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Effects of Chaperones on mRNA Stability and Gene Expression in *Escherichia coli*

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Effects of chaperones on mRNA stability and gene expression were studied in order to develop an efficient *Escherichia coli* expression system that can maximize gene expression. The stability of mRNA was modulated by introducing various secondary structures at the 5'-end of mRNA. Four vector systems providing different 5'-end structures were constructed, and genes encoding GFPuv and endoxylanase were cloned into the four vector systems. Primer extension assay revealed different mRNA half-lives depending on the 5'-end secondary structures of mRNA. In addition to the stem-loop structure at the 5'-end of mRNA, coexpression of *dnaK-dnaJ-grpE* or *groEL-groES*, representative heat-shock genes in *E. coli*, increased the mRNA stability and the level of gene expression further, even though the degree of stabilization was varied. Our work suggests that some of the heat-shock proteins can function as mRNA stabilizers as well as protein chaperones.

Keywords: mRNA stability, gene expression, chaperone

Escherichia coli has been widely utilized for high-level production of recombinant proteins because *E. coli* has many advantages as an expression host [15, 17, 23, 24, 28, 33, 34, 37]. However, not every gene can be expressed efficiently in this organism. Construction of an expression vector requires several elements whose configuration must be carefully considered to ensure the highest level of protein synthesis [1, 8, 10]. Strategies for achieving high-level expression of genes in *E. coli* have been examined, largely in three aspects; increasing transcription activity, enhancing translational efficiency, and protein stabilization [9, 16, 18, 27, 38]. Recently, several researchers proposed that stabilization of mRNA can also enhance the level of gene expression because the stable mRNA has more chances to be translated into protein than unstable ones [2, 6, 21]. Understanding the mechanisms leading to mRNA decay would present a framework to design strategies for improving mRNA stability [35].

Enzymes involved in mRNA decay are largely grouped into exonucleases, which exhibit 3' to 5' processing cleavage activities [30], and endonucleases, which participate in decay-initiating mRNA cleavage. RNase E, a typical endonuclease, requires a free 5'-end to bind to the target mRNA before scanning the transcript for cleavage sites [11, 12]. The RNA degradosome of *E. coli* involved in the degradation of mRNA is a multiprotein complex composed of RNaseE, PNPase, RhlB, and enolase [5], and RNaseE cleavage is generally believed to be the rate-limiting step in mRNA degradation [25]. Hairpin structures at the 5'-end are known to inhibit RNaseE from binding to the free 5'-end of the mRNA [3, 4, 11, 13, 20]. Therefore, rationally designed stem-loop structures at the 5'-end of the transcript can protect mRNA against RNaseE.

The chaperones such as GroEL and GroES are generally known to play a central role in protein folding; partially denatured polypeptides bind to the internal cavity of GroEL and are released in an ATP-dependent reaction regulated by the cofactor GroES that results in folding to the native state [26]. Georgellis *et al.* [14] showed that GroEL was required to form an RNA-binding complex that provides protection against nuclease degradation. Here, we evaluate the function of heat-shock proteins as mRNA stabilizers; *dnaK-dnaJ-grpE* and *groEL-groES* of *E. coli* were coexpressed with reporter genes carrying various hairpin structures at the 5'-end of mRNA, as reported by Carrier and Keasling [6, 7].

Materials and Methods

Bacterial Strains and Plasmids

E. coli DH5α and HB101 were used as recipient strains for transformation. *E. coli* harboring recombinant plasmids was grown

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in TB (tryptone broth, 1% tryptone peptone, and 0.8% NaCl). pBAD18 [19] was modified to insert synthetic DNA at the 5'-end region, and four kinds of sequences producing various secondary structures were introduced into the modified pBAD18 (pBADD). pBADD derivatives involving various synthetic DNAs upstream of P_{BAD} were named pHY1 to pHY4. Two reporter genes were inserted into pHY1 to pHY4; genes encoding endoxylanase and GFPuv were excised from pKJX4 [22] and pGFPuv (CLONTECH Laboratory, Inc.), respectively, and ligated with digested pHY vectors producing pHY1E/2E/3E/4E and pHY1G/2G/3G/4G individually. Coexpression of heat-shock proteins was performed with pG-KJE6 and pGro7 [32]. The DnaK-DnaJ-GrpE chaperone team was expressed under the control of the P_{BAD} promoter in pG-KJE6, and the GroEL-GroES team was also controlled by P_{BAD} in pGro7.

Secondary Structure Design for 5'-End Hairpin Structure

Both strands of insert DNA were synthesized by an automated DNA synthesizer (BIONEER Co.) and followed by an annealing reaction [36]. The synthesized DNA inserts were ligated with pBADD through SalI and XhoI to provide hairpin structures at the 5'-end of mRNA when transcribed. Predicted secondary structures of 5'-UTR were generated using the RNA draw program [29].

RNA Extraction

Cells were cultivated in TB, and L-arabinose of 0.002% was added when the OD_{600} reached 0.1 for the induction of the P_{BAD} system. Rifampicin of 250 μg/ml was added at OD_{600} of 0.2 to inhibit further mRNA synthesis. Cells were incubated at 37°C and harvested periodically after rifampicin addition. Rifampicin treatment time was regarded as time point "0". RNAs were extracted with Trizol solution (Invitrogen Life Technologies, Inc.). After centrifugation, the aqueous phase was precipitated with isopropanol, washed with 75% EtOH, and the RNA pellet was resuspended in RNase-free water.

Primer Extension Analysis

To analyze the level of mRNA, primer extension assay was performed. Purified γ-^{32}P-end labeled primers specific to each structural gene were coprecipitated with 10 μg of total cell RNA and the pellet was resuspended in 20 μl of hybridization buffer containing 250 mM KCl, 2 mM Tris-HCl, pH 7.9, and 0.2 mM EDTA. Primers used in

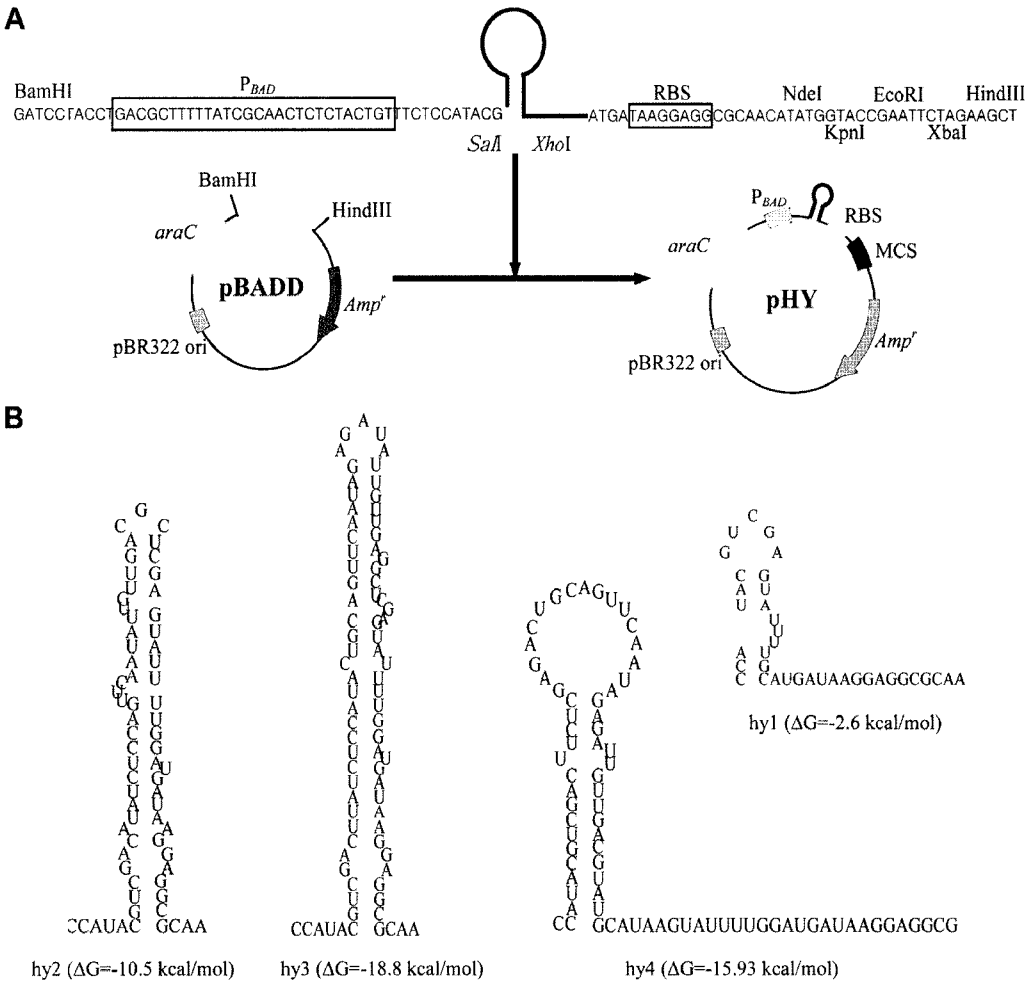

Fig. 1. Scheme of pHY construction, and 5'-end secondary structures of mRNA made from pHY vectors.
A. Synthetic DNA containing P_{BAD}, hairpin strucures, RBS, and multicloning sites was ligated with pBADD digested with BamHI and HindIII. pBADD is the derivative of pBAD18 of which the NdeI site was eliminated. Hairpin structures were designed to be replaced using SalI and XhoI sites. **B.** When transcribed to mRNA, variable secondary structures from hy1 to hy4, predicted by the RNA draw program of Sweden Ole Matzura, are generated at the 5'-end of the mRNA.

primer extension analysis are endo (5'-GTA ATC TGT GCC AGC TGC AGA GGC GGT TGC-3') for endoxylanase mRNA and gfp (5'-GTG CCC ATT AAC ATC ACC ATC TAA TTC AAC-3') for *gfpuv* mRNA. Primer extension reactions were done as described by Ryu and Garges [36]. mRNA half-lives were estimated by calculating slopes from a log plot of the relative intensities of mRNA. Relative mRNA intensities were obtained by measuring the reverse transcriptional signals at serial time points using a phosphor-image analyzer (Fujifilm BAS-2500).

Enzyme Assay and Protein Quantification

Quantitative endoxylanase activity of cell extract was determined by the DNS method using xylan as a substrate [22, 31]. Fluorescence intensity from cells containing various recombinant plasmids, pHY1G to pHY4G, was measured by a fluorescence spectrophotometer (Hitachii model F4500).

Results and Discussion

Construction of pHY Vectors Making Various Hairpin Structures at 5'-End of mRNA

A pHY vector system was constructed to insert the sequences providing stem-loop structures at the 5'-end of mRNA. pHY is based on pBAD18, which has a P_{BAD} promoter and a regulator gene *araC* offering tight regulation. The synthesized DNA fragment containing P_{BAD}, hairpin-producing sequences, RBS, and MCS orderly from 5'-end to 3'-end was ligated with modified pBAD18, pBADD, through BamHI and HindIII sites, and resulted in the pHY vector (Fig. 1A). Design of secondary structures at the 5'-end of mRNA was based on data of Carrier and Keasling [7]. The characteristics of the 5'-end affecting mRNA stability includes the length of stem structure, the size of loop structure, the folding energy, and the length of linear strand prior to the stem structure [2]. It has been known that the length of the single strand mRNA upstream of the hairpin structure should be less than 4 bases in order for the hairpin structure to inhibit access of RNaseE to mRNA [11].

The secondary structures of the 5'-end of mRNA produced from pHY vectors, predicted with the RNA draw program [29], are shown in Fig. 1B. The base lengths prior to stem structures made from pHY1 and pHY4 are 0 and 1 base that are short enough to prevent RNaseE binding to mRNA. On the other hand, mRNA made from pHY2 and pHY3 have a long stem and low folding energy, but the linear strand prior to the stem is 5 nucleotides long. The 5'-end hairpin structures made from each vector were named as hy1, 2, 3, and 4, respectively.

Effects of Secondary Structure at 5'-End of mRNA on mRNA Stability

The mRNA stability was compared by measuring mRNA half-lives of two genes cloned into pHY vectors, one encoding endoxylanase (pHYE) and the other encoding GFPuv (pHYG), with primer extension analysis. Endoxylanase mRNA from four pHYE constructs showed different half-lives depending on the 5'-end hairpin structures (Fig. 2A). Endoxylanase gene with hy4 hairpin produced the most stable mRNA with a half-life of 2.79 min, and the mRNA with hy2 hairpin had the shortest mRNA half-life of 0.52 min. The mRNA with hy1 or hy3 hairpin structures showed moderate half-lives that were longer than hy2 but shorter than hy4. Therefore, the order of stabilizing effects was hy4, hy1, hy3, and hy2. The same pattern of mRNA stabilization by hairpin structures was also seen in pHYG constructs containing a gene encoding GFPuv. The half-life of mRNA with hy4 structure was unusually long with 14.4 min, whereas that of mRNA with hy1 was 3.6 min. The half-life of mRNA with hy2 was 1.1 min, and 3.2 min for hy3 (Fig. 3A). In the case of the *gfpuv*

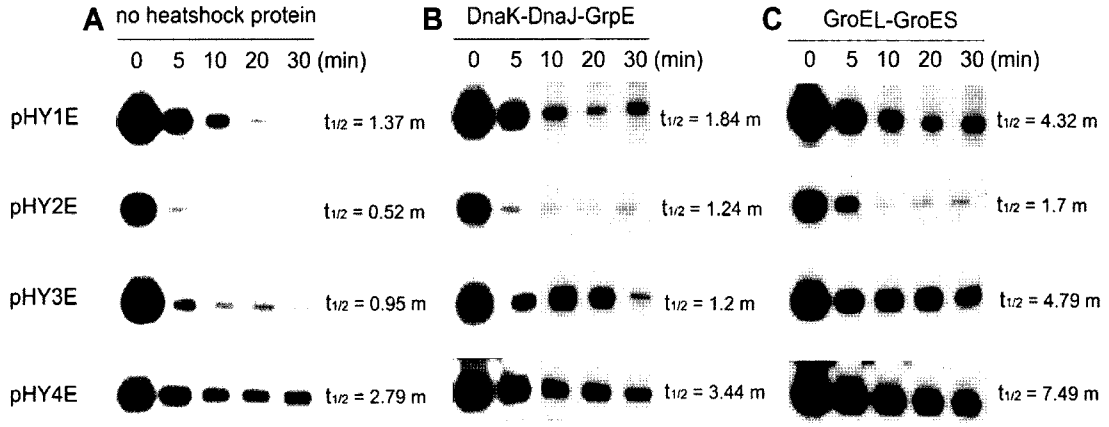

Fig. 2. Half-lives of endoxylanase mRNA depending on the secondary structures at the 5'-end and heat-shock proteins.
To assess the influences of hairpin structures at the 5'-end on mRNA stability, half-lives of endoxylanase mRNA were examined in pHY1E/2E/3E/4E containing various hairpin structures at the 5'-end (**A**). The effects of heat-shock proteins on mRNA stability were investigated by coexpression of *dnaK-dnaJ-grpE* (**B**) and *groEL-groES* (**C**). Heat shock genes from pG-KJE6 and pGro7 [32] were induced by the addition of 0.002% arabinose. Primer extension experiments were performed at least three times using independently prepared RNAs.

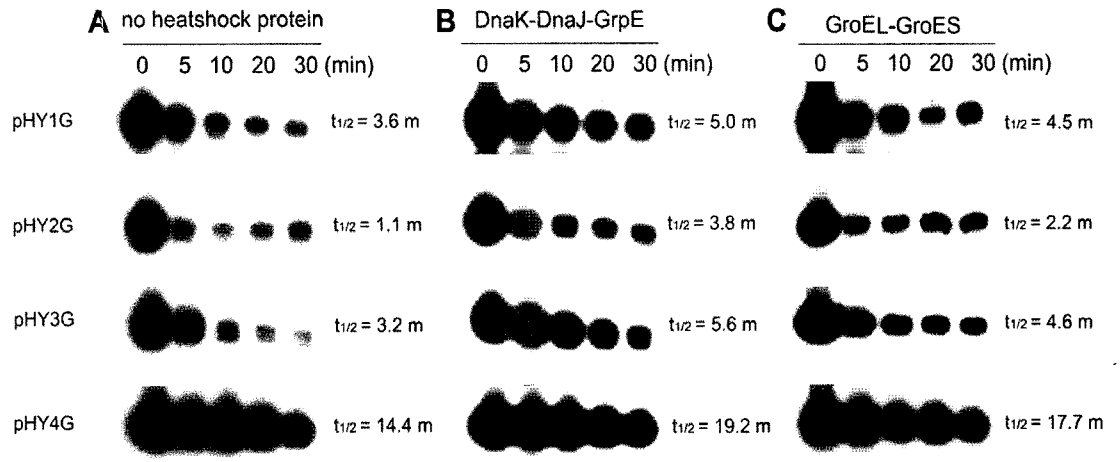

Fig. 3. Half-lives of *gfpuv* mRNA with different secondary structures at the 5'-end and in the presence/absence of heat-shock proteins. To assess the influences of hairpin structures at the 5'-end on mRNA stability, half-lives of *gfpuv* mRNA were examined in pHY1G/2G/3G/4G containing various hairpin structures at the 5'-end (**A**). The effects of heat-shock proteins on mRNA stability were investigated by coexpression of *dnaK-dnaJ-grpE* (**B**) and *groEL-groES* (**C**). Primer extension experiments were performed at least three times using independently prepared RNAs.

gene, the order of stabilizing effects was also hy4, hy1, hy3, and hy2.

The longer half-lives of hy1 and hy4 compared with those of hy2 and hy3 suggests that the number of linear nucleotides between the transcription start point and the stem structure is surely the most important element in mRNA stability, as proposed previously by others [4]. The longer mRNA half-lives observed with hy3 and hy4, when compared with half-lives of hy1 and hy2, respectively, may be attributed to the long and large stem-loop structures resulting in a more stable secondary structure of mRNA.

Effects of Heat-Shock Proteins on mRNA Stability

As an additional stabilizing element, effects of heat-shock proteins on mRNA stability were tested. pG-KJE6 and pGro7 [32] expressing *dnaK-dnaJ-grpE* and *groEL-groES* were introduced respectively into *E. coli* harboring pHYE or pHYG and the stability of mRNA was compared. In the case of pHYG4, which showed the strongest stabilization effect of hairpin structure with half-life of 14.4 min, coexpression with *dnaK-dnaJ-grpE* or *groEL-groES* resulted in further stabilization of mRNA with half-lives of 19.2 min and 17.7 min, respectively (Fig. 3). In the other pHYG constructs and pHYE constructs, mRNA half-lives were also increased by coexpression of heat-shock genes (Figs. 2 and 3). However, the degree of stabilization by the DnaK-DnaJ-GrpE team and the GroEL-GroES team was different depending on the genes tested; endoxylanase mRNA was stabilized more by GroEL-GroES, but *gfpuv* mRNA showed a longer half-life in the presence of DnaK-DnaJ-GrpE.

Table 1. Strains and plasmids.

Strain and plasmid	Relevant genotype	Reference
Escherichia coli		
DH5α	*supE*44 *hsdR*17 *recA*1 *gyrA*96 *thi*-1 *relA*1	
HB101	*supE*44 *hsdS*20(*rB*- *mB*-) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1 *leuB*6 *thi*-1	
Plasmid		
pBAD18	Amp^r pBR322 ori P_{BAD} *araC*	[19]
pBADD	pBAD18 derivative devoid of NdeI recognition site	This study
pHY1	pBADD derivative containing hy1	This study
pHY2	pBADD derivative containing hy2	This study
pHY3	pBADD derivative containing hy3	This study
pHY4	pBADD derivative containing hy4	This study
pKJX4	Amp^r P_{lac} P_B endoxylanase gene	[24]
pGFPuv	Amp^r puc19 ori P_{lac} *gfpuv*	CLONTECH Lab. Inc.
pG-KJE6	Cm^r pACYC ori *dnaKJ-grpE*	[32]
pGro7	Cm^r pACYC ori *groELS*	[32]

Expression of both *dnaK-dnaJ-grpE* and *groEL-groES* was not studied because overexpression of both groups of chaperone genes impaired cell growth significantly (data not shown).

Both DnaK-DnaJ-GrpE and GroEL-GroES could stabilize the mRNAs with different stability tested in this study. GroEL has been known to be an element to constitute an RNA-binding complex, which provides mRNA protection against RNaseE [14]. However, the mRNA protection by DnaK-DnaJ-GrpE was not reported. These results suggest that at least these chaperones can have dual functions of protecting mRNA from nuclease attack and assisting protein folding.

Influence of mRNA Stability on Gene Expression

The stabilization of mRNA by heat-shock proteins resulted in higher production of protein, as higher activities of endoxylanase and GFPuv were detected in the presence of heat-shock proteins (Fig. 4), even though the protein activities were not exactly proportional to the degree of stabilization of mRNA by heat-shock proteins. Endoxylanase activities from the four pHYE constructs were measured by the DNS method. *E. coli* HB101 harboring pHY4E making the most stable endoxylanase mRNA (Fig. 2A) expressed the highest enzyme activity (comparing white bars in Fig. 4A) and the lowest activity was detected in HB101 harboring pHY2E. These results imply that the stabilizing effects of individual hairpin structures resulted in higher protein expression. The protein expression patterns, proportional to mRNA stability, were more evident in pHYG constructs (Fig. 4B).

As shown in Fig. 4, heat-shock proteins enhanced the mRNA stability and resulted in higher protein production, suggesting that the heat-shock proteins tested in this study can function as a controller of gene expression on the level of mRNA chaperones as well as on protein synthesis. Taken together, both 5'-end structures of mRNA and chaperones can affect protein synthesis by stabilizing mRNA, and these can be considered as one of the factors influencing protein expression when an overexpression system in *E. coli* is to be developed.

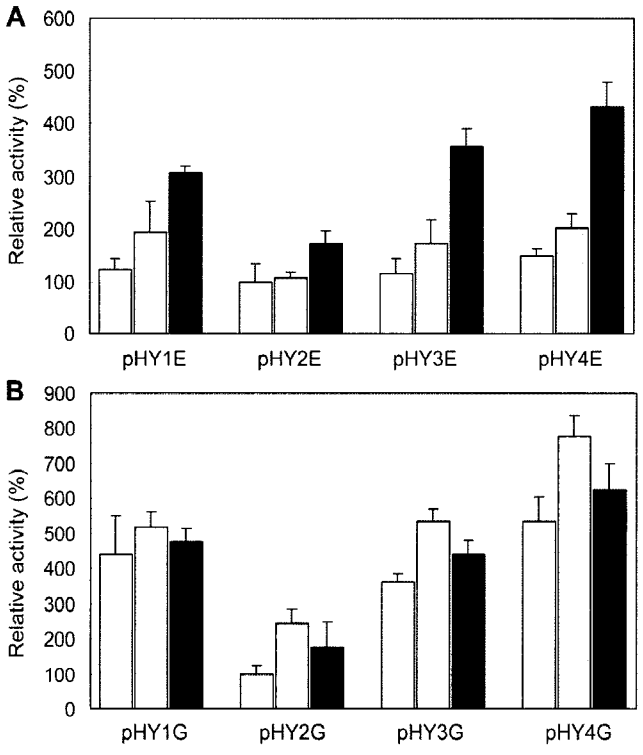

Fig. 4. Endoxylanase and GFPuv activity under various conditions affecting mRNA half-lives.

E. coli harboring pHYE or pHYG was cultivated in tryptone broth containing L-arabinose. Endoxylanase activity (**A**) and GFPuv activity (**B**) were measured from cells grown to mid-logarithmic phase and early stationary phase, respectively. The activities of endoxylanase and GFPuv depending on hairpin structures are depicted by white bars, and the effects of DnaK-DnaJ-GrpE (grey bars) and GroEL-GroES (black bars) were examined by coexpression of pG-KJE6 and pGro7, respectively, under 0.002% arabinose addition. In both cases, the activity was expressed relatively by dividing each value by the lowest value under each experimental condition. The values are the means of three independent assays from different cultures.

Acknowledgments

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