CHAPTER
FIVE
5.1 INTRODUCTION:

A full understanding of the mechanism of osmotic induction of \textit{proU} expression will involve: (i) identification of the necessary and sufficient \textit{cis} elements mediating \textit{proU} induction; (ii) identification of the \textit{trans}-regulatory factors that affect the various \textit{cis}-mechanisms; and (iii) understanding the interaction between the \textit{cis} elements and \textit{trans}-factors on one hand and between the \textit{trans} factors and the environmental signal regulating \textit{proU} expression, on the other.

Previous studies on \textit{cis} regulation of \textit{proU} in \textit{E. coli} had indicated the presence of two promoters, P1 and P2, transcription from each of which are osmotically induced by about 4- and 8-fold respectively \cite{41}. This study mainly dealt with identification of the \textit{trans} factors mediating \textit{proU} induction and the \textit{cis} mechanisms affected by each of these \textit{trans}-factors.

Data from the present study suggested that transcription from promoter P1 is dependent on RpoS, the stationary phase specific sigma factor of RNA-polymerase. It was also shown that \textit{rpoS} does not mediate osmotic inducibility of the promoter P1, but that stationary-phase-induction of P1 does depend on RpoS.

In this study a role for the nucleoid proteins HU and IHF in activating expression from \textit{proU} promoter P2 was also established. It was shown that the effects of HU and IHF on \textit{proU} expression is mediated through the P2R mechanism. On the other hand, effect of a third nucleoid protein HNS on \textit{proU} expression was shown to to be mediated through the NRE.

In this section the results obtained in this study have been integrated with what is known about \textit{cis} regulation of \textit{proU} and about the signal mediating \textit{proU} expression, and a new comprehensive model for \textit{proU} regulation has been presented. Also critically appraised, in brief, are the two existing alternative models of \textit{proU} regulation that invoke, respectively, \textit{in vivo} DNA supercoiling and intracellular concentration of potassium glutamate as signals for \textit{proU} regulation.
5.2 SUPERCOILING-EFFECTS ON proU REGULATION:

Higgins and coworkers had proposed that the increase in proU expression at high osmolarity is determined by an increase in negative superhelical density of DNA (98). Increased DNA supercoiling could conceivably activate promoter P2 expression by facilitating DNA melting and formation of the open complex between RNA polymerase and the promoter. On the other hand, the P2 promoter has only a 16-bp spacer between its -10 and -35 regions (1-bp less than the optimal spacer length of 17 bp), and mutations that increase the spacer length to 17 bp render the P2 promoter active even at low osmolarity (174). An increased supercoiling will be expected to increase DNA twist and therefore to place the -10 and -35 regions further out of register, thus inhibiting promoter-activity (52). At the NRE, increased supercoiling will be expected to favor substitution of HNS by the binding of HU or IHF because the latter two constrain DNA supercoils whereas the former do not; in this manner, increased supercoiling will favor NRE-mediated derepression of proU expression. Indeed Ueguechi and Mizuno (232) have observed in an in vitro system that the inhibitory effect of HNS on proU transcription is dependent on the level of DNA superhelical density of the template.

Nevertheless, experiments from several laboratories indicate that changes in in vivo levels of DNA supercoiling affect proU expression. Mutation in topA, that leads to increased DNA supercoiling, results in increased proU expression (45, 98). Conversely, inhibition of DNA gyrase activity, that results in decreased supercoiling, leads to reduced proU expression (98). In vitro studies have also shown that transcription from promoter P2 is activated by an increase in negative supercoiling of the template (185, 188, 232). Although it must be noted that this finding by itself has little value in discussing environmental regulatory mechanisms, because a majority of promoters are activated in vitro by increased supercoiling of the template.

Higgins et al (98) had initially proposed that the effect of mutation in hns (osmZ) on proU expression is mediated by changes in cellular level of DNA supercoiling in the mutants. However, it must be emphasized here that in demonstrating this correlation they have employed changes in linking number

97
(ΔLk) of reporter plasmids as a measure of superhelical density changes. First, as discussed in detail below, it is not clear that the observed changes in linking number of reporter plasmids in hns mutants are reflective of changes in DNA supercoiling. Second, it is well established now that the repressing effect of HNS on proU expression is mediated through its binding to the NRE, and thus that the altered linking number of reporter plasmid and increased proU expression in hns strains do not probably have a cause-and-effect relationship.

Both Dattananda et al (41) and Owen-Hughes et al (174) have shown a greater reduction in topological linking number of plasmids carrying the cloned NRE compared to those that do not when isolated from cells grown at high osmolarity, and there is evidence that this is an HNS mediated effect. The observed changes in ΔLk now can be explained as a change in constrained DNA supercoiling as binding of HU or IHF leads to constraint of negative DNA supercoiling whereas binding of HNS does not, and thus the dissociation of HNS from the NRE at high osmolarity and its passive replacement by HU or IHF would result in increased constrained DNA supercoiling.

Indeed, it is possible that several of the associations reported by Higgins and coworkers between ΔLk variations and particular mutations (notably hns) or environmental conditions (notably high osmolarity) reflect alterations in constrained DNA supercoiling alone. This would explain several discrepancies in the literature with regard to the proposed correlation between increased supercoiling and proU activation. Thus, (i) different hns mutations that derepress proU expression to similar extents cause varying degrees of alteration in ΔLk (100); (ii) for the same hns mutation, ΔLk alterations for different plasmids (after correction for size) are different (174); (iii) introduction of an hns mutation in S. typhimurium causes ΔLK to change in an opposite direction compared to that in E. coli, even though the regulation of proU in both species (and the effect of hns mutation on such regulation) is similar (106); (iv) likewise, environmental conditions such as growth temperature alter ΔLk in opposite directions in E. coli and S. typhimurium or S. flexneri (57); (v) hns mutations increase both proU and bgl expression, whereas inhibition of DNA gyrase activity (which would directly affect unconstrained superhelical tension) is associated with a decrease in proU and increase in bgl expression (98, 46); and (vi) tolC mutations also increase proU expression, but
otherwise such mutations relax DNA and phenotypically suppress the growth defect associated with hns mutations (49).

So far there is only one study to show that unconstrained supercoiling in vivo increases upon instantaneous osmotic upshock, but even in this study the reported results were obtained in cultures previously treated with chloramphenicol and also subjected to plasmolysis (152). In a recent report Miller and Simons (162) have used a gyrA-lac fusion, expression of which is known to be sensitive to supercoiling, to monitor changes in in vivo DNA supercoiling under different environmental conditions. Expression of this fusion during steady-state growth of cells in medium of high osmolarity was similar to that in cells grown in low-osmolar medium, suggesting that the steady-state level of DNA supercoiling is the same in cells grown at low- and at high-osmolar media. As proU remains induced under steady state growth at high osmolarity, it is unlikely that supercoiling is the regulator of proU expression.

Thus, although DNA supercoiling has an effect on proU expression, it is doubtful whether supercoiling changes are the primary determinants of osmoreponsivity of proU transcription.

5.3 POTASSIUM GLUTAMATE-EFFECTS ON proU REGULATION:

K\(^+\) is the major intracellular cation in E. coli. Intracellular concentration of K\(^+\) is known to increase as a result of osmotic upshock, and cytoplasmic [K\(^+\)] has been proposed to be a key osmoregulatory signal in E. coli (25, 26, 61).

Ionic strength can conceivably affect gene expression by affecting any of several parameters. The structure of DNA is itself sensitive to ionic strength; for example B-to-Z transition in DNA or a change in DNA twist can be brought about by changes in ionic strength, and such changes in template structure could affect transcription-initiation or -elongation. DNA-protein interactions are also sensitive to ionic strength (189); thus, promoter -recognition by RNA polymerase or the binding to DNA of regulatory proteins could be affected by ionic strength. Thus intracellular [K\(^+\)] as a signal for osmotic regulation of gene expression remains an interesting possibility.
A role for $K^+$ in $proU$ regulation was first suggested by Higgins and coworkers (97, 215) based on their findings that osmotic induction of $proU$ is sharply curtailed under $K^+$-limiting conditions of growth. Studies by Villarejo and others (185) have shown that in an in vitro system containing DNA template and purified RNA polymerase, and where no other trans factors (other than a RNase inhibitor) are present, $proU$ transcription can be activated by the addition of potassium glutamate in the system.

As mentioned earlier, the P2 promoter has a 16-bp spacer between its -10 and -35 hexamer boxes, which is 1 bp less than the optimum for $\sigma^{70}$-RNA polymerase-dependent promoters; mutations that increase the spacer length to 17 bp render the P2 promoter active even at low osmolarity (174). Wang and Syvanen (234) have argued that accumulation of potassium glutamate under conditions of high-osmolarity-growth would increase DNA twist such as to bring the -10 and -35 regions of the P2 promoter into optimal alignment for transcription initiation. Results from this study suggest that the HU and IHF proteins may also play a role in this process, but the precise mechanisms remain to be determined.

As described in chapter IV, there exist a pair of identical sequence stretches at the -10 and -35 regions of the promoters $proU$ P1 (41) and $gltS$ P2 (93), both of which are RpoS controlled. Interestingly, the spacer length between the conserved pair of sequence motifs in the $proU$ P1 promoter is 1 base-pair less (16 bp) than that in $gltS$ P2 (17 bp); it is therefore possible that osmotic activation of $proU$ P1 too is mediated by potassium glutamate accumulation and increased DNA twist.

Furthermore, potassium glutamate may also play a role in $proU$ regulation mediated through the NRE. Ueguchi and Mizuno (232) have been able to reconstitute HNS-mediated repression of $proU$ transcription in vitro, and have shown that addition of potassium glutamate to this system relieves this repression. Increased accumulation of potassium glutamate in vivo under high-osmolar growth conditions may thus play a role in dissociation of HNS from the NRE leading to induction of $proU$. 

90
5.4 THE MODEL:

A model for proU regulation based on data obtained in this study and in other laboratories has been presented below. This model tries to account for the effects of various mutations and other factors that modulate proU expression or affect proU osmotic induction. It differs from those proposed by other workers mainly in that it invokes distinct modular cis mechanisms (P1R, P2R, NRE), and it also defines the role of various trans factors at each of these cis elements. A schematic presentation of this model is given in Fig. 5.1.

As shown in this study and according to this model, promoter P1 expression depends on the alternate sigma factor RpoS that controls the expression of genes expressed during entry into stationary phase. However, osmotic induction of promoter P1 does not depend on RpoS. As mentioned above, possibly an increased twist of DNA caused by increased accumulation of K⁺ under high-osmolar conditions, helps bring the -10 and the -35 regions of this promoter, in correct orientation for optimal initiation of transcription from this promoter.

The nucleoid proteins HU and IHF affect the P2R regulation and act (directly or indirectly) as positive activators of transcription from promoter P2. It is proposed here that potassium glutamate may also play an activating role in promoter P2 expression by increasing DNA twist during growth at high osmolarity: the spacer length for promoter P2 is 16, 1 bp less than the optimum 17 bp spacer.

As discussed above, the repressing effect of HNS on proU expression is mediated by its binding to the NRE, and in vitro this repression can be relieved by the presence of potassium glutamate in the system. It is proposed here that increased accumulation of potassium glutamate under high-osmolar-growth conditions leads to dissociation of HNS from the NRE and consequent induction of proU. Thus, potassium glutamate may have distinct roles to play at the three cis mechanisms: an activating role at the P1R and P2R through increased DNA twist, and a derepressing role at the NRE caused by dissociation of HNS from the NRE.
Fig. 5.1. A model for modular mechanisms in osmotic regulation of proU operon in E. coli. The P1R, P2R and NRE mechanisms are schematically illustrated. The postulated roles of supercoiling, HU, IHF, HNS, RpoS (σ^ς) and K^+ion in modulating each of these mechanisms are indicated; the symbols + and - denote activating and repressing mechanisms respectively.