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Promoter upstream bent DNA activates the transcription of the *Clostridium perfringens* phospholipase C gene in a low temperature-dependent manner

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The phospholipase C gene (*plc*) of *Clostridium perfringens* possesses three phased A-tracts forming bent DNA upstream of the promoter. An *in vitro* transcription assay involving *C.perfringens* RNA polymerase (RNAP) showed that the phased A-tracts have a stimulatory effect on the *plc* promoter, and that the effect is proportional to the number of A-tracts, and more prominent at lower temperature. A gel retardation assay and hydroxyl radical footprinting revealed that the phased A-tracts facilitate the formation of the RNAP–*plc* promoter complex through extension of the contact region. The upstream (UP) element of the *Escherichia coli rrnB* P1 promoter stimulated the downstream promoter activity temperature independently, differing from the phased A-tracts. When the UP element was placed upstream of the *plc* promoter, low temperature-dependent stimulation was observed, although this effect was less prominent than that of the phased A-tracts. These results suggest that both the phased A-tracts and UP element cause low temperature-dependent activation of the *plc* promoter through a similar mechanism, and that the more efficient low temperature-dependent activation by the phased A-tracts may be due to an increase in the bending angle at a lower temperature.

Keywords: bent DNA/*Clostridium perfringens*/curved DNA/gene expression/phospholipase C

Introduction

Sequence-directed DNA curvature, which is also referred to as an intrinsic DNA bend, results when special sequence motifs such as A-tracts (Crothers *et al*., 1990; Pérez-Martín *et al*., 1994) and GC-type elements (Bolshoy *et al*., 1991; Brukner *et al*., 1994) are repeated in phase with the DNA helical repeat. A characteristic feature of bent DNA is the anomalous gel mobility at low temperature (Wu and Crothers, 1984; Diekmann, 1987; Koo and Crothers, 1988). Bent DNA sequences are preferentially located upstream of promoters (Plaskon and Wartell, 1987; Tanaka *et al*., 1991; Helmann, 1995). Furthermore, transcriptional regulation by bent DNAs has been demonstrated in a number of cases (McAllister and Achberger, 1989; Ellinger *et al*., 1994; Pérez-Martín *et al*., 1994; Brahms *et al*., 1995; Kim *et al*., 1995). Promoter-proximal and -distal bends tend to facilitate the formation of closed and open complexes, respectively, although there is no clear-cut boundary between their positions (Pérez-Martín *et al*., 1994).

AT-rich sequences were also noted upstream of several strong promoters in *Escherichia coli* (Galas *et al*., 1985; Deuschle *et al*., 1986) and other bacteria (Banner *et al*., 1983; Graves and Rabinowitz, 1986; McAllister and Achberger, 1988; Frisby and Zuber, 1991). The AT-rich sequence located upstream of the *E.coli rrnB* P1 promoter has been shown to stimulate transcription through contact with αCTD, the C-terminal domain of the α subunit of RNA polymerase (RNAP) (Ross *et al*., 1993; Blatter *et al*., 1994; Busby and Ebright, 1994; Jeon *et al*., 1995). Such an AT-rich sequence, known as an upstream (UP) element, has also been demonstrated for other promoters (Rao *et al*., 1994; Fredrick *et al*., 1995; Négre *et al*., 1997), and its consensus sequence has been determined (Estrem *et al*., 1998). A proximal bend and an AT-rich sequence are often located at similar positions (between –60 and –40 bp relative to the transcription start site) and both show phase-dependency (McAllister and Achberger, 1989; Newlands *et al*., 1992). Thus, it is difficult to distinguish the effects on promoter activity of bent DNA and an AT-rich sequence when they overlap.

Phased A-tracts appear to share some features with AT-rich sequences. They are preferentially bound by some DNA-binding proteins, such as histone-like protein HU and integration host factor (IHF) (Shimizu *et al*., 1995). AT-rich sequences are flexible, adopting a bent conformation (Grosschedl *et al*., 1994), which facilitates the recognition and binding of HMG-like domains (Miao and Wang, 1996). Both AT-rich sequences and phased A-tracts show supercoiling-dependent flexibility so as to be localized preferentially in the terminal loops of superhelical domains (Tsen and Levene, 1997). They exhibit characteristic properties, strand opening or transition to a pre-melting state, under low ionic conditions or upon binding to proteins (Chan *et al*., 1993; Bowater *et al*., 1994; Economides *et al*., 1996; Miao and Wang, 1996). Such similarities have raised controversy as to whether bent DNA is or is not responsible for the functions suggested so far.

The phospholipase C gene (*plc*) of *Clostridium perfringens* has three phased A-tracts upstream of the *plc* promoter (Figure 2A). We have previously shown that *plc* gene transcription is stimulated in the presence of A-tracts more prominently at lower temperature than at higher temperature (Matsushita *et al*., 1996). Although this low temperature-dependent stimulation seems to be related to the role of bent DNA, the promoter upstream region (–66 to –40 with respect to the transcriptional initiation site) is rich in A+T (96.3%). Thus, elucidation of the underlying

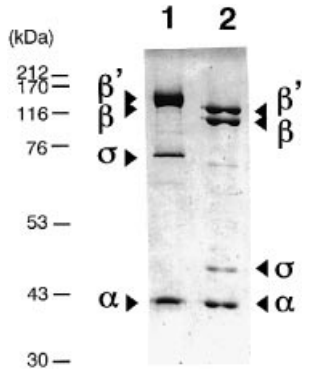

Fig. 1. SDS–PAGE of RNAPs from *E.coli* and *C.perfringens*. EcRNAP (lane 1) and CpRNAP (lane 2) were purified as described in Materials and methods. One microgram of each sample was subjected to SDS–PAGE on a 10% polyacrylamide gel. The numbers on the left are the molecular masses (in kDa) of the markers. The positions of the subunits are indicated by α, β, β′ and σ.

mechanism would solve the controversy as to the role of bent DNA in the activation of the promoter. In this study, we examined the mechanism by means of the following approaches: (i) analysis of the temperature effect on the *in vitro* transcriptional activity of the *plc* promoter with and without phased A-tracts; (ii) determination of the stability of the RNAP–*plc* promoter complex by means of a gel shift assay; (iii) analysis of the complex by hydroxyl radical footprinting; and (iv) comparison of the temperature effects on the transcriptional activation between bent DNA and an AT-rich UP element using the *plc* and *rrnB* P1 promoters. This paper shows that bent DNA enhances the formation of the RNAP–*plc* promoter complex, and that bent DNA of the *plc* gene, but not the UP element of *rrnB* P1, has a low temperature-dependent stimulatory effect on the downstream promoter. Evidence has also been presented that the UP element of *rrnB* P1 has a low temperature-dependent stimulatory effect on the *plc* promoter, although less prominently than the three phased A-tracts. Based on these results, we discuss the mechanism(s) underlying the similar temperature-responsive stimulatory effects of the phased A-tracts and UP element on the *plc* promoter, and also the possible involvement of the conformational transition of the bent DNA in the additional effect shown by the three phased A-tracts.

Results

Purification of RNAP from C.perfringens and E.coli cells

Clostridium perfringens RNAP (CpRNAP) of high purity was obtained by a modification of the method of Garnier and Cole (1988). The present method yielded 0.25 mg of RNAP holoenzyme/liter of culture, of which the specific activity was 489 U/mg of protein. Analysis by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that no other major peptide than the α-, β-, β′- and σ-subunits was present, although only contaminating levels of other peptides were still present in the CpRNAP preparation (Figure 1). The identity of the σ-subunit was verified by its cross-immunoreactivity with *E.coli* σ^{70} on immunoblotting (data not shown). The σ^{70} *E.coli* RNAP holoenzyme (EcRNAP) was also purified to homogeneity (Figure 1). Its specific activity was 393 U/mg of protein, this being comparable with that of CpRNAP.

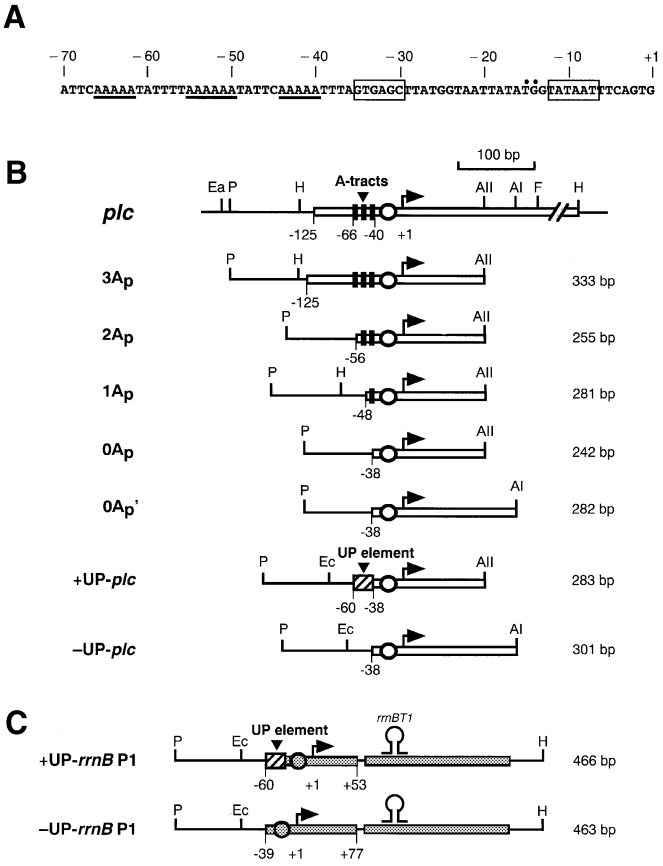

Fig. 2. Nucleotide sequences of the *plc* promoter and its upstream regions, and physical maps of the DNA fragments used in this study. (**A**) Nucleotide sequences of the *plc* promoter and its upstream regions. The –35 and –10 regions are boxed and the transcription start site is indicated as +1. The TG motif in the 'extended –10 element' is denoted by closed circles. The three phased A-tracts are underlined. (**B**) Physical maps of the *plc* gene and its fragments: *plc*, the *plc* gene cloned into pJIR418 (Matsushita *et al*., 1996); $3A_p$, $2A_p$, $1A_p$ and $0A_p$, fragments containing 3, 2, 1 and 0 A-tracts, respectively, which were used as templates for the *in vitro* transcription assay; $0A_p'$, a fragment containing no A-tract, which was used as a template for the promoter competition assay; and +UP-*plc* and –UP-*plc*, fragments with and without the UP element of *E.coli rrnB* P1 promoter. The filled and shaded blocks represent the A5-6-tract of the *plc* gene and the UP element of the *E.coli rrnB* gene, respectively. The *plc* and *rrnB* P1 promoters (open circles) and the regions derived from the vectors (vertical line) are also indicated. The transcriptional start site is indicated by the arrow. The sizes of the fragments are indicated by the numbers on the right. (**C**) Physical maps of *E.coli rrnB* gene-containing fragments: +UP-*rrnB* P1 and –UP-*rrnB* P1, fragments with and without the UP element, respectively. The regions derived from the *rrnB* gene and vectors are indicated by filled boxes and vertical lines, respectively. Abbreviations: AI, *Acc*I; AII, *Acc*II; Ea, *Ear*I; Ec, *Eco*RI; F, *Fok*I; H, *Hin*dIII; P, *Pvu*II.

Temperature effect on the activity of the plc promoter with different numbers of A-tracts

For the *in vitro* transcription assay involving the *plc* promoter with different numbers of A-tracts, template DNAs containing 3, 2, 1 and 0 A-tracts were constructed and designated as $3A_p$, $2A_p$, $1A_p$ and $0A_p$, respectively (Figure 2B). First, we calculated the bending angles of these template DNAs with the published program (Shpigelman *et al*., 1993), since curvatures predicted

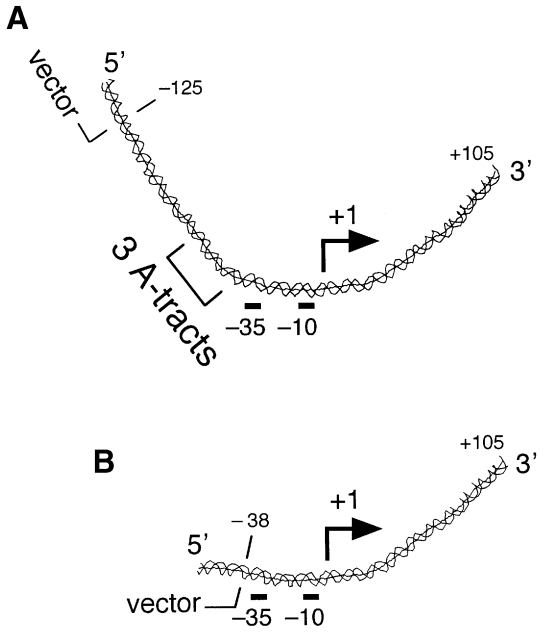

Fig. 3. Predicted DNA paths of fragments $3A_p$ and $0A_p$ used as DNA templates. The two DNA fragments were derived from pCMΔT and pCM0AΔT as described in the Materials and methods and Figure 1. (**A**), $3A_p$; (**B**), $0A_p$. The predicted DNA paths were calculated using the CURVATURE program (Shpigelman *et al*., 1993).

Table I. Effect of the RNAP concentration on the transcriptional activation by bent DNA in the promoter competition assay

CpRNAP (U)	Relative ratio of mRNA levels ($3A_p/0A_p'$)
0.05	174.5
0.1	27.7
0.2	10.3
0.4	4.9
1.0	2.9

The *in vitro* transcription assay was performed in a reaction mixture containing equimolar amounts of the $3A_p$ and $0A_p'$ templates (0.2 pmol each), 50 mM KCl, and various amounts of CpRNAP at 25°C for 10 min.

by computer analyses are fairly consistent with those determined experimentally from the anomalous electrophoretic mobility. The magnitude of the bending near the promoters $3A_p$ and $0A_p$ are predicted to be ~40° and ~0°, respectively (Figure 3). Those of $2A_p$ and $1A_p$ are predicted to be ~30° and ~10°, respectively. Thus, the magnitude of the bending is roughly proportional to the number of A-tracts.

An *in vitro* transcriptional assay was performed using $3A_p$, $2A_p$, $1A_p$ and $0A_p$ at 25 and 45°C (Figure 4). In the case of CpRNAP, the stimulatory effect was more prominent with an increase in the number of A-tracts. Furthermore, the effect was more prominent at 25 than at 45°C. On the other hand, EcRNAP did not show such A-tract- or low temperature-dependent stimulation. Thus, the effect is determined by the RNAP species, being dependent on the number of A-tracts and low temperature.

Promoter competition study on plc promoters with and without A-tracts

A promoter competition assay was performed to compare the strengths of the *plc* promoters with and without three

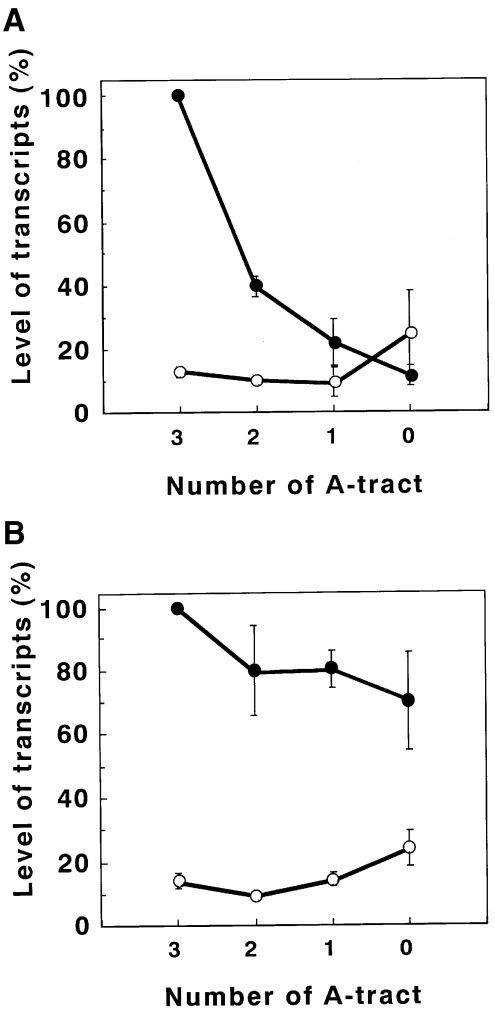

Fig. 4. The effect of the number of A-tracts on the transcriptional activity of the *plc* promoter. $3A_p$, $2A_p$, $1A_p$ and $0A_p$, containing 3, 2, 1 and 0 A-tracts, respectively, were used as templates for the *in vitro* transcription assay. The assay was performed with CpRNAP (closed circles) and EcRNAP (open circles) at 25 (**A**) and 45°C (**B**). The level of each transcript was determined by measuring the intensity of the band corresponding to each transcript, and expressed as a percentage of that of the transcript from the wild-type template ($3A_p$). The means and standard deviations of three determinations are plotted. Some of the error bars are too small to be distinguishable.

A-tracts. To distinguish two transcripts, a *Pvu*II–*Acc*I fragment ($0A_p'$) and a *Pvu*II–*Acc*II fragment ($3A_p$) were used as the template DNAs (Figure 2B). Equimolar amounts of the $3A_p$ and $0A_p'$ templates were incubated at 25°C under various conditions, and then the levels of their transcripts were determined. The transcript ratio, $3A_p$ versus $0A_p'$, increased as the RNAP concentration decreased, and as the KCl concentration increased (Tables I and II). Thus, the magnitude of the upstream region-dependent stimulation is greatest when the concentration of RNAP is limiting, because of either lowering of the concentration of RNAP or increasing of the concentration of KCl. This suggests that the $3A_p$ template can sequester RNAP from the $0A_p'$ template.

Affinity of RNAP to the plc promoters with and without A-tracts

The relative affinities of the *C.perfringens* and *E.coli* RNAPs to $3A_p$ and $0A_p$ were measured at 25°C by means

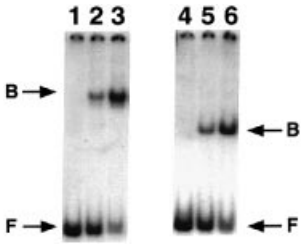

Fig. 5. Relative binding affinity assay of CpRNAP and EcRNAP as to $3A_p$. The ^{32}P-labeled $3A_p$ fragment (5 fmol) was mixed with 30 μl of the reaction buffer containing various amounts of RNAPs. The amounts of EcRNAP in lanes 1–3 were 0, 0.015 and 0.03 U, respectively. Those of CpRNAP in lanes 4–6 were: 0, 0.005 and 0.01 U, respectively. After incubation at 25°C for 15 min, the samples were loaded and run on an 8% polyacrylamide gel at 25°C. The intensities of the band corresponding to the RNAP–DNA complex (bound form, indicated by B) and that corresponding to unbound DNA (free form, indicated by F) were measured as described in the text.

Table II. Effect of the KCl concentration on the transcriptional activation by bent DNA in the promoter competition assay

KCl (mM)	Relative ratio of mRNA levels ($3A_p/0A_p'$)
20	4.2
50	26.1
100	267.2

The *in vitro* transcription assay was performed in a buffer containing equimolar amounts of the $3A_p$ and $0A_p'$ templates (0.2 pmol each), 0.1 U CpRNAP, and various concentrations of KCl at 25°C for 10 min. Determination of the mRNA levels was performed as described in the relevant section of Materials and methods.

Table III. Gel mobility shift assaying of the $3A_p$–RNAP and $0A_p$–RNAP complexes

RNAP	Template	Amount of RNAP required to retard 50% of DNA ± SD (U)	Ratio
CpRNAP	$0A_p$	0.053 ± 0.005	5.3
	$3A_p$	0.010 ± 0.001	1
EcRNAP	$0A_p$	0.067 ± 0.008	2.3
	$3A_p$	0.029 ± 0.001	1

Templates $0A_p$ and $3A_p$ (5 fmol each), which were 5′ end-labeled with ^{32}P as described in the text, were incubated with various concentrations of RNAP at 25°C for 15 min. The reaction mixture was electrophoresed on a 4% polyacrylamide gel at 25°C, followed by autoradiography. The band intensity of the unbound template, which was separated from the bound one, was measured as described in Materials and methods.

of a bandshift assay using ^{32}P-labeled $3A_p$ and $0A_p$ (Figure 2B). A discrete bandshifted RNAP–DNA complex increased as the amount of RNAP increased (Figure 5). The amount of CpRNAP required to retard $0A_p$ by 50% was ~5-fold higher than that of $3A_p$ (Table III), indicating that transcriptional activation occurs mainly, if not wholly, at the level of binding of CpRNAP to the promoter. EcRNAP bound more preferentially and/or tightly to $3A_p$ than to $0A_p$ (Table III). Nonetheless, EcRNAP transcribed the former less efficiently than the latter. The reason for this discrepancy cannot be explained at present but it could be related to an inhibitory effect on promoter clearance (see Discussion).

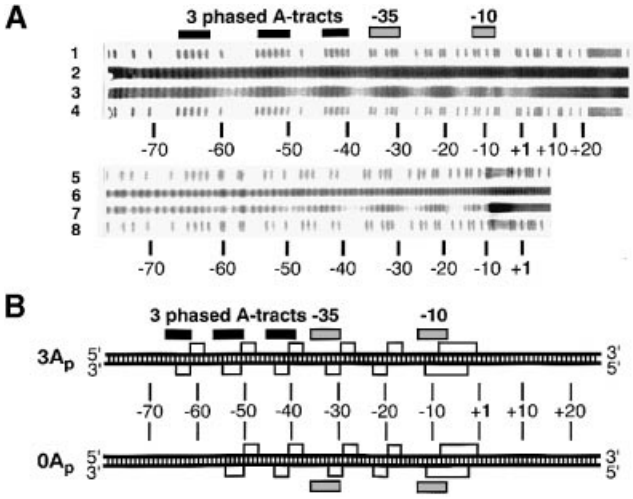

Fig. 6. Hydroxyl radical footprinting analysis of the $3A_p$–CpRNAP and $0A_p$–CpRNAP complexes. (**A**) Autoradiographs of hydroxyl radical footprinting of the $3A_p$–CpRNAP and $0A_p$–CpRNAP complexes. The footprint of the bottom strand is not shown. Lanes: 1, 4, 5 and 8, A and G chemical sequencing ladder; 2, $3A_p$ DNA alone; 3, $3A_p$ DNA plus CpRNAP; 6, $0A_p$ alone; and 7, $0A_p$ DNA plus CpRNAP. The location of the three phased A-tracts, and the –35 and –10 regions are also shown by filled and dotted boxes, respectively. (**B**) A schematic representation of the footprints of the $3A_p$–CpRNAP and $0A_p$–CpRNAP complexes. Open rectangles represent regions of the DNA duplex protected from hydroxyl radical cleavage due to RNAP binding. Note that RNAP binds to the face of the DNA helix opposite to the three phased A-tracts.

Hydroxyl radical footprinting of the RNAP–plc promoter complex

Complexes between RNAP and $3A_p$ or $0A_p$ DNA were formed at 25°C, and then footprints of the resulting binary complexes were determined with hydroxyl radicals. A selection of results is shown in Figure 6. For $0A_p$, CpRNAP affords protection to five sets of bases, i.e. from –50 to –48, –40 to –38, –29 to –27, –19 to –17 and –8 to –1, on the top strand (note that these coordinates are with respect to the *plc* start site). In contrast, hydroxyl radical cleavage of the $3A_p$–CpRNAP complex generated one additional set of bases, from –61 to –59, on the top strand. Footprints of the bottom strand revealed that the 3′ end of the protected region was –54 for $0A_p$, while it was extended further upstream to –64 for $3A_p$ (data not shown). These additional protected regions lie within a region containing three phased A-tracts. The vectors of the bending created by the compression of the minor groove due to the propeller-twist of an A–T base pair were estimated for this region (from –66 to –36), as described by Katahira *et al*. (1990). The results indicate that the bending is directed toward one face, and that this direction is in good agreement with that predicted with the CURVATURE program (Shpigelman *et al*., 1993). When the protected bases are plotted, they reside on the face toward which the bending is directed. Thus, the bent DNA seems to adopt a favorable conformation to facilitate binding to and complex formation with CpRNAP by wrapping around CpRNAP. The patterns of hydroxyl radical generated bands of EcRNAP–$3A_p$ and EcRNAP–$0A_p$ complexes were similar to those of CpRNAP–$3A_p$ and CpRNAP–$0A_p$ complexes, respectively, in the upstream region, although both the downstream protected regions of the EcRNAP complexes were extended to +24, unlike those

Table IV. Effects of temperature on the transcriptional activation of *plc* and *rrnB* P1 promoters by phased A-tracts and *rrnB* P1 UP element in the promoter competition assay

Temperature (°C)	Relative ratio of mRNA levels ± SD			
	$(3A_p/0A_p')$[a]	(+UP-*rrnB* P1/–UP-*rrnB* P1)[b]	(+UP-*plc*/–UP-*plc*)[a]	$(2A_p/0A_p')$[a]
25	30.8 ± 4.3	26.4 ± 2.1	13.5 ± 1.4	13.2 ± 3.9
30	ND	28.5 ± 2.3	8.2 ± 0.8	5.0 ± 1.1
37	4.6 ± 0.8	34.4 ± 2.0	3.8 ± 0.5	3.4 ± 0.9
41	ND	30.8 ± 4.2	3.4 ± 0.5	2.2 ± 0.3
45	1.9 ± 0.2	28.6 ± 5.6	3.5 ± 0.7	1.9 ± 0.6

The *in vitro* transcription assay was performed in a reaction mixture containing two templates (0.2 and 0.4 pmol, respectively), 50 mM KCl, and 0.1 U of CpRNAP or EcRNAP at the indicated temperatures for 10 min. The transcripts in the reaction mixtures (40 or 80 μl) were applied to a 6% polyacrylamide gel containing 7 M urea and then electrophoresed. Determination of the mRNA levels was performed as described in the relevant section of Materials and methods.
[a]CpRNAP was used.
[b]EcRNAP was used.
ND, not determined.

of the CpRNAP ones (data not shown). This suggests that EcRNAP is in contact with the bent DNA similar to CpRNAP in the promoter upstream region.

Promoter competition study on the rrnB P1 promoter with and without the UP element

The low temperature-dependent activation of the wild-type *plc* promoter seems to be due to the phased A-tracts. It is equally possible that the upstream AT-rich sequence is responsible for this activation. To assess these possibilities, we performed two sets of *in vitro* promoter competition experiments, one involving EcRNAP, and wild-type (+UP-*rrnB* P1) and mutant (–UP-*rrnB* P1) *rrnB* templates, and the other involving CpRNAP, and $3A_p$ and $0A_p'$ *plc* templates. The transcript ratio of $3A_p$ versus $0A_p'$ increased with a decrease in temperature, indicating that the extent of the stimulation is correlated with that of bending (Table IV). On the other hand, the transcript ratio of +UP-*rrnB* P1 versus –UP-*rrnB* P1 was nearly constant at all temperatures within the range of 25–45°C, although the effect of UP was maximum at 37°C (Table IV). This suggests that the activation of the *plc* promoter is due to the bent DNA. However, this may not only reflect the difference in the promoter upstream sequence between the *plc* and *rrnB* P1 templates, since they also differ in their downstream sequences including the core promoter sequence. To assess the possible effect of the downstream region, we constructed +UP-*plc*, a chimera of the *rrnB* P1 UP element and the *plc* promoter (Figure 2), and examined transcriptional activity at various temperatures by means of the *in vitro* competition assay. The stimulation of the *plc* promoter by the UP element was greater at lower temperatures than at higher temperatures. The stimulatory effect at 25°C was much lower than that of $3A_p$, being comparable with that of $2A_p$ (Table IV). This indicates that both the *rrnB* P1 UP element and the three phased A-tracts cause low temperature-dependent stimulation of the *plc* promoter, the stimulatory effect of the latter being more prominent than that of the former.

Discussion

Our previous *in vivo* study showed that *plc* gene expression is stimulated by lowering of the temperature (Matsushita *et al*., 1996). The present *in vitro* study has revealed evidence that bent DNA stimulates the *plc* promoter activity depending on the number of phased A-tracts and low temperature. These two factors should increase the bending angle, as rationalized by the junctional model (Koo *et al*., 1986), and exemplified by the anomalous mobility of bent DNA in the gel matrix and by the circular permutation assay (Wu and Crothers, 1984). Therefore, it is highly possible that bent DNA modulates the *plc* gene expression by sensing an alteration in temperature. On the contrary, the activation of *rrnB* P1 promoter by the UP element is temperature-independent, at least between 25 and 45°C.

The results of the experiment involving the chimeric template indicated that the UP element stimulates the *plc* promoter in a low temperature-dependent manner, similarly to the two phased A-tracts ($2A_p$). This suggests possible involvement of the downstream region in the low temperature-dependent stimulation of the *plc* promoter. A *gal* P1 promoter, which consists of the –35 region unrelated to the consensus –35 hexamer and the 'extended –10' element, i.e. 5′-TGnTATAAT-3′ (Barne *et al*., 1997), has been shown to form an open complex at low temperature. On the contrary, a modified *gal* P1con promoter containing the consensus –35 hexamer, 5′-TTGACA, requires a higher temperature to form an open complex (Grimes *et al*., 1991). Furthermore, the open complex formation of the *gal* P1 promoter at lower temperature requires the upstream sequence around –50, which is thought to bend or distort the DNA of the intermediate prior to open complex formation (Grimes *et al*., 1991; Burns *et al*., 1996). The extended –10 element is also present in the *plc* promoter (Figure 2A), suggesting that a similar mechanism underlies the open complex formation of the *plc* promoter. The difference between $3A_p$ and $0A_p$ in the transcriptional activity was more than that in the binding affinity to RNAP. This may be due to the difference in the ease with which the two templates form an open complex at low temperature. Whether or not the phased A-tracts require the extended –10 element to cause the temperature responsive stimulation remains to be determined.

The finding that the UP element exhibited almost the same effect on the *plc* promoter as the two phased A-tracts raises the question of whether the temperature responsiveness is specified by DNA bending or the sequence of the upstream region. The major bent DNA has been shown by electrophoretic analysis to be located at ~100 bases upstream of the transcriptional initiation site, and not just upstream of the –35 hexamer of the *rrnB* P1 promoter (Gaal *et al*., 1994). Furthermore, the phased A-tracts have been shown to activate the downstream promoter through interactions with the RNA polymerase α subunit, similar to UP elements (Aiyar *et al*., 1998). Thus, one likely explanation is that the phased A-tracts exhibit a low temperature-dependent stimulatory effect like the UP element, possibly through interactions with αCTD. However, this does not explain the difference in the extent of the low temperature-dependent stimulation

between the three phased A-tracts and the UP element: the stimulation range between 25 and 45°C is 30.8–1.9 for the former, while it is 13.5–3.5 for the latter (Table IV). Therefore, it seems also possible that the bending of the upstream region is responsible at least for the additional effect of the three phased A-tracts. Recently, a UP element consensus sequence for bacterial promoters was determined by the SELEX procedure to be –59 nnAAA (A/T)(A/T)TTTTnnAAAAnnn –38 (Estrem *et al*., 1998). Thus, there is no doubt that the UP element is a sequence-specific site for αCTD binding. Considering the results of all the analyses together, we speculate that the promoter upstream phased A-tracts function simply as UP elements at higher temperature, while they stimulate promoter activity efficiently at lower temperature, possibly by increasing the bending angle. A more precise study involving constructs with various AT-rich sequences and the *plc* promoter is necessary to prove our hypothesis.

The band shift experiments revealed that bent DNA stabilizes the CpRNAP–*plc* promoter complex. The results of the hydroxyl radical footprinting indicated that the presence of bent DNA extends the contact region in the complex to nucleotide position –64. This extension seems to stabilize the complex through the wrapping of the bent DNA around CpRNAP (Nickerson and Achberger, 1995). The downstream end of the contact region differs from that observed for the EcRNAP–*plc* promoter complex. To focus on the difference in the stable complex formation, the complex was formed under the conditions used for the band shift experiments, which differ from those for the analysis of the sequential steps on transcription initiation. Thus, we cannot explain the difference in the downstream contact regions. Clarification of this will require more detailed biochemical analysis, especially with respect to the nature of CpRNAP and the kinetics of its transcription initiation steps. It should be noted that the *plc* gene transcription by EcRNAP is repressed in the presence of bent DNA, despite the more efficient binding to EcRNAP observed for $3A_p$ than for $0A_p$. The difference in the effect of the bent DNA between the two RNAPs may be related to the finding by other workers (Ellinger *et al*., 1994) that facilitated RNA polymerase binding in the presence of A-tracts stimulates binding-limited promoters but inhibits the promoter function in which polymerase escape and promoter clearance are rate limiting.

The induction or stimulation of protein synthesis by lowering of the temperature has been reported for some proteins. The low temperature-dependent expression of CspA, an *E.coli* major cold-shock protein, is mainly due to increased mRNA stability at low temperature (Brandi *et al*., 1996; Goldenberg *et al*., 1996). The activation of certain promoters in response to low temperature and dehydration in *Arabidopsis thaliana* results from the interaction of the *cis*-acting C-repeat/dehydration responsive element (DRE) with activator protein CBF1 (Stockinger *et al*., 1997). The activation of the lambda P_L promoter by lowering of the temperature has been suggested to involve an architectural alteration of the promoter region due to the intrinsic properties of the promoter as well as the binding of IHF (Giladi *et al*., 1995). In contrast to these examples, the temperature-responsive regulation of *plc* gene expression only involves the *cis*-element. It does not require the co-evolution of a *trans*-acting factor(s) and hence may be one of the most primitive forms of gene regulation.

Many bacterial virulence genes are expressed on a shift from an ambient low temperature to the body temperature upon entering a host (Mekalanos, 1992; Abe *et al*., 1997; Ohlsen *et al*., 1997). Why is PLC, a major virulence factor of *C.perfringens*, downregulated at high temperature? Type A of this organism inhabits the large intestines of humans and other animals, and also lives in soil and water, while the four other types, B through E, live exclusively in the guts of animals. The *plc* genes from all the types share the promoter upstream phased A-tracts (Tsutsui *et al*., 1995). When living symbiotically at 37°C, *C.perfringens* produces low levels of PLC, a potent phospholipase C, as this would be detrimental to the host. When present in the soil, or following the death of its host, the bacterium must adapt to lower temperatures and adverse conditions, and thus induces PLC which degrades cell membranes or other available lipids to generate a source of carbon and energy for growth.

Materials and methods

Purification of RNAP

The σ^{70} *E.coli* RNAP holoenzyme was purchased from Boehringer Mannheim. It was purified further by chromatography on a DNA–cellulose column (denatured DNA; Pharmacia Biotech) according to the method of Lowe *et al*. (1979). CpRNAP was purified from *C.perfringens* NCTC 8237 by the method of Garnier and Cole (1988) with a slight modification. Briefly, the cell extract was treated with polyethyleneimine (pH 8.0) at a final concentration of 0.5% to precipitate nucleic acids. Proteins were eluted from the precipitate, and then reprecipitated with ammonium sulfate (65% saturation). This fraction was subjected to gel filtration on a Sephacryl S-200 column (Pharmacia Biotech), chromatography on a heparin–agarose column (prepacked type II; Sigma Biochemical), FPLC on a MonoQ column (Pharmacia Biotech), and chromatography on a DNA–cellulose column.

DNA

A 333-bp *Pvu*II–*Acc*II fragment containing the *plc* promoter with the three A-tracts was prepared from plasmid pCMΔT (Matsushita *et al*., 1996), this fragment being designated as $3A_p$ (Figure 2B). The fragments with two, one and no A-tracts, which were designated $2A_p$, $1A_p$ and $0A_p$ (Figure 2B), respectively, were prepared from plasmids pCM2AΔT, pCM1AΔT and pCM0AΔT (Matsushita *et al*., 1996), respectively. A 282-bp *Pvu*II–*Acc*I fragment containing the promoter without the A-tracts was prepared from pCM0AΔT, and was designated as $0A_p'$ (Figure 2B). This was used as the template in the promoter competition experiments. For hydroxyl radical footprinting, ^{32}P-labeled DNA fragments were prepared as follows. A 305-bp *Hin*dIII–*Fok*I fragment containing the three A-tracts was labeled at the 5′ end with [γ-^{32}P]dATP (4500 Ci/mmol, ICN Biochemicals) using a Megalabel™ kit (Takara Shuzo) according to the protocol recommended by the supplier. The 5′ end of the ^{32}P-labeled antisense strand was removed by cutting the labeled fragment with *Acc*II. The resulting 242-bp *Hin*dIII–*Acc*II fragment was used as the $3A_p$ template DNA for hydroxyl radical footprinting (Figure 2B). A 254-bp ^{32}P-labeled $0A_p$ fragment was likewise prepared except that a 423-bp *Ear*I–*Dra*I fragment from pCM0AΔT was labeled with ^{32}P, followed by cutting with *Acc*II (Figure 2B).

DNA fragments containing the *rrnB* P1 promoter with and without the UP element were constructed as follows. A 113-bp PCR product, which contained the *rrnB* P1 promoter region spanning bases –60 to +53, was generated using *E.coli* DH5α DNA, 5′-CAGAAAATT-ATTTTAAATTTC, 5′-CTCAGGAGAACCCCGCTTGAC-3′, as the template, and forward and reverse primers, respectively. This fragment was cloned into the *Eco*RV site of the pT7Blue T-Vector (Novagen), the resultant plasmid being designated as pUP8. The nucleotide sequence of the insert in pUP8 was confirmed to be identical with that reported for the *rrnB* gene (Brosius *et al*., 1981). To place the terminator downstream of the insert, a 179-bp *Eco*RI fragment containing *rrnB*T1

was obtained from pKK232–8 (Pharmacia Biotech). The fragment was filled in at both ends, followed by ligation into the *Nde*I site of pUP8, which was blunt-ended and dephosphorylated. The resulting plasmid was designated as pUP9. A 466-bp *Pvu*II–*Hin*dIII fragment of pUP9 was used as the *rrnB* P1 promoter containing the UP element (+UP-*rrnB* P1; Figure 2C). A 116-bp PCR product, which contained the promoter region of the *rrnB* P1 promoter spanning bases –39 to +77, was generated using *E.coli* DH5α DNA and two primers (5′-CTCTTGTCAGGCCGGAATAAC-3′ and 5′-ATTTTTGCTTTCT-CTGCCGGA-3′). This product was then cloned into the pT7Blue T-Vector as described above. The resultant plasmid, named pUP10, was digested with *Nde*I, blunt-ended, and then dephosphorylated. The 179-bp fragment of pKK232-8 containing *rrnB*T1 was filled in and ligated into the blunt-ended *Nde*I site of pUP10, the resultant plasmid being designated as pUP11. A 463-bp *Pvu*II–*Hin*dIII fragment of pUP11 was used as the *rrnB* P1 promoter lacking the UP element (–UP-*rrnB* P1; Figure 2C).

DNA fragments containing the *plc* promoter with and without the UP element were constructed as follows. A 230-bp PCR product, which contained the UP element spanning bases –60 to –38 and the *plc* gene spanning bases –37 to +170, was generated using pCMΔT, and 5′-CAGAAAATTATTTTAAATTTCCTTAGTGAGCTTATGGTAATTA-TATGG-3′ and 5′-ATCAATCTTTCCATCCCAAGC-3′, as the template, and forward and reverse primers, respectively. This fragment was cloned into the *Eco*RV site of the pT7Blue T-Vector, the resultant plasmid being designated as pUP12. A 283-bp *Pvu*II–*Acc*II fragment was prepared from pUP12 and used as a chimeric promoter consisting of the UP element and *plc* promoter (+UP-*plc*; Figure 2B). A 301-bp *Pvu*II–*Acc*I fragment for use as a chimeric *plc* promoter lacking the UP element (–UP-*plc*; Figure 2B) was prepared in the same manner except that 5′-TTAGTGAGCTTATGGTAATTA was used as the forward primer.

DNA manipulations and sequencing

Restriction endonucleases were purchased from Takara Shuzo, Toyobo, and New England Biolabs. The Klenow enzyme and DNA ligation kit were products of Takara Shuzo. All recombinant DNA procedures were carried out as described by Sambrook *et al*. (1989). Nucleotide sequencing was performed as described previously (Matsushita *et al*., 1998).

DNA band shift studies

Two DNA fragments, $3A_p$ and $0A_p$, were labeled at their 5′ ends with [γ-^{32}P]ATP (4500 Ci/mmol, ICN Biochemicals) using a Megalabel kit. These labeled fragments (5 fmol) were incubated with various amounts of RNAP in 30 µl of buffer consisting of 12 mM HEPES (pH 8.0), 4 mM Tris–HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% (v/v) glycerol, 0.3 mg/ml of bovine serum albumin (BSA), and 0.033 mg/ml of poly(dI–dC) (Pharmacia Biotech) at 25°C for 15 min. The reaction mixture was loaded and run on an 8% polyacrylamide gel at 25°C. The temperature during the electrophoresis was kept constant by means of an apparatus with a device for temperature regulation (Hoefer SE650, Pharmacia Biotech). The band intensity was visualized by autoradiography. The relative amounts of the unbound and bound forms of DNA were determined using a BAS1000 Bio-Imaging Analyzer (Fuji Photo Film) and Image GaugeTM ver 3.0 software on a Power Macintosh computer. The bound form of DNA was determined by subtracting the amount of the unbound form of DNA in the presence of RNAP from that in the absence of RNAP. The amount of RNAP required to bind to one-half of the DNA fragment was determined from a calibration curve, which was made using three different concentrations of RNAP at each temperature.

In vitro transcription assay

The *in vitro* transcription assay was performed as described elsewhere (Matsushita *et al*., 1996), except that 0.2 pmol of template DNA was incubated for 10 min with 0.1 and 0.01 U of RNAP at 25 and 45°C, respectively. In promoter competition assays, two different templates were transcribed in the same mixture to compare their promoter activities. After incubation, the transcripts were applied to a 6% polyacrylamide gel containing 7 M urea and then electrophoresed. The relative amounts of the transcripts were determined with a BAS1000 Bio-Imaging Analyzer as described above. The relative ratios of the transcripts were subsequently determined with correction for the number of UMP residues.

Hydroxyl radical footprinting

DNA–RNAP complexes were formed by incubating ^{32}P-labeled DNAs (30 ng) with CpRNAP (0.2 and 1 U for the $3A_p$ and $0A_p$ templates, respectively) under the same conditions as described for the DNA band shift studies. To remove glycerol, 30 µl of the incubation mixture was placed on a Millipore VS filter membrane (0.025 µm; Millipore), and then dialyzed against 30 ml of 8 mM Tris–HCl (pH 7.9) for 1 h at 25°C (Schickor and Heumann, 1994). The protein–DNA complexes were digested with hydroxyl radicals as described by Schickor and Heumann (1994). After digestion for 2 min at 25°C, the reaction was quenched by adding 14 µl of 0.1 M thiourea-20 mM EDTA (pH 8.0) to 51 µl of the reaction mixture. The samples were subsequently precipitated with ethanol, resuspended in an appropriate volume of formamide gel loading buffer and then run on a 6% sequencing gel. The bands were visualized and analyzed as described above.

Enzyme assay, protein determination, SDS–PAGE and immunoblotting

The enzymatic activity of RNAP was determined using [^{3}H]dUTP (35 Ci/mmol, ICN Biochemicals) and poly(dA–dT) DNA (Pharmacia Biotech) as the labeled nucleotide and template DNA, respectively, as described by Pich and Bahl (1991). One unit of RNAP activity caused the incorporation of 1 nmol of UMP into RNA in 10 min at 37°C. Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad Laboratories) with BSA as a standard. SDS–PAGE was performed on a 10% polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue R as described previously (Jin *et al*., 1996). For immunoblot analysis of the σ-subunit of CpRNAP, the purified CpRNAP was electrophoresed on an SDS–10% polyacrylamide gel, and then electroblotted onto a nitrocellulose membrane (Advantec Toyo). Antigens were visualized using antiserum against *E.coli* σ^{70}, which was donated by C.A.Gross (Department of Bacteriology, University of Wisconsin, Madison, WI), and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Promega).

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