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# DNA twist, flexibility and transcription of the osmoregulated *proU* promoter of *Salmonella typhimurium*

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**Transcription from many bacterial promoters is sensitive to the level of DNA supercoiling. We have investigated the mechanism by which environmentally induced changes in DNA supercoiling might regulate transcription. For the *proU* promoter of *Salmonella typhimurium*, osmotically induced changes in DNA topology appear to play a primary regulatory role. Changes in DNA supercoiling (linking number; ΔLk) are partitioned into changes in the winding of the strands of the double helix about themselves (twist; ΔTw) and/or elastic deformations or flexibility of the DNA helix (writhe; ΔWr). Mutations of the *proU* promoter were isolated *in vivo*, or generated *in vitro*, which altered the spacing between the –10 and –35 motifs. Studies on these mutant promoters, both *in vivo* and *in vitro*, exclude models in which changes in DNA twist play a regulatory role. Instead, our data suggest that increased DNA flexibility, reflecting the osmotically induced increase in negative supercoiling of DNA, is required for promoter activation.**

*Keywords*: chromatin/DNA supercoiling/histone-like protein/H-NS/osmoregulation

## Introduction

Transcription from many promoters is sensitive to the level of supercoiling of the DNA template (Brahms *et al.*, 1985; Borowiec and Gralla, 1987; Pruss and Drlica, 1989). In bacteria, DNA supercoiling varies in response to environmental signals such as osmolarity, temperature, oxygen availability and composition of the growth medium (Goldstein and Drlica, 1984; Balke and Gralla, 1987; Dorman *et al.*, 1988; Higgins *et al.*, 1988; McClellan *et al.*, 1990; Hsieh *et al.*, 1991) Furthermore, these environmentally induced changes in DNA topology play a role in regulating gene expression (Dorman *et al.*, 1988, 1990; Higgins *et al.*, 1988; Tobe *et al.*, 1995).

*proU* provides the paradigm for a promoter regulated by environmentally induced changes in DNA topology. The *proU* operons of *Salmonella typhimurium* and *Escherichia coli* encode transport systems for the osmoprotectant glycine betaine, and transcription from the *proU* promoter is increased up to 100-fold by increased extracellular osmolarity (Cairney *et al.*, 1985; Dunlap and Csonka, 1985; Gowrishankar, 1985; May *et al.*, 1986). Although the mechanisms of osmoregulation have yet to be elucidated fully, transcription from the *proU* promoter is sensitive to factors which perturb DNA topology (Higgins *et al.*, 1988; Ni Bhriain *et al.*, 1989; Hulton *et al.*, 1990). As cellular DNA supercoiling varies in response to medium osmolarity (Higgins *et al.*, 1988; McClellan *et al.*, 1990; Dayn *et al.*, 1991; Hsieh *et al.*, 1991), it seems probable that changes in DNA topology play a central role in *proU* regulation. Several observations are consistent with this hypothesis. (i) No sequence-specific regulatory proteins which act at the *proU* promoter have been identified, despite intensive searches (Druger-Liotta *et al.*, 1986; Higgins *et al.*, 1988; Lucht and Bremer, 1991; Manna and Gowrishankar, 1994; Stephen *et al.*, unpublished data). (ii) The only *trans*-acting factor which substantially influences *proU* transcription is the histone-like protein H-NS (Higgins *et al.*, 1988; Hulton *et al.*, 1990; May *et al.*, 1990; Hinton *et al.*, 1992), although the other histone-like protein, HU, may have a minor effect in *E.coli* (Manna and Gowrishankar, 1994). Mutations in the *hns* gene alter the supercoiling of DNA isolated from the cell (Higgins *et al.*, 1988; Hinton *et al.*, 1992; Owen-Hughes *et al.*, 1992), and purified H-NS can constrain DNA supercoils *in vitro* (Tupper *et al.*, 1994). (iii) The only *cis*-acting sequences which play a significant role in osmoregulation (the downstream regulatory element; DRE) are located some distance downstream of the transcription start site, within the *proV* coding sequence (Dattananda *et al.*, 1991; Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992; Lucht *et al.*, 1994). The DRE includes intrinsically curved DNA, and the binding of H-NS to the DRE appears to mediate repression of transcription at low osmolarity (Owen-Hughes *et al.*, 1992).

Changes in plasmid DNA linking number (ΔLk) provide a convenient indicator of changes in DNA supercoiling, and it has been demonstrated that changes in linking number induced by osmolarity do indeed reflect changes in supercoiling *in vivo* (McClellan *et al.*, 1990; Dayn *et al.*, 1991). ΔLk can be partitioned into two distinct geometric conformations of the DNA, twist (Tw) and writhe (Wr), according to the equation ΔLk = ΔTw + ΔWr. Twist describes how the two individual DNA strands coil around each other; writhe describes how the helix axis is deformed in space (i.e. how it bends or flexes). In this study, we have sought to elucidate which of these components, twist or writhe, is most significant in the osmotic regulation of the *proU* promoter.

It has been hypothesized previously that changes in DNA twist between the –10 and –35 motifs of the *proU*

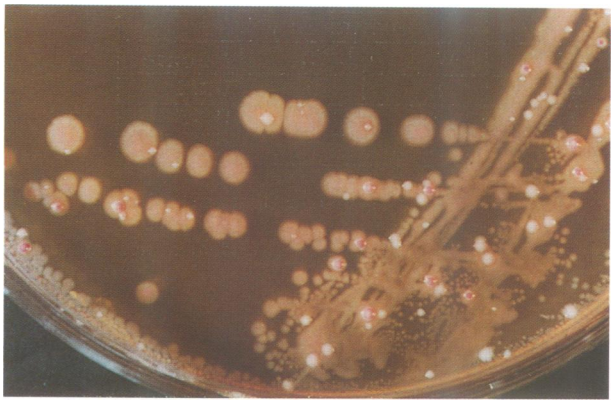


**Fig. 1.** Photograph of faster-growing pink, Lac$^+$ bacteria that arise as spontaneous mutants when *S.typhimurium* strains containing a *proU*::MudJ fusion are streaked out onto MacConkey–lactose plates.

promoter might be the primary mechanism of osmotic regulation of *proU* (Wang and Syvanen, 1992). To test this hypothesis, we isolated or constructed *cis*-acting promoter mutations which altered this spacing between the –10 and –35 motifs. Characterization of these mutant promoters, *in vivo* and *in vitro*, effectively excludes a role for changes in DNA twist but, instead, implicates changes in writhe in the osmoregulation of *proU* transcription.

## Results

### Selection for proU promoter mutations

*Salmonella typhimurium* strains CH1102 and CH1512 carry MudJ-mediated *proU–lacZ* transcriptional fusions on the chromosome (Stirling *et al.*, 1989; Waddell, 1989). As *proU* is only expressed when cells are grown in media of high osmolarity, these strains form white (Lac$^-$) colonies on MacConkey–lactose plates, and pink (Lac$^+$) colonies on MacConkey–lactose plates containing 0.3 M NaCl. In order to isolate *proU* promoter mutations, these strains were streaked onto MacConkey–lactose plates. Pink (Lac$^+$) papillae which arose spontaneously within individual white (Lac$^-$) colonies were able to grow more rapidly than the parental strain by virtue of utilizing lactose as a carbon source (Figure 1). One hundred and fifty one papillae were single-colony purified and shown to be Lac$^+$ (pink) by re-screening on MacConkey–lactose plates. To identify those harbouring mutations in the *proU* promoter, the MudJ element from each strain was transduced into the wild-type strain LT2, selecting for Kan$^r$, and the transductants screened for their Lac phenotype on MacConkey–lactose plates. The mutants fell into three distinct classes. Class I mutants (9/151) gave only Lac$^-$ (white) colonies when transduced into LT2 and, hence, carried mutations unlinked to the *proU* locus; these *trans*-acting mutations were all in the *hns* gene (Hinton *et al.*, 1992) and are not considered further here. Class II mutants were most common (140/151) and gave rise only to Lac$^+$ (pink) colonies when transduced into LT2, showing that the mutation is 100% linked to the MudJ element. These were assumed to be mutations or rearrangements within the *lacZ* gene of the MudJ element itself and were discarded. Class III mutants (2/151) gave a mixture of ~95% Lac$^+$ (pink) and 5% Lac$^-$ (white) transductants, showing they harbour mutations closely linked to the *proU–lacZ* fusion. These were putative *proU* promoter mutations and were designated *proU1718* and *proU1719*, and the strains harbouring them CH1973 and CH1974, respectively.

β-Galactosidase expression from the chromosomal *proU–lacZ* fusions in CH1973 and CH1974 was assayed with or without osmotic upshock (Figure 2). As expected, the parental strain showed normal osmotic induction of *proU* expression (24-fold). In contrast, *proU* expression in CH1973 and CH1974 was near constitutive, with only 1.6- and 1.5-fold induction at high osmolarity, respectively. This was due, in large part, to derepression of expression at low osmolarity, although there was also a small (<2-fold) increase in expression at high osmolarity.

### The putative promoter mutations increase transcription

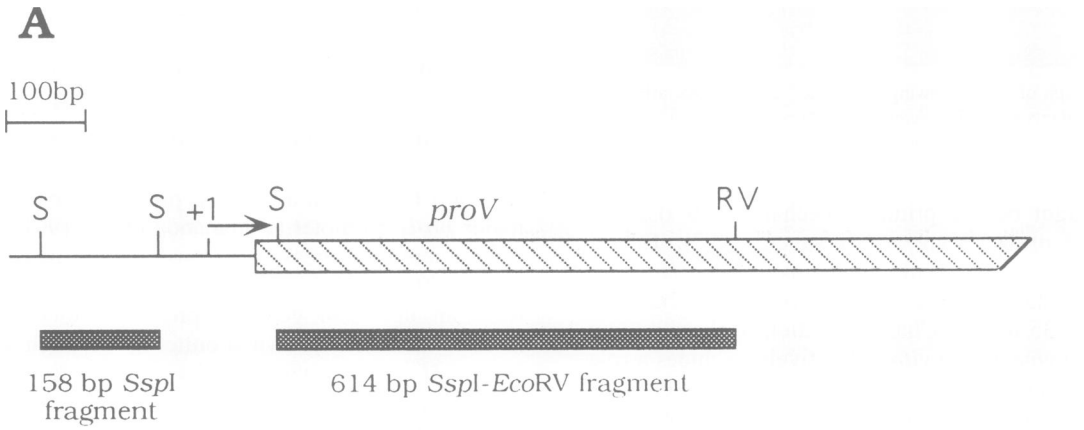
In *E.coli* there is an additional promoter upstream of the major *proU* promoter (Dattananda *et al.*, 1991). To show that increased expression of *proU* in the *proU1718* and *proU1719* mutants was not due to fortuitous readthrough from an upstream gene or promoter, total RNA was isolated from cells grown at either low or high osmolarity and *proU* mRNA quantitated by hybridization. The RNA was hybridized with a 614 bp *Ssp*I–*Eco*RV fragment from downstream of the *proU* promoter (the location of this fragment with respect to the transcription start site is shown in Figure 3A). Compared with the parental strain (LT2), the level of *proU* mRNA at low osmolarity was increased in both mutants (Figure 4). The same blot was also probed with a DNA fragment (the 158 bp *Ssp*I–*Ssp*I fragment; Figure 3A) from immediately upstream of the *proU* promoter; no significant difference in mRNA levels was observed between the mutants and the parental strain (Figure 4). Thus, the *proU1718* and *proU1719* mutations increase transcription from the *proU* promoter rather than increasing readthrough from an upstream gene or promoter. We show below that identical transcription start points are used by the wild-type and mutant promoters.

### Sequences of the mutant promoters

Attempts to clone the *proU* promoter regions from CH1973 and CH1974 onto multicopy plasmids were unsuccessful, probably because these strong promoters are deleterious to the host. Thus, in order to determine the sequence changes in the mutant promoters, they were sequenced directly from chromosomal DNA following PCR amplification using biotinylated primers (Thein and Hinton, 1991). The products of multiple, independent amplifications were sequenced to exclude PCR-induced artefacts. In the region from base –598 to +1159 (+1 is the start of the transcription; Stirling *et al.*, 1989) only a single base change was detected in each mutant. The *proU1718* mutation (in CH1973) was a T→A change in the –35 region of the promoter at nucleotide –31, generating a promoter sequence closer to the consensus (Figure 3B). The *proU1719* mutation (in CH1974) was an insertion of a single base pair between nucleotides –27 and –28, increasing the spacing between the –10 and –35 regions of the promoter (Figure 3B). As the spacing in the *proU* promoter is suboptimal at 16 bp, this mutation generates a promoter with an optimal spacing of 17 bp.

| | | | | | +coumermycin | | |
|---|---|---|---|---|---|---|---|
| Strain | *proU* promoter | low | high | **I** | low | high | **$R_{coum}$.** |
| CH1512 | wild type | 10 | 240 | **24** | 8 | 50 | **0.21** |
| CH1973 | *proU1718* | 248 | 400 | **1.6** | 143 | 180 | **0.45** |
| CH1974 | *proU1719* | 293 | 430 | **1.5** | 179 | 293 | **0.62** |

**Fig. 2.** Effect of promoter mutations on osmoregulation of *proU* expression. Cells of *S.typhimurium* strains CH1512 (wild-type), CH1973 (*proU1718*) and CH1974 (*proU1719*), containing a *proU–lacZ* fusion on the chromosome were grown in NB at low or high osmolarity. The induction ratio (I) is the ratio of expression at high and low osmolarity. The coumermycin repression ratio ($R_{coum}$) is the ratio of expression at high osmolarity with and without adding 5 μg/ml coumermycin. Data are given in Miller units (Miller, 1972).




**B**

| -35 | -10 | name of promoter | | spacing |
|---|---|---|---|---|
| NNTTGACANNNNNNNNNNNNNNNNNNTATAATNNNNNNNNN | | Consensus | | 17 bp |
| GGTTGTCTC | AGATTCTGAGTATGTTAGGGTAGAAAAAGG | *proU* wild type | | 16 bp |
| GGTTGACTC | AGATTCTGAGTATGTTAGGGTAGAAAAAGG | *proU1718* | (spontaneous) | 16 bp |
| GGTTGTCTCCAGATTCTGAGTATGTTAGGGTAGAAAAAGG | | *proU1719* | (spontaneous) | 17 bp |
| GGTTGTCTC | AGAΔTCTGAGTATGTTAGGGTAGAAAAAGG | *proU1723* | | 15 bp |
| GGTTGTCTCAGATTCTAGAGTATGTTAGGGTAGAAAAAGG | | *proU1724* | | 17 bp |
| GGTTGTCTCCAGATTCTAGAGTATGTTAGGGTAGAAAAAGG | | *proU1725* | | 18 bp |
| GGTTGTCTCCAGATTCTAGAGTATGGTTAGGGTAGAAAAAGG | | *proU1726* | | 19 bp |

**Fig. 3.** The *proU* promoter of *S.typhimurium*. (**A**) Schematic drawing of the *proU* promoter region. The hatched region indicates the *proV* reading frame. The two DNA fragments used as probes in Northern blot analysis are indicated. The transcription start site is indicated as +1, according to Stirling *et al.* (1989). Restriction endonuclease sites are: S = *Ssp*I; RV = *Eco*RV. (**B**) Sequence of the wild-type and mutant *proU* promoters used in this study. The consensus sequence for $\sigma^{70}$ promoters is given for comparison. The two spontaneous promoter mutations are indicated. The –35 and –10 regions are underlined and nucleotides in bold indicate insertions or substitutions that differ from the *proU* wild-type sequence. Deletions are shown by the symbol Δ. The spacing between the –35 and –10 regions of the promoter is given in bp.

### *The proU promoter-up mutations enhance RNA polymerase binding and open complex formation*

The *proU1718* and *proU1719* mutations both generate promoters closer to the consensus sequence. The simplest explanation of their effects on *proU* expression is a direct effect on the interaction of RNA polymerase with the promoter DNA. To test this, RNA polymerase binding to the wild-type and mutant promoters was assayed by DNase I footprinting (Figure 5). Both the *proU1718* and *proU1719* mutations enhanced RNA polymerase binding *in vitro*. The mutations also enhanced open complex formation assessed by $KMnO_4$ footprinting (Figure 6, data of *proU1718* mutation not shown). Thus, these promoter mutations alter *proU* expression by directly influencing the efficiency with which RNA polymerase interacts productively at the promoter.

### *The proU promoter is not activated by changes in DNA twist between the –35 and –10 regions*

It has been suggested that osmotically induced changes in DNA twist play a central role in the osmotic regulation of the *proU* promoter (Wang and Syvanen, 1992). The spacing between the –10 and –35 motifs of the promoter is 16 bp rather than the optimal 17 bp. As RNA polymerase interacts with both these motifs, their relative orientations will influence the efficiency of RNA polymerase inter-

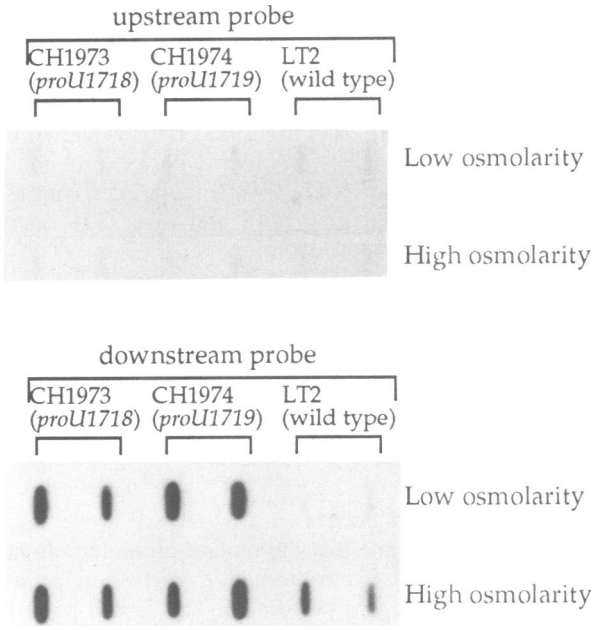



**Fig. 4.** RNA blot of total RNA from *S.typhimurium* strains LT2, CH1973 and CH1974, isolated from cells grown at low or high osmolarity. Total RNA was hybridized with DNA fragments from either upstream or downstream of the *proU* promoter. The locations of the upstream and downstream probes relative to the *proU* promoter are given in Figure 3A.

actions (Borowiec and Gralla, 1987; Auble and deHaseth, 1988): for *proU*, the suboptimal spacing ensures that these two motifs are normally not optimally aligned. A change in DNA twist could potentially restore the orientation of the –10 and –35 regions to a more favourable alignment for RNA polymerase to interact. The finding that the *proU1719* mutation, which alters the spacing between the –10 and –35 motifs, results in essentially constitutive and unregulated expression, is consistent with the 'twist' model. We therefore set out to test this model more rigorously. First, it was necessary to establish that the effect of the *proU1719* mutation was due to an alteration in spacing rather than to a specific base change. Second, the 'twist' model predicts that a further increase in spacing, to 18 bp, would not only render the promoter suboptimal but that such a promoter would respond to changes in osmolarity and increases in negative supercoiling in the opposite manner to the wild-type (16 bp) promoter.

Thus, a series of mutations altering *proU* promoter spacing was constructed (Figure 3B), and these altered promoters were cloned upstream of the *lux* reporter gene. As it is not possible to clone *proU* promoter-up mutations on multicopy plasmids, the low copy number plasmid pHSG575 was used (Takeshita *et al.*, 1987). The *lux* reporter genes from pSB71 (Ahmad and Stewart, 1988) were cloned into pHSG575 as a 2 kb *Eco*RI–*Bam*HI fragment. The wild-type, *proU1718* and *proU1719* promoters, extending from base pairs –217 to +100, were cloned upstream of the *lux* gene as 317 bp *Eco*RI fragments, amplified by the PCR from chromosomal DNA of strains CH1512, CH1973 and CH1974, respectively. Other promoter spacing mutations (Figure 3B) were introduced into the plasmid containing the wild-type promoter by site-directed mutagenesis (see Materials and methods).

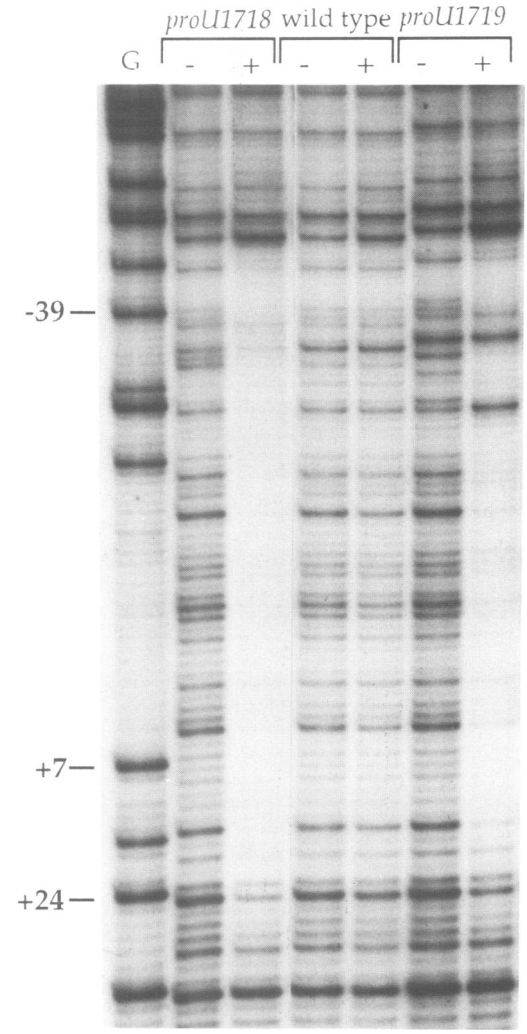



**Fig. 5.** DNase I footprint of RNA polymerase binding to the *proU* wild-type, *proU1718* and *proU1719* promoters. The non-coding strand extending from –217 to +100 was end-labelled. The reactions were in the absence (–) or the presence (+) of 1 unit of RNA polymerase. Chemical cleavage sequencing reactions for G residues, as a marker, were performed on the same DNA fragment encoding the wild-type *proU* promoter (G). The positions of several G residues relative to the transcription start site are indicated to the left.

Each promoter fragment was sequenced to ensure the presence of the desired mutation and the absence of any other changes.

Each plasmid was transformed into *E.coli* strain TG1 and *lux* activity was assayed after growth at both low and high osmolarity (Figure 7A). The wild-type promoter (16 bp spacing) showed 92-fold induction of expression in response to increased osmolarity of the medium, consistent with previous data (Owen-Hughes *et al.*, 1992). The *proU1718* and *proU1719* mutations showed essentially constitutive expression, demonstrating that regulation in multicopy is similar to that in single copy on the chromosome.

The *proU1724* mutation increases the spacing between the –10 and –35 elements from 16 to 17 bp, yet is in a different location to the insertion in the *proU1719* mutation. *proU1724* behaved similarly to *proU1719*, showing that altered spacing, rather than a specific sequence change, results in almost constitutive expression.

Promoters with 15 and 19 bp spacing (*proU1723* and *proU1726*, respectively) exhibited very low activity, not unexpectedly as they deviate substantially from the consensus promoter sequence. Most importantly, the promoter with an 18 bp spacing (*proU1725*) showed essentially normal osmoregulation, behaving similarly to the wild-type (16 bp) promoter. This latter finding cannot be reconciled readily with a role for changes in DNA twist in the osmotic regulation of the *proU* promoter.

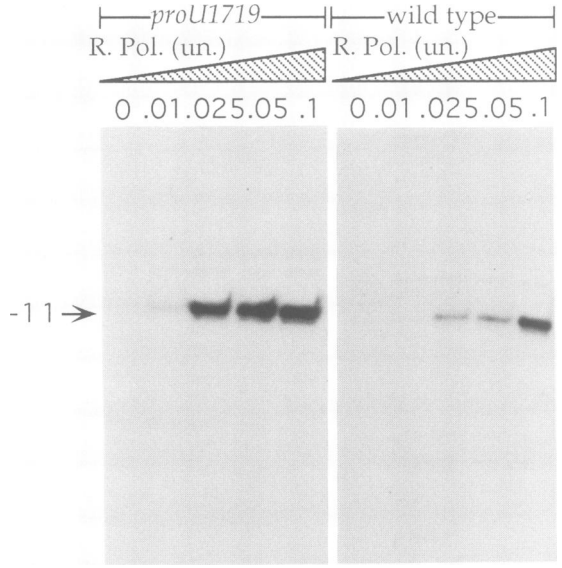



**Fig. 6.** Open complex formation at the *proU1719* and wild-type *proU* promoter. The non-coding strand of DNA fragments from −217 to +225 is end labelled (+1 is the transcriptional start site). Ten ng of DNA was incubated with the indicated amount of RNA polymerase for 15 min, after which open complex formation was measured using $KMnO_4$. The position of nucleotide −11 is indicated.

### *Effect of coumermycin on mutant promoter activity*

Coumermycin is an inhibitor of DNA gyrase which reduces the degree of negative supercoiling and, consequently, *proU* expression (Higgins *et al.*, 1988). This is shown for the chromosomal wild-type promoter whose expression at high osmolarity is repressed by 79% (Figure 2). In contrast, the mutant promoters, *proU1718* and *proU1719*, show less repression by coumermycin.

If changes in twist play a role in regulation of the *proU* promoter, by bringing the −10 and −35 regions into a more favourable alignment, promoters with 16 bp (wild-type) and 18 bp spacing should respond differently to coumermycin. However, this was not observed. Coumermycin reduced *proU* expression, for both the wild-type (16 bp) and *proU1725* (18 bp) promoters (Figure 7B).

### *Transcription start points*

In order to demonstrate that the mutant promoters do not initiate transcription from alternative start sites, primer extension of mRNA was performed. The 5′ end point of mRNA was identical for all mutant promoters (Figure 8A). Furthermore, quantitation of the primer extension products (Figure 8B) gave levels of mRNA consistent with data obtained from *lux* reporter gene assays, confirming that the changes in *proU* expression observed are due to changes in transcription initiation.

### *The effect of increased DNA flexibility between the −35 and −10 motifs on the activity of proU promoters*

The data presented above, for *proU* promoters altered in −10 to −35 spacing, effectively eliminate DNA twist as an important component in the regulation of this promoter. In particular, opposite changes in twist would be required

A

| plasmid | *proU* promoter | spacing | low | high | I |
|---|---|---|---|---|---|
| pTP15 | *proU1723* | 15 bp | 2.3 | 41 | **17.8** |
| pTP16 | wild type | 16 bp | 6 | 554 | **92.3** |
| pTP17 | *proU1719* | 17 bp | 2794 | 2795 | **1** |
| pTP171 | *proU1724* | 17 bp | 2547 | 2775 | **1.1** |
| pTP18 | *proU1725* | 18 bp | 60 | 2035 | **33.9** |
| pTP19 | *proU1726* | 19 bp | 1 | 18 | **18** |

B

| | | | | | | + coumermycin | | |
|---|---|---|---|---|---|---|---|---|
| plasmid | *proU* promoter | spacing | low | high | I | low | high | $R_{coum.}$ |
| pTP15 | *proU1723* | 15 bp | 0.9 | 10 | **11.1** | 1.7 | 6 | **0.60** |
| pTP16 | wild type | 16 bp | 2 | 187 | **93.5** | .5 | 125 | **0.67** |
| pTP17 | *proU1719* | 17 bp | 409 | 3311 | **8.1** | 175 | 1568 | **0.47** |
| pTP18 | *proU1725* | 18 bp | 38 | 2616 | **68.8** | 25 | 1464 | **0.56** |

**Fig. 7.** Luciferase activity of plasmids containing wild-type and mutant *proU* promoters fused to plasmid-encoded *lux* reporter genes. Expression was measured at low osmolarity and after osmotic upshock. The induction ratio (I) is the ratio of expression at high and low osmolarity. The spacing between the −35 and −10 regions of the promoters is given in bp. (**A**) Luciferase activity of wild-type and mutant *proU* promoters in *E.coli* strain TG1. (**B**) Luciferase activity of wild-type and mutant *proU* promoters in *S.typhimurium* strain LT2, with or without adding 5 μg/ml coumermycin. The coumermycin repression ratio ($R_{coum}$) is the ratio of expression at high osmolarity with and without coumermycin.

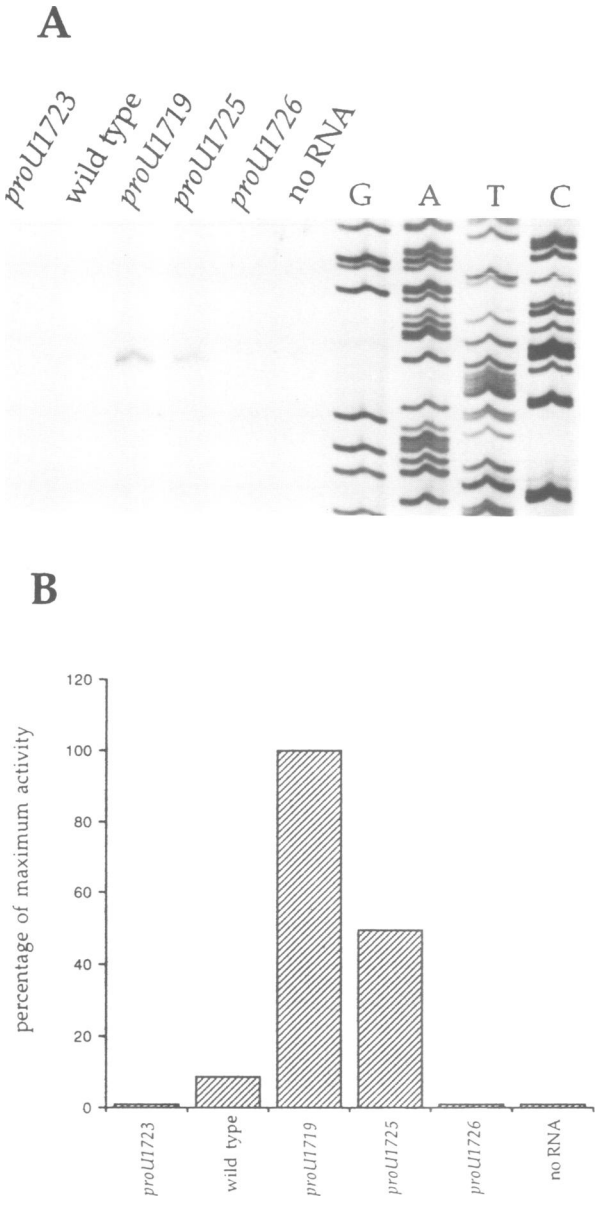



**Fig. 8.** Analysis of RNA by primer extension. (**A**) RNA was isolated from *S.typhimurium* strain LT2 harbouring plasmids containing the wild-type or mutant *proU* promoters as indicated. The 5′ ends of *proU* mRNA were mapped by primer extension. The products were separated by electrophoresis on a denaturing gel, alongside a DNA sequencing reaction on wild-type *proU* promoter DNA carried out with the same primer as used for primer extension. A single transcription start point was identified for all promoters, corresponding to the site mapped previously (Stirling *et al.*, 1989). (**B**) Densiometric analysis of the gel. The intensity of the different extension products is given as a percentage of the extension product of the strongest promoter (*proU1719*). The *proU* promoters present on the plasmids are indicated.

to bring the –10 and –35 regions of the wild-type (16 bp) and *proU1725* (18 bp) promoters into optimal alignment, yet the 18 bp promoter is osmoregulated similarly to the wild-type promoter and the two promoters respond similarly to factors such as coumermycin which perturb DNA supercoiling. However, an increase in DNA flexibility would be predicted to bring the –10 and –35 regions of both the 16 and 18 bp promoters into optimum alignment for RNA polymerase interactions.

In order to address this, promoter flexibility was achieved by introducing a gap into one of the DNA strands between the –35 and –10 motifs. Introducing a gap of one nucleotide dramatically increases the flexibility of DNA (Mills *et al.*, 1994). The DNA fragment from position –91 to +67 was used (the start of the transcription is +1) and the missing nucleotide was nucleotide –22 in the non-coding strand. These gapped promoters were constructed using PCR-based techniques (see Materials and methods, and Figure 9). As a control, the same DNA fragments were generated without a missing nucleotide (non-gapped DNA).

$KMnO_4$ footprinting was used to measure the ability of increasing amounts of RNA polymerase to form open complexes at the different promoters. As expected, RNA polymerase could form open complexes at lower concentrations on the non-gapped *proU1719* promoter (17 bp) than on the non-gapped *proU* wild-type promoter (16 bp; see Figure 10A). Figure 11A shows quantitation of the same gel using a phosphoimager. Importantly, this difference between the promoters was eliminated by introducing flexibility (a gap) into the promoter (Figures 10B and 11B). These data are consistent with a role for increased DNA flexibility in activation of the *proU* promoter at high osmolarity.

## Discussion

The level of DNA supercoiling in bacterial cells responds to environmental signals and plays a role in transcriptional regulation. We have investigated the mechanism by which these topological changes might influence transcription using the *proU* promoter of *S.typhimurium* as a model. This promoter provides a relatively simple *in vitro* system: despite extensive searches, no site-specific activator or repressor has been identified and the available data are consistent with the hypothesis that osmotically induced changes in DNA topology play a primary regulatory role. The data presented here provide direct support for this latter hypothesis. Furthermore, regulation of the *proU* promoter appears unlikely to involve changes in DNA twist: instead, alterations in writhe can account for the observed regulation.

The isolation of *proU* promoter mutations which alter expression *in vivo*, and the correlation of these *in vivo* effects with RNA polymerase binding *in vitro*, demonstrate that *proU* is a $\sigma^{70}$-dependent promoter, consistent with previous results (Jovanovich *et al.*, 1989; Stirling *et al.*, 1989; Ding *et al.*, 1995). Although a minor, *rpoS*-dependent promoter is present upstream of *proU* in *E.coli* (Dattananda *et al.*, 1991), the present data show that it does not play a significant role in the osmotic regulation of this gene in *S.typhimurium*.

The wild-type *proU* promoter has a spacing of 16 bp between the –10 and –35 motifs, compared with the consensus 17 bp. Thus, the relative orientation of the –10 and –35 motifs is suboptimal, and RNA polymerase would be expected to interact inefficiently. This was confirmed *in vitro*: both binding and open complex formation by RNA polymerase were weak at the wild-type (16 bp) *proU* promoter compared with mutant promoters with the consensus spacing of 17 bp. The relative orientation of the –10 and –35 motifs of the wild-type (16 bp) promoter

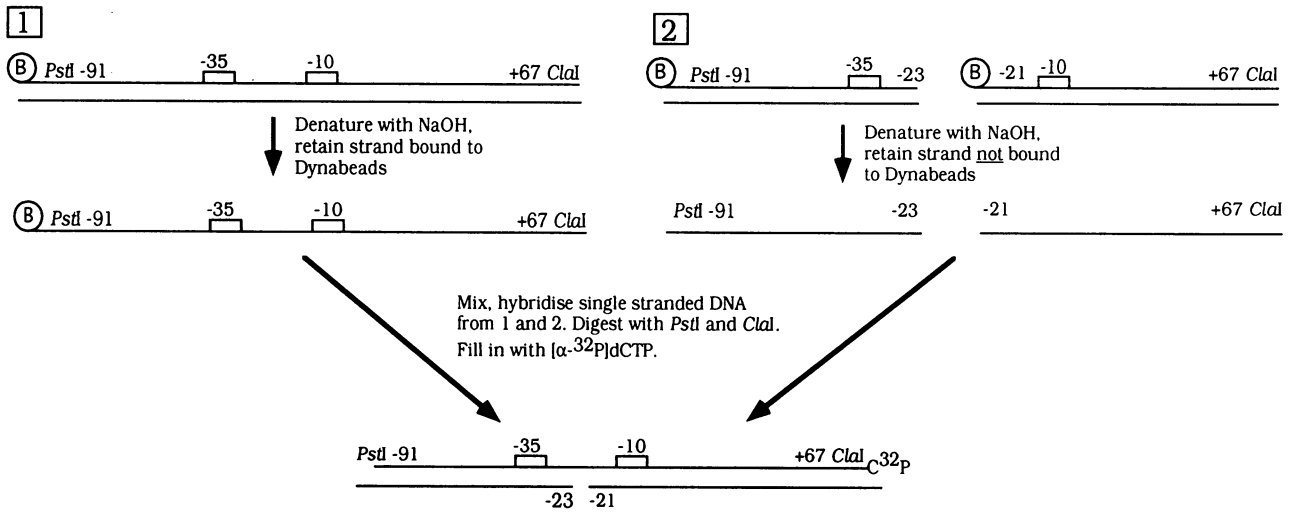



**Fig. 9.** Construction of gapped promoters. DNA from position –91 to +67 missing nucleotide –22 in the non-coding strand was constructed. The coding strand (1) was generated by PCR with the biotinylated –91 primer and the +67 primer. The non-coding strand (2) was generated by PCR with the biotinylated –91 and the –23 primer and with the biotinylated –21 and the +67 primer. PCR products were bound to Dynabeads (B) (DYNAL), denatured and the coding and non-coding strands were hybridized. The resulting gapped DNA was digested with *Pst*I and *Cla*I and filled in with [α-$^{32}$P]dCTP.

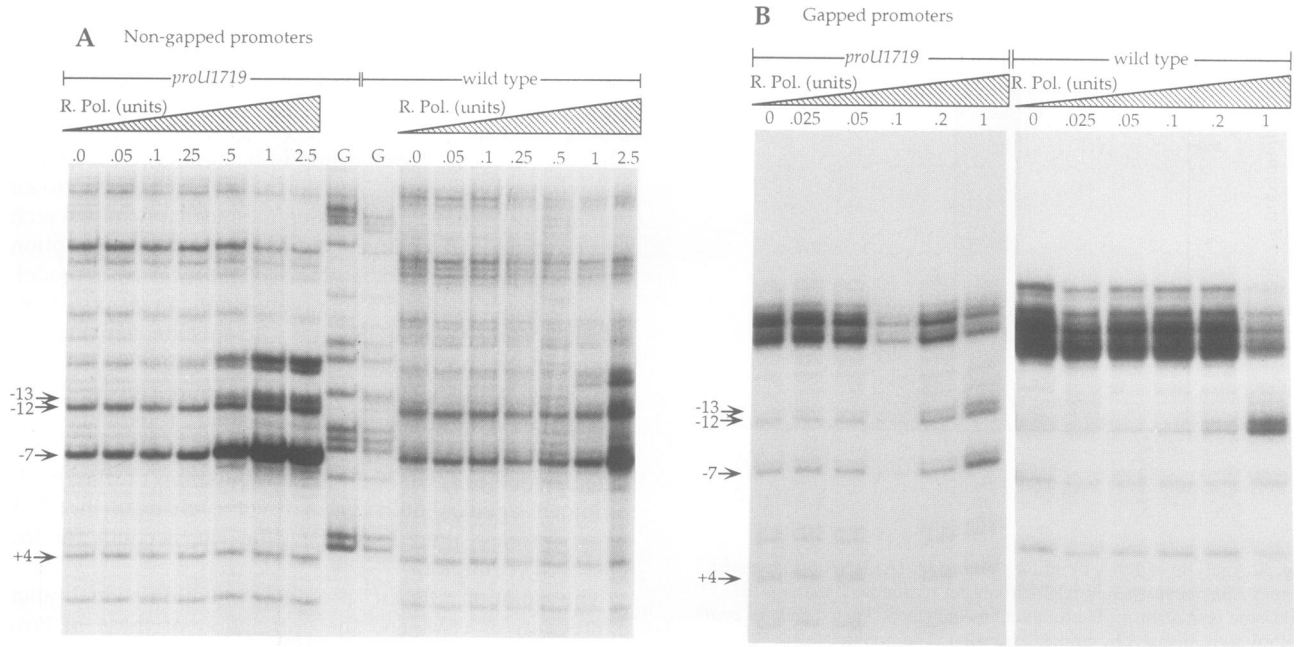



**Fig. 10.** Open complex formation on non-gapped (**A**) and gapped (**B**) *proU* promoters, as measured by $KMnO_4$ footprinting. The coding strand of DNA fragments from –91 to +67 (+1 is the transcriptional start site), containing either the wild-type or the *proU1719* promoter was end-labelled. The gap, when present, was at position –22 in the non-coding strand. DNA (33 ng) was incubated with the indicated amount of RNA polymerase for 15 min, after which the open complex formation was measured. The experiment has been performed several times and similar results were obtained. Chemical cleavage of G residues was performed on the same DNA and run alongside the footprints.

could, potentially, be realigned to optimize RNA polymerase interactions by altering DNA twist. Making reasonable assumptions about the partitioning of linking number changes into twist and writhe (Brady *et al.*, 1987), the change in rotational angle between the –10 and –35 motifs, caused by a physiologically relevant change in linking number (McClellan *et al.*, 1990), could be 34°. This is similar to the change in rotational angle effected by introducing a single base pair (Wang and Syvanen, 1992). Thus, it has been suggested that osmotically induced changes in DNA topology might, primarily, be partitioned into twist and, hence, regulate *proU* expression (Wang and Syvanen, 1992). However, mutants with altered spacing between the –10 and –35 motifs effectively exclude twist as a regulatory factor. Opposite changes in DNA twist are required to optimize the orientation of the –10 and –35 motifs of the wild-type promoter (16 bp) compared with a mutant promoter in which the spacing is too large (18 bp). Yet, an 18 bp promoter showed essentially normal osmoregulation and responded to factors which alter DNA supercoiling (gyrase inhibitors) in a manner indistinguishable from the wild-type (16 bp) promoter. Thus, the hypothesis that osmotically induced changes in DNA twist activate the *proU* promoter can be discounted.

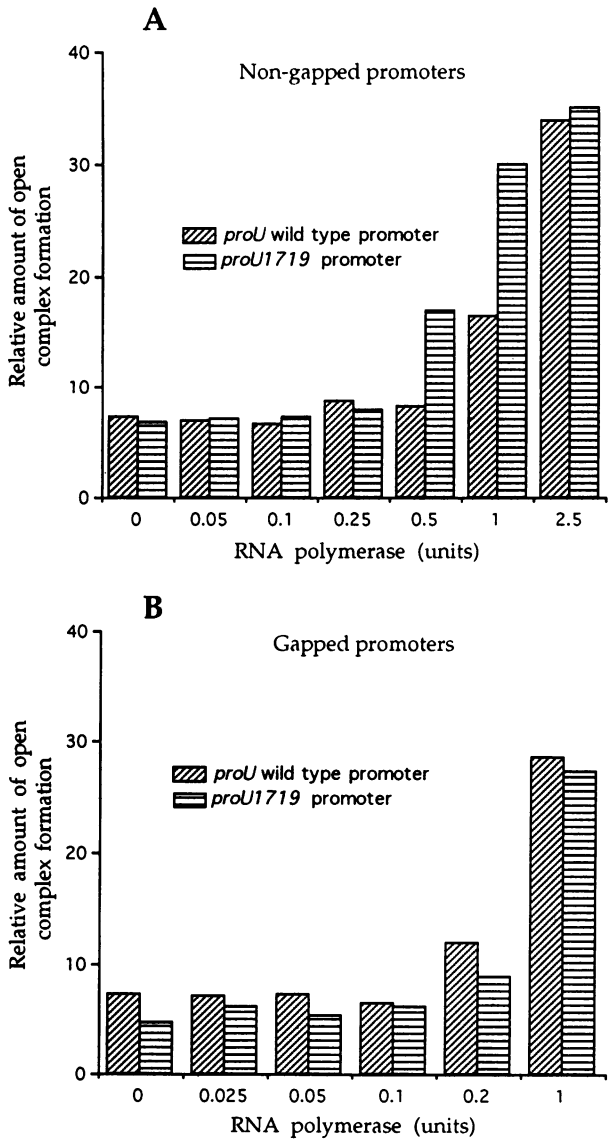



**Fig. 11.** Quantitation of open complex formation using non-gapped (**A**) and gapped (**B**) *proU* promoters. The total amount of radioactivity in the bands corresponding to positions –7, –12 and –13 (which is a measure of the open complex formation) was determined using a phosphoimager and divided by the amount of radioactivity in the band corresponding to +4 (which is independent of open complex formation), see Figure 10A and B. This 'Relative amount of open complex formation' is plotted against the units of RNA polymerase used.

If DNA twist is not involved in the regulation of the *proU* promoter then osmotically induced changes in writhe must facilitate the correct orientation of the –10 and –35 motifs. The wild-type (16 bp) and mutant 18 bp promoters require opposite changes in the orientation of the DNA helix in order to achieve optimal configurations to interact with RNA polymerase. Yet, both promoters respond similarly to osmotic upshock and changes in supercoiling. This apparent paradox could be resolved if osmotic upshock results in increased flexibility of the DNA, permitting opposite changes in writhe to occur with equal facility. This was tested, increasing the flexibility of the DNA by introducing a gap in one of the strands of the promoter between the –10 and –35 regions. This increased flexibility eliminated the differences in efficiency with which RNA polymerase interacted with promoters of different spacing. The data, therefore, support a model in which osmotically induced changes in DNA supercoiling are manifest as changes in DNA flexibility, facilitating the interaction of RNA polymerase at the *proU* promoter.

The experiments described here were carried out with the supercoiling-sensitive *proU* promoter. Other experiments with gapped promoters have been performed with promoters whose supercoiling sensitivity is not known (Ayers *et al.*, 1989; Werel *et al.*, 1991). The experiments in this paper may have implications for other supercoiling-sensitive promoters. If osmotic upshock can increase DNA flexibility in the vicinity of the *proU* promoter, similar effects may occur elsewhere in the genome. It is perhaps significant that many supercoiling-sensitive promoters have a suboptimal spacing between the –10 and –35 motifs. For example, the *bgl* promoter, which is topologically sensitive and *hns* regulated, has a spacing of 16 bp (Higgins *et al.*, 1988; Lopilato and Wright, 1990), and the supercoiling-sensitive *his* operon has an 18 bp spacing (Rudd and Menzel, 1987; Carlomagno *et al.*, 1988). The osmotically regulated *osmC* and *osmY* promoters have spacings of 16 bp (Gutierrez and Devedjian, 1991; Yim and Villarejo, 1992) and the *lac* promoter has a spacing of 18 bp and is more sensitive to DNA topology than the mutant *lacΔ1* promoter with a spacing of 17 bp (Borowiec and Gralla, 1987). Thus, it seems reasonable to suppose that changes in DNA flexibility, facilitating writhe, may be important for the regulation of many promoters whose expression is sensitive to environmentally induced changes in DNA topology.

How might this change in promoter flexibility be achieved following osmotic upshock? The best characterized promoter which is regulated by changes in the orientation of the –10 and –35 motifs is the *mer* $P_T$ promoter. The MerR regulatory protein binds between the –10 and –35 motifs and, in the presence of mercury, distorts the promoter region and activates transcription (Ansari *et al.*, 1992, 1995). Furthermore, many *hns*-sensitive promoters are activated by site-specific regulatory proteins (e.g. Jordi *et al.*, 1992). For *proU*, however, no regulatory protein has been identified despite much searching, and mutagenesis of the spacer region argues against a site-specific regulatory protein binding to this region of the promoter (Mellies *et al.*, 1994; this study). One of the initial responses to osmotic upshock is the accumulation of potassium glutamate in the cytoplasm and this is required for the osmotic induction of *proU* transcription (Sutherland *et al.*, 1986). Potassium ions can influence the conformation of the DNA template directly (Anderson and Bauer, 1978; Brady *et al.*, 1987). However, models in which potassium glutamate directly stimulates the interaction of RNA polymerase with the *proU* promoter (Ramirez *et al.*, 1989; Prince and Villarejo, 1990), are too simple to explain osmoregulation (Csonka *et al.*, 1994).

However, *in vivo*, H-NS also plays a role. All available data are consistent with the hypothesis that, at low osmolarity, H-NS reduces the flexibility of sequences around the *proU* promoter, maintaining it in a repressed state. An increase in intracellular ionic strength following osmotic upshock alters the interaction of H-NS with DNA, increasing flexibility and, hence, productive RNA polymerase interactions. The interaction of H-NS with

**Table I.** Bacterial strains

| Strain | Relevant genotype | Source |
|---|---|---|
| *Salmonella typhimurium* | | |
| LT2 | Wild-type | B.N.Ames |
| CH1102 | *proU1714*::MudJ | Stirling *et al.* (1989) |
| CH1512 | *proU1707*::MudJ | Waddell (1989) |
| CH1973 | *proU1718 proU1707*::MudJ | this work |
| CH1974 | *proU1719 proU1714*::MudJ | this work |
| SA4105 | *hns101*::IS*10 zde-1710*::Tn*10*Δ16Δ17 | K.Sanderson |
| *Escherichia coli* | | |
| TG1 | *supE* Δ(*hsdM-mcrB*)*5* ($r_k^-m_k^-$McrB$^-$) *thi* Δ(*lac-proAB*) F′ [*traD36 lacI*$^q$ Δ(*lacZ*) *M15 pro* $A^+B^+$] | Gibson (1984) |

All *Salmonella* strains are derivatives of LT2.

DNA is modulated by ionic strength *in vitro* (Ueguchi and Mizuno, 1993; Tupper *et al.*, 1994), although it is not known whether this involves dissociation of H-NS from the DNA or an alteration of the H-NS–DNA complex.

In conclusion, the data in this paper provide strong support for the hypothesis that the *proU* promoter is regulated, directly, by environmentally induced changes in DNA topology, and that this involves altered DNA flexibility. The hypothesis that these changes in flexibility are caused by $K^+$-mediated changes in the interaction of H-NS with DNA in the vicinity of the *proU* promoter is currently being tested.

## Materials and methods

### Bacterial strains and growth conditions

Bacterial strains used in this study, and their genotypes, are listed in Table I. A high transducing derivative of phage P22*int-4* was used for transductions in *S.typhimurium*, as described by Roth (1970). Bacteria were grown aerobically at 37°C in LB broth or on LB plates (Bertani, 1951) unless otherwise specified. For assay of reporter gene activity, cells were grown in nutrient broth (NB, Difco) as a low osmolarity medium, or in nutrient broth with 0.3 M NaCl added, as a high osmolarity medium. MacConkey–lactose indicator plates (Miller, 1972) were used where indicated. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml) or tetracycline (50 μg/ml) were added to the growth media for strains expressing the respective antibiotic resistance genes. Where specified, coumermycin was added to the media at a concentration of 5 μg/ml.

### Reporter gene assays

Bacterial strains containing the appropriate reporter gene fusions were grown to $OD_{600\ nm}$ = 0.3 in NB (Difco) at 30°C. NaCl was added to half of the culture, to a final concentration of 0.3 M (high osmolarity), while the other half was left untreated. The cells were incubated for a further 20 min before assaying reporter gene activity. The fact that osmotic induction was achieved by transient upshock excluded complications, such as change in plasmid copy number, that might arise from differences when cells are grown continually in media of different osmolarity. β-Galactosidase activity was measured as described by Miller (1972). Luciferase activity was measured as described by Owen-Hughes *et al.* (1992). All data are the results of multiple independent determinations and are presented after correction for background expression using data obtained for identical plasmids lacking a promoter upstream of the reporter gene.

### DNA manipulations

DNA manipulations were performed according to Sambrook *et al.* (1989). PCR amplifications of DNA used $Vent_R$ DNA polymerase (New England Biolabs). DNA sequences were determined using the Sequenase™ Version 2.0 DNA sequencing kit (USB). Direct sequencing of biotinylated PCR fragments was according to Thein and Hinton (1991).

### mRNA isolation, slot-blot hybridization and primer extension

Cells were grown in NB, to $OD_{600\ nm}$ = 0.2, with or without osmotic induction (see reporter gene assays). Ten ml of cells were pelleted by centrifugation and resuspended in 2 ml of $H_2O$, 250 μl of lysis buffer (0.5 M Tris–HCl pH 7.4, 20 mM EDTA, 10% SDS) was added immediately and samples were snap-frozen in dry ice/ethanol. After thawing, the samples were incubated at 100°C for 2 min, 250 μl of 2 M acetate added, extracted with 1 ml of phenol ($H_2O$-saturated) and 1 ml of chloroform, and then with 2.5 ml of phenol ($H_2O$-saturated)/chloroform (1:1). RNA was precipitated with ethanol, dissolved in 400 μl of DNase I buffer (Boehringer), and incubated at 37°C for 20 min with 180 units of RNase-free DNase I (Boehringer). The samples were then extracted with 200 μl of phenol ($H_2O$-saturated) and 200 μl of chloroform. RNA was precipitated with ethanol and dissolved in $H_2O$. Samples were stored at −70°C.

Slot-blots were performed by binding 20 μg of RNA to nitrocellulose filters and hybridizing the filters with $^{32}$P-labelled DNA probes by standard procedures (Sambrook *et al.*, 1989).

Primer extension was performed by mixing 20 μg of RNA with 10 pmol of oligonucleotide, end-labelled with [γ-$^{32}$P]dATP using polynucleotide kinase (Boehringer). Samples were heated to 70°C for 10 min and then transferred to ice. Five μl of 16 mM Tris–HCl pH 8.3, 40 mM KCl, 2 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1.6 mM dNTPs and 10 units of AMV reverse transcriptase (Promega) was added. After 30 min incubation at 37°C, 40 μl of loading dye (5 mM EDTA, 0.1% bromophenol blue, 0.1% orange G in deionized formamide) was added and the samples were electrophoresed in a denaturing polyacrylamide (sequencing) gel. Primer extension products were visualized by autoradiography and quantified by densitometry.

### Plasmid construction and site-directed mutagenesis

The low copy number plasmid pHSG575 (Takeshita *et al.*, 1987) was digested with *Eco*RI and *Bam*HI and the vector fragment purified from an agarose gel. Plasmid pSB71 (Ahmad and Stewart, 1988) was also digested with *Eco*RI and *Bam*HI, the 2 kb fragment containing the *lux* reporter genes from *Vibrio fischeri* isolated and cloned into pHSG575. The product of the cloning, pTP10, was used as the *lux* vector into which several 317 bp *proU* promoter fragments were cloned. The wild-type *proU* promoter, and the mutant promoters from strains CH1973 and CH1974, were amplified by PCR using chromosomal DNA as a template. Primers used were the −217 primer (5′-GCGGCGGAATTCA-GATCTGCATGCATTACAACATGTCCTACACT-3′) and the +100 primer (5′-GCGAATTCCATGGTACCATTTTATACAGATTCTTCAC-TTC-3′). The resulting fragments were digested with *Eco*RI, isolated from an agarose gel, and cloned into the *Eco*RI site of pTP10.

Additional promoter mutations were generated by site-directed mutagenesis using a modification of the procedure described by Landt *et al.* (1990). An initial PCR used the −217 primer (see above), biotinylated at the 5′ end, and a mutagenic primer complementary to the *proU* promoter (apart from the introduced mutation). The amplified fragment was isolated from an agarose gel and the biotinylated strand separated from the non-biotinylated strand using NaOH and streptavidin-coated Dynabeads® (DYNAL; Thein and Hinton, 1991). The single-stranded DNA was released from the Dynabeads® by incubating at 100°C for 30 min with shaking, and the beads were sedimented using a magnetic particle concentrator (Dynal MPC®). The supernatant was used as one

primer in a second PCR, with the +100 primer (see above) as the second primer. The amplified fragments were cleaved with *Eco*RI, isolated from an agarose gel, and cloned into the *Eco*RI site of pTP10. The entire inserts of all the plasmids generated were sequenced to ensure that the appropriate mutations were present and that no other changes had been introduced.

### *Footprinting with DNase I and $KMnO_4$*

DNA templates were generated using the PCR. DNA fragments were end-labelled by using a primer which had been labelled with [$\gamma$-$^{32}$P]dATP and polynucleotide kinase, or by filling in a restriction site using [$\alpha$-$^{32}$P]dCTP and Sequenase™ Version 2.0 DNA polymerase. Restriction sites were generated by using primers designed to generate the sites at the ends of the amplified DNA fragments. Different primers were used to generate templates of different lengths as appropriate: the –217 primer (5′-GCGGCGGAATTCAGATCTGCATGCATTACAACA-TGTCCTACACT-3′), the –91 primer (5′-AAAACTGCAGATGGGAA-ATCACAGCCGAT-3′), the +67 primer (5′-CCATCGATGCCATGC-AATAGAATGATTCC-3′), the +100 primer (5′-GCGAATTCCATGGT-ACCATTTTATACAGATTCTTCACTTC-3′) and the +225 primer (5′-CGCGAATTCCATGGTACCCGCCTTCTTCAATGGC-3′). The numbers refer to the position of the primer with respect to the *proU* transcriptional start point. Binding reactions were in 20 µl binding buffer (10 mM Tris–HCl pH 7.6, 1.5 mM potassium glutamate, 0.2 mM spermidine, 0.05 mM EDTA) with the indicated concentrations of RNA polymerase (Pharmacia). RNA polymerase was diluted in RNA pol dilution buffer (Pharmacia). After 15 min incubation at 37°C, 3 µl of DNase I (Boehringer) was added (~0.0016 U/µl). The precise concentration of DNase I required to nick each DNA molecule only once, on average, was determined for every batch of DNA. After 1 min incubation, 200 µl of DNase I stop solution (1% SDS, 10 mM EDTA, 50 mM NaCl, 5 µg/ml salmon sperm DNA, 100 µg/ml dextran, 300 mM sodium acetate pH 5.2) was added, the DNA precipitated with ethanol and resuspended in 10 µl of loading buffer (40% formamide, 5 M urea, 5 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol).

For $KMnO_4$ footprints, after the binding reaction, 1 µl of freshly prepared $KMnO_4$ (200 mM) was added to the DNA and incubated for 4 min at 37°C. Fifty µl of $KMnO_4$ stop solution (3 M ammonium acetate, 0.1 mM EDTA, 1.5 M β-mercaptoethanol) was added and samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol and dissolved in 100 µl of 10% piperidine (freshly diluted). After 30 min incubation at 95°C, the samples were quenched on ice, centrifuged for 10 s and transferred to a new tube. One ml of 1-butanol was added, the samples mixed, centrifuged for 2 min and the supernatant removed. The 'pellets' were dissolved in 100 µl of 1% SDS and 1 ml of 1-butanol added, mixed, centrifuged for 2 min, and the supernatant removed. Dry 'pellets' were dissolved in 10 µl of loading buffer (see above). Maxam–Gilbert 'G' chemical cleavage sequencing reactions, for use as size markers, were generated as described by Maxam and Gilbert (1980).

### *Construction of gapped promoters*

DNA fragments containing the *proU* wild-type and *proU1719* promoters missing nucleotide –22 in the non-coding strand, were constructed using PCR techniques. In Figure 9, the procedure by which this was done is presented schematically.

DNA was amplified by PCR, using the biotinylated –91 primer and the +67 primer (see above). Twenty µg of DNA was isolated from NuSieve GTG (FMC) agarose gels using β-agarase (New England Biolabs) and bound to Dynabeads (DYNAL). DNA was denatured and the biotinylated strand isolated according to Thein and Hinton (1991). This gave the coding strand.

The two DNA fragments which were used to generate the non-coding gapped strand were amplified by PCR. The upstream fragment was generated using the biotinylated –91 primer (see above) and either the –23 (5′-AATCTGAGACAACCCTG) or the –23m (5′-AATCTGGAGA-CAACCCTG) primer as a second primer. The –23m primer was used to generate the non-coding strand of a DNA fragment containing the *proU1719* promoter, whereas the –23 primer was used to generate a DNA fragment containing the *proU* wild-type promoter. The downstream fragment was generated by using the biotinylated –22 primer (5′-TGAGTATGTTAGGGTAGAA) and the +67 primer (see above). Twenty µg of each DNA was isolated from NuSieve GTG (FMC) agarose gels using β-agarase (New England Biolabs) and bound to Dynabeads (DYNAL), The DNA was denatured and the non-biotinylated strand was isolated according to Thein and Hinton (1991).

Equal amounts of the biotinylated, single-stranded DNA from –91 to +67 and the non-biotinylated strands from –91 to –23 (or –23m, for the *proU1719* promoter) and –21 to +67 were hybridized overnight in 6× SSC, 0.1% SDS at 42°C. The resulting gapped DNA fragments were sedimented and washed three times in TE 0.1 M NaCl using a magnetic particle concentrator (Dynal MPC®). DNA was dissolved in 80 µl of TE, digested with *Pst*I and *Cla*I, isolated from NuSieve GTG (FMC) agarose gels, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and end-labelled by filling in the *Cla*I site with [$\alpha$-$^{32}$P]dCTP using Sequenase™ Version 2.0 DNA polymerase. The resulting gapped promoters were used as templates for $KMnO_4$ footprints.

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