Expression of Recombinant Proteins in *Escherichia coli* Under
the Transcriptional Control of the Cold-Shock Inducible *cspA* Promoter

by

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Abstract

Expression of Recombinant Proteins in *Escherichia coli* Under the Transcriptional Control of the Cold-Shock Inducible *cspA* Promoter

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Aggregation and proteolytic degradation can significantly decrease the recovery yields of recombinant proteins overproduced in the gram-negative bacterium *Escherichia coli*. Expression at low growth temperatures holds great promise in alleviating these problems since proteolysis is reduced and proper folding is facilitated under these conditions. Unfortunately, the transcriptional efficiency of traditional promoters decreases with the cell growth temperature. When *E. coli* is transferred from a physiological temperature of 37°C to the 10-15°C temperature range, the production of most cellular proteins stops while a handful of cold-shock proteins, that are virtually undetectable under normal growth conditions, become synthesized at very high levels. Because the major *E. coli* cold shock protein, CspA, is primarily regulated at the transcriptional level, we examined the advantages and limitations of the *cspA* promoter in directing the expression of recombinant proteins at reduced growth temperatures.

Transcriptional gene fusions between the *cspA* promoter and either the *lacZ* gene or a gene encoding the tripartite fusion protein preS2-S'-β-galactosidase were constructed to characterize the strength, regulation and inducibility of the major cold shock promoter and to delineate its usefulness for the production of aggregation-prone recombinant proteins. The *cspA* promoter was well repressed at 37°C and efficiently directed gene expression upon temperature downshift to the 30-10°C temperature range for 60-90 min, although it became repressed at later time points. Using the *cspA* promoter at 10°C, the aggregation-prone protein preS2-S'-β-galactosidase could be produced in a completely soluble form at 5-fold higher levels than obtained with the chemically-inducible *tac* promoter at the same temperature. Fermentation, strain and genetic engineering approaches were also investigated to determine if promoter repression following prolonged incubation at low temperatures could be circumvented. While fermentation engineering provided modest improvements on the recovery yields of enzymatic activity from a plasmid-encoded *cspA-lacZ* gene fusion, sustained synthesis of β-galactosidase for about 7 h and 3-fold higher levels of activity were observed in a *rbfA* host strain. Deletions in the 5' untranslated region of the *cspA* RNA further showed that this region plays a fundamental role not only in the low-temperature repression but also in the high temperature repression and cold-shock induction of the promoter.

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Chapter 1. Introduction and Background

1.1. Introduction

The gram-negative bacterium *Escherichia coli* remains the most widely used microorganism for the production of recombinant proteins despite recent advances in alternative expression systems such as mammalian cells, insects cells, yeast or algae (Goeddel *et al.*, 1991). Even though *E. coli* is unable to perform certain types of post-translational modifications (e.g. glycosylation) and synthesizes endotoxins which may contaminate the desired gene product, it offers numerous advantages for recombinant protein production. For instance, *E. coli* can grow at high density on inexpensive substrates, its genetics have been extensively studied and a number of cloning vectors and strains have been developed which allow the high level production and facilitate the purification of heterologous proteins.

Although many recombinant proteins can be produced in a biologically active form in *E. coli*, the overexpression of heterologous proteins often results in the proteolysis or the incorrect folding of the protein of interest. Misfolded proteins may be degraded or form insoluble aggregates known as inclusion bodies. Because inclusion bodies are enriched in the target protein of interest, their formation can be used to facilitate purification schemes (Georgiou and Bowden, 1990). In addition, aggregation may be beneficial if the protein of interest is highly susceptible to proteolysis or toxic to the cell. In some cases, purified inclusion bodies can be treated with strong chaotropic agents such as guanidine hydrochloride or urea in order to fully unfold the proteins. Subsequent slow dilution of these agents may yield biologically active polypeptides in a pure form. Unfortunately, *in vitro* refolding is a difficult and labor intensive task and the final recovery yields of recombinant protein may be disappointing. In addition, there is some evidence that proteases can co-aggregate with the protein of interest and degrade it

during the refolding process (Babbitt *et al.*, 1990). As a result, a large amount of effort has been directed at maximizing the formation of correctly folded recombinant proteins in *E. coli.*

Because growth at low temperatures is one of the simplest and most powerful approaches to favor the proper folding of aggregation-prone recombinant proteins in *E. coli* (Table 1.1), we have started to investigate the potential of low temperature-inducible (cold-shock) promoters in directing the synthesis of heterologous proteins at reduced growth temperatures. The goal of the present research is to use a combination of genetic and fermentation engineering approaches to maximize the recovery yields of recombinant proteins placed under the control of the *cspA* promoter.

1.2. Maximizing recombinant protein production *in vivo*

Since the process of reactivating recombinant proteins through *in vitro* refolding techniques has several drawbacks, there is a great impetus to develop a number of processes to combat the problems associated with overexpressing recombinant proteins *in vivo*. The following is an overview of many methods currently used to increase protein solubility and stability, including: 1) protein engineering; 2) genetic engineering; and 3) optimizing fermentation conditions.

1.2.1. Protein engineering

The manipulation of the primary sequence of a polypeptide by genetics techniques can have a significant impact on protein aggregation. Inclusion bodies are thought to arise from the association of partially folded polypeptides that have become kinetically trapped in an unproductive branch of their folding pathway (Mitraki and King, 1989). As a consequence, mutations which alter the stability of these folding intermediates can have a positive effect on solubility. Indeed, this is the case for several proteins (Wetzel

et al., 1991; Mitraki and King, 1992; Chrunyk *et al.*, 1993; Wetzel and Chrunyk, 1994). However, the effects of specific mutations are difficult to predict and, without knowledge of the crystal structure and/or the folding pathway of a protein, may often lead to increased aggregation rather than enhanced solubility. Another approach is to fuse the protein of interest to a "solubilizing partner" (e.g. thioredoxin (Invitrogen Co.)) which favors the proper folding of the desired gene product (reviewed in LaVallie and McCoy, 1995). Such "solubilizing partners" can be cleaved from the desired polypeptide through the use of sequence-specific peptidases and proteases in order to allow the recovery of the target protein. However, such fusions may have deleterious effects on protein solubility and their usefulness often needs to be evaluated on a case by case basis (LaVallie and McCoy, 1995).

1.2.2. Manipulation of the cellular environment

In addition to inclusion body formation, one of the major problems associated with the production of heterologous proteins in *E. coli* is their susceptibility to degradation by host proteases. Although this problem can be partially alleviated by making use of host strains deficient in cellular proteases (Baneyx and Georgiou, 1990; Baneyx and Georgiou, 1991; Baneyx and Georgiou, 1992; Maurizi, 1992) and, in certain cases, by optimizing the composition of the growth medium (Baneyx *et al.*, 1991), this approach is not without limitations since only a small number of proteases can be deleted without significantly affecting cell growth.

A promising method to minimize inclusion body formation consists in overproducing molecular chaperones together with the protein of interest. Molecular chaperones are highly conserved proteins which have been isolated in all microorganisms and cellular compartments examined to date (Ellis and van der Vies, 1991). They bind to non-native polypeptides and help them progress towards a

biologically active conformation or the correct cellular location without becoming part of the final structure (Ellis and van der Vies, 1991). Molecular chaperones appear to improve correct protein folding by reducing the net concentration of aggregation-prone folding intermediates the cellular milieu (Gething and Sambrook, 1992). A number of reports have demonstrated that an increase in the intracellular concentration of specific molecular chaperones can greatly improve the folding of a number of functionally and structurally unrelated proteins both *in vitro* (Gething and Sambrook, 1992) and *in vivo* (Hockney, 1994; Wall and Plückthun, 1995; Georgiou and Valax, 1996). In many instances, however, chaperone overproduction is not sufficient to completely abolish aggregation. Furthermore, a cellular imbalance of chaperones may be detrimental to the cell (Kalbach and Gatenby, 1993). Finally, the co-overexpression of specific molecular chaperones operons can lead to a substantial reduction in the production rates of the target protein (Caspers *et al.*, 1994; Dale *et al.*, 1994).

1.2.3. Fermentation engineering

Extensive research in a number of laboratories indicates that a decrease in the fermentation temperature or the induction of a chemically-inducible promoter with a suboptimal concentration of gratuitous inducer can substantially reduce the aggregation of recombinant proteins (reviewed in Schein and Noteborn, 1988; Hockney, 1994; Georgiou, 1995). It is believed that these approaches exert their beneficial effect, at least in part, by reducing the intracellular concentration of aggregation prone folding intermediates, since induction with suboptimal concentrations of inducer reduces the synthesis rate by lowering the concentration of target mRNA, while growth at low temperature is accompanied by a decrease in transcriptional, translational, and elongation rates (Broeze *et al.*, 1978). Table 1.1 lists a number of cases for which cell growth at reduced temperature significantly reduces protein aggregation. A number of

in vitro studies have also demonstrated that low temperatures can facilitate the refolding of purified proteins exhibiting a high propensity to aggregate at room temperature (Sugihara and Baldwin, 1988; van der Vies *et al.*, 1992). These results can be explained by a reduction in hydrophobic interactions at low temperatures. Such conditions would confer a longer half-life to folding intermediates allowing proper subdomain-subdomain interactions to take place and facilitating correct folding (Gething and Sambrook, 1992). For instance, it is likely that, when produced at low levels in the cell cytoplasm, aggregation-prone proteins are more efficiently engaged by the host molecular chaperone machinery with the net result of improved folding (Thomas and Baneyx, 1996b; Thomas and Baneyx, 1996a). It is finally important to bear in mind that several *E. coli* proteases (e.g. Lon and Clp) are maximally active at physiological temperature and specifically induced under stress conditions, including the overexpression of recombinant proteins, while their basal level is reduced at lower temperatures (data not shown, Baneyx and Georgiou, 1992; Maurizi, 1992). Thus, recombinant protein production at low temperatures appears to be a simple and powerful tool to maximize the production of soluble and active polypeptides. Although reducing promoter induction and low temperature can lead to a substantial improvement in protein solubility, they are also accompanied by a reduction in the overall production of the recombinant protein of interest.

In addition to inducer concentration and growth temperature, several fermentation parameters can affect inclusion body formation in *E. coli*, including pH, medium composition and aeration levels (Georgiou and Bowden, 1990; Hockney, 1994; Georgiou, 1995). Finally, the nature of the host strain may also influence recombinant protein folding (Browner *et al.*, 1991). Unfortunately, the improvements in protein solubility obtained by altering the above parameters are usually modest and it is often necessary to optimize each variable on a case by case basis.

1.3. *E. coli* promoters

The synthesis of cellular proteins is controlled at the transcriptional level by promoters which are DNA sequences located upstream of the structural genes (for review see Record *et al.*, 1996). Promoter regions are recognized by complexes between the core RNA polymerase and a σ factor which confers the specificity for certain promoters. To date, the six known *E. coli* σ factors and the areas they regulate genes in include: (i) σ^{70}, vegetative expression; (ii) σ^{32} and σ^{24} [σ^{E}], heat-shock response; (iii) σ^{54}, nitrogen assimilation; (iv) σ^{28}, flagellum gene expression; (v) σ^{38}, stationary-phase expression (for review see Record *et al.*, 1996). Upon binding of the holoenzyme (core RNA polymerase+ σ) to the promoter region, a "closed" binary complex is formed in which the promoter DNA remains entirely double helical in structure. This complex is converted to an "open" form through the melting of a short DNA region. Next a ternary initiation complex is formed following incorporation of two complimentary ribonucleotides and formation of a phosphodiester bond. Transcription initiation may progress following the binding of the next ribonucleotide or it may reverse to remove the terminal ribonucleotide. Alternatively, the initiation complex may revert to the open form by releasing the entire nascent oligo mRNA chain (abortive initiation). Once a 7 to 12 nucleotide long RNA has been synthesized by RNA polymerase, the σ factor is released, RNA clears the promoter and elongation of the mRNA chain proceeds. Until the state of processive elongation is reached, all initiation steps are highly reversible.

For biotechnology applications, two of the most important characteristics of a promoter are its inducibility and strength. The inducibility of a promoter is defined as the level of maximal induction versus the level of maximal repression of the target protein. The strength of a promoter can be regarded as either the amount of mRNA

transcribed following induction or the concentration of protein translated from the mRNA. The compilation of the DNA sequences of a wide variety of σ^{70} controlled *E. coli* promoters has revealed the consensus sequence shown in Figure 1.1.

In general, mutations improving promoter function involve the replacement of a non-consensus base pair by a consensus base pair at that position, while the converse is true for mutations that decrease promoter function (Record *et al.*, 1996). However a high level of homology to the consensus sequence does not always guarantee promoter strength. The nature of the DNA sequences located upstream, downstream, and between the consensus -35 and -10 boxes can also significantly affect the strength of a promoter. Promoters can direct transcription constitutively, or may be regulated positively through the binding of an activating protein or molecule, or negatively through the binding of a repressor protein or molecule, or both (Record *et al.*, 1996).

1.4. *E. coli* promoters used for recombinant protein expression

It is well established that the effectiveness of a promoter in directing soluble recombinant protein expression can vary from protein to protein (Goeddel *et al.*, 1991). As a result, several systems are usually tested to identify an optimal promoter for the expression of the desired gene product. An ideal promoter for the soluble expression of cytoplasmic proteins should present the following characteristics. It should (i) be reasonably strong so as to yield a sizable amount of recombinant proteins but not so strong that the products misfold or misassemble in the cell; (ii) display tight regulation to allow the cytoplasmic expression of toxic products, but be inducible without having to resort to expensive means; (iii) direct transcription under conditions favorable for the proper folding of translated proteins; and (iv) it should be straightforward to scale-up. The main characteristics of promoters routinely used for the expression of recombinant proteins in *E. coli* are described in the following sections. Two promising promoters

that respond to changes in the dissolved oxygen concentration or the pH of the environment are also described.

1.4.1. The *lac* promoter and its derivatives

In the *E. coli* chromosome, the lactose promoter (*lac*) directs the transcription of the lactose operon which consists of three structural genes responsible for lactose metabolism. The *lac* operon is preceded by a regulatory gene, *lacI*, which is controlled by its own promoter. The *lacI* gene product is a repressor protein which binds to the operator site of the *lac* promoter thereby preventing RNA polymerase from transcribing the *lac* genes. When lactose is present in the medium it associates with the repressor, yielding a complex unable to bind to the operator. Transcription of the *lac* operon is also positively regulated by the cellular concentration of cAMP which, when complexed with the cAMP receptor protein, CAP, stimulates CAP as an apoactivator of the *lac* operon (Beckwith, 1987). If glucose or one of its precursors is present in the medium, the intracellular concentration of cAMP decreases and repression inhibits expression from the *lac* promoter. When lactose is present in the medium and the cAMP concentration is high, RNA polymerase is then able to productively interact with the *lac* promoter and synthesis of the polycistronic *lacZYA* mRNA takes place.

The *lac* promoter has been cloned and used to direct the expression of several recombinant proteins. Typically, the non-hydrolyzable lactose analog isopropyl-β-D-thiogalactopyranoside (IPTG) is substituted for lactose to induce the transcription of heterologous genes placed under control of the *lac* promoter. Since the *lac* promoter can be leaky when no inducer is present, it is common to use strains carrying the $lacI^q$ allele (either plasmid encoded or under the form of a F'$lacI^q$ episome) which produce higher levels of the lacI repressor protein and thus repress the promoter more efficiently.

lacUV5 is a modified *lac* promoter which is controlled uniquely by the lactose repressor protein. This promoter can therefore be induced with IPTG even when cells are grown in the presence of glucose. Since the *lac* and the *lacUV5* promoters are relatively weak promoters, their usefulness for recombinant protein expression is somewhat limited. A popular choice for biotechnology applications is the synthetic *tac* promoter which consists of the -35 box from the tryptophan (*trp*) promoter and the -10 box and operator region from the *lacUV5* promoter (Amann *et al.*, 1983; de Boer *et al.*, 1983). This promoter is quite strong and it is not uncommon that gene products placed under *tac* control accumulate as 20-30% of the total cellular protein. The *tac* promoter was further modified to increase the 16 bp spacing to the 17 bp consensus, yielding the *trc* promoter (Amann and Brosius, 1985). This modification did not, however, lead to increased promoter strength and the *tac* and *trc* systems are usually used interchangeably. Another modification of the *tac* promoter was made by repositioning the *lac* operator between the -10 and -35 elements. The strength of the resulting sterically repressed promoter (*srp*) was unaffected although the pre-induction levels of recombinant proteins were reduced approximately 50% relative to *tac* promoter. This modification can be quite advantageous since a reduction in promoter leakiness significantly increased host stability when a toxic protein was produced (Ezaz Nikpay *et al.*, 1994).

1.4.2. λP_L promoter

The λP_L promoter was isolated from bacteriophage λ, a temperate phage that can enter either a lysogenic or lytic pathway. In the lysogenic mode, the phage integrates within the host chromosome and conditions leading to the lytic cycle are suppressed by expression of the cI repressor protein. This protein tightly regulates a number of promoters, including λP_L, and effectively represses gene transcription. The cI repressor

protein can be inactivated in a number of ways in order to derepress the λP_L promoter. One mechanism involves the induction of the SOS response by addition of nalidixic acid (Walker, 1987). This pathway results in the degradation of the cI repressor protein. However, the most commonly used approach for the induction of heterologous genes cloned downstream of the λP_L promoter, involves the use of a temperature sensitive mutant of the cI repressor encoded by the λcI_{857} gene. At 30 °C, the mutant repressor protein is fully active and the promoter is repressed. However, when the growth temperature is shifted to 42 °C, the cI_{857} protein is inactivated, and transcription can take place. The λcI_{857} gene is usually provided from a lysogenic strain, on a compatible plasmid, or on a plasmid that also contains a λP_L promoter followed by a multiple cloning site suitable for the insertion of the gene of interest.

The strength of the λP_L promoter can be enhanced by production of the *E. coli* integrated host factor protein (IHF), which, among its many tasks, binds with high affinity to two tandem sites upstream of the λP_L promoter and stimulates transcription by enhancing closed complex formation between the RNA polymerase and the promoter (Giladi *et al.*, 1990). Interestingly, the λP_L promoter was recently found to be induced by growing cultures at low temperatures or by shifting exponentially growing cultures from 37 to 20 or 15 °C (Giladi *et al.*, 1995). Since the strains used in this study did not encode the cI repressor protein, the basal level of protein expression was high. A modified λP_L promoter was obtained by changing four base pairs around the -50 site and one at the -9 site. λP_L-9G-50 was 26 fold less leaky than its native counterpart four hours after temperature shift from 37 to 15 °C. However, this was not without expense, since the strength of the promoter decreases 10-fold with the modifications (Giladi *et al.*, 1995).

1.4.3. T7 promoter systems

A number of other phage promoters can be used to control the expression of recombinant proteins in *E. coli*. One of the most popular systems is the bacteriophage T7 promoter which is not recognized by the *E. coli* RNA polymerase. Plasmids containing genes under T7 promoter control are generally transformed into lysogenic strains that encode the T7 RNA polymerase gene under the control of IPTG-inducible promoter (e.g. lacUV5; Studier and Moffatt, 1986)). Upon addition of the inducer to the culture medium, the *lacUV5* promoter is derepressed and synthesis of T7 RNA polymerase takes place. In this fashion, very large amounts of gene products (up to 50% of the total cellular protein) can be obtained. Unfortunately, this level of induction can lead to ribosomal failure and cell death, and the desired protein is usually found mostly in an inclusion body form (Dong *et al.*, 1995).

1.4.4. Oxygen-responsive promoters

The oxygen-dependent promoter of the *Vitreoscilla* hemoglobin (*VHb*) gene has been shown to be effective in directing the expression of recombinant proteins in *E. coli* at levels that can represent 10% of the total cellular protein (Khosla *et al.*, 1990). The *VHb* promoter is efficiently repressed when cultures are grown under well-aerated conditions [dissolved oxygen (DO) concentration, greater than 60% air saturation]. Decreasing the DO concentration to less than 40% by sparging with nitrogen induces the promoter. Optimal induction conditions were found to occur at DO levels below 5% air saturation. However, strict anaerobic conditions led to the almost complete inhibition of the *VHb* promoter (Khosla and Bailey, 1989).

Recently Lee and coworkers (1996) evaluated the usefulness of the oxygen-dependent *nar* promoter for recombinant protein production in *E. coli*. Like the *VHb*

promoter, the *E. coli nar* promoter is induced under near anaerobic conditions (Iuchi and Lin, 1991). It directs the transcription of several genes including a nitrogen reductase which generates oxygen by converting nitrate to nitrite. The generation of oxygen by the nitrogen reductase can suppress induction of the *nar* promoter, hence autoregulating the operon. Therefore, for biotechnology applications, the chromosomal *nar* operon was insertionally mutated so that no functional nitrogen reductase was produced and autoregulation was eliminated. Recombinant protein expression under control of a plasmid-encoded *nar* promoter was comparable to that obtained under *tac* promoter control when 0.5-1.0% nitrate was included in the medium (Lee *et al.*, 1996).

Another promoter that responds to DO concentrations is that of the *E. coli cadA* gene, which encodes lysine decarboxylase. Besides being responsive to changes in oxygen levels, the promoter is extremely sensitive to changes in environmental pH (Tolentino *et al.*, 1992). The pH-inducible portion of the promoter can be turned on and off by adding bulk chemicals that affect the pH of the growth medium. For instance, the *cad* promoter is induced 200-fold when the pH is lowered from 8 to 6.5 and can direct recombinant proteins at a level of 10-15% of the total cellular proteins (San *et al.*, 1994).

1.4.5. Limitations of currently used promoters systems

Although they have been widely used for production of many heterologous gene products, the promoter systems described in the above paragraphs (e.g. *lac*, *tac*, λP_L and T7 promoters) suffer from a number of drawbacks. The *lac* promoter is relatively weak (typically the target protein only represents 5% of the total cellular protein) and somewhat leaky in the absence of inducer. The latter problem can be alleviated by using strains carrying the *lacI*^{q} allele. Although the *tac* and *trc* promoters are stronger than *lac* , they are still induced by IPTG, a relatively expensive chemical. The λP_L promoter

system must be derepressed by transiently raising the growth temperature to 42 °C. Such a temperature increase also induces the *E. coli* heat-shock response leading to higher synthesis levels of σ^{32}-dependent proteases (e.g. the *lon* and *clp* gene products; Baneyx and Georgiou, 1992; Maurizi, 1992) which may degrade the protein of interest. The recent discovery that the λP_L promoter is induced at low temperatures suggests an alternative approach for its induction. However, more rigorous research will be needed before this method is viable for recombinant protein expression. Finally, strong promoters (e.g. that of bacteriophage T7) may result in the synthesis of sizable amounts of heterologous proteins which can induce the heat shock response at 37 °C. More importantly, the massive amount of proteins synthesized from bacteriophage promoters are often unable to fold properly and accumulate as inclusion bodies.

1.5. Cold-Shock response in *E. coli*

Upon rapid transfer of mid-logarithmic phase W3110 *E. coli* cells from 37 to 10 °C, a four to five hour lag phase is observed before exponential growth resumes with a generation time of 24 hours (Jones *et al.*, 1987). Immediately after temperature downshift, the synthesis of most cellular proteins, including that of heat-shock proteins, becomes repressed, while the production of approximately 20 proteins, collectively known as cold-shock proteins, is selectively induced (Jones *et al.*, 1987; Jones *et al.*, 1992a; Jones and Inouye, 1994). Figure 1.2 shows the pattern of gene expression associated with a sudden temperature downshift in *E. coli*. Whereas a small number of proteins are highly induced at early times, induction of most cold-shock polypeptides reaches a maximum three hours after transfer to low temperature (Jones and Inouye, 1994). Resumption of cell growth and a rise in heat-shock protein synthesis occurs during the next hour (Taura *et al.*, 1989). The cold-shock response is induced by temperature downshifts of 7 °C or greater and the level of cold-shock induction appears

to be proportional to the magnitude of the temperature decrease (Jones and Inouye, 1994; Vasina and Baneyx, 1996).

1.5.1. Cold-shock proteins

Upon transfer of exponentially growing *E. coli* cells from 37 °C to the 10 - 15 °C temperature range, thirteen of the known cold-shock proteins undergo a two- to eleven-fold induction compared to their synthesis rate at 37 °C (Fig. 1.2). On the basis of their migration pattern in 2D gels, and through additional studies, the following polypeptides have been identified as cold-shock proteins: NusA, RecA, GyrA, H-NS, polynucleotide phosphorylase (PNP), initiation factor 2α, initiation factor 2β, dihydrolipoamide acetyltransferase, pyruvate dehydrogenase (lipoamide), Hsc66 (a DnaK homolog), the *hscA* gene product, RbfA, and CsdA (Jones *et al.*, 1987; Lelivelt and Kawula, 1995; Jones and Inouye, 1996; Jones *et al.*, 1996). While these proteins in general are maximally produced three to four hours after temperature downshift (Fig 1.2; La Teana *et al.*, 1991; Lelivelt and Kawula, 1995), three additional cold-inducible polypeptides, CspA, CspB, and CspG, are expressed at very high levels at much earlier time points (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Nakashima *et al.*, 1996) and are dicussed in further detail in section 1.5.2.

Most low temperature inducible proteins are not unique to cold-shock since they are also synthesized at physiological temperatures where they are involved in a number of cellular processes ranging from DNA repair to translation (for review see Jones and Inouye, 1994; Graumann and Marahiel, 1996). Proteins interacting with DNA include RecA, which plays a role in the SOS response and in recombination, Gyrase A, the α-subunit of the topoisomerase DNA gyrase, and H-NS, a nucleoid-associated DNA-binding protein. Both NusA, which is involved in transcription termination and anti-termination and PNP, which has been implicated in mRNA degradation, regulate

mRNA synthesis or stability (Jones *et al.*, 1987; Jones and Inouye, 1994). Initiation factors 2α and 2β, along with CsdA and RbfA, two proteins that were recently shown to be cold-shock inducible play a role in translation initiation. CsdA preferentially associates with the ribosome at low temperature (Jones *et al.*, 1996) while RbfA has been implicated in the adaptive response to low temperature (Jones and Inouye, 1996). RbfA has also been shown to be required for the induction of "traditional" heat-shock proteins (e.g. GroEL and DnaK) once cold-shock adaptation has been completed (Jones and Inouye, 1996). Although the synthesis of heat-shock proteins is repressed during the early stages of the cold-shock response, two homologs of the DnaK and DnaJ heat-shock proteins (the *hscB* and *hscA* gene products, respectively) are induced by cold-shock but not by heat shock (Lelivelt and Kawula, 1995; Kandror and Goldberg, 1997). The unidentified cold-shock proteins from the original two dimensional gel of Jones *et al.* (1987) have coordinates F84.0, G 41.2, G55.0 and G74.0. These polypeptides are likely to correspond to some of the recently discovered cold-shock proteins including CspB, CspG, RbfA, CsdA, Hsc66, HscA, and other cold-shock proteins identified by the use of Tn5-*lac* transposons (Qoronfleh *et al.*, 1992).

1.5.2. The Csp family of cold-shock proteins

The most abundant cold-shock protein is CspA (a.k.a. CS 7.4) which is rapidly, but transiently induced following transfer of mid-exponential phase cells from 37 to 10 °C. This 7.4 kDa hydrophilic polypeptide is virtually undetectable in cultures grown at 30 or 37 °C, but its synthesis rate at 10 °C is 100 - 200-fold higher than at 37 °C (Goldstein *et al.*, 1990). Ninety minutes following temperature downshift, CspA becomes the most abundant protein produced by *E. coli* although its synthesis becomes repressed 2 h after cold-shock (Goldstein *et al.*, 1990). To date, eight CspA homologs (termed CspB to CspI) have been identified either directly or through homology

searches (Yamanaka and Inouye, 1997). CspB, CspC, CspG, and CspE are more than 69% identical to CspA while CspD and CspF share 45% identity with the major cold-shock protein (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Yamanaka *et al.*, 1994; Nakashima *et al.*, 1996). The *cspC* and *cspE* genes were isolated as multicopy suppressors of the temperature-sensitive *mukB106* mutation and are constitutively expressed at all temperatures (Yamanaka *et al.*, 1994; Yamanaka and Inouye, 1997). Similarly, CspD does not appear to be cold-shock inducible. Its synthesis is however upregulated when *E. coli* cells growing at physiological temperatures enter stationary-phase (Yamanaka and Inouye, 1997). This process does not rely on the stationary-phase sigma factor σ^S (Yamanaka and Inouye, 1997). CspB and CspG are induced by cold-shock. Interestingly, the promoter regions of all three genes have nearly identical putative -35 and -10 consensus sequences (Fig. 1.3). Furthermore *cspA*, *cspB*, and *cspG* are transcribed with a ca. 160 nucleotide long 5' extension that is not translated (Nakashima *et al.*, 1996). To date, no information is available on the regulation or physiological function of CspF, CspH and CspI.

CspA homologs have also been identified in other molecular organisms including the CspB cold shock protein from *Bacillus subtilis* (Willimsky *et al.*, 1992) and a 7.0 kDa protein from *Streptomyces clavuligers* (Av Gay *et al.*, 1992). These proteins share 61 and 56% identity with CspA, respectively. More importantly, CspA is also highly homologous to a domain of the eukaryotic Y-box binding proteins (Sommerville and Ladomery, 1996). The Y-box family of proteins are a ubiquitous set of transcriptional factors that contain a highly conserved domain recognizing the ATTGG motif in the Y-box sequence, CTGATTGGCAA (for review see Sommerville and Ladomery, 1996). Y-box proteins have been shown to interact with other promoter elements exhibiting strong purine-pyrimidine asymmetry (Sommerville and Ladomery, 1996). Binding of Y-box proteins to Y-box promoter elements can upregulate the transcription of specific

sets of genes. However, Y-box proteins have also been shown to mask mRNA thereby preventing translation (Ranjan *et al.*, 1993). The Y-box protein domain responsible for binding to Y-box elements is 40% identical to CspA. In this respect it is interesting to note that CspA has been implicated in the transcriptional activation of two cold-shock genes, *gyrA* and *hns* (La Teana *et al.*, 1991; Jones *et al.*, 1992b). Gel mobility shift experiments revealed that CspA could bind to the three ATTGG elements located upstream of the *gyrA* promoter (Jones *et al.*, 1992b). However, replacing the native *E. coli hns* promoter with that from *Proteus vulgaris* which does not contain the ATTGG motif, did not affect the cold-shock induction of the *hns* gene (Brandi *et al.*, 1994). Thus, the ATTGG motif present in the *E. coli hns* promoter does not appear to be necessary for transcriptional activation.

Recently Jiang and coworkers (1997) used gel retardation assays to demonstrate that CspA is not capable of binding double-stranded DNA. However at concentration greater than 2.7×10^{-5} M, CspA cooperatively binds heat-denatured nonspecific single-stranded RNA (ssRNA) sequences larger than 74 bases. This concentration of CspA is much lower than the estimated cellular concentration (10^{-4} M) in cold-shocked cells (Jiang *et al.*, 1997). The elucidation of the tertiary structure of CspA by X-ray crystallography (Schindelin *et al.*, 1994) and nuclear magnetic resonance (NMR`, Newkirk *et al.*, 1994) provide additional evidence that CspA is capable of binding ssRNA or single-stranded DNA (ssDNA). CspA consists of five antiparallel β-sheets arranged in a closed five stranded β-barrel. NMR studies have shown a high frequency of aromatic side chains on the surface of the protein and it was further demonstrated that purified CspA could form a complex with a single-stranded oligodeoxyribonucleotide corresponding to the 5' untranslated region of its own mRNA (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994).

The finding that CspA is able to bind ssRNA, along with the fact that it has been shown through RNase susceptibility assays to interact with RNA secondary structures

led Jiang and co-workers (1997) to suggest that CspA is a RNA chaperone. The fact that, the *cspA* structural gene contains the RNP1 RNA binding sequence motif found in eukaryotic proteins displaying a RNA chaperone ability supports this hypothesis (Dreyfuss *et al.*, 1993; Jones and Inouye, 1994). CspA may therefore function by unwinding or preventing the formation of secondary structures in RNA molecules. Such RNA chaperone function would indeed be particularly useful at low temperatures, since mRNA secondary structures are stabilized under these conditions. Maintaining the mRNA in an unfolded state may be necessary for the efficient translation of mRNAs at low temperatures and could potentially have an effect on transcription. Interestingly, CsdA, the major ribosomal associating cold-shock protein, possesses an ATP-independent helix destabilizing activity (Jones and Inouye, 1996). It is therefore conceivable that CspA could cooperatively bind to mRNAs unwound by CsdA to prevent them from re-annealing. Alternatively, CspA could first bind to RNA and assist CsdA mRNA unwinding (Jones *et al.*, 1996; Jiang *et al.*, 1997).

In addition to its possible RNA chaperone function, CspA has been proposed to play an essential role in activating the transcriptional machinery at low temperature by converting the transcription initiation closed complex into an open complex (section 1.3; Jones and Inouye, 1994). CspA and its homologs may also repress the translation of certain genes by directly binding to their mRNAs (Ranjan *et al.*, 1993). This possibility is further discussed in section 1.6.

1.5.3. Heat-shock gene expression during cold-shock

While heat-shock proteins are initially repressed when actively growing *E. coli* cultures are transferred from 37 to 15 °C, their expression is significant upregulated within 2.5 hours of the temperature downshift (Taura *et al.*, 1989; Jones *et al.*, 1996). This observation is consistent with the fact that the *rpoH* mRNA, which encodes the heat

shock transcription factor σ^{32}, displays extensive stable secondary structure at low temperatures that should prevent its translation and thus repress transcription from heat shock gene promoters (Straus *et al.*, 1987; Nagai *et al.*, 1991). It has also been shown that overproduction of molecular chaperones GroEL-GroES, or that of heat-shock proteins in general, reduces cell viability at 4 °C (Kandror and Goldberg, 1997). To date, however, the mechanisms leading to the derepression of the heat-shock response remains obscure. Since cells carrying a deletion in the *csdA* gene are unable to derepress GroEL and DnaK synthesis following cold shock adaptation, it has however been proposed that the destabilizing activity of CsdA (perhaps in concert with CspA) can eventually unfold the *rpoH* mRNA, thus allowing the high level expression of heat shock proteins (Jiang *et al.*, 1997).

A number of heat-shock proteins are molecular chaperones, which help other polypeptides reach a proper conformation or cellular location without becoming part of the final structure (Ellis and van der Vies, 1991). Since heat-shock proteins are initially repressed following cold-shock, one may speculate that they are not necessary for cold adaptation. Nevertheless, homologs of the DnaK and DnaJ heat shock proteins that are cold-shock inducible have been isolated but there is no proof that either of these proteins exhibit molecular chaperone activity (Lelivelt and Kawula, 1995). In addition, the presence of the peptidyl-prolyl cis/trans isomerase trigger factor is essential for maintaining cell viability at 4 °C (Kandror and Goldberg, 1997).

1.5.4. Role of (p)ppGpp in the cold-shock response

The levels of cellular guanosine 5'- 3'-diphosphate and guanosine 5' triphosphate 3'-diphosphate (collectively abbreviated (p)ppGpp) vary with the growth temperature and affect protein synthesis in *E. coli*. Whereas an increase in growth temperature results in higher concentrations of (p)ppGpp, temperature downshifts lead to lower intracellular

concentration of (p)ppGpp and the magnitude of the decrease is proportional to the temperature drop (Pao and Dyess, 1981). Shifting a *relA spoT* strain, which does not produce detectable levels of (p)ppGpp, from 37 to 10 °C resulted in the higher induction of many cold-shock proteins, as well as that of certain transcriptional and translational proteins, and growth continued without the usual lag period, albeit at a slower rate (Jones *et al.*, 1992a). On the other hand, overproduction of (p)ppGpp prior to temperature downshift resulted in a decreased induction of the cold-shock response and a longer lag period before growth resumed (Jones *et al.*, 1992a). Taken together, the above results suggest that the decrease in the levels of (p)ppGpp that occurs upon temperature downshift positively affects the expression of translational, transcriptional and cold-shock proteins.

Since a nutritional upshift also results in a decrease in the cellular concentration of (p)ppGpp, conditions under which the supply of charged tRNA exceeds the capacity of the translational machinery may trigger a decrease in (p)ppGpp levels and a corresponding change in gene expression (Jones *et al.*, 1992a). It was therefore proposed that the decrease in translational efficiency which accompanies cold-shock (Broeze *et al.*, 1978) is responsible for the lower cellular (p)ppGpp concentrations and the repression of vegetative protein synthesis (Jones *et al.*, 1992a).

1.5.5. Ribosomes as sensors of cold-shock

Shifting cells to low temperature is not the only signal inducing the cold-shock response in *E. coli.* For instance, certain inhibitors of translation (e.g. chloramphenicol, tetracycline and fusidic acid) have been shown to stimulate the synthesis of cold-shock proteins (VanBogelen and Neidhardt, 1990). Nutritional upshift can also induce the promoter of the major cold-shock protein, CspA (unpublished data). These results, along with the observation that shifting cells to lower temperatures has inhibitory effects

on protein translation, led VanBogelen and Neidhardt (1990) to propose that the state of the ribosome is the physiological sensor for the induction of the response. Since many inhibitors of translation that induce the cold-shock response also decrease the level of (p)ppGpp (Lund and Kjeldgaard, 1972), this model is consistent with the proposed involvement of (p)ppGpp levels in the cold-shock response.

In current models (Jones and Inouye, 1996), ribosomes are believed to be able to translate all cellular mRNAs at physiological temperatures. Upon temperature downshift, the translational capacity of the cell decreases due to a reduction in the number of polysomes, an increase in the concentration of 50S and 30S ribosomal subunits and a rise in the amount of 70S monosomes which are not actively transcribing (Jones and Inouye, 1996). Under these conditions, ribosomes are believed to be unable to initiate translation of mRNAs, with the exception of cold-shock mRNAs, but still capable of translating all mRNAs. Cold-shock gene products in particular RbfA, CsdA and initiation factors 2α and 2β, are believed to associate with ribosomes and change their state from cold-sensitive non-translatable to cold-resistant translatable. Finally, it has been proposed that an increase in the concentration of cold-adapted ribosomes serves as a signal for the repression of the cold-shock response. However, the above model does not address 1) how the ribosomes are able to specifically recognize cold-shock mRNAs when they are not in a cold-resistant state, and 2) how the genes encoding cold-shock proteins are induced upon temperature downshift (Jones and Inouye, 1996).

1.6. Regulation of *cspA* expression

The rate of CspA synthesis reaches 13% of the total protein 60-90 minutes following temperature shift from 37 to 10 °C before dropping to a basal level (Goldstein *et al.*, 1990). A similar expression pattern is observed in the case of the *cspB* and *cspG* gene

products, most likely due to the fact that all of these genes have highly similar promoter regions (Fig 1.3; Lee *et al.*, 1994; Etchegaray *et al.*, 1996; Nakashima *et al.*, 1996). Although a great deal of attention has recently been paid to the regulation of *cspA* expression, the induction and repression mechanisms remain poorly understood. *In vivo* footprinting experiments have shown that a region located between base pairs -75 and -35 in the *cspA* promoter was protected from nuclease digestion 15 minutes after shift from 37 to 14 °C, but not in cultures maintained at 37 °C (Fig. 1.4; Tanabe *et al.*, 1992). It was also demonstrated that, when subjected to cold-shock, cell extracts contain factor(s) able to bind *cspA* promoter fragments located in the -92 and -63 bp region (Tanabe *et al.*, 1992). Taken together, these experiments suggest that one or several agents are synthesized *de novo* upon cold-shock and bind upstream of the *cspA* promoter in order to activate transcription of the *cspA* gene (Tanabe *et al.*, 1992). CspA itself appeared to be a good candidate for the binding factor, since it is highly homologous to the Y-box DNA binding family of proteins and some data suggested that the binding factor was a protein. However, no cold-shock specific bands were identified when purified CspA was used in a gel retardation assay involving DNA fragments located upstream of its promoter. This result is in agreement with the recent finding that CspA can only bind ssRNA and ssDNA but not dsDNA (Tanabe *et al.*, 1992; Jiang *et al.*, 1997). Nevertheless, the possibility that CspA requires additional factors in order to associate with DNA regions located upstream of its own promoter cannot be excluded.

Following rapid and high level induction upon temperature downshift, the levels of cellular CspA synthesis decrease after 1 - 2 h incubation at low temperature. Since the intracellular concentration of *cspA* mRNA follows the same pattern, *cspA* expression is likely to be regulated at the transcriptional level (Tanabe *et al.*, 1992; Jiang *et al.*, 1993). Several lines of evidence indicates that the extensive 5' untranslated region (UTR) of the *cspA* transcript plays an important role in the repression of CspA synthesis in the later

stages of the cold-shock response (Jiang *et al.*, 1996). For instance, overexpression of a plasmid-encoded *cspA* 5' UTR mRNA lacking the structural *cspA* gene, results in the prolonged synthesis of cold-shock proteins when transformants are shifted to low temperature (Jiang *et al.*, 1996). Under these conditions, the lag period is significantly increased (Jiang *et al.*, 1996). Similarly, overexpression of the *csdA* 5' UTR under control of the *cspA* promoter derepresses the synthesis of cold-shock proteins at low temperatures (Jiang *et al.*, 1996). Interestingly, the 5' UTRs of CspA (Tanabe *et al.*, 1992), CspB (Lee *et al.*, 1994), CspG (Nakashima *et al.*, 1996) and CsdA (Jones *et al.*, 1996), which are 159, 161, 160, and 226 bases in length, respectively, contain a highly homologous sequence of 11 bases which has been termed "the cold box" (Fig. 1.3; Jiang *et al.*, 1996). The above observations have led to the hypothesis that the cold-box is the binding site of a putative cold-shock inducible repressor that either inhibits further transcription of cold-shock genes and/or destabilizes their mRNAs (Jiang *et al.*, 1996). The latter possibility is supported by the fact that the stability of the *cspA* mRNA decreases with time after cold-shock (Goldenberg *et al.*, 1996). Co-overproduction of CspA together with the 5' UTR lead to slightly higher synthesis levels of CspA, but does not affect the cold-shock response, suggesting that CspA may be involved in the function of the repressor (Jiang *et al.*, 1996).

The expression of *cspA* is heavily dependent on the stability of its transcript which is strongly influenced by temperature. The *cspA* transcript appears to be specifically stabilized at low temperatures, since it is able to direct protein synthesis longer than the average bulk mRNAs at 10 °C (Brandi *et al.*, 1996). Interestingly, the half-life of the *cspA* mRNA at 10 °C is 60-fold greater than at 37 °C (Goldenberg *et al.*, 1996). This phenomenon was attributed to the presence of a destabilizing secondary structure at the 3' end of the transcript, owing to the fact that a *lacZ* fusion to the 346th bp of the *cspA* transcript (removing 26 bp from the *cspA* gene) stabilized the mRNA (Goldenberg *et*

al., 1996). In addition, Fang and coworkers (1997) recently found that a three base substitution around the Shine-Delgarno sequence of *cspA* stabilized the mRNA 150-fold and led to the constitutive production of CspA at 37 °C. The stabilization of the transcript was partially attributed to resistance against RNase E. As further evidence that transcript stability is a major factor is the synthesis of CspA, replacing the native *cspA* promoter upstream of the UTRwith the non-cold-shock inducible promoter *lpp*, resulted in a cold-shock inducible gene (Fang *et al.*, 1997).

1.7. Significance of the current research

The need for refolding many aggregation prone recombinant proteins *in vitro*, following isolation of inclusion body material from bacterial cells, may be the single most important contributor to the production cost of many biopharmaceuticals. In an effort to reduce these costs and increase product yields, a large number of investigations have focused on improving the yields of correctly folded proteins *in vivo*. Cultivating the host cells at low temperature is a simple solution to this problem, that has proven effective in increasing the solubility of a large number of structurally and functionally unrelated polypeptides (Table 1.1). Thus, the further development of promoter systems that are specifically induced upon temperature downshift, such as the *cspA* promoter, may be particularly valuable for the production of aggregation-prone gene products at low temperatures.

Table 1.1. Effect of the growth temperature on inclusion body formation.[a]

Conditions	Protein and Effect	Reference
42 or 37 °C Minimal medium P_L promoter	**Ricin A chain** Soluble and fully active at 37 °C Aggregated at 42 °C	(Piatak *et al.*, 1988)
37 or 30 °C LB medium T7, colE1 or *amp* promoter	**Human interferon-α2** 2.2- to 3.5-fold increase in soluble material in cells grown at 30 °C relative to 37 °C	(Schein *et al.*, 1988)
37 or 30 °C LB medium *trp* promoter	**Human interferon-γ** 16.5-fold less insoluble at 30 °C relative to 37 °C	(Schein *et al.*, 1988)
37 or 30 °C LB medium *trp* promoter	**Murine protein Mx** 50% soluble at 30 °C while insoluble at 37 °C	(Schein *et al.*, 1988)
37 or 23 °C M9 medium *lpp-lac* promoter	***B. subtilus* subtilisin E** Extensive aggreation in the periplasm at 37 °C with induction at 2 mM IPTG; 16-fold increase in activity with growth at 23 °C and induction with 5 μM IPTG	(Takagi *et al.*, 1988)
37, 30 or 21 °C M9 medium *trp* promoter	**Fab fragments** 10-fold increase in soluble protein in cells growing at 30 or 21 °C relative to cells growing at 37 °C	(Cabilly, 1989)
37 or 23 °C LB medium T7 promoter	***V. harveyi* luciferase αβ fusion** 50,000 fold increase in luminescence at 23 °C relative to 37 °C	(Escher *et al.*, 1989)
37 to 22 °C LB medium *tac* promoter	***S. cerevisiae* α-glucosidase PI** 5-fold increase in activity at 22 °C relative to 37 °C	(Kopetzki *et al.*, 1989)
42, 37 or 28 °C LB medium *tac* promoter	**Fusion protein between the hepatitis B surface antigen preS2-S' domains and β-galactosidase** 7-fold increase in activity at 28 °C relative to 42 °C	(Lee *et al.*, 1990)
37-20 °C M9 medium *tac* promoter	**TEM β-lactamase** 90% decrease in periplasmic inclusion bodies and concomitant increase in activity at 20 °C relative to 37 °C	(Chalmers *et al.*, 1990)
37, 20 and 15 °C 2x YT broth T7 promoter	**Soybean lipoxygenase L-1** 3.5-fold increase in activity at 15 °C relative to 37 °C	(Steczko *et al.*, 1991)

Table 1.1. Continued

Conditions	Protein and Effect	Reference
37 and 23 °C M9ZB medium T7 promoter	**Kanamycin nucleotidyltransferase** 10-fold increase in specific activity at 23 °C relative to 37 °C	(Liao, 1991)
37 and 22 °C LB medium T7 and *tac-tac* promoters	**Rabbit muscle glycogen phosphorylase** Complete aggregation at 37 °C; 50% of the protein soluble in T7 driven constructs induced with 25 μM IPTG at 22 °C; 50-90% of the protein soluble in *tac-tac* driven constructs depending on the host strain.	(Browner *et al.*, 1991)

[a]. (Adapted from Baneyx, submitted)

TTGACA - N_{17} - TATAAT - N_{5-7} - start

- 35 box - 10 box

Figure 1.1. Consensus sequence of the *E. coli* promoters utilizing the σ^{70} factor (Hawley and McClure, 1983). N denotes any nucleotide and the subscripts indicate the number of nucleotides; "start" corresponds to the transcription start point.

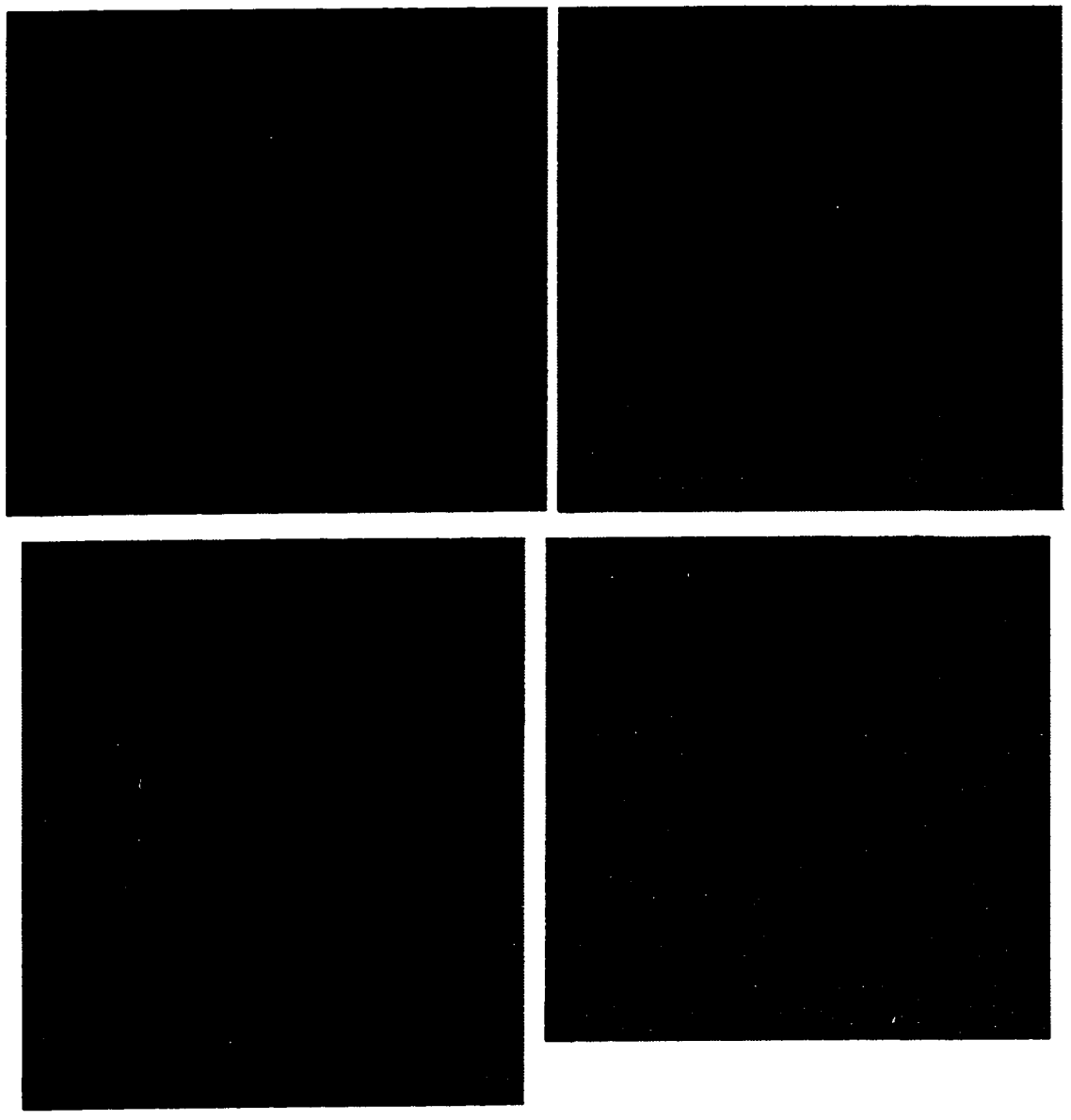

Figure 1.2. Synthesis of individual proteins following a shift from 37 to 10 °C. Two dimensional gels were run using from total cell extracts labeled with [S^{35}]methionine: (A) 5 min prior to shift; (B) 0 to 30 min postshift; (C) 120 to 150 min postshift; (D) 240 to 270 min postshift. The numbered proteins (Jones *et al.* 1987; La Teana *et al.* 1991) are: 1, ribosomal protein L7; 2, ribosomal protein L12; 3, trigger factor; 4, NusA; 5, ribosomal protein S1; 6, ribosomal protein S6B; 7, EF-Ts; 8, RecA; 9, dihydrolipoamide acetyltransferase; 10, polynucleotide phosphorylase; 11, ribosomal protein S6A; 12, D74.0; 13, EF-G; 14, GyrA; 15, β-subunit of RNA polymerase; 16, EF-Tu; 17, CspA; 18, HNS; 19, F24.5; 20, F43.8; 21, F84.0; 22, pyruvate-dehydrogenase-lipoamide; 23, intiation factor 2β; 24, G41.2; 25, G50.5; 26, G55.0; 27, G74.0; 28, initiation factor 2α. Adapted from Jones *et al.* (1987).

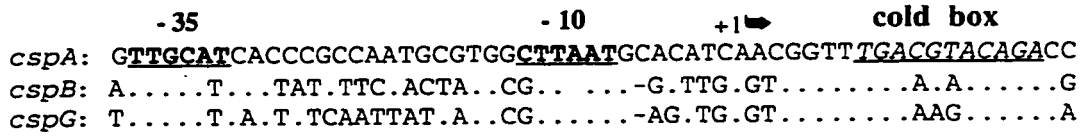

Figure 1.3. Nucleotide sequence of the cold-shock promoters of the *cspA*, *cspB*, and *cspG* genes. The -35 and -10 boxes are underlined and in boldface. The transcription starting site is denoted by an arrow. The putative "cold-box sequence" is italicized and underlined (Jiang *et al.*, 1996). Identical nucleotides are denoted by dots. Dashes show gaps in the alignment.

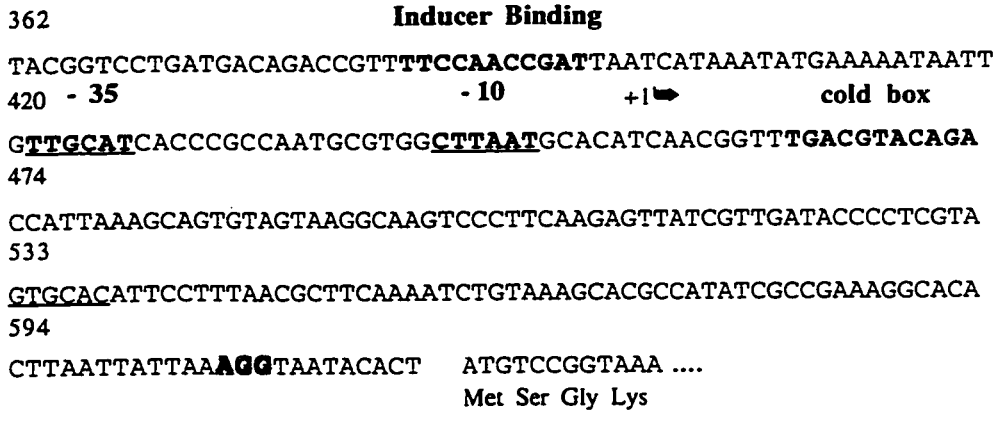

Figure 1.4. DNA sequence of the promoter region of the *cspA* gene. The -35 and -10 boxes are in boldface and underlined. The start of transcription is indicated by an arrow and the ribosome binding site is in outlined letters. The sequences bound by putative inducer and repressors (see text) are shown in bold characters. The start of the *cspA* gene and corresponding first amino acids are also shown. The *Apa*LI site is underlined. The figure is adapted from (Tanabe *et al.*, 1992) and the nucleotides are numbered according to (Goldstein *et al.*, 1990).

Chapter 2. Recombinant protein expression at low temperature under the transcriptional control of *cspA*

2.1. Introduction

In this chapter, the efficiency of the *tac* promoter in directing the synthesis of β-galactosidase was found to significantly decrease as the cellular growth temperature was lowered. The usefulness of the major *E. coli* cold-shock promoter for the production of recombinant proteins at low temperature in *E. coli* was therefore investigated, using a plasmid-encoded transcriptional gene fusion between the *cspA* promoter and the *lacZ* gene. While only basal levels of β-galactosidase were present in cells incubated at 37 or 42 °C, synthesis of the enzyme was rapidly induced upon transfer to low temperatures. However, the induction was highly transient and the intracellular levels of β-galactosidase activity decreased 1 - 2 hours after temperature downshift as a result of efficient promoter repression and cell division.[1]

2.2. Materials and Methods

Strains, plasmids and growth conditions

E. coli JM109 (*gyrA96*(Nal^r) *supE44* e14$^-$(McrA$^-$) *relA1 recA1 thi endA1 hsdR17*(r_K^- m_K^+) Δ(*lac-proAB*) [F' *traD36 lacI^q* Δ(*lacZ*)*M15 proA$^+$B$^+$*]) (Yanisch Perron *et al.*, 1985) and Top 10 (F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80Δ*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ$^-$ *rpsL endA1 nupG*) (Invitrogen) were used in this work. Plasmids pBR322 (Bolivar *et al.*, 1977), pT6 (Lee *et al.*, 1990), pTBG (Lee *et al.*, 1990) and pJJG02 (Goldstein *et al.*, 1990) have been described. All transformants were obtained by the $CaCl_2$ method or by electroporation. Shake flask

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[1] part of Chapter 2 is modifed from (Vasina and Baneyx, 1996)

cultures (30 ml) were grown in LB medium supplemented with 0.2% glucose and 50 µg/ml ampicillin. Labeling experiments were performed in M9 medium supplemented with 1% methionine assay medium (Difco), 0.2% glucose, 0.1 mM $CaCl_2$, 2.5 mM $MgSO_4$ and 50 µg/ml ampicillin.

Plasmid constructions

Plasmid pCSBG, which encodes the *lacZ* gene under the control of the *cspA* promoter was constructed as follows. Plasmid pBR322 was digested with *Eco*RI and *Eco*RV, the large fragment was isolated following low melting point (LMP) agarose electrophoresis with the Qiaquick kit (Qiagen), repaired with Klenow and ligated. Top10 transformants harboring the resulting plasmid (pJAV1) were selected for Amp^r and Tet^s. Plasmids pJAV1 and pTBG, a pBR322 derivative encoding the *lacZ* gene under the control of the *tac* promoter (Lee *et al.*, 1990), were digested with *PstI* and *SalI*. The 983 bp fragment from pJAV1 and the 5,742 bp pTBG backbone were isolated on LMP agarose, ligated, and transformants were selected for Amp^r. The resulting plasmid (pTBGM) contains a promoterless *lacZ* gene. The *cspA* promoter region followed by its authentic ribosome binding site and the first 24 nucleotides of the *cspA* open reading frame was obtained on a 586 bp fragment by PCR amplification of plasmid pJJGO1, which encodes native *cspA* (Goldstein *et al.*, 1990). The forward primer 5'-CCAATGCATGCGCTGTTGAGCAG-3' was designed to introduce a *SphI* site at position 70 and the reverse primer 5'-TACCAGTCGACT TACCGGACATAG-3' to add a *SalI* site at position 632 (Fig. 2.3). Amplification was performed on a Perkin Elmer 480 thermocycler using 25 cycles of incubation at 90 °C for 2 min, 50 °C for 2 min and 22 °C for 2 min. The 586 bp fragment was isolated following LMP agarose electrophoresis and directly subcloned into pT7blue (Novagen). White Top10 transformants were selected on LB/X-gal plates. The presence of the correct insert was

confirmed by sequencing of both strands using commercial primers (Novagen), and the Applied Biosystems dideoxy terminator sequencing kit. The pT7blue derivative and pTBGM were then digested with *Sph*I and *Sal*I. The *csp*A promoter fragment and the pTBGM backbone were recovered following LMP agarose electrophoresis and ligated to yield plasmid pCSBG (Fig. 2.3). Transformants were selected for their ability to produce dark blue colonies on LB/X-gal plates which had been incubated at 37 °C overnight and shifted to 15 °C for at least five hours to induce the cold-shock promoter. All DNA manipulations were confirmed by restriction analysis.

β-galactosidase assays

All enzymatic assays were performed on cultures grown in 125 ml shake flasks containing 30 ml of supplemented LB medium and inoculated with 1 ml of overnight LB cultures. The cells were grown at 37 °C to mid-log phase (OD_{600} = 0.4-0.5) with shaking, and either returned to 37 °C or transferred to water baths maintained at various temperature. In the case of pTBG, chemical induction was performed by addition of 1 mM IPTG. Samples (3 ml) were collected from triplicate cultures immediately prior to induction and at various thereafter. The cells were centrifuged at 8,000 x g for 8 min, resuspended in 50 mM potassium phosphate monobasic (pH 6.5) and lysed by French pressing at 10,000 psi. The samples were centrifuged at 10,000 x g for 10 min to pellet the insoluble fraction. The soluble fractions were assayed for β-galactosidase activity in triplicate using the chromogenic substrate ONPG at 28 °C as described by Miller (Miller, 1972). For specific activity experiments, the protein concentration was estimated in duplicate using the BioRad protein assay with BSA as a standard. Enzymatic activities are reported in ΔOD_{420}/mg of protein/hr (Units) or in Miller units (Miller, 1972).

Labeling experiments

A portion (0.8 ml) of a mid-log phase JM109(pCSBG) culture growing at 37 °C in supplemented M9 medium was labeled with 10 µCi of [^{35}S]-methionine (10 µCi/µl; Amersham) for a 10 min period prior to temperature shift, and for 15 min at various times after transfer to 15, 20 or 37 °C baths. At the end of the labeling period, the samples were immediately precipitated by addition of an equal volume of 10% ice cold trichloroacetic acid, pelleted by centrifugation at 10,000 x g for 5 min and washed with 80% ice cold acetone. The pellet was resuspended in 50 µl of 1X Sodium Dodecyl Sulfate (SDS) loading buffer. Aliquots corresponding to 2x10^5 cpm were heated to 95 °C for 5 min and resolved by 7% SDS-PAGE. The gels were treated with En^{3}Hance (DuPont), and exposed to X-Ray film for 1 to 3 hours at -80 °C. The autoradiograms were digitized using a Sharp JX-325 color scanner with transparency attachment, and densitometry was performed using the NIH Image 1.56 software. The intensity of the β-galactosidase peaks was measured on films obtained with different exposure times in order to remain in the dynamic range of the scanner, and these values were normalized to the sum of the intensities of the labeled proteins in each lane.

RNA isolation and northern blot hybridization

All RNA manipulations were performed according to standard protocols (Ausubel *et al.*, 1987). Aurintricarboxylic acid, a RNase inhibitor, was added to most solutions at a 10 µg/ml concentration. Shake flask cultures (25 ml) were grown to mid-log phase in supplemented LB medium and either transferred to 15 or 20 °C, or incubated at 37 °C for 45 min. Total cellular RNA was extracted using the rapid isolation method and the RNA content was quantified by measuring the absorbance at 260 nm (Ausubel *et al.*, 1987). Aliquots corresponding to 7.5 µg of total RNA were loaded in duplicate on a

1.0% agarose-formaldehyde gel. After electrophoresis half of the gel was stained with ethidium bromide to confirm that equivalent amounts of RNA were present in all samples (data not shown). The other half was washed as described for Northern hybridization and the RNA was transferred to a Nytran Maximum Strength membrane (Schleicher and Schuell). The RNA was cross-linked to the membrane by exposure to 120 mJ/cm^2 of UV radiation. A 617 bp *Bst*XI fragment mapping in the coding region of *lacZ* was isolated from pTBG, and labeled using the Random Primed DNA labeling kit (Boehringer Mannheim) with 50 µCi of [α-^{32}P]-dCTP (10 µCi/µl; Amersham). Unincorporated nucleotides were removed by chromatography on a NICK column (Pharmacia). Hybridization was performed overnight at 42 °C. The membrane was then washed twice for 5 min at room temperature with 2X SSC, 0.1% SDS, twice for 15 min at 42 °C with 0.2X SSC, 0.1% SDS, and exposed to X-ray film for 7 hours.

Determination of plasmid copy number

Total DNA was isolated by the method of Xia *et al.* (1993) from 5 ml aliquots of JM109(pTBG) or JM109(pCSBG) cells grown two hours post-temperature shift in LB medium. Genomic and plasmid DNA (approximately 2.5 µg) were separated on 1% agarose gels for 16 hours at 50 V. The gels were stained with ethidium bromide, photographed, and scanned as described above. Following integration, the plasmid peaks were summed and normalized to the genomic DNA band to yield the relative copy number.

Immunoblotting

Shake flask cultures of JM109(pCSBG) were grown to mid-log phase in supplemented LB medium at 37 °C. Samples corresponding to identical amount of cells were collected before temperature shift and 2 hours after the cells had been transferred to

various temperatures. Each sample was immediately centrifuged at 10,000 x g for 5 min and the cell pellet was resuspended in 1X SDS loading buffer and incubated 5 min at 95 °C. Aliquots corresponding to approximately 20 µg (Coomassie stained gels) or 5 µg (Western blotting) of total protein were resolved by 7% SDS-PAGE. Following transfer to nitrocellulose, β-galactosidase was detected by colorimetric reaction using mouse polyclonal anti-β-galactosidase (Sigma) and alkaline-phosphatase-conjugated goat anti-mouse IgG (Sigma).

2.3. Results

2.3.1. Deleterious effect of cold-shock on the expression of a *tac-lacZ* transcriptional fusion

Although the expression of a number of aggregation-prone recombinant proteins in a soluble form has been shown to be favored by cultivating the cells at low temperature, the influence of suboptimal growth temperatures on the efficiency of recombinant protein synthesis from commonly used promoters has not been well documented. As an initial step in this study, we used a plasmid-encoded transcriptional gene fusion between the synthetic *tac* promoter (Amann *et al.*, 1983) and the *lacZ* gene (Lee *et al.*, 1990) to investigate the effect of various temperature downshifts on the synthesis of enzymatically active β-galactosidase. JM109 cells harboring plasmid pTBG were grown to mid-exponential phase at 37 °C, induced with 1 mM IPTG and either returned to this temperature or immediately transferred to water baths held at 15 or 20 °C. The β-galactosidase specific activity present in clarified cell extracts was measured at various time points following induction and the result of this analysis is shown in Fig. 2.1. At 37 °C, the β-galactosidase specific activity increased steadily with time and an activity plateau corresponding to about 33,000 U was reached three hours post-induction

(Fig. 2.1A, Δ). When IPTG induced cultures were transferred to 20 or 15 °C (Fig. 2.1A, ■ and ○), the initial rates of accumulation of active enzyme decreased approximately 10-fold, and the steady-state levels of β-galactosidase specific activity were more than 3-fold lower compared to those measured at 37 °C. The observed decrease in the β-galactosidase production rates was not the result of a gene dosage effect since the relative plasmid copy number was comparable at all three temperatures (data not shown). However, pulse-labeling experiments (Fig. 2.2) showed that the lower levels of β-galactosidase accumulation following temperature downshift could be accounted for by a reduction in the relative synthesis rates of the enzyme at low temperatures. SDS-PAGE fractionation of identical amounts of labeled proteins synthesized in the 25 to 30 minutes period following induction and transfer to various temperatures showed that approximately 2 or 3.5 times as much β-galactosidase was translated at 37 °C compared to 20 or 15 °C, respectively. Thus, a decrease in the growth temperature to the 15-20 °C range represses the synthesis of both host (Jones *et al.*, 1987; Jones and Inouye, 1994) and recombinant proteins, even when the gene of interest is present on a multicopy plasmid under the control of a strong promoter.

2.3.2. Construction of a *cspA-lacZ* transcriptional gene fusion

Since the transfer of exponentially growing *E. coli* cultures to low temperatures has been shown to induce the synthesis of several cold-shock proteins (Jones *et al.*, 1987; Lee *et al.*, 1994), we investigated the usefulness of a temperature-responsive promoter for the production of recombinant polypeptides at reduced growth temperatures. The promoter of the major *E. coli* cold-shock protein, CspA, was selected for this work since it is efficiently repressed at 37 °C but directs the rapid synthesis of large amounts of this polypeptide immediately after temperature downshift (Fig 1.2; Jones *et al.*, 1987; Goldstein *et al.*, 1990; Tanabe *et al.*, 1992; Jiang *et al.*, 1993). The pTBG derivative

pCSBG encodes a *cspA-lacZ* transcriptional gene fusion which uses the ribosome binding site and the start codon of authentic *cspA* (Fig. 2.3). The translated protein consists of four N-terminal residues from CspA followed by a four amino-acids linker region derived from pTBG and the authentic β-galactosidase amino acid sequence starting at codon nine (Fig. 2.3). About 350 bp upstream of the *cspA* -35 box were necessary to maintain cold-shock inducibility and attempts to use shorter 5' regions failed (data not shown).

2.3.3. Effect of the downshift temperature on the synthesis of β-galactosidase

The ability of the *cspA* promoter to direct the synthesis of β-galactosidase following increasingly large temperature downshifts was examined by measuring β-galactosidase specific activities in soluble extracts two and three hours after transfer to low temperatures. While only basal production levels were detected in the absence of a temperature shift, the β-galactosidase specific activity present in cell extracts increased 4-fold upon transfer from 37 to 30 °C (Fig. 2.4). Maximum specific activities, corresponding to approximately 5-fold higher steady-state level of β-galactosidase relative to the 37 °C value, were observed when the cells were shifted between 15 and 25 °C. However, the magnitude of the response decreased with larger temperature downshifts. At 10 °C, the increase in production was about 3-fold, while transfer to 7.5 or 5 °C only led to a 1.5-fold induction (Fig. 2.4). Interestingly, there was no significant difference in the activity profiles obtained two or three hours following temperature downshift in the 15-30 °C range (compare Δ and ● in Fig. 2.4), suggesting that the induction of β-galactosidase synthesis from the *cspA* promoter was transient under all temperature shift examined.

SDS-PAGE and Western analysis of whole cell extracts collected two hours after transfer to various temperatures confirmed that synthesis of full-length β-galactosidase

was induced at 15, 20 and 30 °C (Fig. 2.5A and B, lanes 3 to 5). Under these conditions, β-galactosidase represented 3 to 5% of the total cellular protein as estimated from videodensitometric scanning of 15% polyacrylamide gels (not shown). JM109(pCSBG) cells also synthesized a small amount of β-galactosidase before temperature shift (lane 2), and following two hours incubation at 37 °C (Fig. 2.5A and B, lane 6) or 42 °C (lane 7) suggesting that the *cspA* promoter was not fully repressed at these temperatures.

2.3.4. Northern analysis

A mild induction of CspA following temperature shift from 37 to 24 °C has previously been detected by two-dimensional gel electrophoresis (Jones *et al.*, 1992a). As a result, the recovery of β-galactosidase activity following transfer of 37 °C exponential cultures to the 20 to 30 °C range was not totally unexpected. Nevertheless, the accumulation of comparable levels of β-galactosidase specific activities over a wide range of temperature (15-30 °C, see Fig. 2.4) was somewhat surprising. A possible explanation for this result would be that the same amount of *lacZ* mRNA is transcribed over the 15-30 °C temperature range and that translational efficiency remains unchanged. Alternatively, less mRNA could be synthesized at the higher temperatures (20-30 °C) but translation would be more efficient. In an effort to discriminate between these possibilities, total RNA was extracted from cells grown to mid-exponential phase at 37 °C, and either maintained at this temperature or shifted to 15 or 20 °C for 45 minutes. Identical amounts of total RNA were then electrophoresed, transferred to nitrocellulose and probed with a [^{32}P]-labeled 600 bp *Bst*XI fragment isolated from *lacZ*, as described in Materials and Methods. Figure 2.6 shows that the probe hybridized to a band migrating at about 3 kb in the RNA isolated from cold-shocked cells (lanes 1 and 2), but did not appreciably bind to the RNA extracted from cells held at 37 °C (lane 3). As the

calculated length of the *cspA-lacZ* mRNA is 3,263 nucleotides, we take this band to be the full-length target transcript. Extensive degradation of the *cspA-lacZ* mRNA was observed in the cold-shocked samples (but again not in the 37 °C sample), as judged by the hybridization of the probe to low molecular weight regions of the blot. The binding was not reduced by using more stringent wash conditions (data not shown). The amount of probe specifically bound to the intact *cspA-lacZ* mRNA was quantified by (i) videodensitometric scanning of the autoradiograms and (ii) scintillation counting of the excised bands. Both techniques indicated that about 1.7-fold more full-length mRNA was present in the cells cold-shocked at 15 °C relative to those incubated at 20 °C. From the above results, we conclude that the transcription of *cspA-lacZ* mRNA increases with the magnitude of the cold-shock, and that the comparable levels of β-galactosidase activity present in cells shifted to the 15 to 30 °C temperature range result from the more efficient translation of smaller amounts of mRNA at higher temperatures.

2.3.5. Kinetics of β-galactosidase synthesis at low temperatures

In order to gain a better understanding of the mechanism of induction of the *cspA* promoter at low temperatures, two shift temperatures corresponding to optimum levels of β-galactosidase production (15 and 20 °C) were selected for further characterization. Figure 2.7A shows that the *cspA* promoter was rapidly induced upon temperature shift to 20 °C (■) or 15 °C (○) and that it became efficiently repressed as the incubation time at 37 °C increased (Δ).

At 15 °C, the production of β-galactosidase exhibited a biphasic behavior. Least-squares fitting of early kinetics data (Fig. 2.7B) yielded an initial synthesis rate of 16 $U.min^{-1}$, and a half-time to reach the activity plateau of about 45 minutes. A similar half-time value was obtained by normalized videodensitometric scanning of SDS time course gels (data not shown), suggesting that all the β-galactosidase synthesized at

15 °C is assembled in a tetrameric and biologically active conformation. The rate of accumulation of β-galactosidase specific activity sharply decreased between one and two hours following temperature downshift and little increase in specific activity was observed at later time points (Fig. 2.7A). After 24 hours at 15 °C, the specific activity in cell extracts had reached 4,100 U.

Transfer to 20 °C resulted in faster accumulation rates of β-galactosidase specific activity with a half-time to reach maximum activity of about 20 minutes as deduced from initial kinetics data (Fig. 2.7B) and SDS-PAGE time course experiments (data not shown). The initial synthesis rate was 34 $U.min^{-1}$, approximately twice that measured at 15 °C. However, after about three hours, the specific activities started to decrease, eventually reaching 1,400 U after 24 hours of incubation at 20 °C. This behavior can be attributed to (i) increased degradation of β-galactosidase at 20 °C relative to 15 °C, and/or (ii) shut-down of transcription and subsequent dilution of the specific activity as a result of cell division at 20 °C. β-galactosidase is however proteolytically stable (Goldschmidt, 1970) and it is unlikely that its susceptibility to degradation is enhanced at lower temperatures. Growth and pulse-labeling experiments were performed to address the second possibility.

Upon transfer to 15 °C, JM109(pCSBG) cells entered a lag period of about 40 minutes and resumed growth after 60 minutes with a specific growth rate (μ) of 0.03 h^{-1}, compared to 0.46 h^{-1} at 37 °C. When the cells were shifted to 20 °C, the lag period was shortened to 20 minutes and growth resumed with a μ value of 0.09 h^{-1}. Thus, dilution of specific activity would occur faster at 20 °C than at 15 °C if the transcription of *cspA-lacZ* stopped at the same time.

Pulse-labeling experiments were carried out to investigate the mechanism of repression at low temperatures (Fig. 2.8). In agreement with the activity data and immunoblotting experiments, a small amount of β-galactosidase was detected in the

37 °C pre-shift samples (lane 1), but synthesis became negligible at later time points, and undetectable after overnight incubation at 37 °C (data not shown). When the cultures were transferred to 15 °C, a clear induction of the promoter, and concomitant synthesis of β-galactosidase, occurred for up to three hours post-shift (Fig. 2.8A and C). Thereafter, synthesis was sharply reduced although residual translation of β-galactosidase was still evident after overnight incubation at 15 °C (Fig. 2.8A lane 7). Similar pulse-labeling experiments showed that the induction/repression behavior closely paralleled that of native CspA (data not shown; Goldstein *et al.*, 1990). Upon transfer to 20 °C, a larger amount of β-galactosidase was synthesized in the 15-30 minutes interval, compared to 15 °C (Fig. 2.8B and C). However, translation essentially stopped after two hours incubation at this temperature (Fig. 2.8B). Taken together with the growth rate data, the above results indicate that the progressive decrease in enzymatic specific activities observed upon prolonged incubation at 20 °C (Fig. 2.7) can be attributed to the efficient repression of the *cspA* promoter and the subsequent dilution of enzymatic activity upon resumption of cell growth.

2.4. Discussion

The synthesis of recombinant proteins at reduced growth temperatures presents the combined advantages of increasing the solubility of aggregation-prone recombinant proteins (see Table 1.1), and limiting their degradation by heat shock proteases that are induced under overexpression conditions (Baneyx and Georgiou, 1992). Nevertheless, a sudden decrease in the growth temperature has severe consequences on *E. coli* physiology including a decrease in the saturation of fatty acids and the inhibition of DNA, RNA and protein synthesis (Shaw and Ingraham, 1967; Broeze *et al.*, 1978). The results of Fig. 2.1 further demonstrate that the initial rates of accumulation and final yields of plasmid-encoded β-galactosidase placed under the control of the *tac* promoter

are also significantly reduced upon temperature downshift. Since the plasmid copy number remains unchanged at low temperatures, this effect cannot be attributed to gene dosage. It is however likely that the observed reduction in the levels of accumulation of enzymatic activity results mainly from the inefficient transcription/translation of the *lacZ* mRNA at low temperatures, as evidenced by pulse labeling experiments (Fig. 2.2). Such results are consistent with the general silencing of the translation of most host proteins upon cell exposure to low temperatures (Jones *et al.*, 1987), and suggest that promoters which are routinely used to direct the transcription of recombinant genes at physiological temperatures may not be appropriate for low-temperature synthesis.

Since a subset of *E. coli* proteins is actually induced following temperature downshift, we decided to investigate the potential of cold-shock inducible promoters in directing the expression of recombinant proteins at low temperatures. Because most cold-shock proteins are also synthesized at 37 °C and only weakly induced by low temperature cultivation conditions (Jones *et al.*, 1987; La Teana *et al.*, 1991; Jones *et al.*, 1992b), we turned our attention to the promoter region of the major *E. coli* cold shock protein CspA. Previous data suggested that the *cspA* promoter possessed many attractive features that could facilitate the expression of recombinant proteins at low temperatures, including the virtual absence of the CspA protein at 37 °C and the strong induction of its synthesis at the transcriptional level upon temperature downshift (Goldstein *et al.*, 1990; Tanabe *et al.*, 1992; Jiang *et al.*, 1993). In an effort to further characterize the behavior of this promoter, a transcriptional gene fusion between the *cspA* promoter and the *lacZ* gene was constructed (Fig. 2.3). The results presented in Fig. 2.4 and 5 show that the 400 bp region located upstream of the *cspA* open reading frame is sufficient to confer cold shock inducibility to the *lacZ* gene upon temperature downshifts of only 7 °C. The promoter displayed the highest efficiency when the cells were shifted in the 15 to 20 °C temperature range with a 5-fold increase in the

accumulation of enzymatically active β-galactosidase. However, when the cultures were exposed to temperatures lower than 15 °C, the steady-state levels of β-galactosidase specific activity decreased (Fig. 2.4). A possible explanation for this effect is a progressive inhibition of translation initiation as the incubation temperature is reduced (Broeze *et al.*, 1978). Furthermore, since *E. coli* is unable to grow below 8.5 °C (Jones *et al.*, 1987), it is likely that protein translation is completely inhibited under this threshold, and that the small amount of induction observed at 7.5 and 5 °C takes place during the transient cooling of the cultures.

In the absence of a temperature downshift, only low levels of β-galactosidase were present in JM109 cells as judged by activity (Fig. 2.4 and 7), immunoblotting (Fig. 2.5) or pulse labeling experiments (Fig. 2.8), and the protein was rapidly diluted as the incubation was pursued, presumably as a result of cell division. Northern analysis further showed that very little *cspA-lacZ* mRNA was transcribed by the cells after 45 minutes incubation at 37 °C, while the transcript was produced in large amounts upon cold shock (Fig 2.6). Taken together, these results suggest that either (i) the *cspA* promoter is efficiently repressed at a transcriptional level at physiological temperatures, or (ii) that the *cspA-lacZ* mRNA is short-lived at 37 °C. The simplest mechanism for a tight repression at 37 °C would be the binding of a protein to the *cspA* promoter region. On the basis of footprinting and gel retardation experiments, Tanabe and co-workers (Tanabe *et al.*, 1992)have shown that a protein factor synthesized *de novo* upon cold shock binds to the -92 to -63 region of the *cspA* promoter at low temperatures, but not at 37 °C. In addition, since a temperature shift from 42 to 36 °C induces the production of small amounts of CspA (Jones *et al.*, 1992a), it is unlikely that the *cspA* promoter is subject to negative regulation as a result of protein binding at 37 °C. The second possibility for the low levels of *cspA-lacZ* mRNA at 37 °C would be transcript instability, as has been shown in the case of authentic CspA (Tanabe *et al.*, 1992). Since

the *tac-lacZ* mRNA appears to be relatively long-lived at 37 °C, as judged by the linear kinetics of accumulation of β-galactosidase specific activity at early time points (Fig. 2.1, Δ), the 159 nucleotide-long non-coding sequence present at the 5' end of the *cspA-lacZ* mRNA (see Fig. 2.3) may target the transcript for degradation at 37 °C.

In an effort to further characterize the mechanism of cold-shock induction, a more detailed analysis of the kinetics of β-galactosidase synthesis was performed at two of the optimal production temperatures, 15 and 20 °C. In both cases, a rapid induction of β-galactosidase synthesis was observed immediately following cold-shock and the levels of enzymatic activity increased steadily for one hour following transfer to low temperatures (Fig. 2.7 and 8). These results are consistent with the proposed model of a positive induction of *cspA* transcription resulting from the binding of an enhancer protein slightly upstream of the promoter region (Tanabe *et al.*, 1992). Interestingly, the initial rates of accumulation of β-galactosidase specific activity were reproducibly higher at 20 °C than at 15 °C. Since 1.7 fold full-length *cspA-lacZ* mRNA were present in cells cold-shocked at 15 °C for 45 minutes than in cells shifted to 20 °C (Fig 2.6) this result can be explained by the fact that a more efficient initiation of translation compensates for the lower levels of mRNA at 20 °C.

Repression of *cspA-lacZ* expression was obvious from both activity (Fig. 2.7) and pulse-labeling experiments (Fig. 2.8), and occurred about two hours following cold-shock to 20 or 15 °C. At present, the mechanisms leading to repression remain unclear, but are likely to operate at a transcriptional level. The DNA region preceding the promoter region and/or the 5' non-coding region of the mRNA are probably involved in low temperature repression, since the synthesis of both authentic CspA and the β-galactosidase transcriptional fusion are identically repressed (Fig. 2.7, reference (Goldstein *et al.*, 1990). A possible explanation would be that, at high enough concentrations, CspA binds to its own promoter region to shut down its transcription.

Nevertheless, purified CspA does not form a complex with DNA fragments isolated from the 5' region located upstream of its open reading frame (Tanabe *et al.*, 1992). Furthermore, the protein appears to be specific for single-stranded nucleic acids (Schindelin *et al.*, 1994). The observation that the *Xenopus* Y box binding protein FRGY2, a eukaryotic CspA homolog, masks maternal mRNAs during oogenesis and prevents their translation (Ranjan *et al.*, 1993) suggests an alternative explanation. CspA may be able to interfere with the translation of its own (or the *cspA-lacZ*) mRNA by binding to the 5' non-coding sequence when present in high enough concentration in cold shocked cells. Such binding could mask the ribosome binding site either directly through steric hindrances, or indirectly by stabilizing elements of mRNA secondary structure in the vicinity of the ribosome binding site. In support of this hypothesis, purified CspA has been shown to bind to a 24-base synthetic oligodeoxyribonucleotide corresponding to part of its 5' non-coding sequence (Newkirk *et al.*, 1994).

Overall, the results of this chapter demonstrate the usefulness of the *cspA* promoter for the synthesis of recombinant protein at low temperatures. The main advantages of this system are: (i) a relatively efficient repression of protein synthesis at physiological temperatures; (ii) a rapid induction of protein expression following temperature downshift; and (iii) an induction mechanism that does not rely on the addition of chemical inducers. Nevertheless, we also found that the accumulation of β-galactosidase at low temperature was limited by the repression of the *cspA* promoter one to two hours after transfer to low temperatures. As a result, although the production of β-galactosidase from the *cspA* and *tac* promoters was comparable at early time points following cold-shock (compare Fig. 2.1 and 7), higher specific activities were obtained with the *tac* promoter when the incubation was continued for longer periods of time.

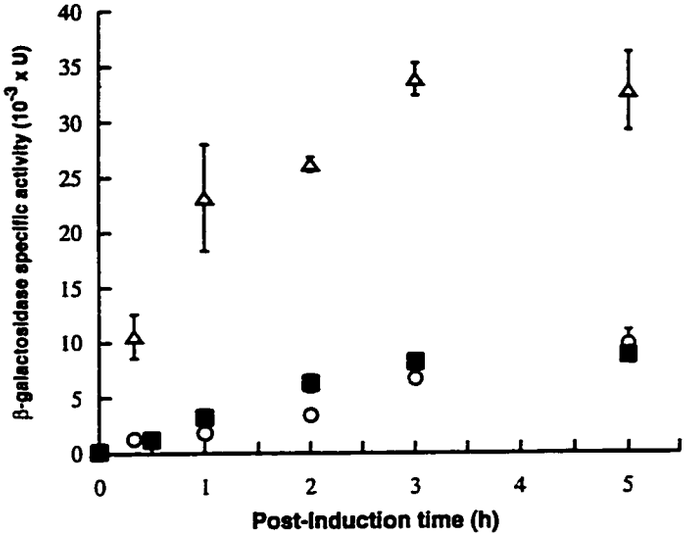

Figure 2.1. Kinetics of β-galactosidase synthesis from the *tac* promoter at various temperatures. JM109(pTBG) cells were grown to mid-exponential phase in LB medium at 37 °C, induced with 1 mM IPTG, and either returned to the same temperature (Δ) or transferred to water baths maintained at 20 °C (■) or 15 °C (○). The β-galactosidase specific activities in soluble extracts were measured at various times after the temperature shift as described in Materials and Methods and are expressed in Units.

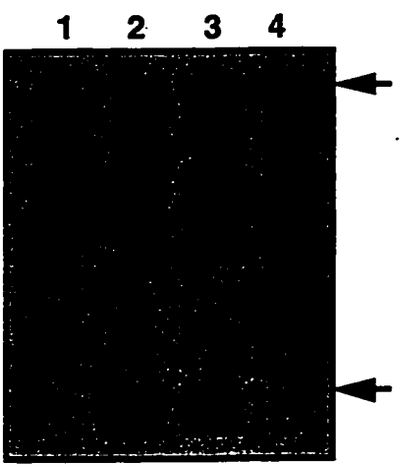

Figure 2.2. β-galactosidase synthesis rates from the *tac* promoter decrease with decreases in the incubation temperature. JM109(pTBG) cells were grown at 37 °C to mid-exponential phase in minimal labeling medium and labeled for 5 minutes either before chemical induction (lane 1), or after addition of 1 mM IPTG and 25 minutes incubation at 15 °C (lane 2), 20 °C (lane 3) or 37 °C (lane 4). Aliquots corresponding to identical amounts of counts were resolved on 15% SDS gels and exposed to X-ray film. The positions of β-galactosidase (top arrow) and authentic CspA (bottom arrow) are indicated.

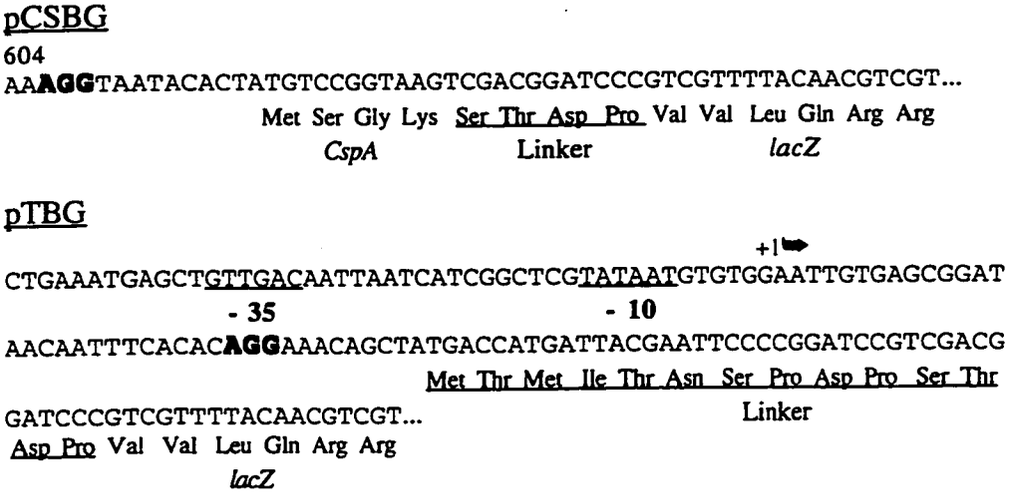

Figure 2.3. Nucleotide and amino acid sequences of the *cspA-lacZ* and *tac-lacZ* transcriptional gene fusions. The complete *cspA* promoter sequence is given in Fig. 1.2. The *tac* promoter sequence and fusion to *lacZ* are shown in the lower figure . The -35 and -10 boxes are shown, the transcription starting point is indicated by a +1. The ribosome binding sites of each promoter are in shadowed type.

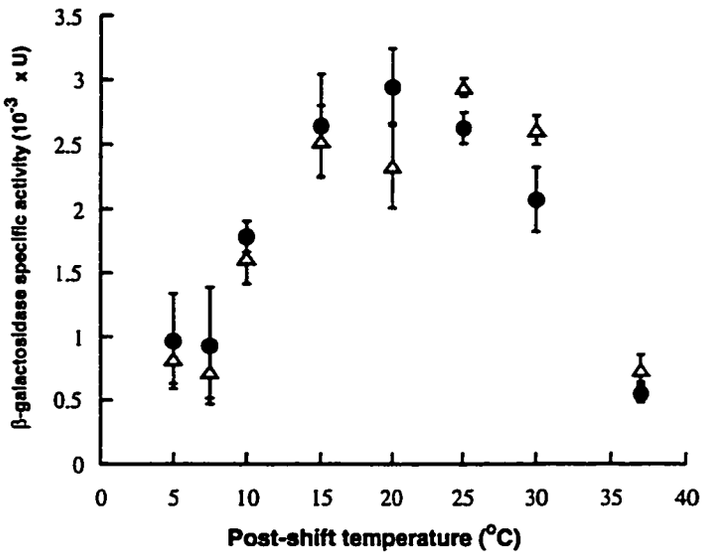

Figure 2.4. Influence of the downshift temperature on the synthesis of β-galactosidase from the *cspA* promoter. JM109(pCSBG) cells were grown to mid-exponential phase in LB medium at 37 °C and transferred to water baths maintained at the indicated temperatures. The β-galactosidase specific activities in soluble extracts were measured two (Δ) or three hours (●) after the temperature shift as described in Materials and Methods and are expressed in Units.

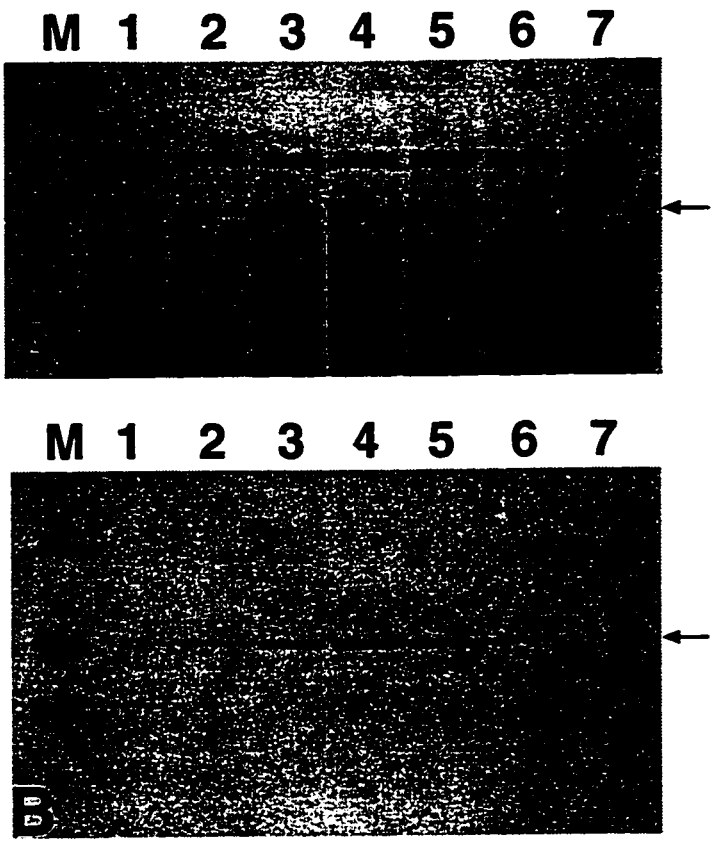

Figure 2.5. Full-length β-galactosidase is synthesized from the *cspA* promoter at various temperatures. JM109 cells transformed with either pCSBG or the vector control pT6 were grown to mid-exponential phase in LB medium at 37 °C, transferred to the indicated temperature and whole cells were resolved by SDS-PAGE (Top) or immunoblotted (Bottom) as described in Materials and Methods. Lanes: M, molecular weight marker, 206, 117 and 89 kDa; 1, JM109(pT6) after 2 hours incubation at 37 °C; 2, JM109(pCSBG) 37 °C mid-exponential culture before temperature shift; 3-7, JM109(pCSBG) 2 hours after transfer to 15 °C (lane 3), 20 °C (lane 4), 30 °C (lane 5), 37 °C (lane 6), or 42 °C (lane 7). The position of β-galactosidase is indicated by the arrow.

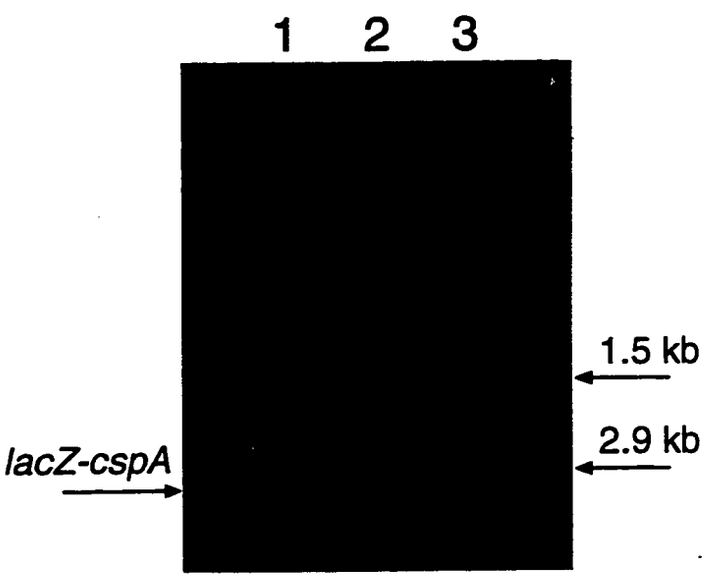

Figure 2.6. Northern blot analysis of *cspA-lacZ* mRNA cellular levels. JM109(pCSBG) cells were grown to mid-log phase in LB medium at 37 °C and incubated for 45 minutes at 15 °C (lane 1), 20 °C (lane 2) or 37 °C (lane 3). Total cellular RNA was extracted and the *cspA-lacZ* mRNA was detected with a *lacZ*-derived probe. The positions of the *cspA-lacZ* mRNA and the 23S and 16S rRNA are indicated by arrows.

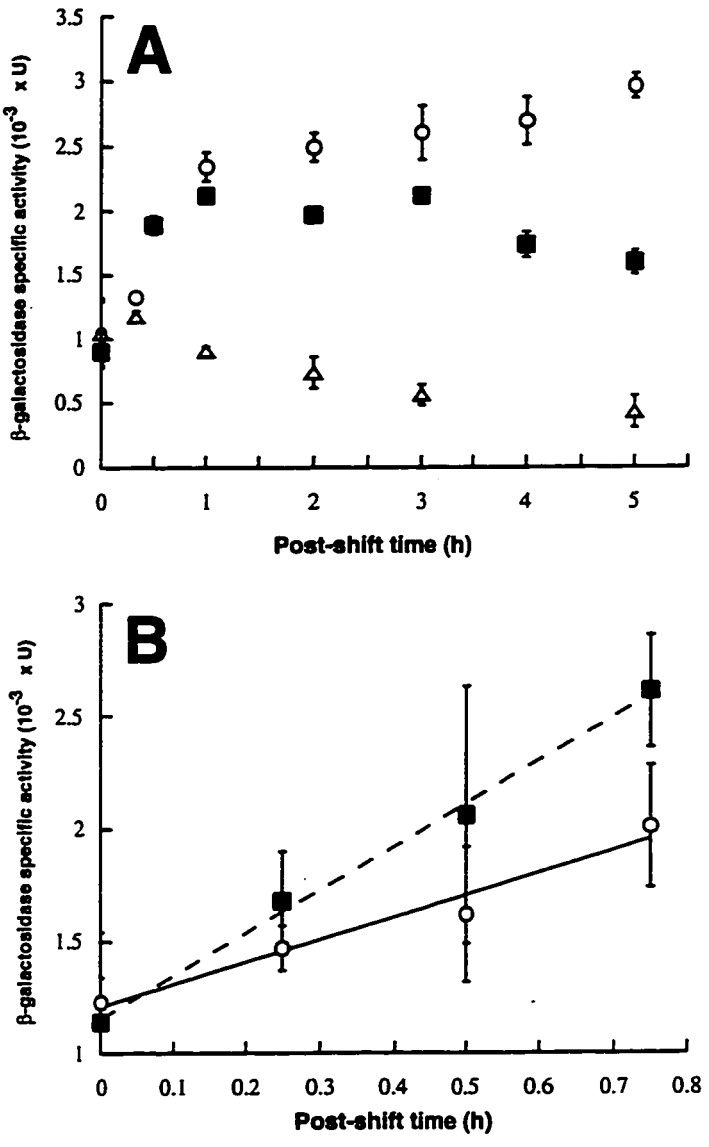

Figure 2.7. Kinetics of β-galactosidase synthesis from the *cspA* promoter at various temperatures. *Panel A*: JM109(pCSBG) cells were grown to mid-exponential phase in LB medium at 37 °C and either returned to the same temperature (Δ) or transferred to water baths maintained at 20 °C (■) or 15 °C (○). The β-galactosidase specific activities in soluble extracts were measured at various times after the temperature shift as described in Materials and Methods and are expressed in Units. *Panel B*: Initial kinetics of β-galactosidase accumulation at 20(■) or 15 °C (○). Data points were obtained from several separate experiments.

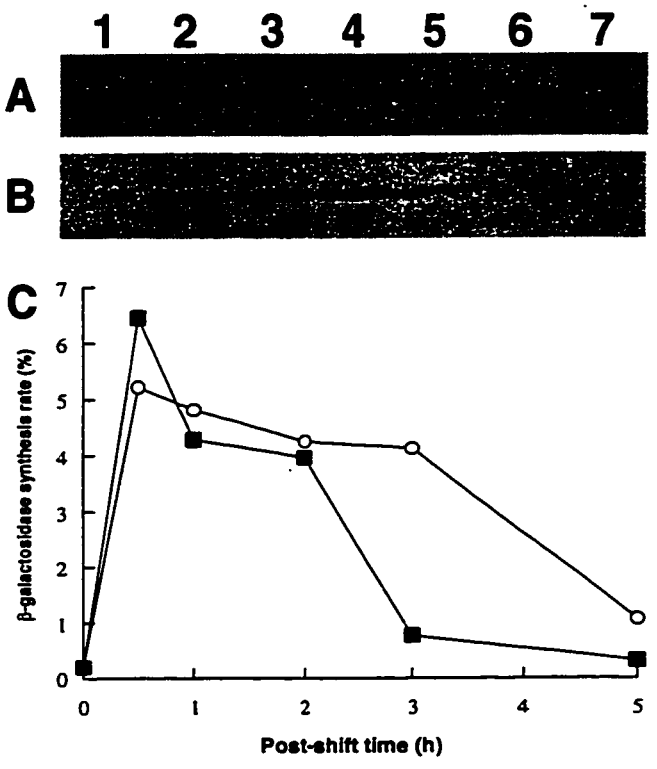

Figure 2.8. Repression of β-galactosidase synthesis is less efficient at low temperatures. JM109(pCSBG) cells were grown to mid-exponential phase in minimal labeling medium at 37 °C and either returned to the same temperature or transferred to water baths maintained at 15 °C (*Panel A*) or 20 °C (*Panel B*). An aliquot from each cultures was labeled for 10 minutes before shift (lane 1), or for 15 minutes after temperature shift at the following times: 15-30 min (lane 2); 45-60 min (lane 3); 105-120 min (lane 4), 165-180 min (lane 5); 285-300 min (lane 6); 1425-1440 min (lane 7). The TCA precipitated samples were fractionated by SDS-PAGE and exposed to X-ray film. Only the part of the autoradiograms corresponding to the β-galactosidase band are shown. *Panel C*: the β-galactosidase synthesis rates were determined at 20 °C (■) or 15 °C (○) by videodensitometric scanning of the autoradiograms as described in Materials and Methods.

Chapter 3. Expression of aggregation-prone recombinant proteins at low temperatures using the *E. coli cspA* and *tac* promoter systems

3.1. Introduction

In Chapter 2, we reported on the construction of a transcriptional gene fusion between the *cspA* promoter region and the *lacZ* gene, and showed that the synthesis of tetrameric β-galactosidase was rapidly and efficiently induced when the cells were shifted from 37 °C to the 15-30 °C temperature range (Fig. 2.4). However, the real advantage of this system lies in the production of heterologous proteins which cannot properly fold at physiological temperatures in *E. coli*. Therefore, in this chapter we extend these result by comparing and contrasting the efficiencies of the *cspA* and *tac* promoters in directing the expression of an aggregation-prone β-galactosidase fusion protein[2].

3.2. Materials and Methods

Strains and media

E. coli JM109 (Yanisch Perron *et al.*, 1985) which carries the Δ(*lacZ*)M15 deletion and contains a F' episome coding for the LacI repressor protein was used for all expression studies. *E. coli* Top 10 (Invitrogen) was used for cloning purposes. Transformants were obtained by the $RbCl_2$ method or by electroporation. Cultures were inoculated at a 1:50 dilution in LB medium supplemented with 0.2% glucose, 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, when appropriate. Labeling experiments were conducted in M9 salts supplemented with 0.1 mM $CaCl_2$, 2.5 mM $MgSO_4$, 0.2% glucose, 1% methionine labeling medium (Difco) and the appropriate antibiotics.

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[2] Chapter 3 is adapted from (Vasina and Baneyx, 1997).

Plasmid constructions

Plasmid pCSBG(H+), which encodes preS2-S'-β-galactosidase under the transcriptional control of the *cspA* promoter was constructed as follows. Plasmids pTBG(H+), an ampicillin-resistant pBR322 derivative encoding preS2-S'-β-galactosidase under *tac* control (Lee *et al.*, 1990), and pJAV1, a pBR322 derivative lacking a *Eco*RI-*Eco*RV fragment (Vasina and Baneyx, 1996), were digested with *Pst*I and *Sal*I. The 983 bp fragment from pJAV1 and the pTBG(H+) backbone were resolved by LMP agarose electrophoresis, isolated with the Qiaquick system (Qiagen), and ligated. The resulting plasmid, pTBGM(H+), encodes a promoterless preS2-S'-β-galactosidase gene. A pT7blue (Novagen) derivative encoding a PCR-amplified *cspA* promoter region (Vasina and Baneyx, 1996) was used as the source of the promoter. Both the pT7blue derivative and pTBGM(H+) were digested with *Sph*I and *Sal*I, and the 586 bp *csp*A promoter fragment was ligated to the pTBGM(H+) backbone to yield plasmid pCSBG(H+). The chloramphenicol-resistant plasmid pNatCspA was constructed by ligating a 819 bp *Sma*I-*Hin*dIII fragment from pJJG02 (Goldstein *et al.*, 1990) encoding the complete *cspA* gene under the control of its native promoter, to the large *Hinc*II-*Hin*dIII fragment from pACYC184 (Yanisch Perron *et al.*, 1985). All DNA manipulations were confirmed by restriction analysis.

Shake flask cultures, cell fractionation and enzymatic assays

Shake flasks containing 30 ml of supplemented LB medium were inoculated in triplicate and grown at 37 °C to mid-exponential phase ($OD_{600} \approx 0.45$). The cultures were either returned to 37 °C or transferred to water baths maintained at the indicated temperatures. JM109 cultures harboring pTBG(H+) were incubated for 5 min at the low temperature to cool the medium before the synthesis of preS2-S'-β-galactosidase was induced with

1 mM IPTG. This point was defined as the zero induction time for JM109[pTBG(H+)]. The zero induction time for JM109[pCSBG(H+)] was taken to be the point at which the cells were transferred to low temperatures due to the rapid activation of the *cspA* promoter upon temperature downshift (Goldstein *et al.*, 1990). Samples corresponding to 1.5 ml or 3 ml of culture were collected immediately before induction and at various time points thereafter. The 3 ml samples were centrifuged at 8,000 x g for 8 min, and the cells were resuspended in 50 mM potassium phosphate monobasic (pH 6.5) before being lysed by French pressing at 10,000 psi. Soluble and insoluble cell fractions were immediately separated by centrifugation at 10,000 x g for 10 min. The soluble fractions were assayed for β-galactosidase activity in triplicate using the chromogenic substrate ONPG, as described in section 2.2.3, and aliquots were precipitated with methanol/chloroform for SDS-PAGE analysis (Thomas and Baneyx, 1996b). The insoluble fractions were directly resuspended in 1X SDS loading buffer and incubated at 95 °C for 5 min. The 1.5 ml whole cell aliquots were resuspended in 1X SDS loading buffer following centrifugation and heated at 95 °C for 5 min. Samples of whole cells, soluble and insoluble fractions corresponding to identical amounts of culture were fractionated on 8% SDS-PAGE gels and visualized by Coomassie blue staining as described (Thomas and Baneyx, 1996b).

Labeling experiments

For the experiments of Fig. 3.5, cultures were grown at 37 °C in labeling medium to mid-exponential phase, transferred to 10, 25 or 37 °C baths, and induced as above. Culture aliquots (0.8 ml) harvested 15 min post-induction, were mixed with 15 µCi of [^{35}S]-methionine (10 µCi/µl; Amersham) and labeled for 15 min at the indicated temperatures. At the end of the labeling period, the proteins were precipitated by addition of an equal volume of 10% ice cold TCA, sedimented by centrifugation at

10,000 x g for 5 min, and washed with 80% ice cold acetone. The pellets were resuspended in 1X SDS loading buffer and aliquots corresponding to 2.6 x 10^4 cpm of acid-precipitable material were heated to 95 °C for 5 min and resolved by 8% SDS-PAGE. The gels were treated with En^{3}Hance (DuPont), and exposed to X-Ray film at -80 °C. The relative synthesis rates of preS2-S'-β-galactosidase from the *tac* and *cspA* promoters were obtained following labeling as above, except that a 200 µl portion of culture was labeled for 40 sec with 25 µCi of [^{35}S]-methionine. After TCA precipitation, the protein pellets were resuspended in 200 µl of 1X SDS loading buffer, heated at 95 °C for 5 min, and 10 µl samples were resolved in duplicate on 8% and 15% SDS-gels. Complete lanes from 15% gels, and the corresponding preS2-S'-β-galactosidase bands from 8% gels were excised and resuspended in 0.6 ml or 0.3 ml of H_2O, respectively. The samples were homogenized, mixed with 0.6 ml of Solvable (DuPont), and incubated for 3 h at 50 °C. Liquid scintillation fluid was added and the samples were counted after overnight shaking at room temperature. Relative synthesis rates are reported as the ratio of the radioactivity present in the preS2-S'-β-galactosidase band to the total labeled protein in the corresponding sample.

Imaging and analysis

SDS-PAGE gels and fluorograms were digitized using a Sharp JX-325 color scanner with transparency attachment. Densitometry analysis of the SDS-PAGE gels was performed with the National Institutes of Health Image 1.59 software on a Power Macintosh 7100/66. The intensity of the preS2-S'-β-galactosidase peaks were determined on gels loaded with different amounts of protein in order to remain in the dynamic range of the scanner.

3.3. Results and Discussion

3.3.1. Construction of a Transcriptional Gene Fusion between the *cspA* Promoter and PreS2-S'-β-galactosidase

PreS2-S'-β-galactosidase, a tripartite fusion protein consisting of the 55-residue preS2 domain and the 30-residue hydrophobic S' domain of HBSAg followed by *E. coli* β-galactosidase (Lee *et al.*, 1990) was chosen as a model for this study since it aggregates extensively in the cytoplasm of *E. coli* cells grown at, or above 37 °C, but is partially soluble when the host strain is cultivated at 30 °C (Lee *et al.*, 1990; Thomas and Baneyx, 1996b). Since the β-galactosidase activity present in cell extracts is directly proportional to the amount of soluble material, preS2-S'-β-galactosidase is a particularly useful substrate for studying protein folding and aggregation in *E. coli* (Thomas and Baneyx, 1996b). Plasmid pTBG(H+) (Lee *et al.*, 1990), encodes the preS2-S'-β-galactosidase gene under the transcriptional control of the IPTG-inducible *tac* promoter (Amann *et al.*, 1983). This plasmid was used as a backbone for the construction of pCSBG(H+) which carries preS2-S'-β-galactosidase under control of the *cspA* promoter (see Materials and Methods). As a result, the copy number of both plasmids was comparable in JM109 transformants grown at physiological or low temperatures (data not shown). Due to construction constraints, the predicted N-terminal sequences of the fusion proteins expressed from pTBG(H+) or pCSBG(H+) differ by a few amino acids (Fig. 3.1). Based on the crystal structure of β-galactosidase (Jacobson *et al.*, 1994), it is unlikely that these minor differences affect the active site. We therefore consider a direct comparison of enzymatic activities to be valid.

3.3.2. Effect of low temperatures on the steady-state accumulation of soluble and active preS2-S'-β-galactosidase

The efficiency of the *tac* and *cspA* promoters in directing the production of soluble and enzymatically active preS2-S'-β-galactosidase was first investigated 2 h following temperature downshift as follows. Shake flask cultures of JM109 cells harboring either pTBG(H+) or pCSBG(H+) were grown to mid-exponential phase at 37 °C and transferred to thermostated shaker baths held between 10 and 37 °C. This time was defined as the zero time point for JM109 [pCSBG(H+)] cultures. Induction of the *tac* promoter was performed by treating pTBG(H+) transformants with 1 mM IPTG five minutes after transfer to guarantee that the medium temperature had equilibrated to the desired value (zero time point in all figures). Culture aliquots harvested 2 h post-induction were disrupted or fractionated into whole cells, soluble or insoluble fractions as described in Materials and Methods.

Fig. 3.2 shows that very low levels of enzymatically active fusion protein were present in either strain if the cells were held at 37 °C. However, two different mechanisms were responsible for this result. While pTBG(H+) transformants synthesized massive amounts of preS2-S'-β-galactosidase, the majority of the protein accumulated in a biologically inactive form in the insoluble fraction of the cells (Fig. 3.3A, lanes *tac*). In contrast, little fusion protein was detected in either the soluble or insoluble fractions of JM109[pCSBG(H+)] transformants (Fig. 3.3A, lanes *cspA*). A possible explanation for this result is that any soluble preS2-S'-β-galactosidase synthesized from the *cspA* promoter is rapidly degraded at 37 °C. This interpretation was however ruled out since soluble preS2-S'-β-galactosidase has a half-life greater than 150 minutes at 37 °C (Lee *et al.*, 1990). The low level of native CspA in *E. coli* cells growing at physiological temperatures has been attributed to the instability of the

cspA mRNA at 37 °C (Tanabe *et al.*, 1992; Brandi *et al.*, 1996; Goldenberg *et al.*, 1996) It was further proposed that the stability determinants are located at the 3' end of the transcript (Goldenberg *et al.*, 1996). Nevertheless, this mechanism cannot account for our results since only 12 nucleotides from the *cspA* open reading frame are present in pCSBG(H+) (Fig. 3.1). Since both the authentic *cspA* and *cspA-preS2-S'-lacZ* mRNAs are transcribed with a non-translated 159 nucleotide-long 5' extension (Tanabe *et al.*, 1992), we propose that this region confers instability to the transcripts at 37 °C. This hypothesis is consistent with the fact that only low concentrations of *lacZ* mRNA can be detected at 37 °C in JM109 cells expressing β-galactosidase under the control of the *cspA* promoter (see Fig. 2.6).

The enzymatic activities present in cultures shifted to sub-optimal growth temperatures for 2 h are also shown in Fig. 3.2. In the case of the *tac* promoter, maximum yields of active preS2-S'-β-galactosidase were obtained when the cells were transferred to 30 °C. However, the β-galactosidase activity decreased linearly when pTBG(H+) transformants were incubated at lower temperatures (Fig. 3.2, O). JM109[pTBG(H+)] cells held at 10 °C for 2 h contained less than 1,000 U of active fusion protein, and only a faint band migrating at the position occupied by preS2-S'-β-galactosidase was visible in Coomassie-stained SDS-gels of 2 h culture samples (Fig. 3.3C, lanes *tac*). The increase in activity in cells cultured between 25 and 30 °C could be readily explained by an increased partitioning of preS2-S'-β-galactosidase in the soluble fraction of the cell (compare Fig. 3.3A and 3B, lanes *tac*). Below this threshold, however, the beneficial effect of low temperature growth on the proper folding of the fusion protein was counterbalanced by a progressive decrease in the efficiency of transcription and translation of the preS2-S'-β-galactosidase gene from the *tac* promoter[3] (Fig. 3.3, lanes w; see below). This result is consistent with the fact that

[3] JAV and FB, manuscript in preparation

the synthesis levels of host proteins and mRNA strongly decrease when *E. coli* is grown at low temperatures (Shaw and Ingraham, 1967; Broeze *et al.*, 1978).

In JM109[pCSBG(H+)] cells, the levels of enzymatic activity increased with larger temperature downshifts due to a more efficient induction of the *cspA* promoter (Fig. 3.2, ●). At the optimum production temperature of 25 °C, the levels of active preS2-S'-β-galactosidase expressed from the *cspA* and *tac* promoters were comparable, and about 50% of the synthesized fusion protein accumulated in a soluble form in both strains (Fig. 3.3B). Incubation of pCSBG(H+) transformants below 25 °C led to a decrease in the concentration of active preS2-S'-β-galactosidase. Unlike the *tac* promoter, however, the cold-shock promoter was still able to direct the efficient synthesis of the fusion protein at 10 °C as judged by both activity (Fig. 3.2) and SDS-PAGE (Fig. 3.3C) experiments.

3.3.3. Kinetics of accumulation of preS2-S'-β-galactosidase

Two shift temperatures, 25 and 10 °C, were selected to further characterize the behavior of the *cspA* and *tac* promoters. At 25 °C, the kinetics of accumulation of active preS2-S'-β-galactosidase were virtually identical for both constructs for up to 2 h following temperature downshift (Fig. 3.4A). Pulse-labeling experiments performed 15 min post-induction revealed that the relative synthesis rate of preS2-S'-β-galactosidase was 6.0% of the total labeled protein when controlled by the *cspA* promoter, compared to 5.5% for the *tac* promoter (Fig. 3.5, lanes 3-4). Nevertheless, while the β-galactosidase activity in JM109[pCSBG(H+)] cells reached a plateau after 2 h, further accumulation of active enzyme was observed with the *tac* promoter (Fig. 3.4A, ○). These data are consistent with previous studies showing that the synthesis of native CspA (Goldstein *et al.*, 1990) or of β-galactosidase placed under control of the *cspA* promoter (see Fig. 2.7) is rapidly induced upon temperature downshift, but

becomes repressed after 1-2 h by an uncharacterized mechanism. The small decrease in activity observed in pCSBG(H+) transformants held at 25 °C for 3 h corresponds to the dilution of enzymatic activity due to the resumption of cell growth, as previously discussed in Chapter 2.

Time-course and labeling experiments were performed to verify that the *cspA* promoter could direct the synthesis of preS2-S'-β-galactosidase at 10 °C. The kinetics of accumulation of enzymatic activity at 10 °C paralleled those observed at 25 °C (Fig. 3.4A-B, ●), except that the concentration of active preS2-S'-β-galactosidase was approximately three-fold lower at all time points in cells grown at the lower temperature. Control fractionation experiments carried out on pre-shift samples showed that all the aggregated preS2-S'-β-galactosidase visible in the insoluble fraction of JM109[pCSBG(H+)] cells incubated at 10 °C (Fig. 3.3C, *cspA*, lane i) accumulated before temperature downshift due to the leakiness of the *cspA* promoter at 37 °C (Fig. 3.3, lane 0 h). We therefore conclude that the fusion protein produced following transfer to 10 °C is almost completely soluble. Pulse-labeling experiments confirmed that preS2-S'-β-galactosidase was synthesized at low levels in pCSBG(H+) transformants held at 10 °C (Fig. 3.5, lane 1). In contrast, the *tac* promoter was very inefficient in directing the synthesis of preS2-S'-β-galactosidase at the same temperature (Fig. 4B, ○), and virtually no fusion protein could be detected in JM109[pTBG(H+)] cells by pulse-labeling (Fig. 3.5, lane 2).

3.3.4. Influence of CspA overproduction

It has been reported that a protein factor synthesized *de novo* following cold-shock binds to the *cspA* promoter, thereby stimulating transcription (Tanabe *et al.*, 1992). The simplest mechanism for such a positive induction scheme would be that CspA binds to its own promoter and upregulates its production following temperature downshift.

Indeed, CspA has been shown to act as a transcriptional activator of the H-NS and GyrA proteins (La Teana *et al.*, 1991; Jones *et al.*, 1992b). In order to investigate the above possibility - and in an effort to increase the strength of the *cspA* promoter - JM109 was co-transformed with pCSBG(H+) and either pACYC184 (vector control) or pNatCspA, a pACYC184 derivative encoding the intact *cspA* gene under the control of its native promoter. Although CspA overproduction could be detected by SDS-PAGE in pNatCspA transformants shifted to 15 °C (data not shown), the magnitude of the induction, and the levels of enzymatic activity were identical to those observed in cells harboring pACYC184 (Fig. 3.6). Since an increase in the *cspA* gene copy number did not affect the synthesis of preS2-S'-β-galactosidase expressed from pCSBG(H+), it is unlikely that CspA acts as its own transcriptional activator.

3.4. Conclusions

Our results indicate that *cspA*, the major *E. coli* cold-shock promoter, is a valuable tool for the production of aggregation-prone or unstable recombinant proteins at low temperatures. The advantages of this system include a relatively efficient repression of protein synthesis at physiological temperatures and an induction mechanism that does not require the addition of chemical inducers or nutritional changes. At 25 °C, the strength of the *cspA* promoter was comparable to that of the synthetic *tac* promoter, one of the most efficient non-bacteriophage promoters. More importantly, while the *tac* system became very inefficient at 10 °C, rapid synthesis of active preS2-S'-β-galactosidase was observed when the transcription of this fusion protein was directed by the *cspA* promoter. Since the translational efficiency of the cell decreases at low temperatures (Shaw and Ingraham, 1967; Broeze *et al.*, 1978), and because four 126 kDa preS2-S'-β-galactosidase monomers must assemble to form an active enzyme, we suspect that the *cspA* promoter will be much more efficient in directing the synthesis of

small monomeric proteins at 10 °C. The major drawback associated with the use of the *cspA* promoter is its repression 1-2 h following temperature downshift. It is likely that an improved understanding of the molecular basis of this mechanism will allow the design of improved cold-shock promoter systems.

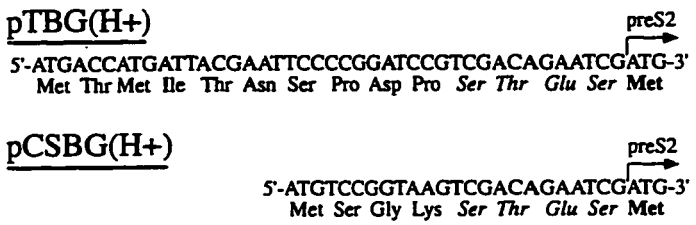

Figure 3.1. Predicted N-terminal sequences of the preS2-S'-β-galactosidase fusion proteins expressed from plasmids pTBGH(+) and pCSBG(H+). The three amino acids following the initiation codon in pCSBG(H+) are derived from the *cspA* open reading frame. The primary sequences of the fusion proteins become identical at the italicized region. The begining of the HBSAg preS2 domain is shown with a broken arrow.

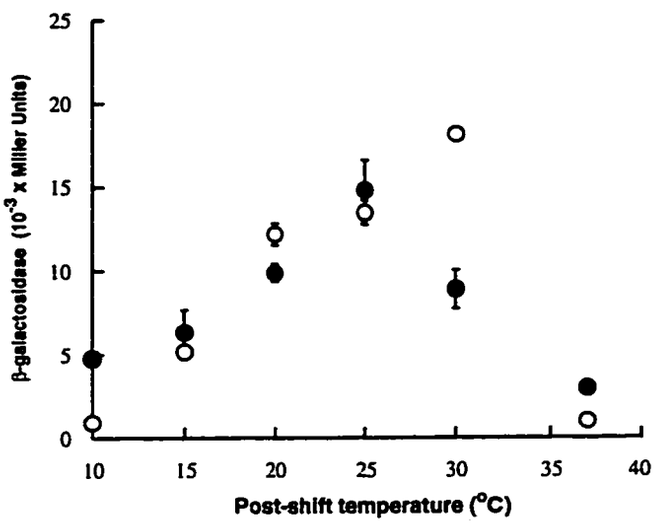

Figure 3.2. Effect of the growth temperature on the production of enzymatically active preS2-S'-β-galactosidase from the *tac* and *cspA* promoters. JM109 transformants harboring pTBGH(+) (○) or pCSBG(H+) (●) were grown to mid-exponential phase at 37 °C, and transferred to shaker baths maintained at the indicated temperatures. Five minute after transfer, pTBG(H+) transformants were induced with 1 mM IPTG. Samples were collected 2 hours after induction and clarified soluble cell extracts were assayed for β-galactosidase activity. Results are reported in Miller units. The pre-shift activity was 250 U in cells carrying pTBG(H+) and 1,500 U in cells containing pCSBG(H+).

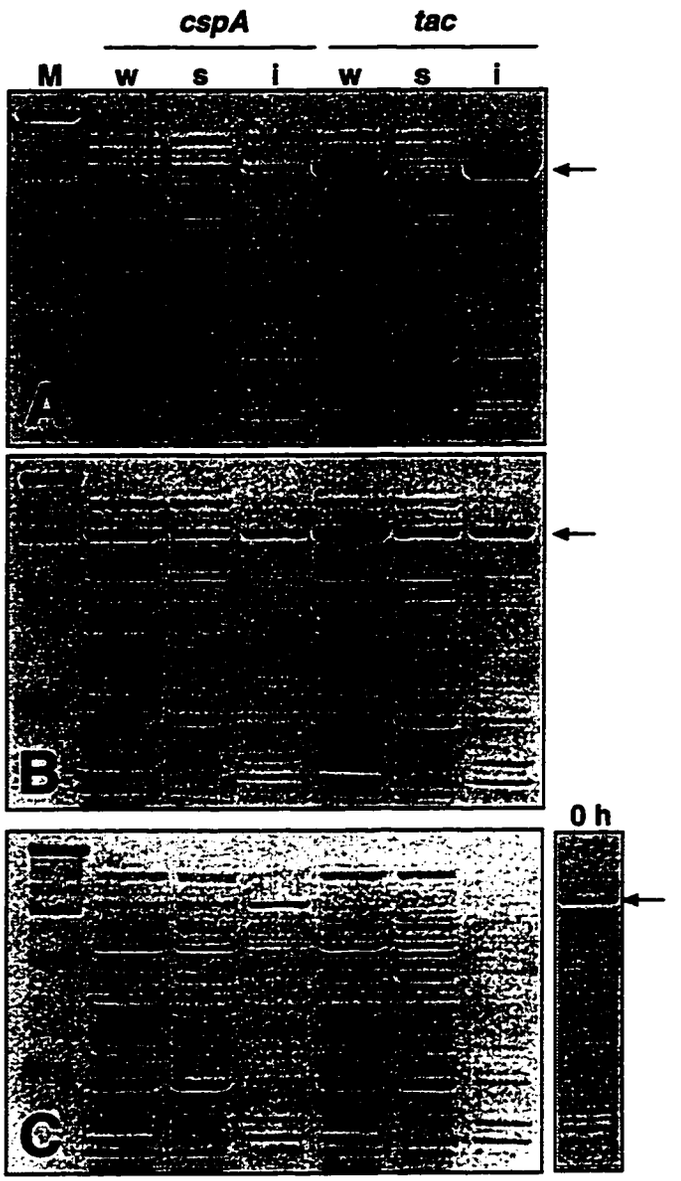

Figure 3.3. SDS-PAGE fractionation of cells incubated for 2 hours at low temperatures. JM109 transformants harboring pTBG(H+) (lanes *tac*) or pCSBG(H+) (lanes *cspA*) were grown as described in the legend of Fig. 2.2 before being returned to this temperature (Panel A), or transferred to shaker baths maintained at 25 (Panel B) or 10 °C (Panel C). Cells harboring pTBG(H+) were induced with 1 mM IPTG after five minutes. Samples harvested 2 hours post-induction were fractionated into whole cells (w), soluble (s) and insoluble (i) fractions, as described in Materials and Methods. The 0 h lane (Panel C) corresponds to the pCSBG(H+) insoluble fraction taken just prior to temperature shift. Aliquots corresponding to equivalent amount of culture were resolved on 8% SDS-minigels. Lanes M contain prestained high molecular weight markers (BioRad): 201, 116, 85 and 50 kDa from top to bottom. The position of preS2-S'-β-galactosidase is indicated by arrows.

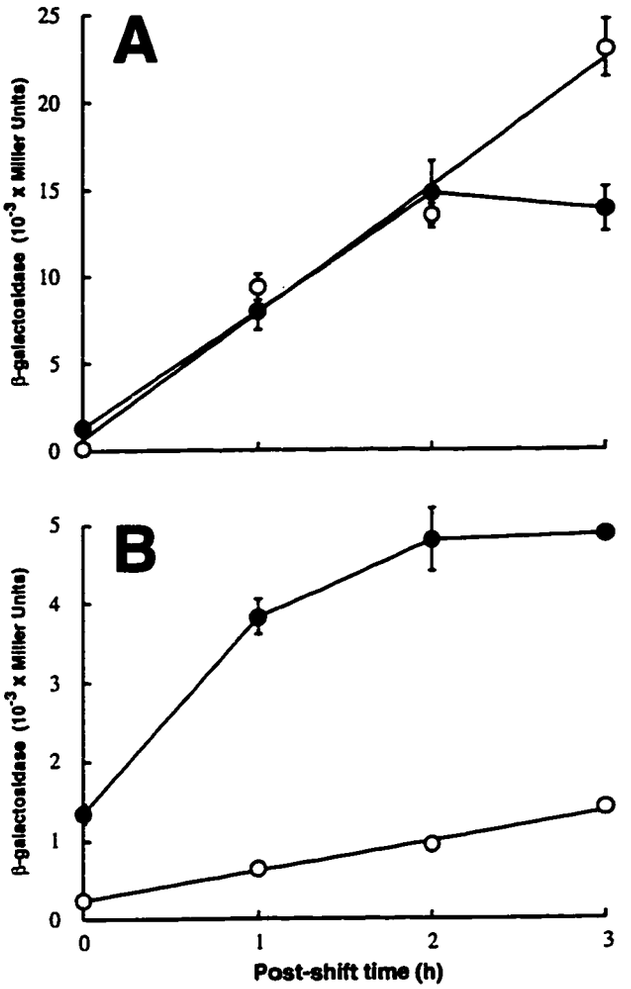

Figure 3.4. Kinetics of accumulation of active preS2-S'-β-galactosidase at 25 and 10 °C. JM109 transformants harboring pTBGH(+) (○) or pCSBG(H+) (●) were grown as described in the legend of Fig. 2.2 before transfer to 25 (Panel A) or 10 °C (Panel B) water baths. After 5 minutes, pTBG(H+) transformants were induced with 1 mM IPTG. Culture samples were collected at the indicated post-induction times and clarified cell extracts were assayed for β-galactosidase activity.

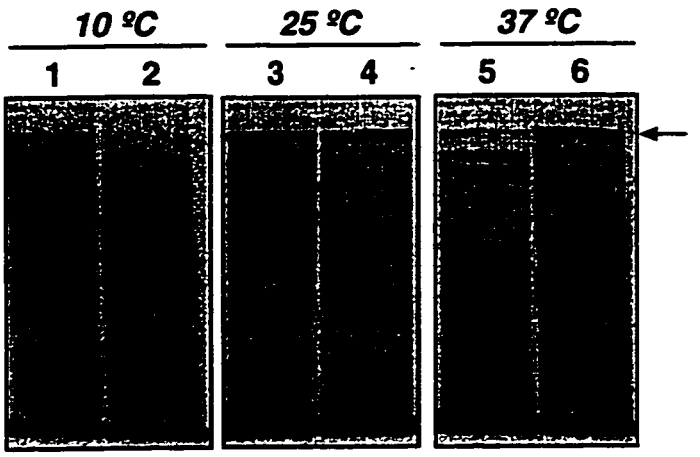

Figure 3.5. Synthesis of preS2-S'-β-galactosidase from the *tac* and *cspA* promoters. JM109 transformants harboring pTBG(H+) (lanes 2, 4 and 6) or pCSBG(H+) (lanes 1, 3 and 5) were grown in labeling medium at 37 °C, and either returned to this temperature or transferred to 25 or 10 °C water baths, as indicated. Chemical induction of pTBG(H+) cells was performed after 5 minutes, and culture samples were labeled with [^{35}S]-methionine 15 minutes post-induction, as described in Materials and Methods. Aliquots corresponding to the same amount of acid-precipitable counts were resolved by 8% SDS-PAGE and exposed to X-ray film.

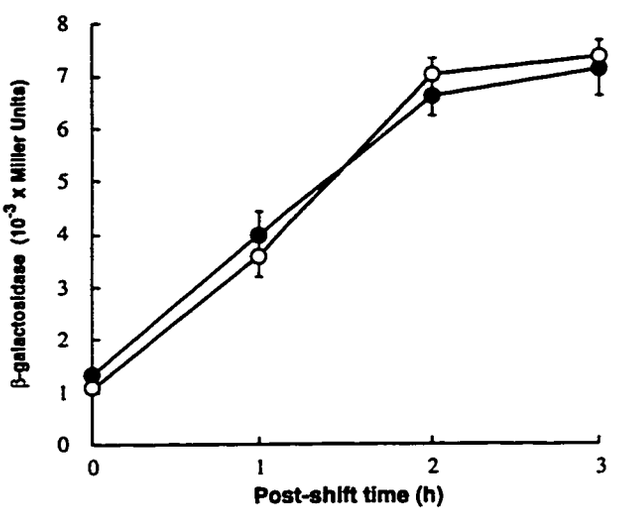

Figure 3.6. Effect of CspA overproduction on the recovery of active preS2-S'-β-galactosidase. JM109 cells harboring pCSBG(H+) and either pACYC184 (○) or pNatCspA (●) were grown as described in the legend of Fig. 2.2 and transferred to 15 °C. Culture samples were harvested at the indicated times and clarified extracts were assayed for β-galactosidase activity.

Chapter 4. Scale-up and Optimization of the Low-Temperature Inducible *cspA* Promoter System

4.1. Introduction

In this chapter, the performance of the major *E. coli* cold shock promoter in directing the synthesis of recombinant proteins at low temperatures was investigated in batch fermentations using a plasmid-encoded transcriptional gene fusion between the *cspA* promoter region and the *lacZ* gene. Rapid synthesis of β-galactosidase was observed when the fermentation broth was chilled from 37°C to lower temperatures using a variety of cooling profiles, including one modeling the heat-transfer characteristics of a 60 L pilot plant unit. As with the shake flask studies described in Chapters 2 and 3, the promoter became repressed 60-120 min after initiation of cooling. Both temperature cycling and stepwise temperature downshifts led to multiple inductions of the *cspA* promoter, but produced only modest gains in β-galactosidase activity accumulation when compared to the optimal single temperature shift.

Promoter repression was abolished in host cells bearing a *null* mutation in the *rbfA* gene, leading to the constitutive and high level expression of β-galactosidase when shake flask cultures were transferred from 42 to 23°C. The suitability of *rbfA* cells for *cspA*-driven recombinant protein production was confirmed in high density fed-batch fermentations.

4.2. Materials and Methods

Strains, plasmids and media

E. coli strains JM109 (Yanisch Perron *et al.*, 1985), CSH142 (*rbfA*$^+$) and CD28 (*rbfA*) (Dammel and Noller, 1995) have been described. Plasmid pCSBG is a pBR322

derivative encoding the *lacZ* gene under*cspA* transcriptional control (Vasina and Baneyx, 1996). Shake flask cultures (25 ml) were grown in LB medium supplemented with 50 µg/ml ampicillin. The growth medium was further supplemented with 0.2% glucose for all batch fermentations. Fed-batch fermentations were carried out in complex batch medium (McDonald *et al.*, 1996) supplemented with 100 µg/ml carbenicillin.

Shake flask cultures

Shake flasks were inoculated in triplicate at a 1:50 dilution with overnight cultures of CSH142(pCSBG) or CD28(pCSBG) and grown at 42°C to mid-exponential phase ($A_{600} \approx 0.45$). A 1 ml sample was harvested and the cultures were either returned to 42°C or transferred to water baths maintained at 23°C (time zero in Fig. 4.6). Samples (1 ml) were collected at indicated time points thereafter. The cells were harvested by centrifugation at 10,000 x g for 2 min and the pellets were stored at -20°C to await further processing.

Batch Fermentations

BioFlo 3000 fermentors (New Brunswick Scientific) cooled by VWR model 1172 refrigeration units held at 7°C were filled with 2.5 L of LB medium supplemented with 0.2% glucose, 50 µg/ml ampicillin and 100µl of antifoam (Sigma 289) and inoculated at a 1:50 dilution with overnight seed cultures of JM109(pCSBG). The pH of the fermentors was maintained at 7.0 by automatic addition of 5% NH_4OH or 1M HCl and the aeration rate and impeller speed were set at 1 vvm and 500 rpm, respectively. Cells were grown to mid-exponential phase ($A_{600} \approx 0.45$) at 37°C (time zero in Fig. 4.1 to 4.5). Heat-transfer limiting temperature shifts (Fig. 4.1, 4.2A, 4.3 and 4.4) were performed by programming the desired set-point values in AFS Biocommand software (New Brunswick Scientific) running on a Dell Pentium 133 computer interfaced with

the fermentors. Typically, a shift between 37 and 15°C was complete within 8 min corresponding to an average cooling rate of 2.6°C per min. For temperature ramping experiments, a cooling rate of 0.5°C per min (Fig 4.2B) or 0.3°C per min (Fig 4.2C) was programmed. To approximate the cooling profile of a larger reactor, a MPP-80 fermentation system (New Brunswick Scientific) filled with 60 L of H_2O and operated with an aeration rate of 1 vvm and an agitation speed of 500 rpm was slaved to a Neslab Coolflow HX-200 cooling system held at 7°C and interfaced with the AFS Biocommand software. After completion of steady-state heating to 37°C, a new set-point of 15°C was programmed. The liquid temperature was recorded at one minute intervals and the experiment was repeated. The cooling profile was fitted with the polynomial function $T = - 0.7973t + 0.1150t^2 - 6.254x10^{-5}t^3$, which was next programmed in the AFS Biocommand software for the experiments of Fig. 4.3 and 4.7. Temperature cycling (Fig. 4.4) and successive temperature downshifts (Fig. 4.5) were carried out under heat-transfer limiting conditions with programmed incubation times at intermediates temperatures. When the temperature was reduced to 15°C, the aeration rate and agitation speed were reduced to 0.25 vvm and 250 rpm, respectively, due to excessive foaming. All fermentations were performed in duplicate. Samples (1 ml) were collected at various time points. The cells were recovered by centrifugation at 10,000 x g for 2 min and stored at -20°C.

Fed-batch fermentations

Fed-batch fermentations were performed essentially as described (McDonald *et al.*, 1996). Briefly, glycerol stocks of CD28(pCSBG) were used to inoculate 60 ml of LB medium supplemented with 100 µg/ml carbenicillin and the cells were grown at 42°C to $A_{600} = 0.55$. BioFlo 3000 fermentors containing 1.1 L of complex batch medium (McDonald *et al.*, 1996) supplemented with 100 µg/ml carbenicillin and 100 µl of

antifoam were inoculated with 30 ml of the seed culture. The pH was maintained at 7.0 by addition of 15% NH_4OH or 1M HCl, the aeration rate was set at 1 vvm and the mixing speed was 500 rpm. Cells were grown at 42°C for 10 h to A_{600} = 2.5, at which point feeding was initiated by addition of a concentrated (40X) solution of complex batch media at a flow rate of 0.5 ml/min. Samples (25 ml) were periodically removed to maintain the reactor volume at 1.1 L. The feed rate was adjusted every 30 min to maintain the glucose level (measured off-line with a glucose analyzer) between 2.5 and 5 g/L. Feeding was pursued for 7 h until an A_{600} of 27 was reached (time zero in Fig. 4.7). At this point, the medium was chilled to either 15 or 23°C using the function modeling the cooling profile of the MPP-80 fermentation unit. Concentrated complex batch medium was fed at 0.5 ml/min for 1 h after the temperature shift. Two hours post-induction, feeding was resumed at a flow rate of 0.15 ml/min for the reactor shifted to 15°C and 0.3 ml/min for the fermentor held at 23°C. Samples (1 ml) were harvested at various time points. The cells were recovered by centrifugation and stored at -20°C.

β-galactosidase assays and SDS-PAGE

All cell pellets were resuspended in 50 mM potassium phosphate monobasic (pH 6.5) before being lysed by French pressing at 15,000 psi. Soluble and insoluble fractions were separated by centrifugation at 10,000 x g for 10 min and the supernatant was assayed for β-galactosidase activity in triplicate using the chromogenic substrate ONPG, as described by Miller (Miller, 1972). For comparison purposes, the amount of enzymatic activity present in the cells immediately before temperature downshift were subtracted from the enzymatic activity at subsequent time points to yield Δ Miller Units. Whole cell aliquots were resuspended in 1X SDS loading buffer following centrifugation and incubated at 95°C for 5 min. Samples corresponding to identical amounts of culture were fractionated on 8% SDS-PAGE gels and visualized by

Coomassie blue staining as described (Thomas and Baneyx, 1996b). The gels were digitized using a Sharp JX-325 color scanner with transparency attachment interfaced with a Power Macintosh 7100/66. Videodensitometric analysis was performed using the NIH Image 1.60 software as described (Thomas and Baneyx, 1996b).

4.3. Results and Discussion

4.3.1. Induction of the *cspA* promoter in pilot scale batch fermentations

Plasmid pCSBG is a pBR322 derivative encoding a transcriptional gene fusion between the *cspA* promoter and the *E. coli lacZ* gene (Vasina and Baneyx, 1996). We have previously shown that β-galactosidase synthesis can be rapidly induced by shifting 25 ml shake flask cultures of JM109(pCSBG) cells from 37°C to the 15 -30°C temperature range and that maximum yields of active enzyme are obtained when the cells are held at approximately 20°C (Vasina and Baneyx, 1996). To demonstrate the usefulness of the *cspA* promoter for large scale protein production and characterize its behavior in pilot scale fermentations, JM109 cells harboring pCSBG were grown to mid-exponential phase at 37°C in BioFlo 3000 fermentors operated in batch mode with a 2.5 L working volume. At time zero, the growth medium was chilled to either 29, 20 or 15°C at the maximum permissible cooling rate and samples were assayed for β-galactosidase activity at various time points. In all cases, induction of β-galactosidase synthesis occurred almost immediately following temperature downshift and the initial rates of enzyme accumulation were comparable (Fig. 4.1A). In fermentors cooled to 29°C, an activity plateau of 3,000 U over background levels (Δ Miller Units) was reached within 30 min and the β-galactosidase activity present in the cells progressively decreased after 60 min incubation at this temperature (Fig. 4.1A, ❑). Cooling to 20 (Fig. 4.1A, Δ) or 15°C (●) resulted in approximately 2-fold higher levels of enzymatic

activity relative to 29°C 90-to-120 min after initiation of the temperature shift. Thereafter, the levels of β-galactosidase activity remained constant in fermentors held at 15°C while they started to decline after 3 h in fermentors shifted to 20°C. This behavior closely parallels that observed in shake flask experiments (Vasina and Baneyx, 1996) and can be explained by a balance between promoter repression and dilution of β-galactosidase activity through cell growth. One to two hours after temperature downshift, transcription and translation of *lacZ* from the *cspA* promoter becomes repressed by an unidentified mechanism thus bringing β-galactosidase synthesis to a halt. Because cells held at 29°C maintain healthy growth (Fig. 4.1B, ❑), the progressive increase in biomass concentration leads to a decrease in enzymatic activities (Vasina and Baneyx, 1996). Transfer to 20°C is followed by an acclimation lag phase before active growth resumes with a reduced doubling time, leading to a net decrease in enzymatic activities at late time points (Fig. 4.1B, Δ). At 15°C, however, essentially no growth takes place over the length of the experiment (Fig. 4.1B, ●) and the levels of β-galactosidase enzymatic activity remain at a constant value. Overall, the above data indicate that the *cspA* promoter is fully functional and inducible in a batch fermentation set-up and that the choice of the final downshift temperature affects the accumulation of enzymatic activity in a manner similar to that observed in shake flask cultures.

4.3.2. Influence of the cooling rate on the accumulation of enzymatic activity

To investigate the influence of the cooling rate on the performance of the *cspA* promoter, mid-exponential phase cultures of JM109(pCSBG) cells growing at 37°C in batch mode were chilled to 15°C according to one of the following schemes: heat transfer limited cooling (Fig 4.2A), linear cooling profile at 0.5°C per min (Fig 4.2B), and linear cooling profile at 0.3°C per min (Fig 4.2C). From the data presented in Fig

4.2, it is clear that changes in cooling profiles have no effect on the onset of repression. Nevertheless, the steady-state levels of β-galactosidase activity were reproducibly 30% higher when the reactor was chilled using the intermediate cooling profile (Fig. 4.2B). This behavior appears to contradict the fact that larger amounts of *cspA-lacZ* mRNA are transcribed when JM109(pCSBG) cells are downshifted to low temperatures relative to intermediates ones (Vasina and Baneyx, 1996). Nevertheless, it is important to keep in mind that translational efficiency (and in particular translation initiation) become less efficient as the medium temperature decreases (Shaw and Ingraham, 1967; Broeze *et al.*, 1978). Thus, the higher enzymatic yields in fermentors chilled at 0.5°C per min are likely to reflect an optimum situation in which the lower levels of *cspA-lacZ* mRNA transcription due to the intermediate cooling rate are compensated by more efficient translation. According to this view, rapid chilling (Fig 4.2A) results in lower yields due to inefficient translation of larger amounts of mRNA, whereas slow cooling (Fig 4.2C) has the same net effect due to a reduction in the concentration of *cspA-lacZ* transcripts.

While cooling rates can be easily controlled in fermentors operated with a small working volume, heat transfer limitations are a more serious concern in larger reactors. To address this issue, the temperature response profile of a 60 L fermentor subjected to a step change from 37 to 15°C under heat transfer limiting conditions was recorded (Fig 4.3, ×) and fitted with a polynomial function. The model (Fig 4.3, ----) was then programmed into the control software of a BioFlo 3000 fermentor operated in batch mode with a 2.5 L working volume. Fig 4.3 shows that the reduction in cooling efficiency between 25 and 15°C that is characteristic of heat-transfer limitations in the 60 L fermentor had little influence on the induction of the *cspA* promoter and that the levels of enzymatic activity obtained under these conditions were in fact higher than those reached by making use of a 0.5°C per min linear cooling profile (Fig 4.2B). We therefore conclude that intermediate cooling profiles are optimal for the efficient

expression of recombinant proteins placed under *cspA* transcriptional control and that the performance of the promoter is not affected by heat transfer limitations encountered in typical 60 L reactors.

4.3.3. Effect of temperature cycling on β-galactosidase expression from the *cspA* promoter

Fig 4.1 to 4.3 show that the high level production of recombinant proteins placed under *cspA* transcriptional control is limited by promoter repression 60-120 min after transfer to low temperatures. Although the precise mechanisms responsible for this phenomenon remain obscure, it has been suggested that the accumulation of a cold-shock induced repressor molecule may be responsible for shutting down transcription from the *cspA* promoter or destabilizing cold-inducible mRNAs (Jiang *et al.*, 1996). If the repressor could be cleared by shifting the cultures to a higher temperature where less stringent adaptive demands are placed on the cell, temperature cycling may permit additional cycles of induction of the *cspA* promoter. To test hypothesis, JM109(pCSBG) cells grown to mid-exponential phase in batch fermentors were subjected to heat-transfer limiting temperature downshift and held at 15°C for 30 min after initiation of the shift. This time was selected because the rates of β-galactosidase synthesis decrease significantly with prolonged incubation at 15°C (Fig 4.2). The cultures were next subjected to a series of alternative heating and cooling steps between 15 and 25°C. The latter temperature was chosen for two reasons. First, the growth rate of JM109(pCSBG) cells at 25°C is about 5-fold lower than at 37°C (Vasina and Baneyx, 1996) which limits the dilution of β-galactosidase activity due to cell growth. Second, we anticipate that the *cspA* system will be primarily useful for the production of aggregation-prone recombinant proteins which are likely to have a higher propensity to misfold at 37°C relative to 25°C. Fig 4.4 shows the effect of temperature cycling on the

accumulation of β-galactosidase activity in 2.5 L BioFlo 3000 fermentors programmed to heat and cool the reactor as fast as heat transfer limitations permit (≈ 20 min for a complete heating-cooling cycle) and to hold the medium at 15°C for 30 min after the onset of each cooling cycle. In Fig 4.4A, cooling was initiated as soon as a temperature of 25°C was reached, whereas the cultures were maintained at 25°C for 30 min after initiation of the heating steps in the experiment of Fig 4.4B. Inspection of Fig 4.4A shows that, although multiple inductions of the *cspA* promoter can be achieved through short, successive cycles of heating and cooling, β-galactosidase activity only accumulates at high level during the first temperature pulse. A possible explanation for this result is that low amounts of the putative repressor have been synthesized by the cells at early time points, making the first re-induction of the *cspA* promoter more efficient than the others. This hypothesis is discussed in more detail in the following section. Alternatively, the rapid accumulation of β-galactosidase could be explained by a more efficient translation of the *cspA-lacZ* mRNA synthesized at 15°C when the medium temperature is increased to 25°C. Fig. 4.4B supports the latter explanation since the enzymatic activity increases mostly during the heating phase of the first temperature cycle. Nevertheless, when the same experiment was repeated with 15°C incubation periods of 90 rather than 30 min, the magnitude of the first re-induction was similar to that of the second re-induction in Fig 4.4B (data not shown). Thus, prolonged incubation at low temperatures may lead to the accumulation of a threshold concentration of repressor that destabilizes the *cspA-lacZ* mRNA (or directly or indirectly prevents its translation) and abrogates the beneficial effect of improved translation upon heating to 25°C. This phenomenon would also explain why weak re-inductions of the promoter are observed during the second and third temperature cycles in Fig 4.4. We conclude that efficient re-induction of the *cspA* promoter by temperature cycling is only possible with cells that have not become acclimated to low temperatures and shut down the

production of cold shock proteins. Since we observed little difference in the magnitudes of the second and third re-induction steps by increasing the duration of the incubation period at 25°C (compare Fig 4.4A to 4B), it is likely that cells may have to grow for a number of generations before "forgetting" that they have been exposed to low temperatures.

4.3.4. Effect of stepwise temperature downshifts on β-galactosidase expression from the *cspA* promoter

One of the drawbacks associated with the use of temperature cycling is that incubation of the cells at the intermediate temperature is accompanied by cell growth which leads to dilution of the enzymatic activity accumulated upon transfer to 15°C. In order to circumvent this problem and to gain a better understanding of *cspA* promoter regulation, we investigated whether successive temperature downshifts could be used to maximize the yields of β-galactosidase. This approach appeared feasible since temperature drops as small as 7°C have been shown to be sufficient to induce the *cspA* promoter in shake flask cultures (Vasina and Baneyx, 1996). Fig 4.5A shows that when mid-exponential phase cultures of JM109(pCSBG) growing in 2.5 L batch fermentors were transferred from 37 to 29°C, about 4,000 U of activity accumulated within 1 h before becoming diluted as a result of biomass increase. Under these conditions, the initial rates of β-galactosidase synthesis were comparable to those observed upon single temperature downshift from 37 to 15°C (Fig 4.2A). A second, efficient induction of the *cspA* promoter leading to the synthesis of 3,000 U of activity could however be achieved by chilling the medium to 15°C after 2 h incubation at 29°C. In an effort to optimize this scheme, a three-step cooling protocol (37 to 29°C, 29 to 21°C and 21 to 13°C) was selected and the influence of the length of the temperature step on the production of active β-galactosidase was examined. When the medium was held at intermediate

temperatures for 90 min (Fig 4.5B), three distinct cycles of induction were observed. However, the kinetics of β-galactosidase accumulation were about 5-fold lower in the second and third re-induction cycles relative to the first one. Reducing the length of each plateau to 60 min improved the initial rates of β-galactosidase synthesis in the second induction cycle but led to a sluggish third induction cycle (Fig 4.5C). These results can be interpreted as follows. When given sufficient time to re-establish efficient transcription/translation following temperature downshift, cells repress the high level synthesis of cold shock proteins that are no longer necessary for survival. Subsequent cell division leads to the inactivation of the repression mechanism, perhaps through the dilution of a cold-shock induced repressor molecule. After a few generations, the cells have lost their "memory" of the insult and respond to another temperature downshift by rapidly synthesizing cold shock proteins at high levels. This situation is exemplified in Fig 4.5A where 120 min of incubation at 29°C appear to be sufficient to almost completely "reset" the *cspA* promoter. If the length of the acclimation period is reduced (e.g. to 90 min, Fig 4.5B), cells are unable to turn on the high level synthesis of cold shock proteins when subjected to additional temperature downshifts owing to the fact that the repression mechanism is still functional. This situation translates into weak re-inductions of the *cspA* promoter (Fig 4.5B; also see Fig 4.4). A possible explanation for the fact that a rather efficient second induction cycle is observed when the length of the acclimation period is set to 60 min (Fig. 4.5C) is that a longer period of time (about 90 min) is necessary to fully activate the mechanism responsible for the repression of cold shock protein synthesis (for instance to reach a threshold concentration of repressor molecules). Under these conditions, high level transcription from the *cspA* promoter remains possible following a second temperature downshift. However, as incubation at low temperatures is extended, efficient repression becomes established and an additional temperature downshift only results in a modest induction of the *cspA* promoter. Taken

together, the data presented in Fig 4.4 and 5 indicate that, whereas fermentation engineering approaches lead to a slight improvement (≈ 30-40%) in the production of recombinant proteins placed under *cspA* transcriptional control and reduce the time necessary to reach maximal production levels, they are not suitable to abolish promoter repression.

4.3.5. Effect of the *rbfA* mutation on β-galactosidase expression from the *cspA* promoter in shake flasks and high density fermentations

RbfA is a 15-kDa protein that associates with free 30S ribosomal subunits and has been suggested to function either as a late ribosome maturation factor or as a translation initiation factor (Dammel and Noller, 1995). Strains bearing a *null* mutation in the *rbfA* gene exhibit severe growth defects at low temperatures and an abnormal polysomal profile due to increased levels of 30S and 50S ribosomal subunits (Dammel and Noller, 1995). It was recently reported that chromosomally encoded cold shock and ribosomal proteins are constitutively induced through an obscure mechanism when cultures of *rbfA* mutants are transferred from 42 to 15°C (Jones and Inouye, 1996). In order to explore the usefulness of these cells for the production of recombinant proteins placed under *cspA* transcriptional control, the *rbfA* mutant CD28 and its isogenic wild type CSH142 (Dammel and Noller, 1995) were transformed with plasmid pCSBG and the influence of the mutation on β-galactosidase expression was investigated in shake flask experiments. At 37°C, the specific growth rate of CD28(pCSBG) cultures was about 2-fold lower than that of CSH142(pCSBG) cells and very high basal amounts of β-galactosidase activity (≈ 10,000 U) were present in the cytoplasm of mutant cells (data not shown). It is therefore clear that, even at physiological temperatures, the *rbfA* mutation exerts severe effects on cell growth and the regulation of the cold shock

response. To circumvent the problem of leaky expression, cultures were incubated to mid-exponential phase at 42°C. Under these conditions, the *cspA* promoter was once again efficiently repressed with a basal level of enzymatic activity inferior to 700 U. Transfer of the cultures from 42 to 15°C did not lead to the efficient induction of the *cspA* promoter in *rbfA* mutant cells. Nevertheless, the level of enzymatic activity in CD28(pCSBG) cells were about 6-fold higher than in CSH142(pCSBG) cultures 24 h after initiation of the shift (data not shown). An intermediate temperature of 23°C was therefore selected for further experiments. Fig 4.6A shows that,while no increase in enzymatic activity was observed when wild type or mutant cells were held at 42°C (open symbols), the *cspA* promoter was rapidly induced when mid-exponential phase cultures were shifted to 23°C. In the case of wild type cells (Fig 4.6A, ◆), about 18,000 U-of enzymatic activity accumulated within 1 h before becoming diluted as a result of promoter repression and cell growth. Although genotypic differences are likely to account for the higher production levels of β-galactosidase in CSH142 relative to JM109, the patterns of induction/repression were remarkably similar in both strains (compare Fig 4.6A to Fig 4.1A). In the *rbfA* mutant, however, no repression was observed after 1 h and high level synthesis of β-galactosidase from the *cspA* promoter continued for 7 h following temperature downshift (Fig 4.6A, ●). The maximum yield of active β-galactosidase in *rbfA* mutant cells (reached 7 h post-shift) was approximately 3-fold higher than in its isogenic wild type (1 h post-shift), a result that was confirmed by SDS-PAGE analysis (Fig 4.6B).

To determine the usefulness of *rbfA* mutants as hosts for the high level production of recombinant proteins placed under the transcriptional control of the *cspA* promoter, CD28(pCSBG) cells were grown at 42°C to high density ($A_{600} \approx 27$) in BioFlo 3000 fermentors operated in fed-batch mode. Less than 250 U of activity was present in the cells before chilling. The medium was next shifted to 15 or 23°C using a programmed

temperature profile duplicating the cooling behavior of a 60 L reactor. The turbidity of the culture dropped rapidly in the fermentor cooled to 15°C (data not shown) indicating that *rbfA* cells are not viable when cultivated at high density at this temperature. At 23°C, however, high level production of β-galactosidase was achieved for at least 6 h following temperature downshift leading to the accumulation of about 25,000 U of enzymatic activity (Fig 4.7A). An additional 10,000 U were synthesized with reduced kinetics over the next 15 h. SDS-PAGE analysis showed that β-galactosidase became the major cellular protein 3-to-5 h after temperature downshift (Fig 4.7B). From videodensitometric analysis of diluted samples fractionated on 15% SDS gels, we estimate that the enzyme represents approximately 15% of the total cell protein at the end of the fed-batch run. We conclude that *rbfA* mutants are useful hosts for the high level and unrepressed production of proteins transcribed from the *cspA* promoter following culture cooling from 42°C to the 20°C temperature range.

4.4. Conclusions

We have previously shown that the promoter region of the major *E. coli* cold-shock protein is well suited for the expression of recombinant proteins at low temperatures, and that this system may be particularly valuable to facilitate the production of unstable and/or aggregation-prone polypeptides in a full-length and soluble form (Vasina and Baneyx, 1996; Vasina and Baneyx, 1997). In this work, we demonstrate that the *cspA* promoter performs efficiently in fermentation set-ups and define the optimal conditions for its induction. Although temperature cycling and successive temperature downshifts led to multiple re-inductions of the promoter, fermentation engineering approaches only resulted in limited improvements in the final recovery yields of the model protein β-galactosidase. The underlying problem of promoter repression could be abolished by making use of *rbfA* mutants which were robust enough to be cultivated in high density

fermentors at 23°C. The availability of a genetic background allowing constitutive expression of gene products placed under *cspA* transcriptional control makes the major cold-shock promoter a powerful tool for the production of recombinant proteins at low temperatures.

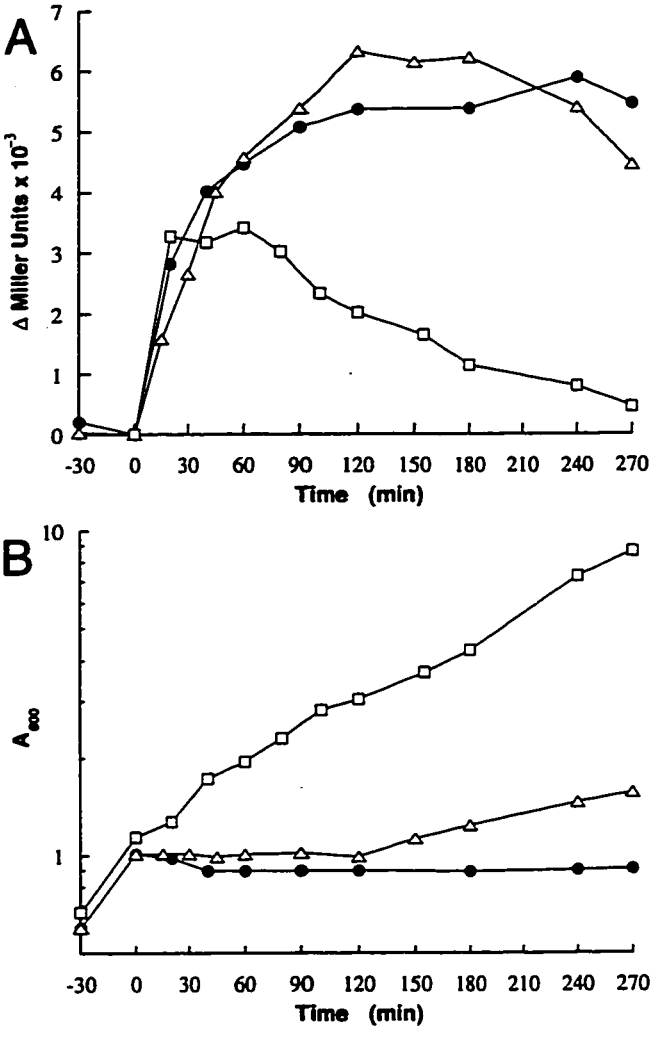

Figure 4.1. Effect of the downshift temperature on the accumulation of β-galactosidase activity (**Panel A**) and the growth of JM109(pCSBG) transformants (**Panel B**) in batch fermentors. Cells were grown to mid-exponential phase at 37°C in 2.5 L BioFlo 3000 fermentors operated in batch mode. At time zero, the temperature set-point was shifted to 15 (●), 20 (Δ), or 29°C (□). The absorbance (A_{600}) of the cultures (natural log scale) and the enzymatic activity present in clarified cell extracts were determined at the indicated time points. Pre-shift enzymatic activities were 2080, 2970, and 2600 Miller Units before cooling to 15, 20 and 29°C, respectively.

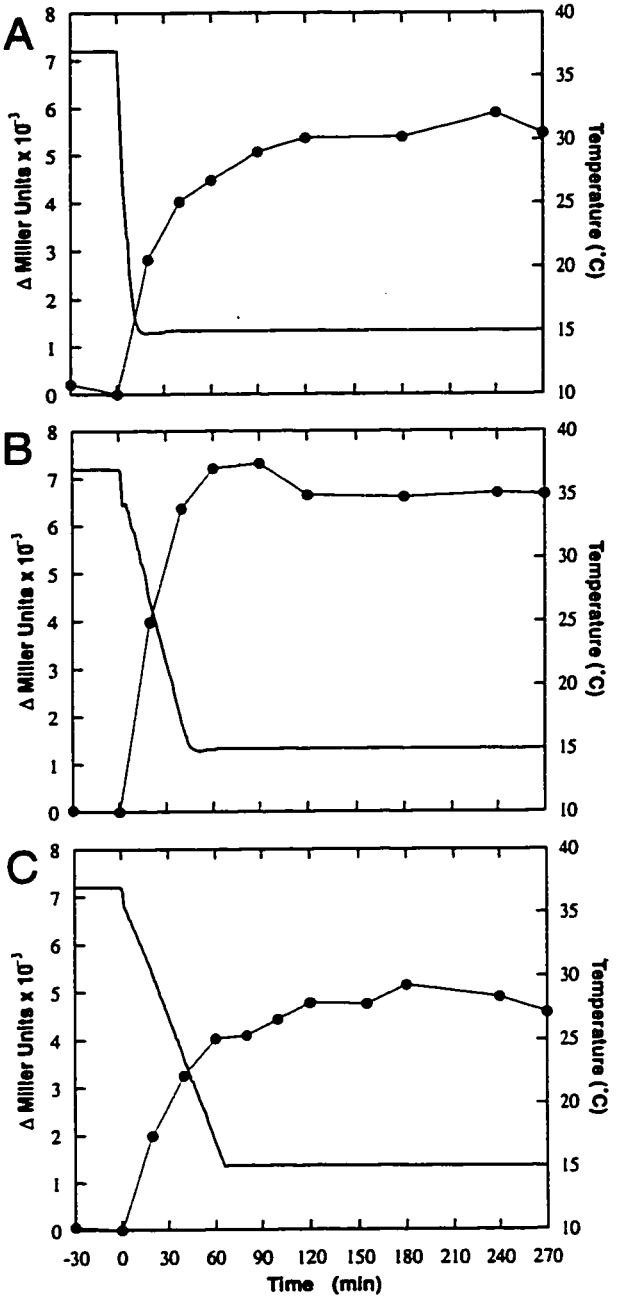

Figure 4.2. Effect of the cooling rate on the accumulation of β-galactosidase activity. JM109 cells harboring pCSBG were grown to mid-exponential phase as described in the legend of Fig. 1. At time zero, the medium temperature was decreased to 15°C under heat transfer limiting conditions (**Panel A**), or by using linear cooling rates of 0.5 (**Panel B**) or 0.3°C per min (**Panel C**). Solid lines show the medium temperature at 1 min intervals. Activities are indicated by filled circles (●). Pre-shift enzymatic activities were 2080, 2430, and 2490 Miller Units for panels A, B and C, respectively.

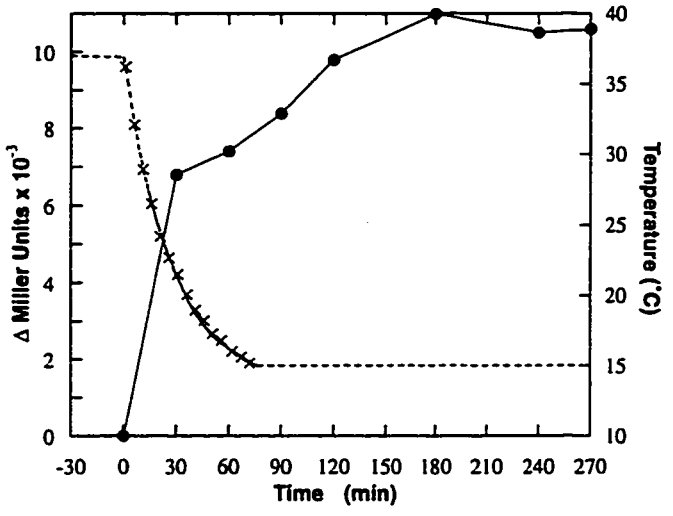

Figure 4.3. Induction of the *cspA* promoter using a cooling profile modeling the heat transfer characteristics of a 60 L vessel. JM109 cells harboring pCSBG were grown to mid-exponential phase as described in the legend of Fig. 1. The medium was chilled to 15°C using a scheme mimicking the cooling behavior of a 60 L fermentor (see Materials and Methods). Solid lines show the medium temperature at 1 min intervals. Actual temperature data gathered at 5 min intervals for the 60 L unit are shown (×). Activities are indicated by filled circles (●). The pre-shift enzymatic activity was 9370 Miller Units.

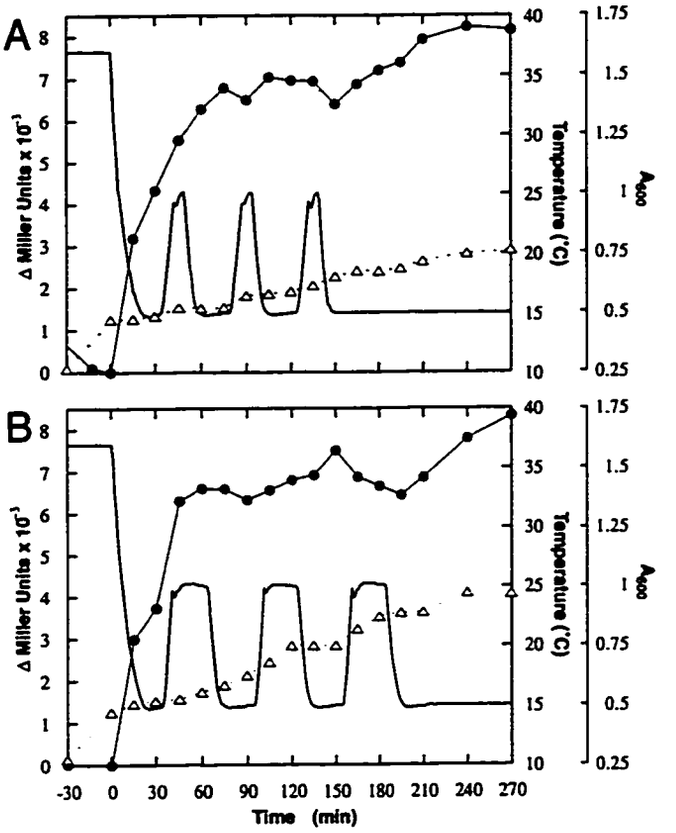

Figure 4.4. Temperature cycling leads to multiple inductions of the *cspA* promoter. JM109 cells harboring pCSBG were grown to mid-exponential phase as described in the legend of Fig. 1 and the medium was cooled to 15°C under heat transfer limiting conditions. The temperature was next cycled between 15 and 25°C with immediate cooling to 15°C when a temperature of 25°C was reached (**Panel A**), or with a 30 min holding period at 25°C after initiation of heating (**Panel B**). In both cases the medium was maintained at 15°C for 30 min after initiation of each cooling cycle. Solid lines show the medium temperature at 1 min intervals. The cultures absorbance (A_{600}, Δ) was noted and the activity (●) was determined. Pre-shift enzymatic activities were 4340 and 4290 Miller Units for panels A and B, respectively.

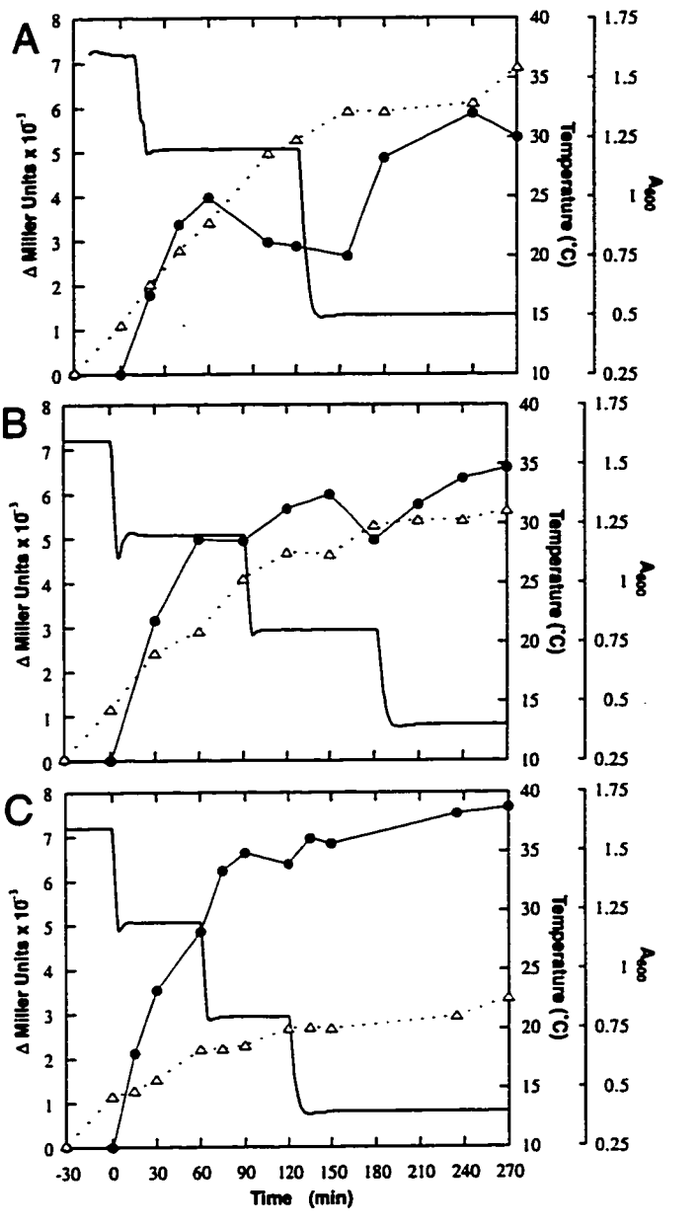

Figure 4.5. Stepwise temperature downshifts lead to multiple inductions of the *cspA* promoter. JM109 cells harboring pCSBG were grown to mid-exponential phase as described in the legend of Fig. 1 before being subjected to successive cooling cycles under heat transfer limiting conditions. **Panel A**: the medium was chilled from 37 to 29°C, held at this temperature for 2 h and further cooled to 15°C. **Panel B**: the medium was subjected to three temperature downshifts (from 37 to 29°C, from 29 to 21°C, and from 21 to 13°C) with 90 min holding periods at each intermediate temperature. **Panel C**: the medium was subjected to the temperature downshifts of Panel B but the length of each holding period was decreased to 60 min. Solid lines show the medium temperature at 1 min intervals. The cultures absorbance (A_{600}, Δ) was noted and the activity (●) was determined. Pre-shift enzymatic activities were 2990, 3180 and 1860 Miller Units for panels A, B and C, respectively.

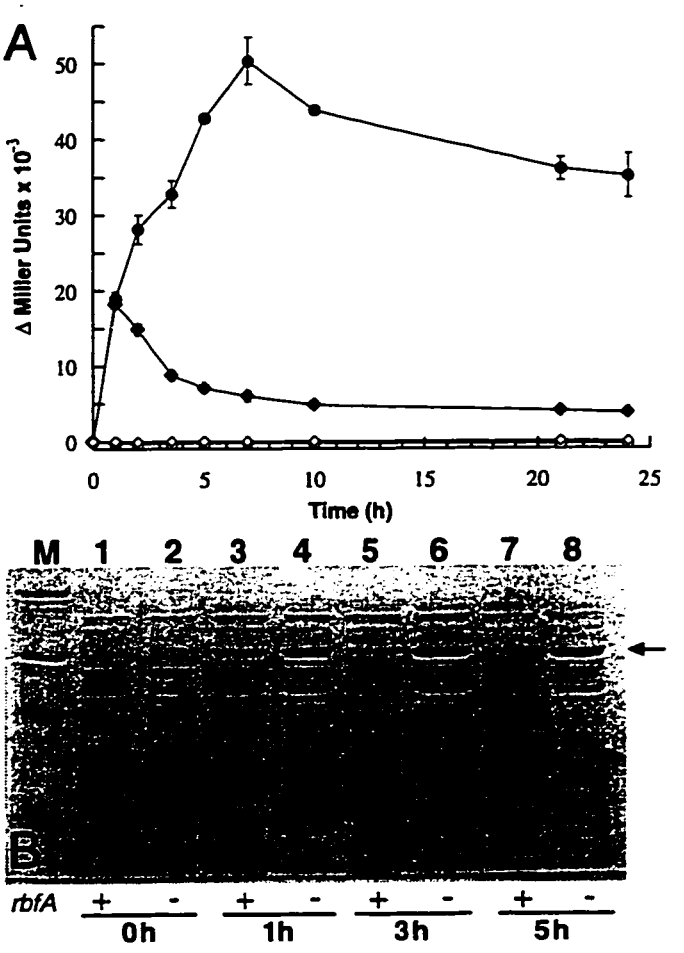

Figure 4.6. Constitutive expression of β-galactosidase from the *cspA* promoter in *rbfA* mutant cells. The *rbfA null* mutant CD28 (●,○) and its isogenic wild type CSH142 (◆,◊) were transformed with pCSBG and grown to mid-exponential phase at 42°C in shake flasks. At time zero, the cultures were either shifted to 23°C (filled symbols) or held at 42°C (open symbols) and the enzymatic activity present in clarified cell extracts was determined at the indicated time points (**Panel A**). Pre-shift enzymatic activities were 150 and 680 Miller Units for CSH142 and CD28 transformants, respectively. Error bars were obtained from triplicate cultures. **Panel B**: Whole cells samples from cultures shifted to 23°C for the indicated times were fractionated by SDS-PAGE. Lane M contains prestained molecular mass markers (BioRad): from top to bottom: 205, 116.5, 89, and 49.5 kDa. The position of β-galactosidase is indicated by an arrow.

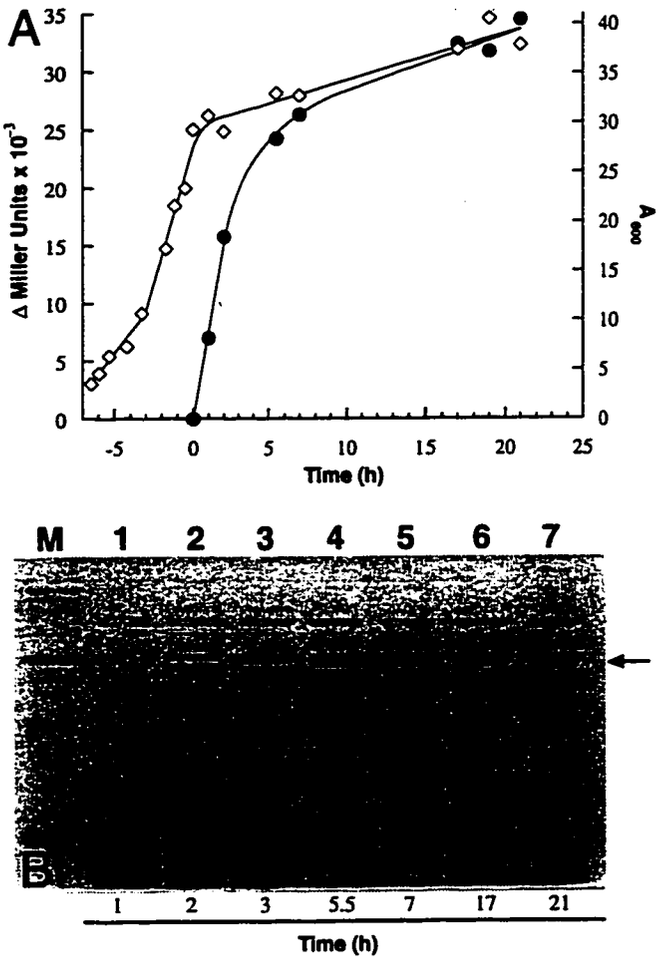

Figure 4.7. *rbfA* mutants are suitable for the high level expression of β-galactosidase from the *cspA* promoter in high density fed-batch fermentations. CD28(pCSBG) cells were grown at 42°C in complex batch medium to A_{600} = 27 as described in Materials in Methods. At time zero, the medium was chilled to 23°C using a scheme mimicking the cooling behavior of a 60 L fermentor. **Panel A**: the absorbance (A_{600}) of the cultures (◊) and the enzymatic activity present in clarified cell extracts (●) was determined at the indicated time points. The pre-shift enzymatic activity was 210 Miller Units. **Panel B**: Whole cells samples from cultures shifted to 23°C for the indicated times were fractionated by SDS-PAGE. Lane M contains prestained molecular mass markers (BioRad): from top to bottom: 205, 116.5, 89, and 49.5 kDa. The position of β-galactosidase is indicated by an arrow.

Chapter 5. Effects of the Untranslated Region of *cspA* mRNA on β-galactosidase from the *cspA* promoter

5.1. Introduction

The 159 bp-long untranslated region (UTR) of the *cspA* mRNA has been proposed to play a crucial role in the regulation of *cspA* synthesis in the adaptive phase of the cold-shock response (Jiang *et al.*, 1996). The aim of this chapter is to improve the current understanding of the regulatory function of the *cspA* UTR and to determine whether modifications in this region may be valuable for the production of heterologous proteins placed under transcriptional control of the *cspA* promoter. In the first part of the study, we examine the effect of deleting either the cold box domain or a 26-nt long region located in the center of the UTR on the synthesis of β-galactosidase by plasmid-encoded *cspA-lacZ* gene fusions. The effects of co-overexpressing part of the UTR and of increasing the gene dosage of the authentic *cspA-lacZ* fusion are also analyzed.

5.2. Materials and Methods

Strains and media

E. coli Top10 (F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80Δ*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL endA1 nupG*) (Invitrogen) was used as a host strain for all recombinant DNA manipulations. Shake flask experiments were conducted in *E. coli* CSH142 (*ara* Δ(*gpt-lac*)5) (Dammel and Noller, 1995) using 30 ml of LB medium supplemented with 100 μg/ml carbenicillin and 34 μg/ml chloramphenicol when appropriate.

Plasmid constructions

The relevant characteristics of the plasmids used in this study are listed in Table 5.1. Plasmids pUCCSBG and pACCSBG are pUC18 and pACYC184 derivatives, respectively, encoding the authentic *cspA-lacZ* transcriptional gene fusion but carrying different origins of replications. Plasmid pCSBG (Vasina and Baneyx, 1996) was digested with *Sph*I and *Bsp*EI and the 4.1 kbp fragment encoding the *lacZ* gene under *cspA* promoter control was recovered following low melting point (LMP) agarose electrophoresis using the Qiaquik kit (Quiagen). Both pTG10, a pACYC184 derived cloning vector (Chang and Cohen, 1978), and pUC18 (Yanisch Perron *et al.*, 1985) were digested with *Sph*I and *Xma*I and the 3.9 kbp and 2.7 kbp backbones were isolated by LMP agarose electrophoresis. The *cspA-lacZ* cassette was ligated with each backbone and Top10 was transformed with the ligation mixture by electroporation. Blue transformants harboring the resulting plasmids (pACCSBG and pUCCSBG) were selected on Ampicillin/X-GAL plates after overnight growth at 37°C and incubation at 4°C for 5 hours.

Plasmid pCSBG2D is a pCSBG derivative encoding the *lacZ* gene under control of a modified *cspA* promoter with an internal deletion centered around the *ApaLI* site in the 5' UTR of the promoter region. In order to construct a plasmid encoding the *cspA* promoter and containing a single *Apa*LI site, both pT7CS, which encodes a PCR amplified *cspA* promoter, and the cloning vector pACYC184, were cut with *Sph*I and *Sal*I. The DNA fragments corresponding to the pACYC184 backbone and the *cspA* promoter were isolated and ligated to generate plasmid pACCS. The plasmid was next linearized with *Apa*LI, incubated with 1 U of Bal-31 exonuclease for one to eight minutes at 30°C, and the DNAs were religated after polishing with T4 DNA polymerase. Top10 cells were transformed with the ligation mixtures by electroporation

and selected for on chloramphenicol plates. Plasmid DNA was isolated from several transformants and the modified *cspA* promoters were released on *Sph*I-*Sal*I fragments and resolved by 0.8% LMP agarose electrophoresis. Since all fragments were found to be approximately of identical size, one plasmid obtained after a 2 min Bal-31 incubation step,, pACCS2D, was selected for further studies. The modified *cspA* promoter region fragment was harvested on a *Sph*I-*Sal*I fragment and cloned into the same sites of pTBGM to yield pCSBG2D. Sequencing of pCSBG2D revealed that 26 bp had been deleted between nucleotides +71 and +98 in the *cspA* UTR (Fig. 5.1).

Plasmid pXCSBG is a pCSBG derivative encoding the *lacZ* gene under control of a modified *cspA* promoter lacking a cold box domain in the 5' UTR of the promoter region. To construct this plasmid, a 5' portion of the *cspA* promoter was amplified by PCR on a 412 bp fragment using plasmid pJJG02 (Goldstein *et al.*, 1990) as a template. The forward primer 5'-CCAATGCATGCGCTGTTGAGCAG-3' was designed to introduce a *Sph*I site at position 70 and the reverse primer 5'-CCCGGGCAATTGCCGTTGATGTGCATTAAGCCACGC-3' to hybridize at position 461 (2 bp past the transcription start site) while simultaneously introducing a 5' tail encoding a *Mfe*I restriction site. The downstream portion of the *cspA* promoter was amplified from pJJG02 on a 171 bp fragment using the forward primer 5'-CGTACAGACAATTGAAGCAGTG-3' to introduce a *Mfe*I site at position 474 and the reverse primer 5'-TACCAGTCGACTTACCGGACATAG-3' to add a *Sal*I site at position 632 (all numbering according to Goldstein *et al.*, 1990). Amplification of the fragments was performed on a Perkin Elmer 480 thermocycler using 25 cycles of incubation at 90°C for 2 min, 50°C for 2 min and 22°C for 2 min. Amplified DNAs were resolved by LMP agarose gel electrophoresis, isolated and ligated into pT7Blue Blunt (Novagen) to yield the plasmids pUP2 (5' promoter region) and pDN2 (3' promoter region). Plasmid pUP2 was digested with *Sph*I and *Mfe*I and pDN2 was

cleaved with *Mfe*I and *Sal* I to liberate the *cspA* promoter fragments. The promoterless *lacZ* plasmid pTBGM was cut with *Sph*I and *Sal*I and a three way ligation between the *cspA* promoter fragments and the pTBGM derived backbone was performed. Top10 transformant harboring the resulting plasmid, pXCSBG, were selected for dark blue colonies on ampicillin/X-GAL plates following overnight growth at 37°C and 5 hours incubation at 4°C.

Plasmid pUTRf encodes the authentic *cspA* promoter together with the first 61 bp of its normally 159 bp-long UTR followed by the native *cspA* transcription terminator. This plasmid was constructed by PCR using plasmid pJJG02 as a template as follows. The forward primer 5'-GCAATTCACGGGCCCGCAGTGTG-3' was designed to introduce an *Apa*I site at position 21 while the reverse primer 5'-CCCGGGATCCCCGCCAAATGGCAGGGATGGTATCAACGATAACTCTTGAAGGG-3' was designed to hybridize to the *cspA* promoter DNA through nucleotide 525 and to simultaneously introduce a 5' tail encoding the native *cspA* transcription terminator hairpin loop (underlined regions). The 550 bp fragment was amplified as above and cloned into pT7Blue Blunt (Novagen) to yield the 3.4 kb plasmid pUTR. Since a *lac* promoter directing transcription in the same orientation and upstream of the *cspA* promoter is present in pUTR, the plasmid was digested with *Afl*III to liberate a 700 bp fragment encoding the *lac* promoter. The staggered ends of the 2.7 kb backbone were polished with T4 polymerase and religated to yield plasmid pUTRf.

The sequence of all PCR products were confirmed by sequencing of both strands using commercial primers (Novagen), and the Applied Biosystems dideoxy terminator sequencing kit. All plasmid constructions were confirmed with restriction enzyme analysis.

Shake flask cultures

Shake flasks were inoculated in triplicate at a 1:50 dilution with overnight cultures of the strain indicated and grown at 42°C to mid-exponential phase ($A_{600} \approx 0.45$). The cultures were either returned to 42°C or transferred to water baths maintained at 23°C (time zero in all Figures). Samples (1 ml) were collected at indicated time points before and after the shift. The cells were harvested by centrifugation at 10,000 x g for 2 min and the pellets were stored at -20°C to await further processing.

mRNA secondary structure predictions

The mRNA secondary structure predictions were carried out using the program Mufold (Zuker, 1989). The program loopDloop, developed by D.G. Gilbert (1992)] was used for drawing mRNA secondary structures. mRNA folding simulations presented in this study and those by Fang *et. al.* (1997) were performed on the 5' UTR only. The influence of the sequence downstream of the UTR was not considered.

β-galactosidase assays and SDS-PAGE

β-galactosidase assays and SDS- PAGE analysis were conducted as described in Section 4.2.

5.3. Results

5.3.1. UTR deletions affect the regulation of *cspA-lacZ* gene fusions

An intriguing feature of cold inducible proteins in *E. coli* is the presence of a 159-nucleotide untranslated region (UTR) at the 5' end of their mRNA which includes a highly conserved cold box motif that has been proposed to be involved in the repression of CspA synthesis during the adaptive phase of the cold-shock response (Fig. 5.1;Jiang

et al., 1996). In an effort to gain further information on the regulatory role of the 5' UTR, two modified *cspA-lacZ* transcriptional gene fusions carrying a deletion in either the 5' terminus or the center of the UTR were generated as described in Materials and Methods (Fig. 5.1). For comparison purposes, the modified promoter regions were subcloned in the pTBGM backbone used for the construction of pCSBG, yielding a set of three isogenic plasmids (Table 5.1): pCSBG (native 159 nt-long UTR), pXCSBG (13 nt-long UTR deletion removing the cold box domain) and pCSBG2D (26 nt-long deletion in the central region of the UTR).

In CSH142(pCSBG) transformants grown at 42°C, β-galactosidase synthesis from the native *cspA* promoter was well repressed at all times as judged by both activity experiments (Fig. 5.2A, ◊) and SDS-PAGE analysis (Fig. 5.2B). Upon transfer of mid-exponential phase cultures to 15°C, the enzymatic activity present in clarified cell extracts rapidly increased from about 70 to 7,000 U in the first 2 h following temperature downshift. Thereafter, β-galactosidase synthesis stopped and the enzymatic activity remained at a constant value of about 6,000 U (Fig. 5.2A, ●). This efficient repression at high temperature and low temperature induction-repression behavior closely parallels that of native CspA (see Section 1.6) and suggests that the *cspA-lacZ* gene fusion is subject to the same regulatory mechanisms as the authentic major cold-shock protein.

In cells harboring pXCSBG, the basal level of β-galactosidase activity at 42°C were 25-to-30 fold higher compared to pCSBG transformants at early time points (Fig. 5.3A). SDS-PAGE analysis confirmed that deletion of the cold-box led to leaky β-galactosidase expression at high temperatures (Fig. 5.3B). As incubation at 42°C was pursued (Fig. 5.3A), the enzymatic activity present in clarified cell extracts decreased monotonously, eventually reaching a constant value of 700 U that was about 14-fold higher than that present in pCSBG transformants at the same time point. In addition to

exhibiting defective repression of *lacZ* expression at 42°C, CSH142(pXCSBG) cells did not rapidly accumulate high levels of β-galactosidase when transferred to 15°C (Fig. 5.3, ●). In fact, 5 h after temperature downshift, the activity level in pXCSBG transformants was 2,500 U, corresponding to a 1.5-fold induction ratio relative to the 0 h time point. Similar experiments performed with CSH142(pCSBG2D) showed that deleting the central domain of the UTR had an intermediate effect on the regulation of *lacZ* expression from the *cspA* promoter. At 42°C, about 325 U of β-galactosidase activity were present at all times in pCSBG2D transformants (Fig. 5.4A, ◊) indicating that repression of *lacZ* expression at high temperature was somewhat compromised. However, the same cells exhibited a more pronounced induction upon temperature downshift relative to pXCSBG transformants (Fig. 5.4A (●) and 5.4B) despite the fact that the induction ratio between 0 and 5 h was only about 3-fold compared to 80-fold for cells harboring pCSBG.

5.3.2 Overproduction of a 69-nt long UTR region does not influence *cspA-lacZ* regulation

The above results suggest that the sequence and/or structure of the UTR play a key role in repressing the synthesis of proteins placed under transcriptional control of the *cspA* promoter at high temperatures, as well as in mediating their effecient production upon temperature downshift. Since, Jiang and coworkers (1996) have also shown that overproducing portions of the UTR RNA containing the cold box domain leads to the sustained synthesis of CspA at low temperature, the UTR also appears to be involved in low temperature repression. To determine how overproduction of the UTR would influence the regulation of *cspA-lacZ* gene fusions, pUTRf, a high copy number derivative of pUC19 encoding the *cspA* promoter through base +69 of the UTR and followed by the native *cspA* transcription terminator was constructed by PCR (see

Materials in Methods). To avoid incompatibility problems, the authentic *cspA-lacZ* gene fusion was excised from pCSBG and subcloned into pACYC184 to yield pACCSBG (Table 5.1). CSH142 cells transformed with pACCSBG and either pUTRf or the control vector pUC18 were grown to mid-exponential phase at 42°C and either held at this temperature or transferred to 15°C. Fig. 5.5 shows that the presence of pUTRf did not affect either the repression of *lacZ* synthesis at high temperatures or its induction upon temperature downshift. Nevertheless, while the β-galactosidase activity in CSH142(pCSBG) cultures declined to about 2,000 U after 26 h incubation at 15°C, pACCSBG/pUTRf co-transformants contained more than 25,000 U of activity 20 h after temperature downshift (Fig. 5.5 ●). While this result is consistant with the report of Jiang *et al.* (1996) indicating that UTR overproduction abolishes the low temperature repression mechanism, a comparable trend was observed in cells harboring the control vector pUC18 (Fig. 5.5. ■).

5.4. Discussion

Jiang *et al.* (1996) have proposed that the cold box domain present in the 5' UTR of the *cspA* mRNA binds a low-temperature induced repressor molecule, thereby preventing CspA synthesis following prolonged incubation at low temperatures. These conclusions are based on the fact that transcription of the first 25 bp of the UTR encoded on a high copy number plasmid, p2JTEK, is sufficient to lead to the constitutive synthesis of chromosomal CspA at low temperature and to increase the length of the growth lag phase (Jiang *et al.*, 1996).

To exploit this observation for the sustained production of heterologous proteins placed under *cspA* transcriptional control, we first attempted to increase the gene dosage of the *cspA-lacZ* fusion by transferring it from a pBR322 backbone (15-20 copies/cell) to a pUC18 backbone (500-700 copies/cell). In doing so, we expected to increase the

intracellular concentration of the *cspA* mRNA UTR (including the cold box) and therefore to titrate out the cellular factor involved in repression at low temperatures. However, when transformed with the high copy number plasmid (pUCCSBG), CSH142 cells were found to (i) exhibit high levels of β-galactosidase activity at 42°C and (ii) rapidly lose their ability to synthesize β-galactosidase when cycled between high and low temperatures, presumably as a result of suppressor mutations. We therefore concluded that high intracellular concentrations of the *cspA* UTR are deleterious to *E. coli*, possibly by titrating RNase E or other RNases involved in the degradation of the mRNA under physiological conditions (Babitzke *et al.*, 1993).

We next investigated the effect of co-overexpressing mRNA encoding the first 61 nt of the *cspA* UTR on β-galactosidase synthesis from a *cspA-lacZ* fusion borne by a pACYC184 derivative (10-12 copies/cell). As expected, co-overproduction of the truncated UTR did not affect the repression of *lacZ* synthesis at high temperature or its induction upon temperature downshift relative to the pUC18 control cells (Fig. 5.8A and B). At late time points, continuous synthesis of β-galactosidase was observed in both pUTRf and pUC18 co-transformants. While this behavior was anticipated in cells overexpressing the UTR fragment, it was totally unexpected in the control strain. Clearly, the gene dosage of *cspA-lacZ* has a profound effect on the low temperature repression of *lacZ* synthesis. Folding simulations indicate that the UTR RNA encoded by pUTRf and pJTEK display a similar structure in the vacinity of the cold box relative to the authentic *cspA* UTR (Fig. 5.6 and 5.7; Jiang *et al.*, 1996). The lack of influence of overexpressing the UTR RNA may therefore be related to its unstability since we did not observe an increase in lag-phase at low temperatures in CSH142 cells harboring pUTRf. Further studies will be necessary to clarify these results.

The cold box domain of the *cspA* UTR appears to be directly or indirectly responsible for interacting with a putative cold-shock induced repressor molecule,

which leads to repression of *cspA* synthesis upon prolonged exposure to low temperature (Jiang *et al.*, 1996). We therefore hypothesized that deleting this region may lead to a more pronounced and/or longer induction of β-galactosidase synthesis following transfer of mid-exponentially cells to low temperatures. However, deletion of the cold box domain increased the basal level of β-galactosidase activity at 42°C by 30-fold and reduced the magnitude of the cold-shock induction by more than 50-fold (Fig 5.2 and 5.3). A random 26-nt-long deletion centered within the 5' UTR led to similar, but marginally less severe results with a 5-fold higher level of β-galactosidase activity prior to temperature downshift, and a 25-fold lower induction compared to the native *cspA-lacZ* fusion (Fig. 5.2 and 5.4). These results highlight the fact that the *cspA* UTR plays a fundamental role not only in low temperature repression, but also in high temperature repression and cold-induction of proteins placed under *cspA* promoter control.

It was recently shown that a 3 base substitution (arrows in Fig. 5.7A and B) near the Shine-Delgarno sequence of the *cspA* mRNA leads to a 75-fold stabilization of the transcript at 37°C and suggested that decreased degradation by RNase E due to structural changes was responsible for this repression (Fang *et al.*, 1997). To determine if the higher levels of β-galactosidase activity present in cells harboring *cspA* deletion constructs and grown at 42°C could be a similarly explained, the secondary structure of native and mutant UTR RNAs was predicted at 15 and 42°C using the method of Zuker *et al.* (1989).

Fig. 5.9A shows that the internal 26 bp deletion leads to significant secondary structure in the putative RNase E recognition sequence, while it is fully exposed in the native UTR structure (Fig. 5.7A). At 15°C, however, the predicted structure of this region remains the same in the deletion mutant whereas the native UTR gains significant secondary structure in its RNase E recognition domain. We speculate that the increased

secondary structure observed at 42°C in the central deletion mutant stabilizes the RNA and leads to leaky production of downstream genes at high temperature. Because it includes the Shine-Delgarno sequence, the presence of the same structure at 15°C is likely to lead to inefficient translation initiation and thus to low-induction upon temperature downshift. Fig. 5.7 and 5.8 show that the secondary structure of the putative RNase E recognition site is the same in both the native UTR and the cold box deletion mutant at 42°C. Since there 30-fold increase in the basal levels of β-galactosidase activity with the latter construct, we speculate that other RNase recognition sites are obscured in the cold box deleted UTR at 42°C due to overall variations in the secondary structure (compare Fig. 5.7 and 5.8). Alternatively, the mutant mRNA may be more efficiently translated at 42°C. From inspection of the secondary structure we cannot ascertain why the cold box deletion lead to virtually no induction of β-galactosidase upon temperature downshift.

Table 5.1. Plasmids used in Chapter 5.

Plasmid	Description[a]	Source or Reference
Cloning vectors and plasmids		
pACYC184	Low copy number cloning vector bearing a p15A origin of replication (Amp^r, Tet^r)	(Chang and Cohen, 1978)
pTG10	pACYC184 derived cloning vector (Chl^r)	(Goloubinoff *et al.*, 1989)
pT7Blue	High copy number pUC19 derivative bearing a ColE1 origin of replication (Amp^r)	Novagen
pUC18	High copy number cloning vector bearing a ColE1 origin of replication (Amp^r)	(Yanisch Perron *et al.*, 1985)
pTBG	pBR322 derivative encoding *lacZ* under *tac* promoter control (Amp^r)	(Lee *et al.*, 1990)
pTBGM	pTBG derivative encoding a promoterless *lacZ* gene (Amp^r)	Chapter 2
Plasmids encoding native or modified versions of the cspA *promoter*		
pJJG02	pUC19 derivative encoding the complete *cspA* gene (Amp^r)	(Goldstein *et al.*, 1990)
PT7CS	pT7Blue derivative encoding a PCR-amplified *cspA* promoter from pJJG02 (Amp^r)	Chapter 2
pACCS	pACYC184 derivative encoding the *cspA* promoter and containing a unique *Apa*LI site in the 5' UTR of the promoter (Chl^r)	This Chapter
pACCS2D	pACCS derivative containing a 26 bp deletion in the 5' UTR of the *cspA* promoter centered around the *Apa*LI site (Chl^r)	This Chapter
pUP2	pT7Blue derivative encoding a PCR-amplified *cspA* promoter fragment upstream of the cold box (Amp^r)	This Chapter
pDN2	pT7Blue derivative encoding a PCR-amplified *cspA* promoter fragment downstream of the cold box (Amp^r)	This Chapter

Table 5.1. Continued

Plasmid	Description[a]	Source or Reference
pUTR	pT7Blue derivative encoding the *cspA* promoter and the first half of the UTR followed by the native *cspA* transcription terminator (Amp^r)	This Chapter
pUTRf	pUTR derivative lacking its upstream *lac* promoter (Amp^r)	This Chapter
Plasmids encoding native or modified cspA-lacZ *gene fusions*		
pCSBG	pTBGM derivative encoding *lacZ* under *cspA* promoter control (Amp^r)	Chapter 2
pACCSBG	pTG10 derivative encoding *lacZ* under *cspA* promoter control (Chl^r).	This Chapter
pUCCSBG	pUC18 derivative encoding *lacZ* under *cspA* promoter control (Amp^r)	This Chapter
pCSBG2D	pTBGM derivative encoding *lacZ* under control of the modified *cspA* promoter from pACCS2D (Amp^r)	This Chapter
pXCSBG	pTBGM derivative encoding *lacZ* under control of a modified *cspA* promoter lacking the cold box region (Amp^r)	This Chapter

[a]. Amp^r, Ampicillin resistant; Chl^r, Chloramphenicol resistant; Tet^r, Tetracycline resistant.

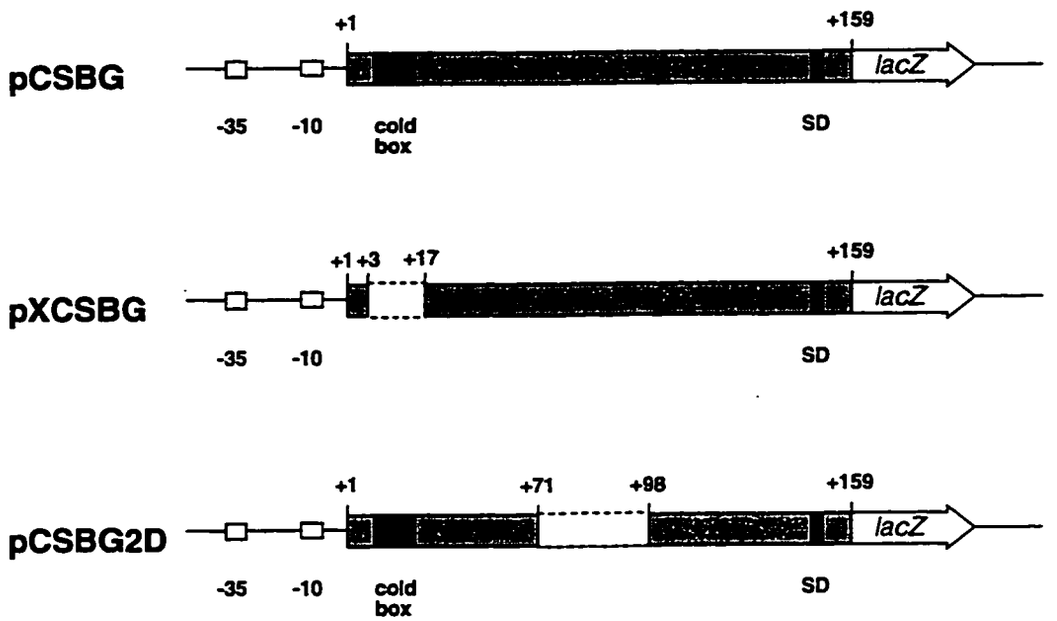

Figure 5.1. Location of deletions in the 5' UTR of *cspA-lacZ* transcriptional gene fusions. Plasmid pCSBG contains the native *cspA* promoter with its authentic 159 bp-long 5' UTR fused to the *lacZ* gene. Plasmid pXCSBG is isogenic to pCSBG but contains a 13 bp deletion near the 5' end of the UTR which removes the cold box region (Jiang *et al.*, 1996). As a result of construction constraints, the UTR contains C → A and A → G mutations at positions +19 and +23 respectively. Plasmid pCSBG2D is isogenic to pCSBG and contains a 26 bp-long deletion in the central region of the UTR. The -35 and -10 boxes and the locations of the cold box and the Shine-Delgarno sequence (SD) are shown. The transcription starting point is denoted by +1 and deletions are indicated by dashed white boxes.

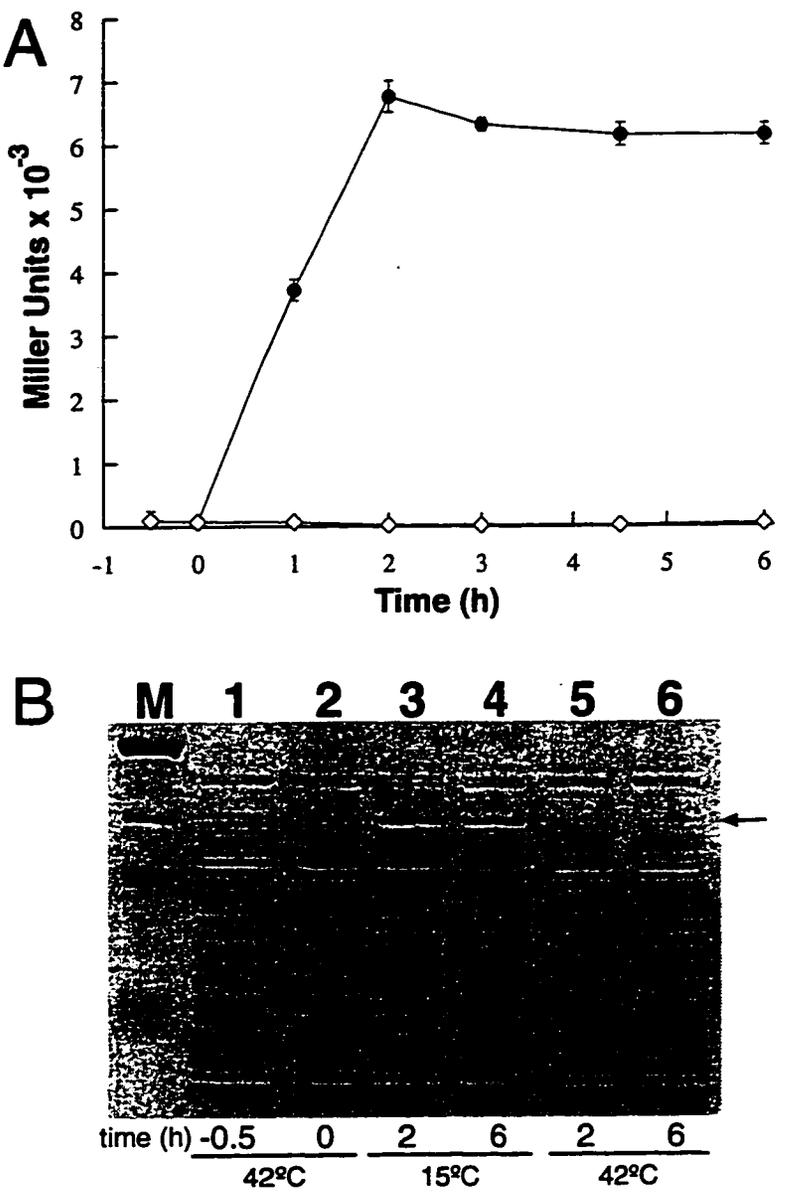

Figure 5.2. Expression of β-galactosidase from the native *cspA* promoter. CSH142 cells harboring pCSBG were grown to mid-exponential phase at 42°C and either shifted to 15°C (●) or held at 42°C (◊) at time zero. **Panel A**: The enzymatic activity in clarified cell extracts was determined at the indicated time points. **Panel B**: Whole cells samples collected at the indicated times and temperatures were fractionated by SDS-PAGE. Lane M contains prestained molecular mass markers (BioRad): 205, 116.5, 89, and 49.5 kDa from top to bottom. The position of β-galactosidase is indicated by an arrow.

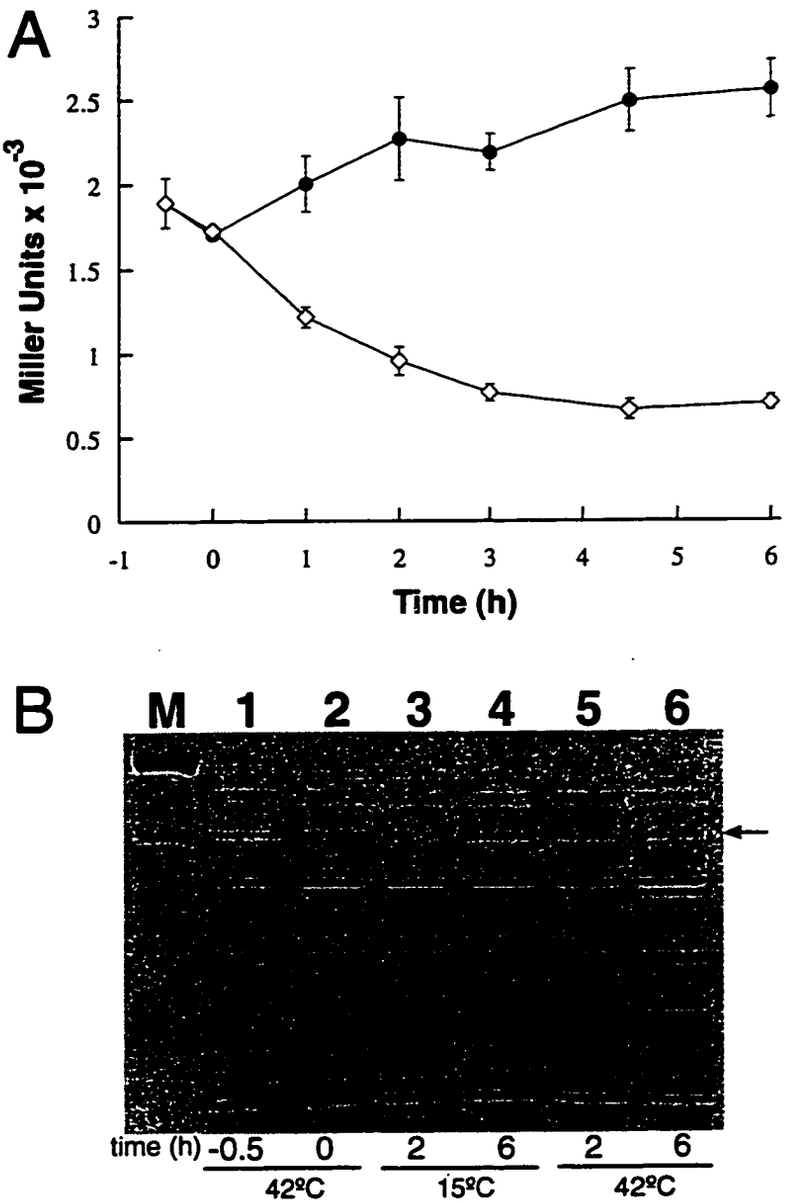

Figure 5.3. Expression of β-galactosidase from a modified *cspA* promoter lacking the UTR cold box region. CSH142 cells harboring pXCSBG were grown to mid-exponential phase at 42°C and either shifted to 15°C (●) or held at 42°C (◊) at time zero. **Panel A**: The enzymatic activity in clarified cell extracts was determined at the indicated time points. **Panel B**: Whole cells samples collected at the indicated times and temperatures were fractionated by SDS-PAGE. Lane M contains prestained molecular mass markers (BioRad): 205, 116.5, 89, and 49.5 kDa from top to bottom. The position of β-galactosidase is indicated by an arrow.

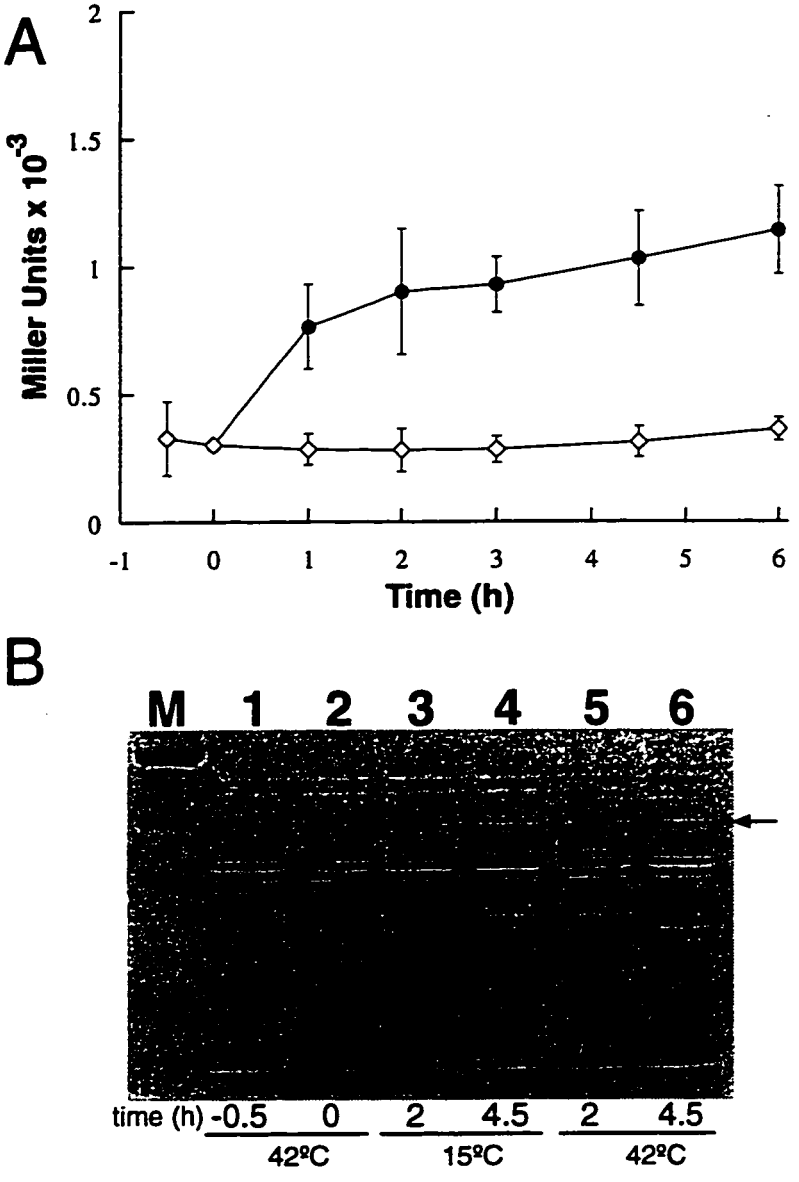

Figure 5.4. Expression of β-galactosidase from a modified *cspA* promoter containing a 26 bp-long deletion in the central region of the UTR. CSH142 cells harboring pCSBG2D were grown to mid-exponential phase at 42°C and either shifted to 15°C (●) or held at 42°C (◊) at time zero. **Panel A**: The enzymatic activity in clarified cell extracts was determined at the indicated time points. **Panel B**: Whole cells samples collected at the indicated times and temperatures were fractionated by SDS-PAGE. Lane M contains prestained molecular mass markers (BioRad): 205, 116.5, 89, and 49.5 kDa from top to bottom. The position of β-galactosidase is indicated by an arrow.

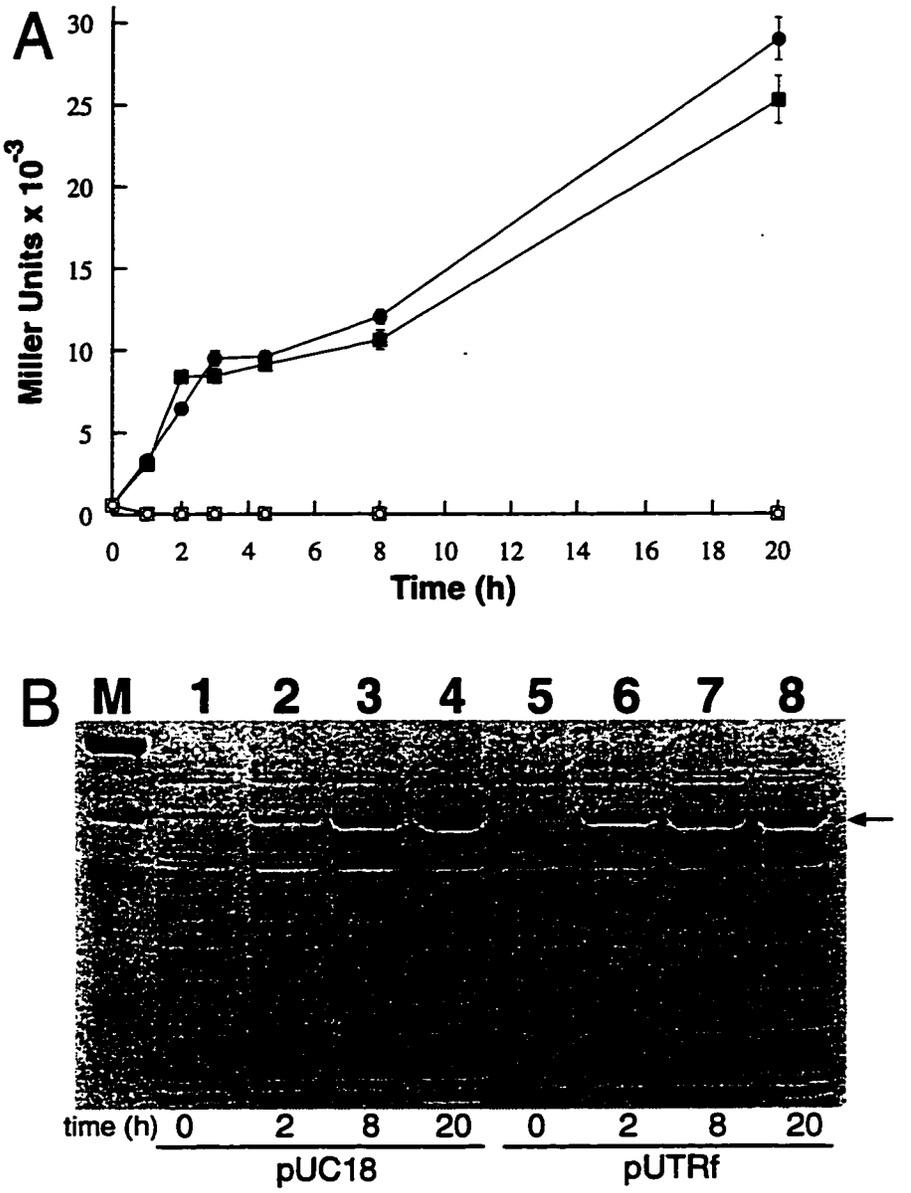

Figure 5.5. Co-overexpression of plasmid-encoded RNA encoding the cold box region does not affect β-galactosidase synthesis from the *cspA* promoter. CSH142 cells harboring pACCSBG and either pUTRf (●, ○) or the control plasmid pUC18 (■, □) were grown to mid-exponential phase at 42°C and either shifted to either 15°C (filled symbols) or maintained at 42°C (open symbols) at time zero. **Panel A**: The enzymatic activity in clarified cell extracts was determined at the indicated time points. **Panel B**: Whole cells samples collected at the indicated times and temperatures were fractionated by SDS-PAGE. Lane M contains prestained molecular mass markers (BioRad): 205, 116.5, 89, and 49.5 kDa from top to bottom. The position of β-galactosidase is indicated by an arrow.

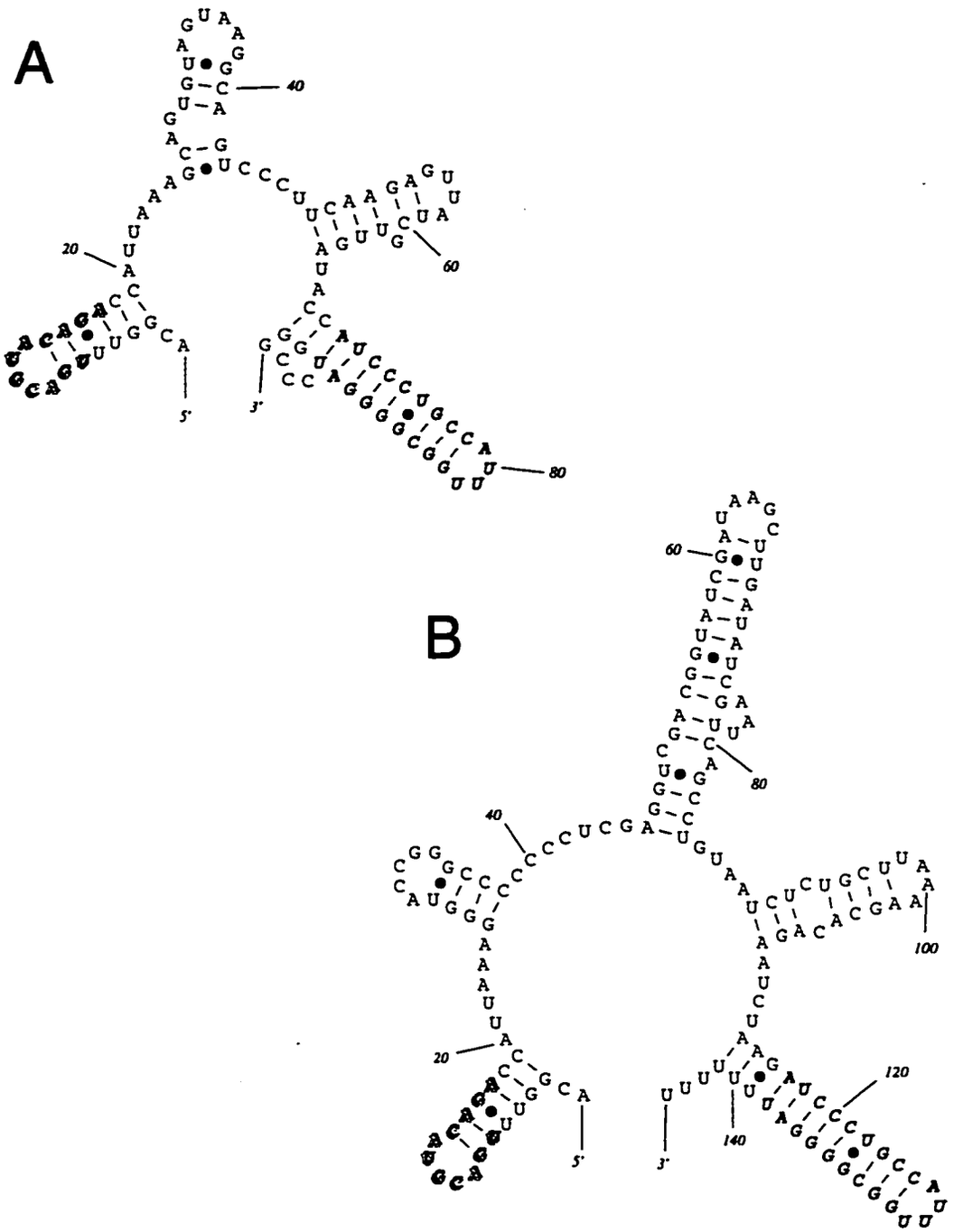

Figure 5.6. Predicted secondary structures of the truncated *cspA* UTR RNA and transcription terminator encoded by plasmids pUTRf (**Panel A**) and p2JTEK (**Panel B**) at 15°C. The nucleotides of the cold box and transcription terminator are shadowed and bold-italics typeface, respectively. The cold box and transcription terminator of each maintained the same structure at 42°C (data not shown). Simulations were performed by the method of Zuker *et al.* (1989) using the program Mufold.

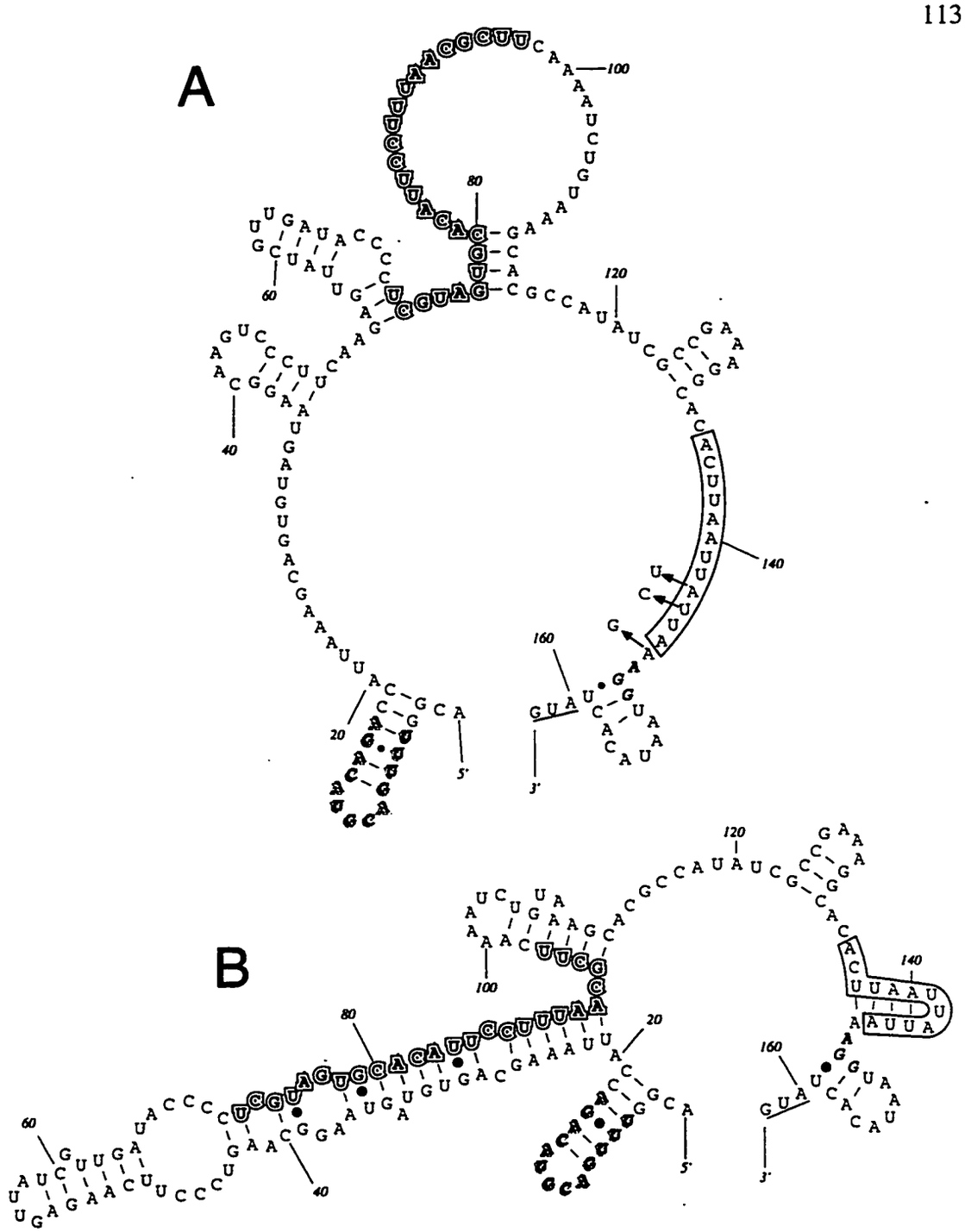

Figure 5.7. Predicted secondary structures of the authentic *cspA* UTR encoded by pCSBG at 42°C (**Panel A**) and 15°C (**Panel B**). The nucleotides deleted in plasmids pXCSBG (cold box deletion) and pCSBG2D (central deletion) are shadowed and outlined, respectively. The proposed RNase E cleavage region (Fang *et al.*, 1997) is boxed, the Shine-Delgarno sequence is shown in bold-italics typeface while the start codon is underlined. Simulations were performed by the method of Zuker *et al.* (1989) using the program Mufold.

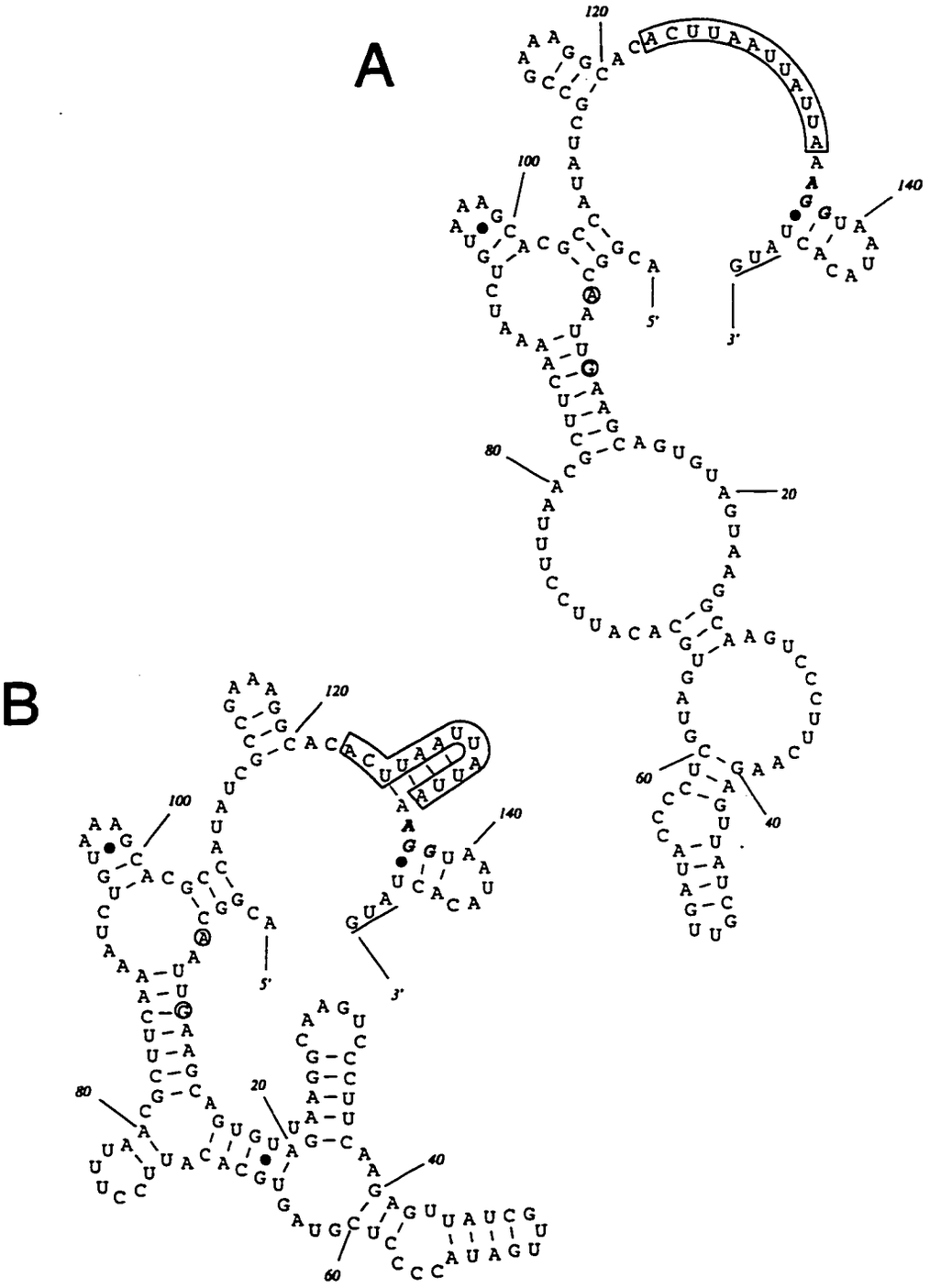

Figure 5.8. Predicted secondary structures of the cold box deleted *cspA* UTR RNA encoded by pXCSBG at 42°C (**Panel A**) and 15°C (**Panel B**). The proposed RNase E cleavage region (Fang *et al.*, 1997) is boxed, the Shine-Delgarno sequence is shown in bold-italics typeface while the start codon is underlined. Simulations were performed by the method of Zuker *et al.* (1989) using the program Mufold.

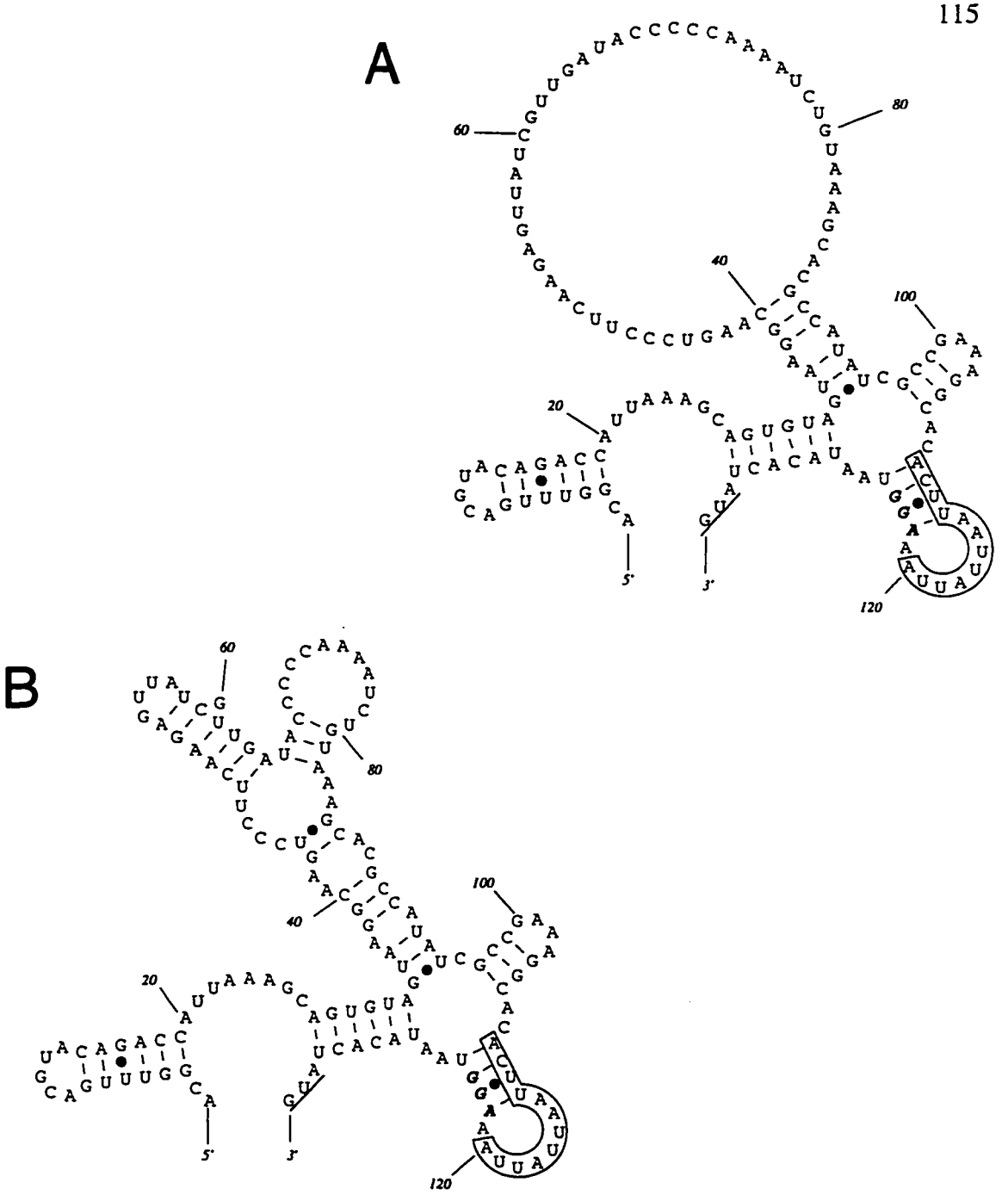

Figure 5.9. Predicted secondary structures of the centrally deleted *cspA* UTR RNA encoded by pCSBG2D at 42°C (**Panel A**) and 15°C (**Panel B**). The proposed RNase E cleavage region (Fang *et al.*, 1997) is boxed, the Shine-Delgarno sequence is shown in bold-italics typeface while the start codon is underlined. Simulations were performed by the method of Zuker *et al.* (1989) using the program Mufold.

References

Amann, E. and Brosius, J. (1985) "ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* **40**, 183-190.

Amann, E., Brosius, J. and Ptashne, M. (1983) Vectors bearing a hybrid *trp-lac* promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene* **25**, 167-178.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

Av Gay, Y., Aharonowitz, Y. and Cohen, G. (1992) contain a 7.0 kDa cold shock like protein. *Nucleic Acids Res* **20**, 5478.

Babbitt, P.C., West, B.L., Buechter, D.D., Kuntz, I.D. and Kenyon, G.L. (1990) Removal of a proteolytic activity associated with aggregates formed from expression of creatine kinase in *Escherichia coli* leads to improved recovery of active enzyme. *Biotechnology N Y* **8**, 945-949.

Babitzke, P., Granger, L., Olszewski, J. and Kushner, S.R. (1993) Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *J Bacteriol* **175**, 229-239.

Baneyx (submitted) *In vivo* Folding of Recombinant Proteins in *Escherichia coli*. In J. E. Davies, A. L. Demain, G. Cohen, C. L. Hershberger, L. J. Forney, I. B. Holland, W. S. Hu and J.-H. D. Wu (eds.), *Manual of Microbiology and Biotechnology*. ASM Press.

Baneyx, F., Ayling, A., Palumbo, T., Thomas, D. and Georgiou, G. (1991) Optimization of growth conditions for the production of proteolytically-sensitive proteins in the periplasmic space of *Escherichia coli*. *Appl Microbiol Biotechnol* **36**, 14-20.

Baneyx, F. and Georgiou, G. (1990) *In vivo* degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J Bacteriol* **172**, 491-494.

Baneyx, F. and Georgiou, G. (1991) Construction and characterization of *Escherichia coli* strains deficient in multiple secreted proteases: protease III degrades high-molecular-weight substrates *in vivo*. *J Bacteriol* **173**, 2696-2703.

Baneyx, F. and Georgiou, G. (1992) Expression of proteolytically sensitive polypeptides in *Escherichia coli*. In T. J. Ahern and M. C. Manning (eds.), *Stability of Protein Pharmaceuticals, Part I*. Plenun Press, New York, pp. 69-108.

Beckwith, J. (1987) The lactose operon. In F. C. Neidhardt (ed.) *Escherichia coli and Salomonella typhimurium Cellular and Molecular Biology*. ASM Press, Washington, D.C., Vol. 1, pp. 1444-1452.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L. and Boyer, H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95-113.

Brandi, A., Pietroni, P., Gualerzi, C.O. and Pon, C.L. (1996) Post-transcriptional regulation of CspA expression in *Escherichia coli*. *Molecular Microbiology* **19**, 231-240.

Brandi, A., Pon, C.L. and Gualerzi, C.O. (1994) Interaction of the main cold shock protein CS7.4 (CspA) of *Escherichia coli* with the promoter region of *hns*. *Biochimie* **76**, 1090-1098.

Broeze, R.J., Solomon, C.J. and Pope, D.H. (1978) Effects of low temperature on *in vivo* and *in vitro* protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. *J Bacteriol* **134**, 861-874.

Browner, M.F., Rasor, P., Tugendreich, S. and Fletterick, R.J. (1991) Temperature-sensitive production of rabbit muscle glycogen phosphorylase in *Escherichia coli*. *Protein Eng* **4**, 351-357.

Cabilly, S. (1989) Growth at sub-optimal temperatures allows the production of functional, antigen-binding Fab fragments in *Escherichia coli*. *Gene* **85**, 553-557.

Caspers, P., Stieger, M. and Burn, P. (1994) Overproduction of bacterial chaperones improves the solubility of recombinant protein tyrosine kinases in *Escherichia coli*. *Cell Mol Biol* **40**, 635-644.

Chalmers, J.J., Kim, E., Telford, J.N., Wong, E.Y., Tacon, W.C., Shuler, M.L. and Wilson, D.B. (1990) Effects of temperature on *Escherichia coli* overproducing β-lactamase or epidermal growth factor. *Appl Environ Microbiol* **56**, 104-111.

Chang, A.C. and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134**, 1141-1156.

Chrunyk, B.A., Evans, J., Lillquist, J., Young, P. and Wetzel, R. (1993) Inclusion body formation and protein stability in sequence variants of interleukin-1 beta. *J Biol Chem* **268**, 18053-18061.

Dale, G.E., Schönfeld, H.J., Langen, H. and Stieger, M. (1994) Increased solubility of trimethoprim-resistant type S1 DHFR from *Staphylococcus aureus* in *Escherichia coli* cells overproducing the chaperonins GroEL and GroES. *Protein Eng* **7**, 925-931.

Dammel, C.S. and Noller, H.F. (1995) Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. *Genes Dev* **9**, 626-637.

de Boer, H.A., Comstock, L.J. and Vasser, M. (1983) The *tac* promoter: a functional hybrid derived from the trp and lac promoters. *Proc Natl Acad Sci U S A* **80**, 21-25.

Dong, H., Nilsson, L. and Kurland, C.G. (1995) Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J Bacteriol* **177**, 1497-1504.

Dreyfuss, G., Matunis, M.J., Pinol Roma, S. and Burd, C.G. (1993) hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem* **62**, 289-321.

Ellis, R.J. and van der Vies, S.M. (1991) Molecular chaperones. *Annu Rev Biochem* **60**, 321-347.

Escher, A., O'Kane, D.J., Lee, J. and Szalay, A.A. (1989) Bacterial luciferase alpha beta fusion protein is fully active as a monomer and highly sensitive *in vivo* to elevated temperature. *Proc Natl Acad Sci U S A* **86**, 6528-6532.

Etchegaray, J.P., Jones, P.G. and Inouye, M. (1996) Differential thermoregulation of two highly homologous cold-shock genes, *cspA* and *cspB*, of *Escherichia coli. Genes to Cells* **1**, 171-178.

Ezaz Nikpay, K., Uchino, K., Lerner, R.E. and Verdine, G.L. (1994) Construction of an overproduction vector containing the novel *srp* (sterically repressed) promoter. *Protein Sci* **3**, 132-138.

Fang, L., Jiang, W.N., Bae, W.H. and Inouye, M. (1997) Promoter-independent cold-shock induction of *cspA* and its derepression at 37°C by mRNA stabilization. *Molecular Microbiology* **23**, 355-364.

Georgiou, G. (1995) Expression of proteins in bacteria. In J. L. Cleland and C. S. Craik (eds.), *Principles and Practice of Protein Engineering*. Butterworths, New York, pp. 101-127.

Georgiou, G. and Bowden, G.A. (1990) Inclusion body formation and the recovery of aggregated recombinant proteins. In A. Prokop, R. K. Bajpai and C. Ho (eds.), *Recombinant DNA Technology and Applications*. McGraw-Hill, New York, pp. 333-356.

Georgiou, G. and Valax, P. (1996) Expression of correctly folded proteins in *Escherichia coli. Curr Opin Biotechnol* **7**, 190-197.

Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355**, 33-45.

Giladi, H., Goldenberg, D., Koby, S. and Oppenheim, A.B. (1995) Enhanced activity of the bacteriophage lambda PL promoter at low temperature. *Proc Natl Acad Sci U S A* **92**, 2184-2188.

Giladi, H., Gottesman, M. and Oppenheim, A.B. (1990) Integration host factor stimulates the phage lambda pL promoter. *J Mol Biol* **213**, 109-121.

Gilbert, D.G. loopDloop, a Macintosh program for visualizing RNA secondary structure. 1992.

Goeddel, D.V., Emr, S.D., Gold, L., Henner, D.J. and Levinson, A.D., eds. (1991). *Gene Expression Technology. Methods in Enzymology, Vol. 185.* Academic Press,

Goldenberg, D., Azar, I. and Oppenheim, A.B. (1996) Differential mRNA stability of the *cspA* gene in the cold-shock response of *Escherichia coli. Molecular Microbiology* **19**, 241-248.

Goldschmidt, R. (1970) *In vivo* degradation of nonsense fragments in *E. coli. Nature* **228**, 1151-1154.

Goldstein, J., Pollitt, N.S. and Inouye, M. (1990) Major cold shock protein of *Escherichia coli. Proc Natl Acad Sci USA* **87**, 283-287.

Goloubinoff, P., Gatenby, A.A. and Lorimer, G.H. (1989) GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in *Escherichia coli. Nature* **337**, 44-47.

Graumann, P. and Marahiel, M.A. (1996) Some like it cold: Response of microorganisms to cold shock. *Archives Of Microbiology* **166**, 293-300.

Hawley, D.K. and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* **11**, 2237-2255.

Hockney, R.C. (1994) Recent developments in heterologous protein production in *Escherichia coli. Trends Biotechnol* **12**, 456-463.

Iuchi, S. and Lin, E.C. (1991) Adaptation of *Escherichia coli* to respiratory conditions: regulation of gene expression. *Cell* **66**, 5-7.

Jacobson, R.H., Zhang, X.-J., DuBose, R.F. and Matthews, B.W. (1994) Three-dimensional structure of β-galactosidase from *E. coli. Nature* **369**, 761-766.

Jiang, W., Fang, L. and Inouye, M. (1996) The role of the 5'-end untranslated region of the mRNA for CspA, the major cold-shock protein of *Escherichia coli*, in cold-shock adaptation. *J Bacteriol* **178**, 4919-4925.

Jiang, W., Jones, P. and Inouye, M. (1993) Chloramphenicol induces the transcription of the major cold shock gene of *Escherichia coli, cspA. J Bacteriol* **175**, 5824-5828.

Jiang, W.N., Hou, Y. and Inouye, M. (1997) CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. *Journal Of Biological Chemistry* **272**, 196-202.

Jones, P.G., Cashel, M., Glaser, G. and Neidhardt, F.C. (1992a) Function of a relaxed-like state following temperature downshifts in *Escherichia coli. J Bacteriol* **174**, 3903-3914.

Jones, P.G. and Inouye, M. (1994) The cold-shock response--a hot topic. *Mol Microbiol* **11**, 811-818.

Jones, P.G. and Inouye, M. (1996) RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. *Molecular Microbiology* **21**, 1207-1218.

Jones, P.G., Krah, R., Tafuri, S.R. and Wolffe, A.P. (1992b) DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli. J Bacteriol* **174**, 5798-5802.

Jones, P.G., Mitta, M., Kim, Y., Jiang, W. and Inouye, M. (1996) Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli. Proc Natl Acad Sci U S A* **93**, 76-80.

Jones, P.G., VanBogelen, R.A. and Neidhardt, F.C. (1987) Induction of proteins in response to low temperature in *Escherichia coli. J Bacteriol* **169**, 2092-2095.

Kalbach, C.E. and Gatenby, A.A. (1993) Stable expression plasmid for high-level production of GroE molecular chaperones in large-scale cultures. *Enzyme Microb Technol* **15**, 730-735.

Kandror, O. and Goldberg, A.L. (1997) Trigger factor is induced upon cold shock and enhances viability of *Escherichia coli* at low temperatures. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **94**, 4978-4981.

Khosla, C. and Bailey, J.E. (1989) Characterization of the oxygen-dependent promoter of the *Vitreoscilla* hemoglobin gene in *Escherichia coli. J Bacteriol* **171**, 5990-6004.

Khosla, C., Curtis, J.E., Bydalek, P., Swartz, J.R. and Bailey, J.E. (1990) Expression of recombinant proteins in *Escherichia coli* using an oxygen-responsive promoter. *Biotechnology N Y* **8**, 554-558.

Kopetzki, E., Schumacher, G. and Buckel, P. (1989) Control of formation of active soluble or inactive insoluble baker's yeast α-glucosidase PI in *Escherichia coli* by induction and growth conditions. *Mol Gen Genet* **216**, 149-155.

La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C.L. and Gualerzi, C.O. (1991) Identification of a cold shock transcriptional activator of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc Natl Acad Sci USA* **88**, 10907-10911.

LaVallie, E.R. and McCoy, J.M. (1995) Gene fusion expression systems in *Escherichia coli. Curr Opin Biotechnol* **6**, 501-506.

Lee, J., Cho, M.H. and Lee, J. (1996) Characterization of an oxygen-dependent inducible promoter system, the nar promoter, and *Escherichia coli* with an inactivated nar operon. *Biotechnology And Bioengineering* **52**, 572-578.

Lee, S.C., Choi, Y.C. and Yu, M.H. (1990) Effect of the N-terminal hydrophobic sequence of hepatitis B virus surface antigen on the folding and assembly of hybrid β-galactosidase in *Escherichia coli. Eur J Biochem* **187**, 417-424.

Lee, S.J., Xie, A., Jiang, W., Etchegaray, J.P., Jones, P.G. and Inouye, M. (1994) Family of the major cold-shock protein, CspA (CS7.4), of *Escherichia coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. *Mol Microbiol* **11**, 833-839.

Lelivelt, M.J. and Kawula, T.H. (1995) Hsc66, an Hsp70 homolog in *Escherichia coli*, is induced by cold shock but not by heat shock. *J Bacteriol* **177**, 4900-4907.

Liao, H.H. (1991) Effect of temperature on the expression of wild-type and thermostable mutants of kanamycin nucleotidyltransferase in *Escherichia coli*. *Protein Expr Purif* **2**, 43-50.

Lund, E. and Kjeldgaard, N.O. (1972) Protein synthesis and formation of guanosinetetraphosphate. *FEBS Lett* **26**, 306-310.

Maurizi, M.R. (1992) Proteases and protein degradation in *Escherichia coli*. *Experientia* **48**, 178-201.

McDonald, J.R., Ong, M., Shen, C., Parandoosh, Z., Sosnowski, B., Bussell, S. and Houston, L.L. (1996) Large-scale purification and characterization of recombinant fibroblast growth factor-saporin mitotoxin. *Protein Expression And Purification* **8**, 97-108.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mitraki, A. and King, J. (1989) Protein folding intermediates and inclusion body formation. *Bio/Technology* **7**, 690-697.

Mitraki, A. and King, J. (1992) Amino acid substitutions influencing intracellular protein folding pathways. *FEBS Lett* **307**, 20-25.

Nagai, H., Yuzawa, H. and Yura, T. (1991) Interplay of two cis-acting mRNA regions in translational control of sigma 32 synthesis during the heat shock response of *Escherichia coli*. *Proc Natl Acad Sci U S A* **88**, 10515-10519.

Nakashima, K., Kanamaru, K., Mizuno, T. and Horikoshi, K. (1996) A novel member of the *cspA* family of genes that is induced by cold shock in *Escherichia coli*. *J Bacteriol* **178**, 2994-2997.

Newkirk, K., Feng, W., Jiang, W., Tejero, R., Emerson, S.D., Inouye, M. and Montelione, G.T. (1994) Solution NMR structure of the major cold shock protein (CspA) from *Escherichia coli*: identification of a binding epitope for DNA. *Proc Natl Acad Sci U S A* **91**, 5114-5118.

Pao, C.C. and Dyess, B.T. (1981) Stringent control of RNA synthesis in the absence of guanosine 5'-diphosphate-3'-diphosphate. *J Biol Chem* **256**, 2252-2257.

Piatak, M., Lane, J.A., Laird, W., Bjorn, M.J., Wang, A. and Williams, M. (1988) Expression of soluble and fully functional ricin A chain in *Escherichia coli* is temperature-sensitive. *J Biol Chem* **263**, 4837-4843.

Qoronfleh, M.W., Debouck, C. and Keller, J. (1992) Identification and characterization of novel low-temperature-inducible promoters of *Escherichia coli. J Bacteriol* **174**, 7902-7909.

Ranjan, M., Tafuri, S.R. and Wolffe, A.P. (1993) Masking mRNA from translation in somatic cells. *Genes Dev* **7**, 1725-1736.

Record, M.T.J., Reznikoff, W.S., Craig, M.L., McQuade, K.L. and Schlax, P.J. (1996) *Escherichia coli* RNA Polymerase ($E\sigma^{70}$), Promoters, and the Kinetics of the Steps of Transcription Initiation. In F. C. Neidhardt (ed.) *Escherichia coli and Salomonella typhimurium Cellular and Molecular Biology*. ASM Press, Washington, D. C., Vol. 1, pp. 792-821.

San, K.Y., Bennett, G.N., Chou, C.H. and Aristidou, A.A. (1994) An optimization study of a pH-inducible promoter system for high-level recombinant protein production in *Escherichia coli. Ann N Y Acad Sci* **721**, 268-276.

Schein, C.H. and Noteborn, M.H.M. (1988) Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperatures. *Bio/Technology* **6**, 291-294.

Schindelin, H., Jiang, W., Inouye, M. and Heinemann, U. (1994) Crystal structure of CspA, the major cold shock protein of *Escherichia coli. Proc Natl Acad Sci U S A* **91**, 5119-5123.

Shaw, M.K. and Ingraham, J.L. (1967) Synthesis of macromolecules by *Escherichia coli* near the minimal temperature for growth. *J Bacteriol* **94**, 157-164.

Sommerville, J. and Ladomery, M. (1996) Masking of mRNA by Y-box proteins. *Faseb J* **10**, 435-443.

Steczko, J., Donoho, G.A., Dixon, J.E., Sugimoto, T. and Axelrod, B. (1991) Effect of ethanol and low-temperature culture on expression of soybean lipoxygenase L-1 in *Escherichia coli. Protein Expr Purif* **2**, 221-227.

Straus, D.B., Walter, W.A. and Gross, C.A. (1987) The heat shock response of *E. coli* is regulated by changes in the concentration of sigma 32. *Nature* **329**, 348-351.

Studier, F.W. and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113-130.

Sugihara, J. and Baldwin, T.O. (1988) Effects of 3' end deletions from the *Vibrio harveyi luxB* gene on luciferase subunit folding and enzyme assembly: generation of temperature-sensitive polypeptide folding mutants. *Biochemistry* **27**, 2872-2880.

Takagi, H., Morinaga, Y., Tsuchiya, M., Ikemura, H. and Inouye, M. (1988) Control of folding of proteins secreted by a high expression secretion vector, pINIIIompA: 16-fold increase in production of active subtilisin E in *Escherichia coli. Bio/Technology* **6**, 948-950.

Tanabe, H., Goldstein, J., Yang, M. and Inouye, M. (1992) Identification of the promoter region of the *Escherichia coli* major cold shock gene, *cspA*. *J Bacteriol* **174**, 3867-3873.

Taura, T., Kusukawa, N., Yura, T. and Ito, K. (1989) Transient shut off of *Escherichia coli* heat shock protein synthesis upon temperature shift down. *Biochem Biophys Res Commun* **163**, 438-443.

Thomas, J.G. and Baneyx, F. (1996a) Protein folding in the cytoplasm of *Escherichia coli*: requirements for the DnaK-DnaJ-GrpE and GroEL-GroES molecular chaperone machines. *Mol Microbiol* **21**, 1185-1196.

Thomas, J.G. and Baneyx, F. (1996b) Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells overproducing heat-shock proteins. *J Biol Chem* **271**, 11141-11147.

Tolentino, J.G., Meng, S.-Y., Bennett, G.N. and San, K.-Y. (1992) A pH-Regulated Promoter for the Expression of Recombinant Proteins in *Escherichia coli*. *Biotechnology Letters* **14**, 157-162.

van der Vies, S.M., Viitanen, P.V., Gatenby, A.A., Lorimer, G.H. and Jaenicke, R. (1992) Conformational states of ribulosebisphosphate carboxylase and their interaction with chaperonin 60. *Biochemistry* **31**, 3635-3644.

VanBogelen, R.A. and Neidhardt, F.C. (1990) Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc Natl Acad Sci U S A* **87**, 5589-5593.

Vasina, J.A. and Baneyx, F. (1996) Recombinant protein expression at low temperatures under the transcriptional control of the major *Escherichia coli* cold shock promoter *cspA*. *Applied And Environmental Microbiology* **62**, 1444-1447.

Vasina, J.A. and Baneyx, F. (1997) Expression of aggregation-prone recombinant proteins at low temperatures: a comparative study of the *E. coli cspA* and *tac* promoter systems. *Protein Expr Purif* **9**, 211-218.

Walker, G.C. (1987) The SOS Response of *Escherichia coli*. *Escherichia coli and Salomonella typhimurium Cellular and Molecular Biology*. ASM Press, Washington, D.C, Vol. 1, pp. 1346-1357.

Wall, J.G. and Plückthun, A. (1995) Effects of overexpressing folding modulators on the *in vivo* folding of heterologous proteins in *Escherichia coli*. *Curr Opin Biotechnol* **6**, 507-516.

Wetzel, R. and Chrunyk, B.A. (1994) Inclusion body formation by interleukin-1 beta depends on the thermal sensitivity of a folding intermediate. *FEBS Lett* **350**, 245-248.

Wetzel, R., Perry, L.J. and Veilleux, C. (1991) Mutations in human interferon gamma affecting inclusion body formation identified by a general immunochemical screen. *Bio/Technology* **9**, 731-777.

Willimsky, G., Bang, H., Fischer, G. and Marahiel, M.A. (1992) Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J Bacteriol* **174**, 6326-6335.

Xia, G., Manen, D., Yu, Y. and Caro, L. (1993) *In vivo* and *in vitro* studies of a copy number mutation of the RepA replication protein of plasmid pSC101. *J Bacteriol* **175**, 4165-4175.

Yamanaka, K. and Inouye, M. (1997) Growth-phase-dependent expression of *cspD*, encoding a member of the CspA family in *Escherichia coli*. *Journal Of Bacteriology* **179**, 5126-5130.

Yamanaka, K., Mitani, T., Ogura, T., Niki, H. and Hiraga, S. (1994) Cloning, sequencing, and characterization of multicopy suppressors of a *mukB* mutation in *Escherichia coli*. *Mol Microbiol* **13**, 301-312.

Yanisch Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Zuker, M. (1989) On finding all suboptimal foldings of an RNA molecule. *Science* **244**, 48-52.

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Vasina, J. A. and F. Baneyx (1997) Expression of Aggregation-Prone Recombinant Proteins at Low Temperatures: A Comparative Study of the *Escherichia coli cspA* and *tac* Promoter Systems. *Protein Expr. Purif.* **9**:211.

Vasina, J. A., M. S. Peterson, and F. Baneyx (1997) Scale-up and Optimization of the Low-Temperature Inducible *cspA* Promoter System. *Bio. Prog.* submitted