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The Expression and Regulation of *cspH* Gene, Encoding the Cold Shock Protein in *Salmonella enterica* Serovar Typhimurium UK-1

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Previously, we showed that *cspH* gene was induced in response to a temperature downshift during exponential phase and its promoter was activated at even 37℃. Here, we investigated the expression mode of the *cspH* gene by DNA gyrase and Fis at 37℃. When cells at stationary-phase are subcultured into a rich medium, its mRNA levels dramatically increased prior to the first cell division. However, when the cells were treated with the DNA gyrase inhibitors, the *cspH* mRNA was not induced upon nutrient upshift. The low level of superhelical density at its promoter affected the expression of *cspH* mRNA *in vitro*. Here, we also showed that a *fis* deficient strain has a low level of *cspH* mRNA than the wild type upon nutrient shift-up. These results suggested that *cspH* expression at 37℃ might be in part mediated *via* changes in the DNA superhelical density and the induction of Fis protein followed by a nutritional upshift.

Introduction

Chromatin structure can affect processes such as transcription, replication and recombination (5, 19, 22), and several environmental factors are known to alter the superhelical density of DNA such as osmolarity, temperature, anaerobiosis, and nutrients (26, 37). The supercoiling level of DNA is homeostatically controlled by cellular DNA topoisomerases. An increase in the superhelical density by DNA gyrase is compensated by activation of *topA* expression and increased levels of topoisomerase I, which relaxes excessive supercoils. Conversely, the decrease of DNA supercoiling in turn activates the *gyrA* and *gyrB* promoters and increases DNA gyrase production, which restores the physiological level of supercoiling (10, 32).

The nutritional shift-up has been shown to cause a very rapid (within 2-3 min) increase of DNA supercoiling followed by relaxation (2). The initial effect is due, at least in part, to an increase in the ATP/ADP ratio required for the activation of DNA gyrase (10, 13, 15, 36, 40).

Fis, a small DNA binding protein, is a versatile transactivator that functions at many different promoters (20). The expression of *fis* sharply increases on nutritional shift-up and its promoter is autorepressed by Fis (3 24, 25). Recent studies strongly suggest that Fis in *Escherichia coli* plays a role in determining the topology of DNA during the exponential growth phase (14, 32, 33, 35). The *fis*-dependent effects on DNA topology during transition to exponential growth phase are due in part to the repression of the *gyrA* and *gyrB* genes and

decreased gyrase activity (32, 33). However, in *S. typhimurium*, study on the homeostatic control of DNA supercoiling by Fis and gyrase has not yet been investigated.

Previously, we identified that the *cspH* gene from *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*), which encodes for the 70 amino acids CspH protein, and this gene was shown to have very different promoter sequence from that in *E. coli*. *S. typhimurium cspH* was induced at normal temperature as well as upon cold shock. The pattern of the expression at 37℃ was dependent on the growth phase, and the maximal induction took place during early exponential phase. The *cspH* promoter has well conserved discriminator sequence (between −10 and +1: GCGCCTC) (16). A single promoter element, the GC-rich discriminator, is known to confer sensitivity to different regulatory mechanisms - stringent control, growth rate-dependent control and alterations of superhelical density (9, 25, 32, 42).

In this study, we have investigated how the *cspH* gene responds to nutrient shift-up similar to the condition at early exponential phase. We showed that the increased superhelicity of DNA accompanied by nutrient shift-up affected the transcription of the *cspH* gene and *fis* gene regulated its transcription at the same condition The analysis on the pattern of *cspH* expression suggested that the homeostatic control of DNA supercoiling by Fis and gyrase in *E. coli* might be present in *S. typhimurium*.

Results

cspH gene from *Salmonella tyhpimurium* was induced by nutrient-shift up.

Previously, we reported that the *cspH* was expressed depending on growth phase with maximal level at early log phase (16). Here, to test whether the expression of the *cspH* gene responds to nutritional shift-up which is similar to the condition at early log phase, total RNA was isolated at intervals after addition of fresh medium to overnight cultured ell and *cspH* mRNA level was measured by Northern blotting (data not shown) and primer extension. Figure 1A showed that the *cspH* mRNA expression was increased dramatically 10 min after nutrient shift-up. The construct of *cspH-lacZ* translational fusion (pYH1) (16) also showed a similar pattern to the expression of *cspH* message (Fig. 1B). These data suggest that the *cspH* is a gene to respond to a physiological state after nutritional up condition in *S. typhimurium*.

DNA gyrase influenced the expression of *cspH* upon nutrient shift-up.

The increase in ATP/ADP ratio through nutritional shift-up activates DNA gyrase. (10, 13, 15, 40). On its promoter, the *cspH* gene has a well-conserved GC-rich discriminator, which is related to growth rate-dependent and stringent control system (7, 9, 38). Recently, Schneider *et al.* (32) reported that the discriminator confers sensitivity to alterations of DNA superhelical density. Therefore, the induction of *cspH* expression upon nutrient shift-up could be an effect by the change of DNA superhelical density through DNA gyrase. To investigate whether DNA gyrase affects the expression of *cspH* upon nutrient shift-up, we first monitored the influence of the gyrase inhibitors, coumermycin and novobiocin on *cspH* mRNA levels by primer extension analysis. As shown in Fig 2, the addition of the inhibitors completely blocked the expression of *cspH* mRNA. The construct of *cspH-lacZ* translational fusion (pYH1) also showed a similar pattern to the expression of *cspH* message (Fig. 2B), indicating that the supercoiling level of the *cspH* promoter

by DNA gyrase could affect its expression upon nutrient shift-up.

Superhelical density of *cspH* promoter influenced its expression.

It has been known that the gyrase, one of the topoisomerases, increases DNA supercoiling. As described above, *cspH* expression was induced upon nutrient shift-up and blocked by addition of gyrase inhibitors. To investigate a relationship between superhelical density and *cspH* expression, we prepared the *cspH* promoter constructs (pYHC) and it was transformed into wild type strain. The plasmid was extracted at stationary phase, upon nutrient shift-up and after addition of novobiocin. Fig. 3A shows that the addition of novobiocin did not increase the superhelical density compared to the nutritional shift-up. Furthermore, the pYHC prepared by nutrient shift-up had more superhelical density than that prepared at stationary phase. With the constructs prepared at the different conditions, we investigated the dependence of the *cspH* promoter activity on DNA supercoiling level by *in vitro* transcription. Afterwards, primer extension reactions were carried out to evaluate the amount of mRNA synthesized from the *cspH* promoter. As shown in Fig. 3B, a relaxation of superhelical density led to decrease of *cspH* mRNA level. However, a difference of superhelical density between lane 1 and 2 Fig. 3B did not affect an expression of *cspH* (see discussion). To test directly whether the expression of *cspH* is dependent upon DNA supercoiling level, we prepared that the topoisomers with different superhelical densities of the *cspH* promoter construct (pYHC) were prepared by DNA topoisomerase I and used for *in vitro* transcription (Fig. 4A). Primer extension reactions were carried out to evaluate the amount of mRNA synthesized from the *cspH* promoter (Fig. 4B). Quantification of the signals obtained demonstrated that the activity of the *cspH* promoter is sensitive to the superhelical density of DNA.

Fis protein affects the expression of *cspH* gene.

Figure 2 showed that gyrase inhibitor blocked completely the transcription of *cspH* gene upon nutrient shift-up. However, the expression of *cspH* by RNA polymerase on topoisomeric constructs was not blocked completely (Fig. 3, lane 3; Fig. 4). Therefore, it was speculated that *cspH* expression *in vivo* might be affected by another factor in addition to the superhelical density. Fis protein, which is induced in early exponential phase or upon nutrient shift-up, is a pleiotropic regulator in *E. coli* (11, 12, 20, 30). The overall *fis* operon organization in *S. typhimurium* is the same as that in *E. coli*, with the deduced Fis amino acid sequences being identical between both species (4, 27). Because *cspH* gene is also expressed during early-exponential growth or upon nutrient shift-up (16), we tested whether Fis affects the expression of *cspH* gene on nutrient shift-up. Figure 5A showed the different induction levels of *cspH* mRNA in Fis+ (wild type) and Fis– strains (YK4240) upon nutrient shift-up, suggesting that Fis might affect the expression of *cspH* during early log phase. We already showed that DNA superhelical density affected the expression of *cspH* upon nutrient shift-up. Therefore, both DNA superhelical density and Fis protein might simultaneously affect the expression of *cspH* upon nutrient shift-up condition.

Recently, Schneider *et al.* (32) reported that *fis* mRNA level in *E. coli* was decreased by addition of coumermycin, gyrase inhibitor, upon nutrient shift-up and was strongly dependent on the superhelical density of DNA. It is also known that Fis, which is induced upon nutrient shift-up, reinforces the repression of the

gyrA and *gyrB* gene on transcriptional level to maintain DNA topology. We already showed that the expression of *cspH* gene was sensitive to DNA superhelical density of its promoter and blocked by gyrase inhibitor (Fig. 2). Previously, we also reported that *cspH* mRNA level was dramatically decreased at $O.D_{600}$ 0.3 comparing that at $O.D_{600}$ 0.15 (16). Taken together both the results in this work and previous study, it was supposed that although the expression of *cspH* in Fis mutant is decreased at early log phase, *cspH* mRNA level could be less repressed after early log phase due to the absence of repression of *gyr* gene by Fis mutation. To test this suppose, we transformed the pYH1 into *fis* mutant (YHF) and measured the expression level of CspH-LacZ at different growth phases. The expression of LacZ in the Fis + strain (YH1) has maximal level at early log phase (O.D 0.15). But, entering mid exponential phase, the expression was decreased by steps. On the other hand, LacZ activity in the Fis- strain was even increased at O.D0.3 comparing to O.D0.15. As CspH-LacZ protein could be stable in *S. typhimurium*, this result should be confirmed by mRNA level. To measure the decreased level of *cspH* mRNA, the primer extension was performed with Fis+ (wild type) and Fis- strains. Interestingly, Fis- and Fis+ strains had a similar level of *cspH* mRNA at O.D 0.3 (Fig. 5C, lane 2 and 4), although the level of *cspH* mRNA in Fis+ strain was higher than that in Fis- strain at O.D 0.15. This indicates that Fis- strain showed less repressed level of *cspH* mRNA between O.D 0.15 and 0.3.

Discussion

In the present paper, we showed that DNA superhelical density and Fis protein simultaneously affected the expression of *cspH* upon nutrient shift-up condition. In *Escherichia coli*, it has been extensively studied about the relationship between DNA superhelicity and physiological state in cell. The supercoiling level of DNA is homeostatically controlled by cellular DNA topoisomerases (for a review, see Drlica, 1992). Recently, Schneider *et al.* (32) suggested that entering nutrient shift-up condition, the increase of supercoiling beyond the physiological level would, at least initially, activate the *fis* promoter and reinforce the repression of *gyrA* and *gyrB* by Fis in *E. coil*. Eventually, the physiological level of supercoiling will be reset and stabilized by steady-state levels by Fis and active gyrase in the cell (32, 36). However, in *S. typhimurium*, a relationship between Fis protein and the level of DNA supercoiling has not been investigated. Here, it was speculated that *cspH* gene could be a good material to examine whether the *fis* from S. *typhimurium* may manage the fine-tuning control mechanism of DNA superhelicity like that in *E. coli* because *cspH* gene is regulated by both DNA gyrase and FIS upon nutrient shift-up and at early exponential phase as described in this paper. As shown in Fig. 5, the expression of the *cspH* in Fis+ strain was induced to maximal level at early exponential phase ($O.D_{600}$ 0.15), but was dramatically reduced afterwards ($O.D_{600} \geq 0.3$). However, at $O.D_{600}$ 0.3, the expression of the *cspH* gene in Fis- strain was less reduced than that in Fis+ strain although the maximal level of mRNA in Fis- strain was less than that in Fis + strain. Therefore, this suggests that the dramatic decrease of *cspH* mRNA, when entering from early log phase to mid log phase, may be in part due to the relaxation of DNA superhelical density through the repression of *gyr* genes and the autorepression of the *fis* transcription by Fis. This also demonstrates that *fis* gene in *Salmonella* may be involved in the 'fine- tuning' homeostatic control mechanism of DNA supercoiling like that in *E. coli.*

Previously, we showed that although the expression of *cspH* at 37℃ was induced at early exponential phase, that was decreased to basal level during stationary phase (16). In this paper, it was also indicated that the *cspH* mRNA was not expressed at stationary phase (Fig. 2B; lane 1, 3, and 5). However, as shown in Fig. 3A, the superhelicity of pYHC extracted at stationary phase was enough to induce the transcription of the *cspH* gene *in vitro*. In this work, we constructed pYH1 derivative, which contains a region to just –44 from transcription start site of *cspH* gene (data not shown). The strain containing the plasmid showed the elevated level of LacZ in stationary phase in opposition to the decrease of LacZ level during that phase (data not shown). These indicate that there might be a factor to repress the expression of *cspH* during a transition from mid-exponential to stationary phase in *S. typhimurium*.

From NMR spectroscopic analysis of CspA (23), the residues that are involved in the formation of the hydrophobic core of the CspA β-barrel structure have been identified. These residues are Val-9, Ile-21, Val-30, Val-32 and Val-51 (indicated by solid circles above the CspA sequence; Fig. 6). As shown in Fig. 6, CspH also contains all these hydrophobic residues, suggesting that CspH could form the CspA β-barrel structure. All CSP members but not CspF and CspH in *E. coli* have highly conserved surface aromatic residues (Phe: F, Tyr: Y, and Trp: W) (Yamanaka *et al.*, 1998). The well-conserved aromatic residues in RNA binding motifs, RNP1 and RNP2, are known to be essential for binding to RNA and ssDNA (1, 28, 39). The RNPs of CspH contain a well-conserved feature as that of CspA, but do not have only aromatic residues in the RNPs. In addition, it has high isoelectric point (pI: 8.96) compared to the pI of CspA. These properties suggest that CspH from *S. typhimurium* might bind to DNA rather than to ssDNA or RNA. Here, we suggest demonstrate that study about function of *cspH* gene during early exponential phase *in vivo* is worth for further investigation.

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