint assays. Figure 5B shows that wild-type RNAP binds very efficiently to the A2c promoter generating a footprint that spans the region from +16 to –57. Particularly, the following positions in the upstream promoter region were protected: –42 until –45, and –56. As explained in the Discussion, the αCTD is responsible for protection of these upstream promoter positions, which can be concluded from ϕ29 promoter A2c studies carried out in our laboratory (40,42).

DISCUSSION

In this work, we demonstrate that the three major early promoters C2, A2b and A2c of the lytic phage ϕ29 contain functional UP elements. However, the strength of the three ϕ29 promoters depends to different extents on the UP element. Whereas the UP element is critical for A2b promoter activity, the UP elements of promoters C2 and A2c stimulate transcription ~2-fold. Although the ~2-fold stimulation of the A2c and C2 promoters is a relatively modest effect, the UP elements most probably will be vital for fitness of the phage. During the ~50 min infection cycle, up to 1000 progeny phages are produced, indicating that the ϕ29 promoters are very strong, allowing synthesis of high levels of phage proteins required for the production of such large numbers of progeny phages. Indeed, quantitative western blotting showed that a single infected cell contained up to 180 000 molecules of protein p16.7 and up to about 2 million copies of the single-stranded DNA-binding protein (SSB) p5 (43,44). Protein p16.7 is expressed from promoter C2 and the SSB p5 is expressed from the tandemly organized promoters A2b and A2c. A 2-fold reduction in promoter strength will affect the production of phage progeny and hence its fitness in natural conditions.

Although the three ϕ29 promoters analysed are very strong, they have different affinities for the RNAP. The C2 and A2c promoters have a high RNAP affinity [see Figs 4 and 5 (45)] but the A2b promoter has a very low affinity for the RNAP and is very salt sensitive [see Fig. 5A (37,41)]. Thus, the C2 and A2c promoters are optimized for RNAP binding and the A2b promoter owes its high promoter activity to efficient conversion of open complexes into elongation complexes (37,41). Interestingly, whereas the presence of an UP element is critical for the weak RNAP affinity promoter A2b, it is far less important for the A2c and C2 promoters having high RNAP affinity. Probably, UP elements are generally more important for promoters having low compared with those having high RNAP affinity. This is supported by the observation that the effect of lack of UP elements on promoter strength is more pronounced at high salt concentrations, which weakens promoter affinity (25,46), and is in agreement with the view that UP elements function primarily by increasing the initial equilibrium constant between RNAP and DNA (10).

A consensus UP element sequence has been determined for *E.coli* promoters (39). The alignment presented in Figure 6 shows that the upstream region spanning positions –59 to –38 of the ϕ29 promoters C2, A2b and A2c shares a high level of similarity with the consensus UP element sequence determined for *E.coli* promoters. The 22 bp *E.coli* UP element sequence was later shown to actually consist of two subsites, each of which can bind a subunit of α. Of these, the proximal subsite (positions –46 to –38) was shown to be far more important for stimulation of *E.coli* promoters than the distal subsite (positions –59 to –46) (14). Figure 6 shows that especially the proximal subsite sequences are highly conserved in the ϕ29 promoters, supporting the view that this subsite is also important in *B.subtilis*.

In this work, we have demonstrated that the upstream sequences of the C2 and A2b promoters are docking sites for αCTD and have determined the promoter A2c upstream positions that are protected by wild-type RNAP. Binding of wild-type, αΔ15 and αΔ59-RNAP to the A2c promoter has been analysed in other studies carried out in our laboratory (40,42). These studies demonstrate that the αCTD is responsible for protection of the A2c upstream promoter positions shown in Figure 5B and, therefore, forms the docking site of the αCTD in this promoter.

The αCTD-binding sites of C2 and A2c are located within the –60 to –40 promoter region. Together with the observation that the presence of this region stimulates the activity of these promoters *in vitro* and *in vivo*, we conclude that these regions constitute the functional UP elements of these promoters. In the case of promoter C2, this conclusion is further supported by the results obtained with the mutant C2mut promoter,

which contains an upstream promoter region that is more similar to the *E.coli* consensus UP element sequence than the native C2 upstream sequence. The C2mut promoter displayed a higher activity *in vivo* and *in vitro* and the RNAP bound more tightly to this promoter compared with the wild-type C2 promoter, especially under stringent conditions.

The –42 to –50, and –49 to –55 upstream region of the C2 and A2c promoters, respectively, are rather inefficiently digested by DNase I, leaving the possibility that the αCTD may contact one or more positions in these regions besides the observed ones. Nevertheless, the contacts made by the αCTD in the upstream regions of promoters A2c and C2 are limited to the –40 to –60 regions, demonstrating that the UP element of these promoters are located just upstream of the –35 box, a situation that is found for most of the UP elements identified so far. On the contrary, however, although the A2b upstream promoter positions –47 and –49 were partially protected, the most prominent positions protected by the αCTD in this promoter were located within the –50 to –70 region (Fig. 5A). The –40 to –50 region of the A2b promoter is very well digested by DNase I, demonstrating that the region contacted by αCTD at the A2b promoter is located further upstream of the –35 box than is usually found in *E.coli* UP element-containing promoters. The A2b upstream promoter region contacted by αCTD is A + T rich and shares a high level of similarity with the consensus *E.coli* UP element sequence. We therefore conclude that the UP element of the A2b promoter is located further upstream of the core promoter, as commonly observed.

The location of the αCTD-docking site at the A2b promoter is possibly related to the p4-mediated regulation of this promoter that includes an intrinsic curvature located at the A2b promoter (41,47). About 20 min after infection, the early A2b promoter becomes repressed due to binding of the ϕ29-encoded protein p4 to its cognate binding site which encompasses the A2b upstream promoter region and its –35 box (see Fig. 6). The RNAP-hypersensitive band at position –40 is located at the middle of the p4-binding site. This DNA region has a static bend of ~45° (41). Here, we show that the αCTD binds to the DNA region just upstream of the intrinsically curved DNA. Probably, the static bend prevents binding of αCTD to the region directly upstream of the –35 box due to alteration of the width of the minor groove in this region and the static bend may facilitate αCTD to bind further upstream.

Generally, the αCTD-binding site of an UP element is located one or one and two helical turns upstream of the –35 box. In the A2b promoter, however, the αCTD-binding site is located two and three helical turns upstream of their respective –35 box. The αCTD is known to interact with DNA and/or activator molecules at different locations upstream of the –35 element (11,13,48–52). The long flexible linker that connects the αCTD to the N-terminal domain (11,53) is believed to allow the positional plasticity of the αCTD. In fact, the observation that the artificial re-location of the *rrnB* P1 UP element by a single turn of DNA helix did not lead to a loss of transcription stimulation (48,54) demonstrated that UP elements can function at different distances from the –35 box.

The αCTD-binding sites of the three promoters analysed in this study share a high level of similarity with the consensus UP element sequence determined for *E.coli* promoters (see above), indicating that the αCTDs encoded by *E.coli* and *B.subtilis* recognize the same DNA sequences. This confirms the prediction made by Estrem *et al.* (39), which was based on the fact that protein sequences of eubacterial αCTDs share a high level of similarity. Therefore, it is likely that they will have very similar structures and, hence, will recognize similar DNA sequences. Indeed, the seven residues of the *E.coli* αCTD that are most crucial for DNA interaction are nearly invariant in bacteria (15,16). These residues reside in two HhH motifs (21), which interact with the UP element DNA in and across the minor groove (17,18). The recently reported high-resolution crystal structure of the *E.coli* αCTD bound to DNA confirmed the roles of the two HhH motifs of αCTD and five of the seven crucial side chains (R265, N268, G296, K298 and S299) in DNA recognition (20). Thus, it is most likely that the eubacterial αCTDs have highly similar structures and that the DNA sequences recognized by α, therefore, are also very likely to be conserved. The results obtained in this work further support this view.

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