SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of *lac* operon

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SsrA RNA of *Escherichia coli*, also known as 10Sa RNA or tmRNA, acts both as tRNA and mRNA when ribosomes are paused at the 3' end of an mRNA lacking a stop codon. This process, referred to as *trans*-translation, leads to the addition of a short peptide tag to the C-terminus of the incomplete nascent polypeptide. The tagged polypeptide is then degraded by C-terminal-specific proteases. Here, we focused on endogenous targets for the SsrA system and on a potential regulatory role of SsrA RNA. First, we show that *trans*-translation events occur frequently in normally growing *E.coli* cells. More specifically, we report that the *lacI* mRNA encoding Lac repressor (LacI) is a specific natural target for *trans*-translation. The binding of LacI to the *lac* operators results in truncated *lacI* mRNAs that are, in turn, recognized by the SsrA system. The SsrA-mediated tagging and proteolysis of LacI appears to play a role in cellular adaptation to lactose availability by supporting a rapid induction of *lac* operon expression.

Keywords: *lac* operon/Lac repressor/tmRNA/
transcriptional roadblock/*trans*-translation

Introduction

SsrA RNA or 10Sa RNA encoded by the *ssrA* gene is a small stable RNA found first in *Escherichia coli* (Subbarao and Apirion, 1989). The RNA is also named tmRNA because it has characteristics of both tRNA and mRNA (Atkins and Gesteland, 1996; Jentsch, 1996; Keiler *et al.*, 1996). The sequence analysis and *in vitro* studies revealed that the *E.coli* SsrA RNA could form an alanyl tRNA-like structure and can be charged with alanine (Komine *et al.*, 1994). The property of SsrA RNA as mRNA was first suggested by the observation that a mammalian protein expressed in *E.coli* had a short tag sequence encoded by the *ssrA* gene at its C-terminus (Tu *et al.*, 1995). Following these observations and based on their own studies, Keiler *et al.* (1996) proposed an intriguing mechanism whereby SsrA RNA acts both as tRNA and mRNA. This model, referred to as *trans*-translation (Atkins and Gesteland, 1996; Jentsch, 1996), suggests that when ribosomes are stalled at the 3' end of a truncated mRNA without an in-frame stop codon, SsrA

RNA charged with alanine binds in the A site of the ribosome, donates alanine to the growing polypeptide chain and then acts as an mRNA to provide the coding sequence for a short peptide. The final translation products are chimeric polypeptides in which a specific 11 residue tag (AANDENYALAA) is attached to the C-terminus of aberrant polypeptides. The tag sequence targets the products of *trans*-translation for degradation through several C-terminal specific proteases such as ClpAP, ClpXP, HflB and Tsp (Keiler *et al.*, 1996; Gottesman *et al.*, 1998; Herman *et al.*, 1998).

Since the proposal of the *trans*-translation model, major studies on SsrA RNA have been performed primarily from the mechanistic point of view, resulting in several important findings. First, *trans*-translation has been shown to occur not only at the 3' ends of stop codon-less mRNAs but also at a run of rare codons (Roche and Sauer, 1999). Secondly, a unique RNA-binding protein, SmpB, has been newly identified and shown to be required for SsrA activity (Karzai *et al.*, 1999). Thirdly, structural and/or sequence determinants of SsrA RNA itself that are responsible for the *trans*-translation reaction were partly elucidated (Nameki *et al.*, 1999a,b; Williams *et al.*, 1999). In addition, RNase E, known to participate in processing of rRNA precursors and degradation of a variety of mRNAs, has been shown to be involved in the maturation of SsrA RNA (Lin-Chao *et al.*, 1999).

The wide distribution of SsrA RNA in many bacterial species certainly suggests an important role of this unusual RNA in cellular activity (Williams, 2000). In fact, bacterial cells lacking a functional SsrA RNA exhibit a variety of phenotypes such as slower growth (Oh and Apirion, 1991; Komine *et al.*, 1994), reduced motility (Komine *et al.*, 1994), inhibition of phage growth (Retallack *et al.*, 1994; Karzai *et al.*, 1999; Julio *et al.*, 2000), induction of Alp protease activity (Kirby *et al.*, 1994), enhanced activity of several repressors (Retallack and Friedman, 1995) and reduced pathogenesis (Julio *et al.*, 2000). In addition, it has been shown that SsrA RNA is essential for the growth of *Neisseria gonorrhoeae* (Huang *et al.*, 2000). The major biological relevance of SsrA-mediated tagging and proteolysis is believed to be providing both a way to clear the ribosome stalled on mRNAs and a quality-control mechanism that allows the cell to eliminate potentially harmful truncated polypeptides (Atkins and Gesteland, 1996; Jentsch, 1996; Keiler *et al.*, 1996). However, it is largely unknown how the SsrA-mediated tagging and proteolysis is related to various cellular phenotypes mentioned above. This is, in part, due to the lack of information on natural mRNA substrates for the SsrA system. In fact, studies on *trans*-translation have been carried out only on foreign or artificial mRNAs both *in vivo* (Keiler *et al.*, 1996; Karzai *et al.*, 1999; Roche and Sauer, 1999) and *in vitro* (Himeno

et al., 1997). Thus, no endogenous mRNA substrates for the SsrA system have been identified yet. The identification of such endogenous targets would certainly be useful to gain insight into the molecular mechanisms underlying various phenotypes observed in the SsrA-defective cells and to understand the full physiological significance of *trans*-translation, including a potential regulatory role.

We report here that the *trans*-translation event occurs quite frequently in cells. Then, we demonstrate that the *lacI* mRNA encoding Lac repressor (LacI) is a specific endogenous target for the SsrA-mediated tagging and proteolysis. The SsrA-mediated tagging and proteolysis of LacI plays a role in the regulation of the expression of the *lac* operon. This is the first instance in which a specific phenotype of SsrA-defective cells may have been linked via a specific target mRNA and protein to the *trans*-translation model.

Results

Effect of *ssrA* mutation on the expression of an artificially tagged model protein

As the first step to evaluate the physiological role of the SsrA system, we examined the effect of *ssrA* mutation on the expression of an artificially tagged model protein in *E. coli* cells. The *crp* gene, encoding the cyclic-AMP receptor protein (CRP), on a plasmid, was genetically manipulated to produce a tagged CRP (CRP-AA) that has a degradation tag at its C-terminus (Figure 1A). Another mutant *crp* gene encoding CRP-DD, in which two aspartates are substituted for two alanines at the very C-terminus of the tag sequence, was also constructed (Figure 1A). The tag sequence with two aspartates at its C-terminus (DD-tag) is known to be resistant to degradation by cellular proteases (Keiler *et al.*, 1996; Gottesman *et al.*, 1998; Herman *et al.*, 1998). Plasmids carrying the wild-type or variant *crp* genes were introduced both into PP47 (*crp*) and PP47 *ssrA* cells. The expression of CRP proteins in two cells was analyzed by western blotting using anti-CRP polyclonal antibody (Figure 1B). The intact CRP and CRP-DD proteins were highly expressed at equivalent levels in both the *ssrA* and its parent cells (Figure 1B, lanes 1, 2, 5 and 6). On the other hand, the level of CRP-AA in cells with the wild-type *ssrA* gene was markedly reduced in comparison with CRP or CRP-DD (Figure 1B, lanes 3 and 4). More interestingly, little CRP-AA was detected with anti-CRP in the *ssrA* cells (Figure 1B, lane 4). Northern blot analysis revealed that the levels of *crp-AA* mRNA were comparable to levels of *crp-DD* in both cells (Figure 1C). Thus, the differences in steady-state protein levels are not due to the differences in mRNA levels. Instead, the results can be easily explained by assuming that CRP-AA is unstable and degraded more efficiently in the *ssrA* cells than in cells with the wild-type *ssrA* gene. The simplest interpretation would be that cells containing SsrA RNA produce abundant endogenous tagged polypeptides through *trans*-translation, which could compete with CRP-AA for proteases, while in the absence of SsrA RNA there is less competition for the proteases due to the lack of endogenous tagged polypeptides and CRP-AA is more susceptible to the tag-specific proteases.

Fig. 1. Expression of artificially tagged CRPs in wild-type and *ssrA* cells. (A) Structure of CRP-AA and CRP-DD. Amino acid sequences of the C-terminal portion of CRP derivatives are shown along with corresponding nucleotide sequences. Asterisks indicate the stop codon. The *Mlu*I site (5'-ACGCGT-3') used to introduce DNA fragments encoding the AA- or DD-tag is underlined. (B) Effect of the *ssrA* mutation on the expression of CRP, CRP-AA and CRP-DD proteins. Total proteins equivalent to OD₆₀₀ = 0.01, prepared from PP47 (lanes 1, 3 and 5) and PP47Δ*ssrA* (lanes 2, 4 and 6) cells harboring pCRP (lanes 1 and 2), pCRP-AA (lanes 3 and 4) or pCRP-DD (lanes 5 and 6), were analyzed by western blotting using anti-CRP antibody. (C) Northern blot analysis of the *crp* mRNAs. Total RNAs (2.5 μg), prepared from PP47 (lanes 1 and 3) and PP47Δ*ssrA* (lanes 2 and 4) cells harboring pCRP-AA (lanes 1 and 2) or pCRP-DD (lanes 3 and 4), were subjected to northern blot analysis using a fluorescein-labeled probe for *crp* mRNAs.

Direct evidence for tagging of endogenous proteins

In order to examine directly whether the SsrA-mediated tagging of cellular proteins indeed occurs, we constructed plasmids pSsr-AA and pSsr-DD, carrying the wild-type and mutant *ssrA* genes, respectively (Figure 2A). The mutant *ssrA* gene (*ssrA*^{DD}) encodes a variant SsrA^{DD} RNA containing a protease-resistant tag sequence in which two alanines at the very C-terminus of the tag sequence have been changed to two aspartates (Roche and Sauer, 1999). These plasmids were introduced into *ssrA* cells and the total proteins were analyzed by western blotting using anti-DD-tag polyclonal antibody. A large number of polypeptides were detected by anti-DD-tag antibody when cells carried pSsr-DD (Figure 2B, lane 3) while most of these bands were absent in *ssrA* cells harboring a control plasmid pSTV28 (lane 1) and in cells carrying pSsr-AA (lane 2). These results clearly indicate that many endogenous cellular proteins were tagged through the SsrA system, resulting in chimeric proteins. The proteins with the wild-type tag sequence would be subsequently degraded by proteases whereas the proteins with the DD-tag may escape from the proteolysis. We conclude that the SsrA-mediated *trans*-translation occurs quite frequently in normally growing cells.

Truncated LacI proteins are generated in *ssrA* cells

Potential targets for *trans*-translation, incomplete mRNAs, can be produced by premature transcription termination before a stop codon or by nuclease cleavage of mRNAs. For example, one can imagine that premature transcription termination may occur when a strong DNA-binding

Fig. 2. (A) Schematic drawing of alanyl-SsrA^{AA} and alanyl-SsrA^{DD} RNAs. The nucleotides mutated in the tag-coding region of SsrA^{DD} are underlined. (B) Detection of tagging of cellular proteins. Total proteins equivalent to OD₆₀₀ = 0.1, prepared from PP47ΔssrA cells harboring pSTV28 (lane 1), pSsr-AA (lane 2) and pSsr-DD (lane 3), were analyzed by western blotting using anti-DD-tag antibody. Lane 4 represents total protein equivalent to OD₆₀₀ = 0.01, prepared from PP47ΔssrA cells harboring pCRP-DD. The arrow indicates the artificially tagged CRP-DD.

protein binds within an open reading frame (ORF). The *E. coli lac* system appears to represent this situation. In the *lac* operon, there are two auxiliary operators, *O*₂ and *O*₃, located 410 bp downstream and 82 bp upstream of the transcription start site, respectively, in addition to the major operator, *O*₁ (Müller-Hill, 1996). It is known that the LacI tetramer binds simultaneously to *O*₁ and to either *O*₂ or *O*₃, forming a DNA loop, to give a stronger repression (Oehler et al., 1990). It should be noted that *O*₃ overlaps the coding sequence for the C-terminus of LacI (Figure 3). Thus, LacI binding to *O*₃ is expected to cause premature termination of *lacI* transcription by forming an obstacle to transcription elongation. In fact, an early study suggested that this might be the case (Sellitti et al., 1987). This raises a possibility that the *lacI* mRNA can be a specific target of the SsrA system.

To examine whether the binding of LacI to the *lac* operators produces truncated *lacI* mRNAs and then

Fig. 3. (A) Schematic drawing of the *E. coli lacPO* region. *O*₁ and *O*₃ are shown as open boxes. *O*₃ partially overlaps the *lacI* ORF. Closed boxes indicate the parts of *lacI* and *lacZ* ORFs. The arrow indicates the transcription start site of *lacP*. (B) Nucleotide sequences of the wild-type and modified *lac* operators. Mutations introduced into the *lacPO* region are indicated by lowercase (*O*₁⁻ and *O*₃⁻) or underlined letters (*O*₃^{id}). The last two amino acid residues of LacI, GQ, were changed to EL in the *O*₃^{id} mutant. Asterisks indicate the *lacI* stop codon.

truncated LacI proteins, we constructed a pBR322-derived pIT613 that contains the entire *lac* region under the control of the *lacI^q* promoter. pIT613 was introduced into *ssrA* cells harboring compatible plasmids pSTV28, pSsr-AA or pSsr-DD. The expression of LacI protein in these cells was analyzed by western blotting using anti-LacI polyclonal antibody. In addition to the intact LacI protein, several prominent bands were observed below the intact LacI in cells with pIT613 in the absence of the *ssrA* gene (Figure 4, lane 2, shown by dotted arrows). These bands were not detected in cells carrying both pIT613 and pSsr-AA (Figure 4, lane 3), suggesting that they are efficiently removed by the SsrA system. No protein was detected with anti-LacI antibody in cells carrying a control plasmid pBR322 instead of pIT613, as expected (Figure 4, lane 1).

Detection of SsrA-mediated LacI tagging

In order to examine whether the SsrA-mediated tagging of LacI occurs, total proteins of the *ssrA* cells harboring both pIT613 and pSsr-DD were also analyzed by western blotting using anti-LacI antibody (Figure 4, lane 4). In addition to the intact LacI protein, a prominent band, which has a higher molecular weight than the intact LacI, and several other weak bands were observed. These bands presumably represent tagged LacI proteins in which the protease-resistant DD-tag peptide was attached to the C-terminal portion of incomplete LacI proteins. If this is the case, the bands should also be recognized by anti-DD-tag antibody in western blotting. In fact, the bands recognized by anti-LacI antibody cross-reacted with anti-DD-tag antibody, indicating that the truncated LacI proteins are efficiently tagged with SsrA^{DD} RNA (Figure 4, lane 5). Interestingly, three strong signals to anti-DD-tag

Fig. 4. Western blot analysis of the LacI protein produced in cells harboring pIT613. MC4100Δ*ssrA* cells harboring pIT613 were transformed with pSTV28 (lanes 2 and 7), pSsr-AA (lanes 3 and 6) or pSsr-DD (lanes 4 and 5). Total proteins equivalent to OD₆₀₀ = 0.01 were analyzed by western blotting using anti-LacI antibody (lanes 1–4) or anti-DD-tag antibody (lanes 5–8). The position of intact LacI is indicated by an asterisk. The dashed arrows indicate major truncated LacI proteins recognized by anti-LacI antibody in Δ*ssrA* cells. The arrow indicates an elongated tagged LacI recognized by anti-LacI antibody. The major tagged LacI proteins recognized by anti-DD-tag antibody are indicated by a bracket. Lanes 1 and 8 represent total proteins equivalent to OD₆₀₀ = 0.01 prepared from MC4100Δ*ssrA* cells harboring pBR322/pSTV28 and pBR322/pSsr-DD, respectively.

Fig. 5. Effect of IPTG on LacI tagging. MC4100Δ*ssrA* cells harboring both pIT613 and pSsr-DD were grown in the presence (lanes 2 and 4) or the absence (lanes 1 and 3) of 1 mM IPTG. Total proteins equivalent to OD₆₀₀ = 0.01 were analyzed by western blotting using anti-LacI or anti-DD-tag antibodies. The position of intact LacI is indicated by asterisks. The arrow indicates an elongated tagged LacI recognized by anti-LacI antibody. The major tagged LacI proteins recognized by anti-DD-tag antibody are indicated by a bracket.

antibody were observed near the position of intact LacI (Figure 4, indicated by a bracket). The largest band corresponds exactly to the extended LacI that is recognized by anti-LacI antibody and separable from the intact LacI on the gel. The remaining two prominent DD-tagged proteins exhibit almost the same electrophoretic mobility as the intact LacI (Figure 4). These two bands are likely to be tagging products of shorter truncated LacI proteins observed below the intact LacI in *ssrA* cells carrying both pIT613 and pSTV28. Furthermore, the tagging of another longer truncated LacI, which was not completely separable from the intact LacI on the gel (Figure 4, lane 2), presumably leads to the production of the extended LacI.

Fig. 6. Effect of operator mutations on LacI tagging. The pIT613 derivatives containing the *lacO* variants indicated were introduced into MC4100Δ*ssrA* cells harboring pSsr-DD. The nucleotide sequences of *lacO* variants are shown in Figure 3. Cells were grown in the presence or the absence of 1 mM IPTG and total proteins equivalent to OD₆₀₀ = 0.01 were analyzed by western blotting using anti-DD-tag antibody. The position of intact LacI is indicated by an asterisk. The major tagged LacI proteins recognized by anti-DD-tag antibody are indicated by a bracket.

The exact assignment of these bands remains to be studied. In any case, however, the anti-DD-tag specific bands were no longer observed when cells carried pSsr-AA (Figure 4, lane 6) or pSTV28 (Figure 4, lane 7). In addition, they were not observed in cells harboring both pSsr-DD and a control plasmid pBR322, except a few, less significant smaller bands (Figure 4, lane 8). These results strongly suggest that truncated LacI proteins are indeed produced and efficiently tagged with the SsrA^{DD} RNA by a *trans*-translation mechanism and that the major tagging sites would be close to the C-terminus of LacI. Few tagged bands were detected when the LacI was co-expressed with the wild-type SsrA RNA (Figure 4, lane 6), suggesting that the LacI proteins containing the wild-type tag sequence were efficiently removed by proteolysis. Although a number of DD-tagged endogenous cellular proteins are produced in cells harboring pSsr-DD as shown in Figure 2, only a few bands were detectable due to the smaller amount of total proteins analyzed (Figure 4, lane 8).

Effects of IPTG and operator mutations on LacI tagging

If the binding of LacI to its operators is responsible for the production of truncated *lacI* mRNAs that are the potential substrates for the SsrA system, one would expect that inactivation of LacI by isopropyl-β-D-thiogalactopyranoside (IPTG) eliminates the LacI tagging. In fact, the presence of IPTG in the culture medium significantly reduced the production of the extended LacI recognized by anti-LacI antibody (Figure 5, lanes 1 and 2) and a series of tagged LacI proteins recognized by anti-DD-tag antibody including three major bands (lanes 3 and 4), indicating that LacI tagging depends on the binding of LacI to the *lac* operator(s).

The dependency of LacI tagging on the binding of LacI to its operators was further analyzed by mutating the *lac* operators. As expected, the mutation of *O3* significantly

Fig. 7. Model for *trans*-translation acting on the *lacI* mRNA. Formation of the *O1*–*LacI*–*O3* repression DNA loop may cause premature termination of transcription resulting in truncated *lacI* mRNAs that would be recognized by the SsrA system.

reduced the extent of LacI tagging (Figure 6, lane 5), indicating that LacI–*O3* interaction is required for efficient tagging. Interestingly, the tagging was more significantly reduced when *O1* was disrupted (Figure 6, lane 3), suggesting that the LacI tagging is dependent upon the specific binding of LacI to both *O1* and *O3*. When *O3* was replaced with the ideal operator (Sadler *et al.*, 1983), the LacI tagging was markedly enhanced and it was still sensitive to IPTG (Figure 6, lanes 7 and 8). Taken together, we conclude that the *O3*–LacI–*O1* repression loop (Oehler *et al.*, 1990) is primarily responsible for the production of truncated *lacI* mRNAs and therefore for the LacI tagging (Figure 7).

Tagging in cells harboring a plasmid containing the wild-type *lacI* gene

So far we focused on cells harboring pIT613 because the LacI protein is efficiently expressed from the *lacI^q* promoter, and this facilitated the analysis of the SsrA-mediated LacI tagging. To examine whether the SsrA-mediated LacI tagging also occurs when LacI is expressed from the wild-type *lacI* promoter, we analyzed proteins produced in cells harboring pIT455 carrying the native *lac* region by western blotting using anti-LacI antibody. Although LacI is poorly expressed from the wild-type *lacI* promoter, the use of a large amount of total proteins allowed us to detect LacI as a major band along with other non-specific cellular bands with anti-LacI antibody (Figure 8). We first examined whether the major truncated LacI proteins could be produced in the absence of SsrA RNA. The presumed truncated LacI proteins were detected along with several non-specific bands (Figure 8, lane 2). These bands were apparently missing in the presence of the wild-type SsrA RNA, suggesting that they were

Fig. 8. LacI tagging in the wild-type *lacI* gene. MC4100Δ*ssrA* harboring pIT455 was transformed with vector plasmid pSTV28 (lane 2), pSsr-AA (lane 3) or pSsr-DD (lanes 4 and 5). MC4100Δ*ssrA* harboring both pBR322 and pSTV28 was also subjected to the analysis as a control (lane 1). Cells were grown in the presence (lane 5) or the absence (lanes 1–4) of 1 mM IPTG and total proteins equivalent to OD₆₀₀ = 0.2 were analyzed by western blotting using anti-LacI antibody. The position of intact LacI is indicated by an asterisk. The arrow indicates an extended tagged LacI recognized by anti-LacI antibody.

removed by the SsrA system (Figure 8, lane 3). When LacI was co-expressed with SsrA^{DD}, extended tagged LacI was clearly detected (Figure 8, lane 4). This band was significantly reduced in the presence of IPTG (Figure 8, lane 5). The extended tagged LacI was no longer detected when LacI was co-expressed with the wild-type SsrA RNA (Figure 8, lane 3) or when LacI alone was expressed (Figure 8, lane 2). These results indicate that the SsrA-mediated LacI tagging and proteolysis indeed occur even when LacI was expressed from the wild-type *lacI* promoter. We also performed western blotting using anti-DD-tag antibody. However, the identification of tagged LacI proteins with anti-DD-tag antibody was difficult due to the presence of a large number of cellular tagged proteins under these experimental conditions (data not shown).

Effect of the *ssrA* mutation on the induction of the *lac* operon

Does SsrA-mediated LacI tagging and proteolysis play any role in the regulation of *lac* operon expression? It was reported previously that the level of β-galactosidase was consistently 10-fold lower in *ssrA* cells in comparison with the wild-type cells at concentrations of IPTG ranging from 0 to 4 mM (Retallack and Friedman, 1995). We re-examined the effect of the *ssrA* mutation on *lac* operon expression. We observed that the level of β-galactosidase was 2- to 3-fold lower in the *ssrA* strain than in the wild-type strain when a low concentration (<0.01 mM) of IPTG was used, whereas essentially no difference in the β-galactosidase activity between two strains was observed at a high concentration (>0.1 mM) of IPTG (data not shown). Thus, the effect of the *ssrA* mutation on the expression of the *lac* operon in an equilibrium state apparently varied depending on the IPTG concentration. However, we found that the *ssrA* mutation affected the kinetics of induction of the *lac* operon upon IPTG addition. Namely, cells lacking SsrA showed a significant delay in the induction of the *lac* operon upon the addition of IPTG (Figure 9A). Furthermore, an extended lag phase (a delay in *lac* operon expression) upon the exhaustion of

Fig. 9. Effect of the *ssrA* mutation on *lac* operon expression. (A) Expression of the *lac* operon in response to the addition of IPTG. W3110 (squares) and its *ssrA* derivative (circles) were grown in LB medium. IPTG was added to the culture at $OD_{600} = 0.3$ to a final concentration of 0.1 mM and the β -galactosidase activity at various time points was measured. (B) Expression of the *lac* operon in W3110 (upper panel) and its *ssrA* derivative (lower panel) in response to the consumption of glucose in the medium. The strains were grown in M9 minimal medium containing 0.04% glucose and 0.2% lactose as carbon sources. Optical density at 600 nm (squares) and β -galactosidase activity (diamonds) of the samples were determined at the time points indicated.

glucose in glucose–lactose diauxic growth was also observed in *ssrA* cells (Figure 9B). These results suggest that the SsrA system allows the cells to use lactose rapidly in response to the environmental conditions (lactose availability) by modulating LacI expression and/or activity (see Discussion).

Discussion

In the present work, we first found that a plasmid-encoded chimeric CRP protein containing the C-terminal degradation tag appears to be degraded less effectively in the presence of the wild-type SsrA RNA than in the absence of this RNA. This was presumably due to the production of a plentitude of tagged cellular proteins through *trans*-translation in cells with the wild-type *ssrA* gene. In fact, we demonstrated directly by western blotting that many cellular polypeptides can be tagged with a mutant SsrA^{DD} encoding a protease-resistant DD-tag. The DD-tagged cellular proteins have been reported previously (Karzai *et al.*, 1999), although the tagging was shown only in higher molecular weight proteins in the previous study. An important conclusion of our experiments is that a *trans*-translation event could occur quite frequently on a variety of cellular mRNAs, resulting in a large number of tagged polypeptides in normally growing cells.

Then we tried to identify the potential natural targets for *trans*-translation and this led us to the major conclusion that *lacI* mRNA is one of the targets for *trans*-translation. We found that truncated LacI proteins were significantly produced in the *ssrA* cells but not in cells with the wild-type *ssrA* gene. The use of a variant *ssrA* gene encoding SsrA^{DD} allowed us to detect the tagged LacI proteins directly by western blotting. We showed that the tagging occurred preferentially at the C-terminus of LacI because the major tagged proteins were larger or similar in size compared with the intact LacI. Furthermore, we demonstrated that the LacI tagging was dependent on the binding of LacI to both *O*1 and *O*3, because inactivation of LacI by IPTG or the disruption of one of two operators markedly reduced the LacI tagging. Our data clearly demonstrate a typical situation where SsrA RNA participates in tagging and proteolytic degradation of incompletely synthesized polypeptides. A strong DNA-binding protein such as LacI bound to sites within or near the ORF may cause premature transcription termination resulting in the truncated mRNAs that can be recognized by the SsrA system.

In addition, our results suggest that the SsrA-mediated tagging and proteolysis of LacI plays a role in the regulation of expression of the *lac* operon in response to lactose availability. How does SsrA affect *lac* expression? It was originally proposed that SsrA RNA, by binding several DNA-binding proteins including LacI and other regulatory proteins, may reduce their free concentrations and thereby reduce their activities (Retallack and Friedman, 1995). However, this competition model fails to explain more recent results regarding SmpB, a newly identified RNA-binding protein essential for the SsrA-mediated tagging activity, because *smpB* cells exhibit the same phenotype as *ssrA* cells even though they have a normal level of SsrA RNA (Karzai *et al.*, 1999). We assume that the lack of SsrA-mediated tagging and subsequent proteolysis of truncated LacI is responsible for the delayed induction of the *lac* operon in the *ssrA* cells. For example, one could argue that the total concentration of LacI would be higher in *ssrA* cells due to the accumulation of truncated LacI proteins, which may lead to the delayed induction of the *lac* operon. More interestingly, our preliminary experiments suggest that

Table I. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and property	Source
Strain		
W3110 <i>ssrA</i>	W3110Δ <i>ssrA</i> ::Kan	Komine <i>et al.</i> (1994)
PP47	<i>crp</i>	Aiba <i>et al.</i> (1982)
PP47 <i>ssrA</i>	PP47Δ <i>ssrA</i> ::Kan	this study
MC4100	Δ <i>lac</i>	Casadaban (1976)
MC4100 <i>ssrA</i>	MC4100Δ <i>ssrA</i> ::Kan	this study
Plasmid		
pHA7	derivative of pBR322 containing the <i>crp</i> expressed from <i>bla</i> promoter	Aiba <i>et al.</i> (1982)
pHA7M	derivative of pHA7 in which a <i>Mlu</i> I site is introduced near last codon of <i>crp</i>	this study
pHA7M-AA	derivative of pHA7M containing a variant <i>crp</i> encoding CRP-AA	this study
pHA7M-DD	derivative of pHA7M containing a variant <i>crp</i> encoding CRP-DD	this study
pSTV28	derivative of pACYC184	Takara Shuzo
pCRP	derivative of pSTV28 containing the <i>crp</i> derived from pHA7M	this study
pCRP-AA	derivative of pSTV28 containing a variant <i>crp</i> encoding CRP-AA	this study
pCRP-DD	derivative of pSTV28 containing a variant <i>crp</i> encoding CRP-DD	this study
pSsr-AA	derivative of pSTV28 containing the wild-type <i>ssrA</i>	this study
pSsr-DD	derivative of pSTV28 containing a variant <i>ssrA</i> encoding SsrA ^{DD} RNA	this study
pTTQ18	derivative of pUC18 containing the <i>lacI</i> ^q	Stark (1987)
pIT613	derivative of pBR322 containing the <i>lacI</i> ^q <i>ZYA</i>	this study
pIT647	derivative of pIT613 in which the <i>lacO</i> 1 is mutated	this study
pIT648	derivative of pIT613 in which the <i>lacO</i> 3 is mutated	this study
pTA100	derivative of pIT613 in which the <i>lacO</i> 3 is changed to the ideal operator	this study
pXX701	derivative of pUC13 containing the wild-type <i>lacIZYA</i>	Niki <i>et al.</i> (1988)
pIT455	derivative of pBR322 containing the wild-type <i>lacIZYA</i>	this study

some truncated LacI proteins lacking several amino acid residues at their C-termini exhibit an inducer-insensitive property (*I*^s phenotype) (T.Abo, T.Inada and H.Aiba, unpublished data). The production of the truncated LacI proteins including the inducer-insensitive variant in the repressed state could explain the observed effects of *ssrA* mutation on the kinetic nature of *lac* operon expression, namely the significant delay of induction in the *ssrA* strain. This is because the truncated LacI proteins would be produced only in the absence of inducer and they will be diluted out over several generations after IPTG addition. This explains well why *lac* operon expression is barely affected by the *ssrA* mutation under the induced state.

The most distinctive phenotype of SsrA-defective cells is a dramatic reduction in the ability to support the growth of λ phage (Retallack *et al.*, 1994; Withey and Friedman, 1999) and mu phage (Karzai *et al.*, 1999). In this respect, it should be noted that the *ssrA* mutation has been shown to cause a delay in the induction of phage P22 lysogen after mitomycin treatment in *Salmonella* (Julio *et al.*, 2000). Thus, there is an apparent similarity between the *lac* and P22 systems with regard to the kinetics of induction. It is interesting to examine whether the SsrA system is involved in the regulation of phage repressor proteins in quantity and/or in quality as in the case of LacI.

Materials and methods

Media and growth conditions

Cells were grown aerobically at 37°C in Luria–Bertani (LB) medium (Miller, 1972). Antibiotics were used at the following concentrations: ampicillin (50 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml). Bacterial growth was monitored by determining the optical density at 600 nm.

Strains and plasmids

The *E. coli* K-12 strains and plasmids used in this study are listed in Table I. P1 phage produced from W3110 Δ*ssrA*::kan (Komine *et al.*,

1994) was used to disrupt the *ssrA* gene of PP47 (*crp*) (Aiba *et al.*, 1981) and MC4100 (Δ*lac*) (Casadaban, 1976). To construct mutant *crp* genes encoding CRP-AA or CRP-DD, a *Mlu*I site was first introduced near the last codon of CRP ORF in plasmid pHA7 (Aiba *et al.*, 1982). The resulting plasmid was designated pHA7M. DNA fragments encoding ANDENYALAA and ANDENYALDD (see Figure 1 for their nucleotide sequences) were prepared by annealing complementary synthetic oligonucleotides and inserted into the *Mlu*I site of pHA7M. The resulting plasmids were designated pHA7M-AA and pHA7M-DD, respectively. The *Eco*RI–*Bam*HI fragments of pHA7M, pHA7M-AA and pHA7M-DD were subcloned into pSTV28 (Takara) to construct plasmids pCRP, pCRP-AA and pCRP-DD, respectively (Figure 1A). pIT613 containing the *lacIZYA* in which the *lacI* is transcribed from the *lacI*^q promoter was constructed by inserting the 1.9 kb *Hind*III–*Eco*RV fragment containing the *lacI*^q promoter and the *lacI*^q ORF derived from pTTQ18 (Stark, 1987), a 1.5 kb *Eco*RV–*Eco*RV fragment containing '*lacI*–*lacZ*' and a 10.5 kb *Eco*RV–*Pst*I fragment with *Pst*I–*Sal*I linker attached containing *lacZ*^q*YA*, both derived from pXX701 (Niki *et al.*, 1988), into pBR322. The pIT613 derivatives with operator mutations were constructed by PCR mutagenesis. pIT455 was constructed by inserting a 12 kb *Pst*I fragment containing the *lacIZYA* region derived from pXX701 into pBR322. The 707 bp DNA fragment containing the *ssrA* gene was amplified by PCR using primers 5'-CCGGAA-TTCTGTTGACCAAGTTCCTCACCG-3' and 5'-CCCAAGCTTAATG-GGCCTAAAAGGTTCCGG-3' from the chromosomal DNA of W3110, digested with *Eco*RI and *Hind*III, and then cloned into pSTV28 to construct pSsr-AA. pSsr-DD was constructed by PCR mutagenesis from pSsr-AA.

Western blotting

Anti-CRP polyclonal antibody was described previously (Ishizuka *et al.*, 1993). Anti-LacI and anti-DD-tag polyclonal antibodies were prepared by immunizing the rabbit with LacI protein purified based on the method described (Müller-Hill *et al.*, 1971) and a synthetic peptide, AAN-DENYALDD, respectively. Bacterial cells grown in LB medium containing appropriate antibiotic(s) were harvested at OD₆₀₀ = 0.8 and suspended in 100 μl of SDS–PAGE loading buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). The sample was heated at 100°C for 5 min. Total cellular proteins of the indicated amount were subjected to 12% acrylamide–0.1% SDS gel electrophoresis and transferred to Immobilon membrane (Millipore). The polypeptides detected by the antibodies were visualized by an ECL system (Pharmacia).

Northern blot analysis

Cells were grown aerobically at 37°C in LB medium and total RNAs were extracted as described (Aiba *et al.*, 1981). The total RNAs were resolved by 1.0% agarose gel electrophoresis in the presence of formamide and blotted onto Hybond-N⁺ membrane (Amersham) as described (Sambrook *et al.*, 1989). The *crp* mRNAs were visualized using the Gene Images labeling and detection system (Amersham). The HindIII-EcoRV fragment derived from pHA7 was used as a probe for *crp* mRNA.

β -galactosidase assay

β -galactosidase activity was determined with permeabilized cells by the method of Miller (1992).

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