ABSTRACT

Osmoregulation is the process by which organisms adapt to growth in environments of varying water activity. A major role of various osmoregulatory mechanisms is the maintenance of cellular turgor. Turgor is controlled by adjusting the total osmotic solute pool of the cytosol in response to changes in external osmolarity.

Central to osmoregulation are the processes of solute accumulation and regulation of such processes. In media of low osmolarity, the osmolarity of the cytosol is accounted largely by ionic solutes; on the other hand, in media of higher osmolarity neutral solutes such as trehalose, glycine betaine, and L-proline make major contributions to the osmolarity of the cytosol.

The proU operon in the Enterobacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (previously called *S.typhimurium*) encodes a binding protein dependent transporter belonging to the family of ABC transporters, and which is involved in active uptake of exogenous glycine betaine and L-proline in response to hyperosmotic stress. It is comprised of three structural genes proV, proW and proX. The proU operon is transcriptionally induced 400-fold by growth in media of elevated osmolarity, which is the highest among the osmoreponsive genes so far identified.

Previous genetic searches for regulatory proteins that mediate osmotic control of proU transcription have not identified any classical regulatory factors such as specific activators or repressors. These experiments, however, showed that several DNA binding proteins which have pleiotropic effects on gene regulation, can affect proU expression. Mutation in topA, the gene for topoisomerase I, increased the expression of proU at low osmolarity, whereas inhibitors of DNA gyrase decreased it at high osmolarity. Mutations at the hns locus (encoding the nucleoid protein H-NS) resulted in derepression of proU expression at low osmolarity. Mutations in the genes for other nucleoid proteins such as IHF or HU led to moderate decreases in the basal and induced levels of proU expression without much alteration of its osmotic regulation. None of the trans-acting mutations abolished the osmotic inducibility of proU operon completely.

Earlier studies have also shown the dependence of proU induction on K⁺ uptake suggesting that potassium glutamate (which is known to accumulate in cells grown at high osmolarity) may have direct effects on proU transcription. This suggestion has also been supported by some in vitro studies. However, other in vitro studies have suggested that stimulation by potassium is non-specific, and that it also increases transcription of
genes that are not osmotically regulated. An alternative model (to the potassium glutamate model) that has been postulated is that the proU operon is regulated by DNA supercoiling, and that the latter increases with increase in medium osmolarity. However, without postulating some specific transcriptional regulatory factor, it is difficult to explain why the proU operon would be uniquely sensitive to supercoiling in vivo. There are a large number of other genes that are equally sensitive to supercoiling in vitro but do not exhibit osmotic control. Neither of the above models, which have invoked either DNA supercoiling or K⁺ ion concentration (that are otherwise known to have pleiotropic effects on gene regulation), explains the fine tuned features of proU expression and its regulation.

The studies reported in the thesis were initiated to understand the mechanism of proU osmotic regulation, by an approach involving the delineation of cis-regulatory elements of the operon and determination of the role of the elements thus identified in conferring osmotic inducibility. For this purpose, a 2.4 kb fragment from the proU locus was cloned into a low copy number promoter probe vector pMU575 (that carries lacZ as reporter gene and trimethoprim resistance gene as an antibiotic marker) to generate plasmid pHYD251. DH5α transformants of plasmid pHYD251 were shown to exhibit a 230-fold inducibility of β-galactosidase expression after growth in medium supplemented with 0.3 M NaCl, comparable with that observed earlier for chromosomal proU-lac strains. The proU insert in plasmid pHYD251 was subjected to progressive deletion at either end by exonuclease III-mediated deletion mutagenesis. Discrete fragments of the proU regulatory region, and nested deletions generated within it by site-directed mutagenesis or PCR mediated deletion mutagenesis were also cloned into the vector pMU575. Members of the panel of pMU575 plasmid derivatives thus generated were assayed for osmoreponsive lacZ expression. These studies showed the presence of two promoters P1 and P2 for proU, with start-sites of transcription situated, respectively, 250 bp and 60 bp upstream of the initiation codon of the first structural gene proV. The sequences immediately around P1 and P2 conferred five- and eight-fold osmotic inducibility on transcription initiated from the respective promoters. The cis sequence and mechanisms mediated via P1 and P2 are referred to as P1R and P2R respectively. Furthermore, a negative regulatory element (NRE) residing in the 600 bp region of sequence downstream of P2 (within the first structural gene proV) was shown to be necessary for repression of proU expression at low osmolarity and to constitute the third
cis regulatory element for proU. The three mechanisms (P1R, P2R and NRE) were shown to contribute independently and additively to the observed 200-fold osmoreponsivity of proU operon.

Delineation of the three cis regulatory elements for proU also led to the identification of trans-acting factors that interacted with these elements. The P1 and P2 promoters were shown to be transcribed by RNA polymerase holoenzyme bearing, respectively, the stationary-phase sigma factor (σ^5) and the housekeeping sigma factor (σ^70). Furthermore, the derepressing effect of mutations in hns was shown to be mediated via the NRE.

A segment of DNA from S.enterica, equivalent to the proU P1 promoter region of E.coli, was PCR-amplified and cloned into plasmid vector pMU575 to obtain plasmid pHYD373. Plasmid pHYD373 was shown not to exhibit promoter activity in vivo, whereas the equivalent plasmids with E.coli proU P1 (pHYD275 and pHYD380) exhibited σ^5-dependent promoter activity in vivo. On the other hand, both E.coli and S.enterica derived fragments exhibited σ^5-dependent P1 transcription in vitro.

A spontaneous Lac^+ mutant of Δlac chromosomal strain MC4100 carrying pHYD373 was selected on minimal A-lactose agar plate supplemented with 0.15 M NaCl. The resulting strain was shown to carry on its plasmid, a σ^5-dependent osmotically regulated in vivo promoter activity equivalent to the E. coli P1 promoter and the mutant plasmid was designated as pHYD374. Nucleotide sequence analysis of plasmid pHYD374 showed the presence of a 22 bp promoter distal deletion spanning from +63 to +84 (relative to the P1 transcriptional start site). Primer extension analysis of RNA isolated from cells carrying the wild-type and mutant S. enterica proU P1-bearing plasmids pHYD373 and pHYD374, respectively, indicated that a primer which hybridizes proximal to +60 is able to detect transcripts from both strains but that a primer which hybridizes distal to +85 is able to do so only from the latter. The results therefore indicated the existence of a σ^5-dependent proU P1 promoter in S. enterica which is rendered cryptic in vivo because of transcription attenuation within a short distance downstream of the promoter start site. The fact that such attenuation occurs only in vivo but not in vitro suggests that it is factor-dependent.

Even in E.coli, although osmotically- and stationary phase-induced σ^5-dependent promoter activity was demonstrated in vivo for proU-lac plasmid constructs carrying only the P1R-related sequences, there was no P1 promoter activity demonstrable in the
plasmid constructs (pHYD394 and pHYD395) that also carried P2R (with site-specific knock out mutations in the -10 region of promoter P2) and NRE sequences. This suggested that the *E. coli* P1 promoter is also cryptic in its native context.

A candidate gene approach was employed in the attempt to identify the factors involved in cryptification of proU P1 promoter in *S. enterica* and *E. coli*. In this approach the effects of mutations in *hns*, *rho* (encoding the transcription termination factor Rho) and *stpA* (encoding the H-NS analog StpA) on proU P1-lac expression from plasmids pHYD373, pHYD374, pHYD275, pHYD380, pHYD394 and pHYD395 were studied. The effects of different environmental conditions on lac expression from these plasmids were also examined in order to detect a possible physiological role of the proU P1 promoter. The two conditions tested were growth of *E. coli* at low temperature (10°C) and as biofilms.

The results demonstrated that: (i) growth as biofilms does not induce proU P1 expression, (ii) low temperature induction of proU P1 promoter of both *E. coli* and *S. enterica* does occur, and is achieved mainly by increase in σ^S^ cellular levels, (iii) the *S. enterica* proU P1 promoter is activated, individually and additively, in a *rho* mutant (which is defective in the transcription termination factor Rho) as well as by growth at 10°C, (iv) the *E. coli* proU P1 promoter, which is cryptic in its native context is activated by *hns* null mutation at 30°C or by a *rho* mutation during growth at 10°C, and (v) mutation in *stpA* has no effect either by itself or in combination with the other mutations *rho* and *hns*. In brief, the results led to the conclusions that proU P1 promoter activity is perhaps modulated both by nucleoid structure and by Rho-mediated transcription attenuation, and that this promoter may be physiologically important for proU operon expression during low temperature growth.

In one of the *E. coli* strains GJ829 in the laboratory, it was fortuitously observed that *E. coli* proU P1 directed lac expression in plasmid pHYD275 is abolished but that it is restored in its isogenic derivative GJ830 which is defective in the *rho* gene. The genetic and molecular characterization of the mutation in GJ829 revealed it to be an IS10 insertion disrupting the 330-codon-long rpoS open reading frame encoding σ^S^, in the region of codons 51-53; the mutation was designated as rpoS365::IS10. By Western blot analysis, it was confirmed that the mutant gene codes for a truncated σ^S^ polypeptide of the expected size, which was designated σ^S^Δ1-50.
The activity of $\sigma^S\Delta1-50$ was tested on several $\sigma^S$-dependent promoters ($proU$ P1, $csiD$, $katE$ and $osmY$) by expressing the truncated protein in vivo either from the chromosomal $rpoS365::IS10$ allele (in rho mutant strains), or from a plasmid-borne arabinose-inducible promoter plasmid (pHYD408). The results suggested that $\sigma^S\Delta1-50$ is as proficient as the wild type in directing transcription of $proU$ P1 promoter but exhibits reduced efficiency at the other three promoters. Both by Western blot analysis and by in vivo measurement of $\sigma^S$-specific promoters it was demonstrated that the cellular levels of $\sigma^S\Delta1-50$, even when expressed from a template that possessed neither the transcriptional nor the translational control elements of wild type $rpoS$, is subjected to regulation by environmental variables such as low temperature, high osmolarity and growth phase, in the same manner and to the same extent as that described earlier for $\sigma^S$. These results suggest that environmental control of the $\sigma^S$ regulon genes is achieved primarily through the regulation of $\sigma^S$ protein degradation.

In summary, the existence of multiple mechanisms in osmotic regulation of $proU$ was demonstrated, mediated via three cis-elements P1R, P2R and NRE. These mechanisms appear to contribute independently and additively to the high level of osmotic inducibility of $proU$ expression. Promoters P1 and P2 are transcribed, respectively, by $\sigma^S$- and $\sigma^70$-bearing RNA polymerase, and the NRE is the site of repressive action of the nucleoid protein H-NS. The $proU$ P1 promoter is subjected to additional controls that involve H-NS, Rho-dependent transcription attenuation, and transcriptional induction during growth at 10°C, but the mechanisms for these are still to be determined. Transcriptional induction of $\sigma^S$-dependent genes in response to environmental conditions such as growth in stationary phase, at low temperature and at high osmolarity, consequent to increase in cellular $\sigma^S$ content, was shown to occur even in the absence of transcriptional or translational control of $rpoS$, suggesting that it is mediated primarily through the regulation of $\sigma^S$ protein degradation.