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RNase III initiates rapid degradation of *proU* mRNA upon hypo-osmotic stress in *Escherichia coli*

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Hyper-osmotic stress strongly induces expression of the *Escherichia coli proU* operon encoding a high affinity uptake system for the osmoprotectants glycine betaine and proline betaine. Osmoregulation of *proU* takes place at the transcriptional level by upregulation of the promoter at high osmolarity and repression of transcription by the nucleoid-associated protein H-NS at low osmolarity. In the present study, we describe an additional level of *proU* osmoregulation that is independent of transcriptional regulation. We show that osmoregulation occurs at a post-transcriptional level involving RNase III. RNase III specifically processes the *proU* mRNA within a conserved secondary structure extending from position +203 to +293 of the transcript. Processing is efficient at low osmolarity, but inhibited at high osmolarity. Blocking of RNase III processing by mutation of the processing site eliminates post-transcriptional osmoregulation of *proU*. Further, the *proU* mRNA is relatively stable at high osmolarity with a half-life of approximately 65 sec. However, upon osmotic downshift, RNase III immediately processes the *proU* mRNA which reduces its half-life to less than 4 sec. The data suggest that the primary role of RNase III-mediated processing of *proU* mRNA is to ensure rapid shutdown of *proU* upon hypo-osmotic stress.

Escherichia coli RNase III is a member of the ubiquitous family of RNase III enzymes.[1] It contains a single ribonuclease domain and a double-stranded RNA binding domain typical for the class I of these enzymes and is active as a dimer. The primary role of RNase III in *E. coli* is maturation of rRNAs and of some tRNAs.[2] However, RNase III also controls the stability and activity of specific transcripts.[2] RNase III specifically processes double-stranded RNA generating 3' hydroxyl overhangs of two nucleotides.[3] RNase III processes specific secondary structures formed within transcripts or generated by pairing of an ncRNA (small regulatory non-coding RNA) with its cognate mRNA.[4-7] Accordingly, in an RNase III mutant the abundance of 12% of the mRNAs is affected.[8]

Examples for processing within secondary structures of mRNAs include autoregulation of RNase III by processing of the *rnc* mRNA within the 5' untranslated leader,[9] and processing of the *rpsO-pnp* operon mRNA encoding ribosomal protein S15 and polynucleotide phosphorylase within the intergenic region.[10] Furthermore, RNase III processes *bdm* mRNA encoding a small protein downregulated in biofilms and induced by osmotic stress.[11,12] Interestingly, environmental cues such as temperature and osmolarity affect processing by RNase III.[12,13] The cold-shock induced protein YmdB interacts with the catalytic region of RNase III and thereby inhibits processing of *rnc* and *pnp* mRNA under cold-shock.[13] Similarly, the activity of RNase III is inhibited at high osmolarity resulting in upregulation of the *rnc*, *pnp*, and *bdm* mRNAs.[12] The mechanism of osmoregulation of RNase III activity is still unclear.[12]

The *proU* (*proVWX*) operon encompasses three genes and encodes a high affinity ABC transport system for the uptake of osmoprotectants such as glycine betaine and proline betaine.[14,15] Osmoregulation of *proU* expression is very tight. It involves induction of the osmo-responsive σ70-dependent *proU* promoter at high osmolarity and repression of transcription by the nucleoid-associated protein H-NS at low osmolarity. Repression by H-NS requires binding sites upstream of the promoter and high affinity nucleation sites located downstream of the promoter.[16-19] In addition, a second σS-dependent promoter as well as additional regulatory elements located upstream and within the *proV* coding region are likely to add to *proU* osmoregulation in *E. coli*.[16,17,20] However, osmotic upshift and growth at high osmolarity results in several 100-fold induction of *proU* expression,[16,17,21] and up to date the molecular mechanisms underlying this high degree of *proU* osmoregulation remains unclarified.

Here we show that osmoregulation of the *proU* operon includes a post-transcriptional level which is based on processing of the

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proU mRNA by RNase III. This additional post-transcriptional level of osmoregulation by RNase III contributes to repression of *proU* under low osmolarity conditions. Furthermore, we show that RNase III-mediated processing triggers immediate degradation of the *proU* mRNA upon shift from high to low osmotic conditions, suggesting that processing is crucial for rapid shutdown of *proU* expression.

Results

Osmoregulation of proU includes a post-transcriptional level. To re-address osmoregulation of the *proU* operon, we constructed reporter fusions, which supposedly carry all elements important for *proU* osmoregulation. These fusions include 315 bp of the upstream regulatory region and the full-length *proV* gene. At the 3' end of the *proV* open reading frame a sequence coding for a HA-tag was added for monitoring ProV protein levels. To quantify steady-state expression levels at various osmolarities, the *lacZ* gene was fused 3' to the $proV_{HA}$ coding region either as a transcriptional or as a translational fusion. The *lacZ* constructs were integrated into the chromosome of a strain in which the native *proU* operon was deleted.

First, we quantified the contribution of H-NS repression and of the *proU* promoter to osmoregulation using the *lacZ* reporter constructs by determining β-galactosidase expression levels of strains grown in low and high salt LB medium (0.01 M and 0.3 M NaCl) (**Fig. 1**). The reporter construct with a translational fusion of *lacZ* to the 3' end of the $proV_{HA}$ gene was expressed at an extremely low level at low osmolarity, and was induced more than 2000-fold in cells grown at high osmolarity (P*proU* Φ*proV-lacZ*

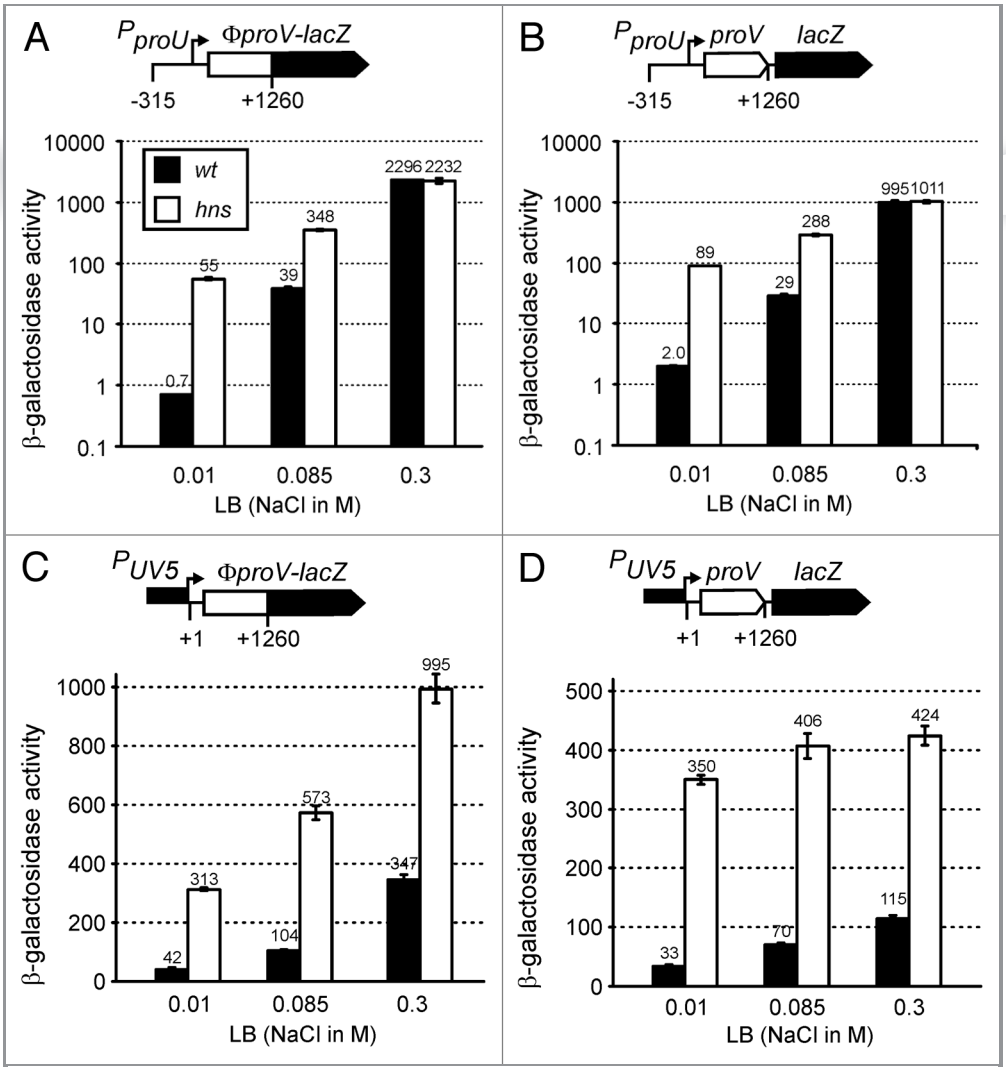

Figure 1. Contribution of the *proU* promoter, H-NS-mediated repression, and post-transcriptional control to *proU* osmoregulation. Expression analyses of translational (A, C) and transcriptional (B, D) *lacZ* reporter constructs integrated into the chromosome. Expression of the reporter constructs directed from the natural *proU* promoter (A, B) and constitutive *lacUV5* promoter (C, D) was determined in the wild type (*wt*) and *hns* mutants. Bacteria were grown in LB with the indicated NaCl concentrations. The expression levels are indicated by bars (black for wild type and white for *hns* mutant). Values are the mean of at least three measurements. Error bars represent standard error of the mean. Strains used: A: *wt* S4068, *hns* S4115; B:, *wt* S4066, *hns* S4113; C: *wt* S4070, *hns* S4117; D, *wt* S4064, *hns* S4111. Note that different scales are used to indicate β-galactosidase activity.

in **Fig. 1A**). In the *hns* mutant osmoregulation of this fusion dropped to 41-fold (**Fig. 1A**), confirming the importance of H-NS repression for osmoregulation. This drop in osmoregulation is due to de-repression of *proU* at low osmolarity in the *hns* mutant (**Fig. 1A**). At high osmolarity no difference between expression in the wild type and the *hns* mutant was detected (**Fig. 1A**), demonstrating that H-NS-mediated repression of *proU* is abrogated at high osmolarity. Substitution of the native osmoresponsive *proU* promoter by the *lacUV5* promoter lead to a big drop in osmoregulation from more than 2000-fold to only 8-fold in case of the translational Φ*proV*$_{HA}$-*lacZ* fusion (compare **Fig. 1A and 1C**). This drop in osmoinduction presumably reflects both the effects of promoter exchange and the loss of H-NS binding sites located within the upstream regulatory element (see also ref. 19).

Surprisingly, we found that expression of the translational Φ*proV-lacZ* fusion remained 3.2-fold osmoregulated even when the *proU* promoter was substituted by the constitutive *lacUV5* promoter and when simultaneously *hns* was deleted (*PlacUV5* Φ*proV-lacZ* in **Fig. 1C**). In contrast, the transcriptional *PlacUV5 proV-lacZ* fusion was not osmoregulated in the *hns* mutant (**Fig. 1D**). Correspondingly, there was a similar 4-fold difference in osmoregulation of the translational vs. the respective transcriptional fusion, whose expression are directed by the native *proU* promoter (compare **Fig. 1A** and **Fig. 1B**). These data suggested that, in addition to H-NS repression and osmoinduction of the *proU* promoter, another independent mechanism contributes to *proU* osmoregulation. This mechanism must be operative on a post-transcriptional level, as it is detected only with the translational but not with the transcriptional *lacZ* reporter gene fusion.

To corroborate the results obtained with the *lacZ* reporter fusions, osmoregulation of *proU* was independently determined by protein gel blots. The *proV*$_{HA}$ gene was expressed from low-copy-number plasmids in wild type and *hns* mutant cells. Again two constructs were used, one with *proV*$_{HA}$ being expressed from the native *proU* promoter and the other with expression being directed by the *lacUV5* promoter. Expression was determined for cells grown under low and high osmolarity conditions, as well as after hyper-osmotic upshift (**Fig. 2**). Strong osmoinduction was observed already ten minutes after hyper-osmotic stress when the amount of ProV$_{HA}$ protein was similarly high as in cells grown under steady-state hyper-osmotic conditions (**Fig. 2**). As expected, osmoregulation of *proV*$_{HA}$ expression was higher when expression was directed by the *proU* promoter than by the *lacUV5* promoter (**Fig. 2**). Importantly, *proV*$_{HA}$ expression was still osmoregulated when expression was directed by the *lacUV5* promoter in *hns* mutant cells (**Fig. 2**). These results are qualitatively in agreement with the results obtained with the *lacZ* reporter fusions and thus support an additional post-transcriptional mechanism of osmoregulation. Quantitative differences may be attributable to a lower reliability of protein gel blot quantification as compared with the wide linear range of β-galactosidase assays.

The proU mRNA is processed by RNase III and this processing is osmoregulated. Post-transcriptional osmoregulation of *proU* expression can be based on various mechanisms including modulation of *proV* mRNA stability, of translation and of protein stability. Intriguingly, within the *proV* coding sequence there is an

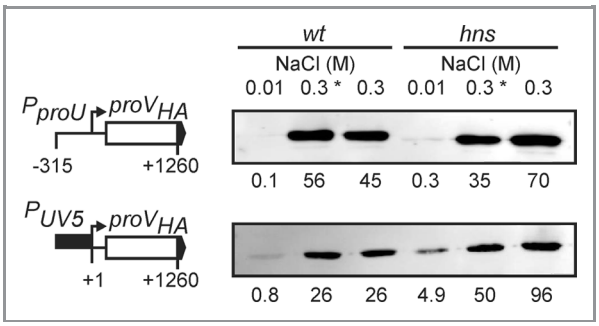

Figure 2. Osmoregulation of *proU* analyzed by western-blot detection of ProV-HA expression. Osmoregulation of *proU* was determined by western-blot analyses using plasmids encoding a tagged *proV*$_{HA}$ gene. Expression was directed from the *proU* promoter (top, plasmid pKEKK11) or the *lacUV5* promoter (bottom, plasmid pKEKK12) in wild-type cells (S3460) and in the *hns* mutant (S4162). Cells were grown under steady-state conditions in LB supplemented with 0.01M or 0.3M NaCl (labeled 0.01 and 0.3 respectively). In addition, cultures were first grown in LB with 0.01M NaCl to OD_{600} of 0.5, followed by addition of NaCl to a final concentration of 0.3M, and samples were taken 10 min later (labeled 0.3*). The Western-blot was probed with HA-tag antibodies and the bands of this representative blot were quantified. The result of the quantification is indicated as arbitrary units underneath the blots.

approximately 100 bp stretch of DNA which is identical in *E. coli* K12 and *Salmonella enterica* serovar Typhimurium str. LT2,[16] and which is highly conserved among the *proU* loci of *Enterobacteriaceae* (**Table 1**). This conserved sequence maps from +203 to +293 relative to the transcription start. Secondary structure prediction using the program *mfold*[22] with various fragments of different length suggests that the *proU* mRNA can form an extensive stem-loop structure (**Fig. 3**). A similar stem-loop structure was predicted before.[16] This potential stem-loop structure likewise is conserved in *Enterobactericeae*, as sequence variations map predominantly to bulges and loops (**Fig. 3**). Additional sequence variations mapping within double-stranded regions of the secondary structure presumably do not disrupt pairing. In most cases these sequence variations concern the exchange of G-C or A-U pairs to the non-canonical G-U, or the exchange of G-C and A-U pairs (**Fig. 3**).

Table 1. Conservation of *proU* +203 to +293 in *Enterobactereaceae*

Homology of *proU* sequence[a]		
Genus (number of strains)	**+1 to +1263**	**+203 to +293**
Escherichia (with *Shigella*) (33)	96–100%	99–100%
Salmonella (17)	82–85%	99–100%
Citrobacter (1)	86%	98%
Enterobacter (2)	82%	95–96%
Sodalis (1)	75%	97%
Klebsiella (2)	82%	92%
Erwinia (1)	76%	93%
Photorhabdus (1)	75%	91%

[a] Percent sequence identity determined using BLAST.

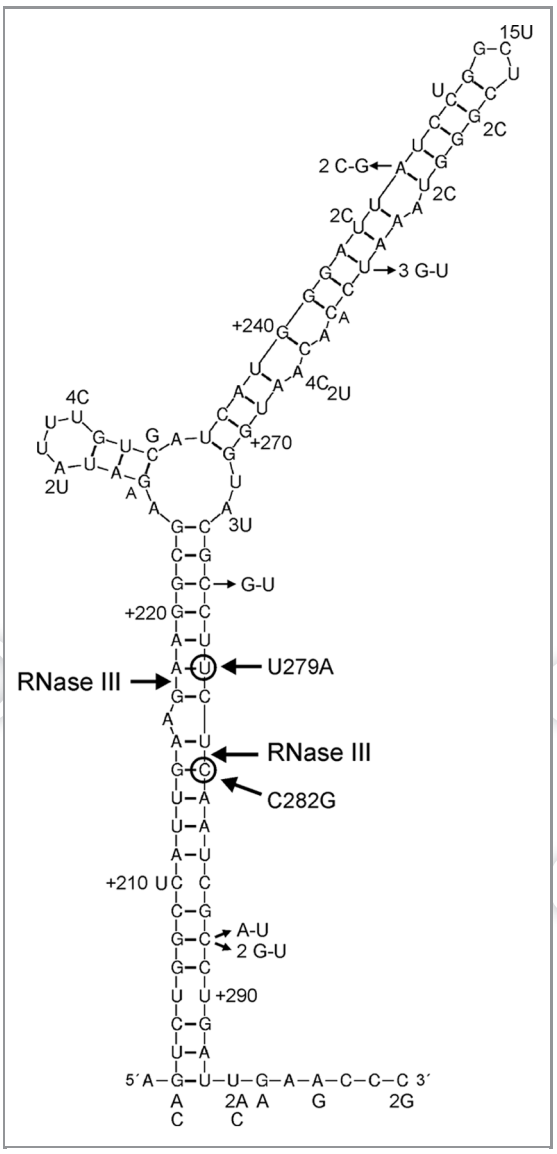

Figure 3. Conserved secondary structure of the *proU* mRNA and RNase III processing sites. The secondary structure of the *proU* mRNA +202 to +300 region from *E. coli* K-12 substr. MG1655 was predicted using *mfold*.[22] Numbers indicate the coordinates in relation to the transcription start (+1). RNase III cleavage sites mapped by 5′ RACE are shown with arrows. Nucleotides mutated to abolish RNase III processing are circled. A BLAST search with the *proU* sequence of *E. coli* K-12 substr. MG1655 against bacterial genomes was performed and single nucleotide polymorphisms (SNPs) found in 57 *Enterobacteriaceae* strains were mapped. The SNPs are shown as single nucleotide or as nucleotide pairs. The following SNPs were found: *Escherichia*, 11 of 33 analyzed strains carry SNP C252U, 1 strain carries SNP C276U; *Salmonella*, 1 of 16 strains carries SNP C252U; *Citrobacter*, 1 strain, 2 SNPs: A228U, C252U; *Enterobacter*, 2 strains, in total 9 SNPs: G203C, A228U, U231C, C234G, A243G, C252U, A267C, A267U, A273U; *Sodalis*, 1 strain, 6 SNPs: U231C, A267U, C288U, U294A, A297G, C300G; *Klebsiella*, 2 strains, both carry the same 7 SNPs: U231C, A243G, U244C, A246C, U258G, A267C, A273U; *Erwinia*, 1 strain, 7 SNPs: C252U, G255C, U258C, C264A, A267C, C288U, U294C; *Photorhabdus*, 1 strain, 11 SNPs: G203A, G208A, C210U, G225A, C249U, G255C, U258C, C288U, U294A, G295A, C300G.

To examine whether the conserved secondary structure represents an RNA processing site, we analyzed the transcripts expressed by the plasmid encoded *PlacUV5 proV*$_{HA}$ reporter construct using 5'-RACE (rapid amplification of cDNA ends).[23] RNA was isolated from transformants of this reporter plasmid, which were grown under low and high osmolarity conditions. For 5' RACE, an oligonucleotide complementary to position +486 to +467 relative to the transcription start was used. When the cells were grown in high osmolarity LB medium, 5' RACE products corresponding to the primary transcript as well as two shorter products were detected (**Fig. 4**). For cells grown at low osmolarity almost no primary transcript was detected, while the two shorter products were apparent (**Fig. 4**). These results suggest that the *proV* RNA is processed and that the processing is osmoregulated. The larger RACE product corresponds to the full-length transcript. Surprisingly, this product was detectable independent of treatment of the RNA with tobacco pyrophosphatase (TAP), which is a required step before ligation of the RNA adaptor to the 5' end of primary transcripts. However, sequencing of several clones suggests that the 5' end of the RNA primary transcript is trimmed in vivo as most clones lacked the first bases irrespective of whether the RNA was isolated from the wild type or *rnc* mutant (**Fig. S1**). For the shorter RACE products sequencing of several cDNA bands revealed that the 5' ends correspond to positions +218 and +282 relatively to the transcription start site (**Fig. S1**). Thus, these presumptive processing sites are located in the double-stranded stem of the putative RNA secondary structure (**Fig. 3**). Importantly, processing at these sites leads to formation of two nucleotide 3' overhangs, which is a typical feature of RNase III processing sites.

To analyze whether RNase III is responsible for processing of the *proV* RNA the 5' RACE experiment was repeated with RNA isolated from an *rnc105* mutant which encodes an inactive RNase III with a G44D exchange.[24] In the *rnc105* mutant only the primary transcript was detected irrespective of whether the transformants with the *proU* reporter plasmid were grown in low or high osmolarity medium (**Fig. 4**). Taken together the data suggest that the *proU* mRNA is processed by RNase III and that this processing is osmoregulated.

Mutation of the RNase III processing sites abolishes post-transcriptional osmoregulation. The importance of RNase III processing for *proU* osmoregulation was further addressed by mutation of the RNase III site and expression analyses of these mutants at low and high osmolarity. Two mutants were constructed, each of which carries a single base mutation expected to disrupt RNA pairing next to the RNase III processing site. These mutants concern the exchange of base C282 to G and of T279 to A, respectively. In both of these mutants the amino acid sequence of ProV remains unchanged.

First, the mutations were introduced into the *PlacUV5 proV*$_{HA}$ reporter plasmid and 5' RACE analysis of the *proU*-C282G and *proU*-T279A mutants was performed. This demonstrated that both of the mutations completely abolished *proV* mRNA processing (**Fig. 4**). Next, we examined the effect of these mutations

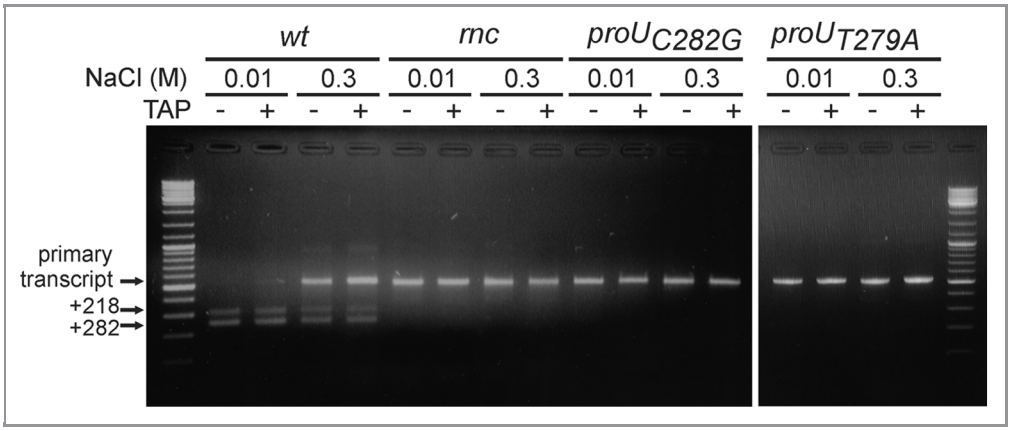

Figure 4. 5′ RACE analysis of *proU* mRNA processing by RNase III. 5′ RACE analysis of *proU* mRNA in the wild type (*wt*) and the *rnc* mutant (*rnc*). Amplification products derived from the primary transcripts and processing products (+218, +282) are indicated. In addition, processing of mRNA of *proU* mutants (C282G and T279A) was analyzed in the wild type strain. Wild type and mutant forms of *proU* transcripts were expressed from the *lacUV5* promoter using low-copy-number plasmids pKEKK12 (wild type *proU*), pKEKK38 for *proU*-C282G, pKEKK36 for *proU*-T279A. Transformants of the wild type strain S3460 and the *rnc* mutant S4023 were grown in LB supplemented with either 0.01M or 0.3M of NaCl. (+) and (-) refers to TAP-treatment prior to ligation of the RNA adaptor.

on osmoregulation with protein gel blot and *lacZ* expression analyses, as well as by qRT-PCR.

Expression analysis by protein gel blots was performed of cells grown at low osmolarity and of cells harvested ten minutes after hyper-osmotic upshift. In the wild-type background both mutations of the RNase III processing site lead to significantly higher expression levels of $ProV_{HA}$ at low osmolarity, but had little effect on $ProV_{HA}$ expression at hyper-osmotic stress (**Fig. 5A and** B). This supports the importance of *proU*-mRNA processing by RNase III. In the *hns* background, in which repression by H-NS is not operative, both mutations of the RNase III processing site caused a significant increase of $ProV_{HA}$ at low osmolarity. Importantly this level was comparable to the expression levels at hyper-osmotic stress. Taken together, these results support the notion that RNase III-mediated processing of *proU* RNA is important for downregulation of *proU* at low osmolarity.

Osmoregulation of the *proU*-C282G and *proU*-T279A mutants was also studied using translational Φ*proV-lacZ* fusions under the control of the *lacUV5* promoter (**Fig. 5C and Fig. S2**). Beta-galactosidase expression levels were determined in wild-type and *hns* mutant cells grown under low and high osmolarity conditions. First, the mutations of the RNase III processing site lead to a significantly higher expression level of all the constructs tested when cells were grown at low osmolarity. In contrast expression in cells grown at high osmolarity was only moderately increased (**Fig. 5C**). As a result, osmoinduction of the *proV*-C282G and *proV*-T279A mutants was 3 to 4-fold lower than of the translational *proV-lacZ* fusion both in the wild type and *hns* mutant (**Fig. 5C**). Furthermore, the mutations of the RNase III processing site completely abolished osmoregulation of the translational *PlacUV5* Φ*proV-lacZ* reporter in the *hns* mutants (**Fig. 5C**). In addition, expression of the translational Φ*proV-lacZ* fusion and its C282G mutant was determined in the *rnc* mutant (**Fig. S2**). Osmoregulation of the *PlacUV5* Φ*proV-lacZ* dropped from 8.3-fold in the wild type to 3.7-fold in the *rnc* mutant, while osmoregulation of the C282G mutant was comparable in the wild type (2.1-fold) and *rnc* mutant (3.2-fold) (**Fig. S2**). (However, these results should be interpreted cautiously, as mutation of *rnc* causes partially non-specific effects on gene expression in *E. coli*[12]). Taken together, these data support the conclusion that RNase III processes the *proU* mRNA at low osmolarity and that this processing is osmoregulated. Further expression analyses using translational Φ*proV-lacZ* fusions under the control of the native *proU* promoter demonstrate that mutation of the processing site results in a 4-fold reduction of osmoregulation irrespective of the promoter and of the presence of H-NS (**Fig. S3**).

Lastly, expression of the *PlacUV5* $proV_{HA}$ reporter plasmid and its C282G mutant was determined by quantitative reverse transcription PCR (qRT-PCR) at steady-state low and high osmolarity conditions (**Fig. 6**). For qRT-PCR two pairs of *proU* primers were used. Primers T520 and T521 were used to amplify the *proU* +171 to +338 cDNA which includes the RNase III processing site and the stem-loop structure. Primers T522 and T523 were used to amplify a *proU* cDNA fragment mapping at the 3' end of the *proV* gene (+1071 to +1212). The relative quantity of *proU* RNAs was normalized to the level of 16S rRNA. In the wild type strain background qRT-PCR revealed a significant (26-fold) osmoregulation of the steady-state amount of $proV_{HA}$ mRNA at the processing site and also at the 3' end of the *proV* gene (8-fold). The mutation C282G drastically reduced osmoregulation to a level of only 2- to 3-fold in the wild type background. In the *hns* mutant the steady-state amount of the wild-type $proV_{HA}$ mRNA was still osmoregulated (12-fold at the processing site, 4-fold at the 3' end of *proV*). Importantly though, in the *hns* background the $proV_{HA}$-C282 mutant was not osmoregulated anymore (**Fig. 6**). These data again support the conclusion that RNase III mediated processing of the *proU* mRNA is osmoregulated.

RNase III initiates rapid degradation of proU mRNA upon osmotic down-shift. Processing of the *proU* mRNA by RNase III

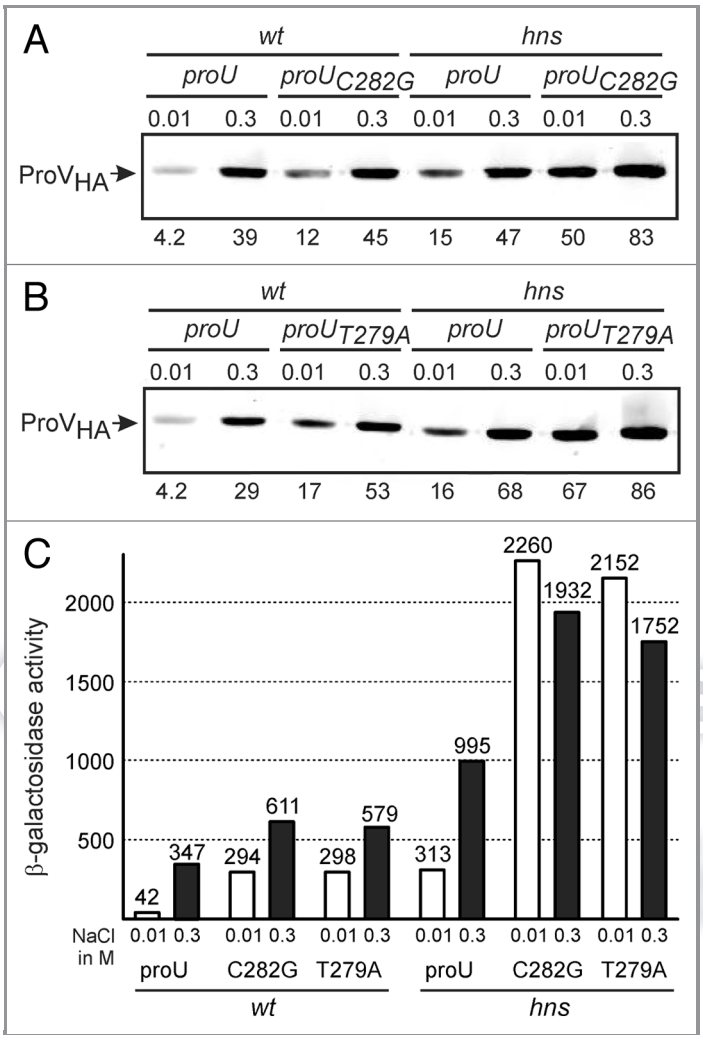

Figure 5. Effect of RNase III processing site mutations on osmoregulation of *proU*. (A and B) Western-blot analyses of expression of $ProV_{HA}$ encoded by wild type *proU*, as well as by the *proU* mutants C282G and T279A. Expression was directed by the *lacUV5* promoter from plasmids pKEKK11 (*proU*, A and B), pKEKK38 (*proU*-C282G), (A), and pKEKK36 (*proU*-T279A), (B) respectively. Transformants of the wild type (S3460) and the *hns* mutant (S4162) were grown in LB supplemented with 0.01M NaCl to OD_{600} of 0.5. Then NaCl was added to a final concentration of 0.3 M and samples were taken immediately before (labeled 0.01) as well as 10 min after this hyper-osmotic stress (labeled 0.3). Total cell lysates were separated by SDS-PAGE, and $ProV_{HA}$ levels (indicated as arbitrary units underneath the blots) were determined using HA-tag specific antibodies. (C) Expression levels of translational *PlacUV5 ΦproU-lacZ* fusion and its C282G and T279A mutant were determined in the wild type and *hns* mutant. Cultures were grown in low and high osmolarity LB medium. The expression levels are given as Miller units and are indicated by bars (white bars LB 0.01M NaCl, black bars for LB 0.3 mM NaCl). Strains used: *proU* (S4070), *proU*-C282G (T811), *proU*-T279A (T887).

is efficient at low osmolarity and inhibited at high osmolarity. Therefore, we hypothesized that the function of RNase III is to initiate fast degradation of the *proU* mRNA upon osmotic downshift, i.e., under physiological conditions at which expression of the *proU* operon must be quickly turned off. To test this hypothesis the stability of the native *proU* mRNA expressed by wild type *E. coli* K12 strain BW30270 under high osmolarity conditions and upon application of hypo-osmotic stress was determined with qRT-PCR. In addition, the C282G mutation was introduced into the chromosomal *proU* locus to block RNase III processing of the *proU* mRNA. The stability of wild type *proU* mRNA and of mutant *proU-C282G* mRNA was compared.

To collect RNA samples for qRT-PCR analysis, the wild type strain BW30270 and the *proU*-C282G mutant strain T1001 were grown in high osmolarity medium (LB 0.3M NaCl) (**Fig. 7**). At mid-exponential growth phase the bacteria were concentrated by centrifugation and re-suspended in a small volume of LB with 0.3 M NaCl. One part of the concentrated bacterial cells was diluted into LB with 0.3 M NaCl and used to determine the *proU* RNA stability at high osmolarity. The other part was transferred to LB containing no NaCl so that the final concentration of NaCl in the medium became 0.01 M to test the *proU* mRNA stability under hypo-osmotic stress. Immediately after transfer of the bacteria to high and low salt LB medium, samples were taken, followed by addition of rifampicin (t = 0) to block transcription. Further samples were taken 30 sec, 1 min, 2.5 min, 5 min and 10 min after rifampicin addition and total RNA was isolated. For qRT-PCR the same two pairs of *proU* primers were used, which include the RNase III processing site and which map to the 3' end of the *proV* gene (+1071 to +1212), respectively (**Fig. 7**). The relative quantity of *proU* RNAs was normalized to the level of 16S rRNA. The normalized values were plotted against the time after rifampicin addition, and the half-life of the RNA was estimated.

The half-life of the *proU* mRNA for region +171 to +338, which reflects the efficiency of RNase III processing, was approximately 65 sec at high osmolarity (**Fig. 7C**). The half-life of the *proU* mRNA decreased dramatically to 4 sec after application of hypo-osmotic stress (**Fig. 7C**). Moreover, samples immediately taken after transfer to low osmolarity medium contained ten-times less RNA than samples immediately taken after transfer to high osmolarity medium (**Fig. 7C**). This demonstrates that the majority of RNA was already degraded during the first few seconds elapsed between transferring cells to low osmolarity medium and taking of the sample for RNA purification. Blockage of RNase III processing by mutation C282G lead to a dramatic increase of the *proU* mRNA half-life under hypo-osmotic stress conditions from 4 sec to 90 sec (**Fig. 7C**). The half-life at high osmolarity was increased from 65 sec to 175 sec (**Fig. 7C**). The dynamics of degradation of *proU* RNA for region +1071 to 1212 was only slightly slower than of the +171 to +338 region (**Fig. 7D**). The half-life of the wild-type mRNA dropped from 75 sec to 7 sec upon application of hypo-osmotic stress. The half-life of the *proU*-C2882G mutant mRNA was determined as 190 sec at high osmolarity and 100 sec under hypo-osmotic stress (**Fig. 7D**). These results support the hypothesis that the function of RNase III processing is to initiate a rapid degradation of *proU* mRNA under hypo-osmotic stress conditions.

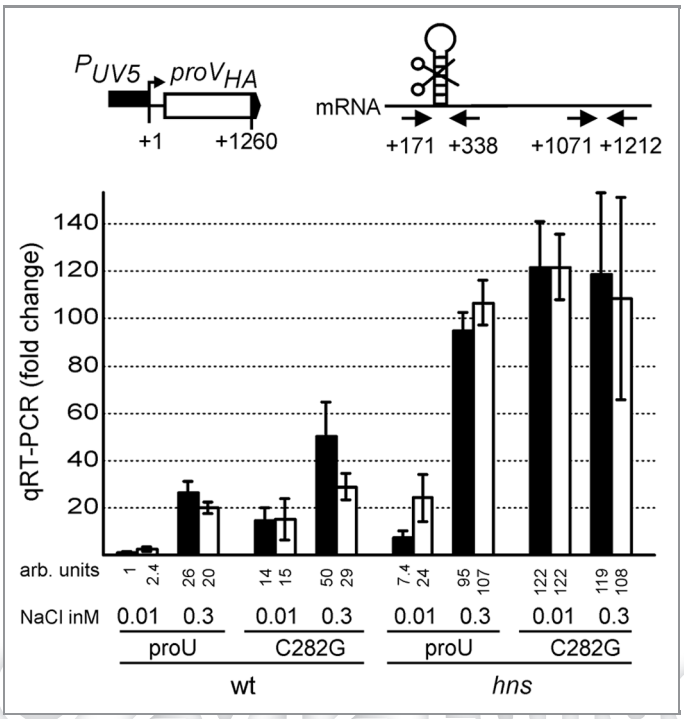

Figure 6. Effect of RNase III processing site mutants on osmoregulation of *proU*. qRT-PCR analyses of expression of plasmidic *PlacUV5 proU*, as well as its mutant C282G. Expression was directed by the *lacUV5* promoter from plasmids pKEKK11 (*proU*) and pKEKK38 (*proU*-C282G), respectively. Transformants of the wild type (S3460) and the *hns* mutant (S4162) were grown in LB supplemented with 0.01 M NaCl or LB with 0.3 M NaCl. The amount of *proU* mRNA was determined with two primer pairs. Indicated by white bars is the relative amount of *proU* +171 to +338 mRNA determined with primers T520 and T521 which flank the RNase III processing site. Black bars indicated the relative amount of the mRNA located at the 3′ end of the *proV* gene (+1071 to +1212) as determined with primers T522 and T523.

Discussion

Expression of the *proU* operon is tightly osmoregulated. Osmoregulation operates at a transcriptional level and, as shown here, at an independent post-transcriptional level. Post-transcriptional osmoregulation is based on RNase III-mediated processing within a conserved stem-loop structure that is predicted to be formed in the 5' part of the *proU* mRNA. RNase III-mediated processing is efficient under low osmolarity growth conditions and inhibited by high osmolarity, and it is independent of osmoregulation of the promoter and H-NS-mediated repression. The primary role of RNase III mediated processing is presumably to initiate immediate degradation of the *proU* mRNA under hypo-osmotic stress. In addition, processing by RNase III supports repression of *proU* at low osmolarity. The independent modes of osmoregulation of *proU* at the transcriptional and the post-transcriptional level are integrated into a model for tight *proU* osmoregulation (**Fig. 8**).

The data show that RNase III is crucial for rapid degradation of the *proU* mRNA under hypo-osmotic stress, and they suggest that processing by RNase III initiates rapid degradation of *proU* mRNA by other RNases. The molecular mechanism underlying osmoregulation of processing by RNase III is open. The extremely fast response to hypo-osmotic stress indicates direct sensing by RNase III or by the *proU* mRNA, either alone or in complex with a regulatory protein or RNA. The rapid response rather excludes de novo synthesis of a co-factor, whether protein or RNA. One possibility is that RNase III activity is controlled by interaction with a protein. Kim et al.[13] identified the cold-shock induced RNase III modulator protein YmdB, which inhibits processing of the *rnc* and *pnp* transcripts by RNase III under cold-shock conditions. Sim et al.[12] proposed that RNase III is inhibited by hyper-osmotic stress, and demonstrated that expression of *rnc*, *pnp*, and *bdm*, which are likewise processed by RNase III, is induced by hyper-osmotic stress.[9,12,25] However, YmdB, the cold-shock-induced inhibitor of RNase III, was not involved in osmoregulation of these genes.[12] Possibly, RNase III is inhibited during hyper-osmotic stress by some uncharacterized protein.[12] Another possibility is that the secondary structure formed by the +203 to +293 region of the *proU* mRNA might function as an osmosensor. Some RNA function as RNA thermometers, pH-responsive or Mg^{2+}-responsive elements that change their conformation in response to one of these environmental signals.[26-28] Similarly, the *proU* stem-loop might potentially change the conformation in response to different osmolarity conditions that would lead to different efficiency of RNase III processing. A further possibility is that pairing of the *proU* mRNA with an unknown ncRNA is osmoregulated and affects processing by RNase III.

Taken together, the combination of osmoregulated transcription and of osmoregulated mRNA degradation may explain tight control of *proU* expression (see model in **Fig. 8**). First, under steady-state low osmolarity growth conditions transcription initiation of the Σ70-dependent *proU* promoter is repressed by H-NS. H-NS binds to high affinity sites downstream of the promoter and then supposedly forms an extended complex by lateral extensions and/or DNA bridging, which prevents open complex formation by RNA polymerase at the promoter. In addition, RNase III based processing of the *proU* mRNA further lowers the background level of expression, as shown here. Second, upon a shift to high osmolarity the activity of the promoter presumably increases. Whether this is based on the influx of potassium ions as a first response to hyper-osmotic stress is still unresolved. Simultaneously, repression by H-NS may decrease. The mechanism of this decrease in repression by H-NS is also still unclear. One aspect is that a moderately increased transcription rate can reduce repression by H-NS.[19] In addition, repression by H-NS is less efficient at high osmolarity even when transcription is directed by the constitutive *lacUV5* promoter (**Fig. 1C and** D), in agreement with less efficient DNA binding of H-NS under high osmolarity conditions.[29] Furthermore, at hyper-osmotic stress, processing by RNase III is inhibited. Third, under steady-state high osmolarity conditions the activity of the *proU* promoter remains high, repression by H-NS is abrogated, and processing of the *proU* mRNA by RNase III is inhibited. Fourthly, upon a shift from high to low osmolarity expression of the *proU* operon must be rapidly shut off. Possibly, the efflux of osmolytes as a first response to hypo-osmotic stress reduces the activity of the promoter and restores repression by H-NS. Importantly, hypo-osmotic stress causes

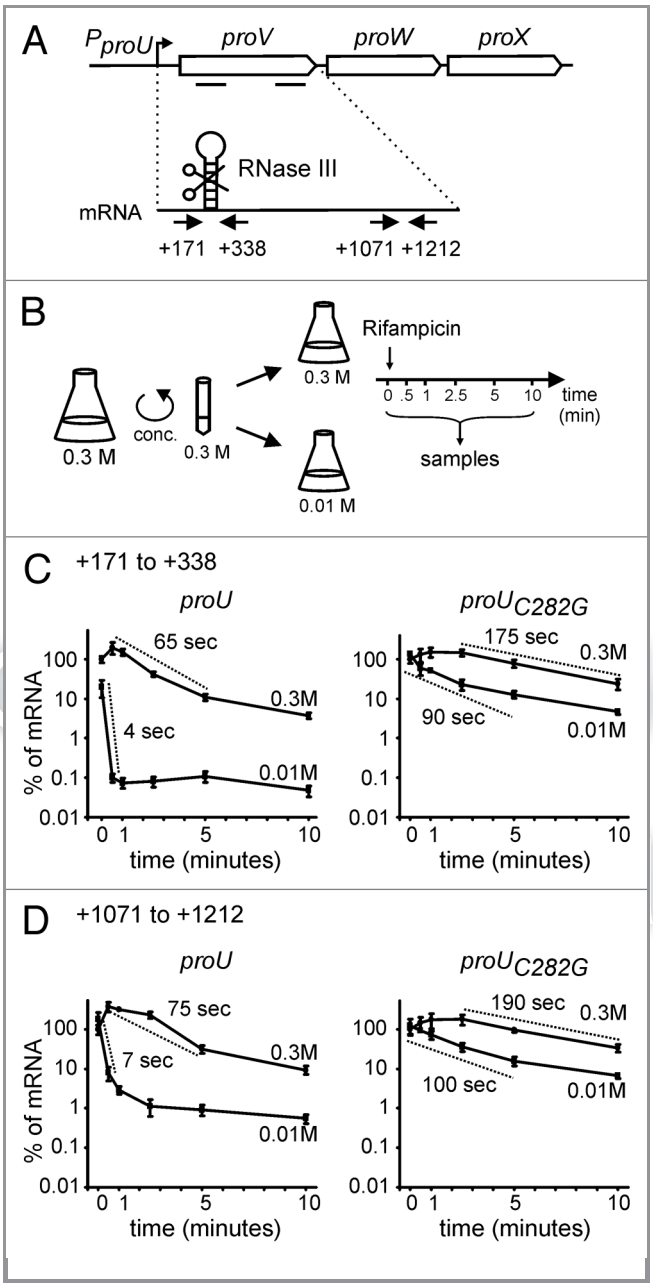

Figure 7. RNase III initiates rapid degradation of *proU* mRNA after hypo-osmotic stress. The stability of *proU* mRNA was determined by qRT-PCR. (A) Schematic representation of the *proU* locus. Coordinates of the amplified regions relative to the transcription start site are shown. (B) Schematic representation of the growth conditions, (C and D) Stability of wild-type *proU* mRNA (strain BW30270) and *proU*-C282G mRNA (strain T1001). The relative amount of RNA was determined by qRT-PCR. The amount of *proU* mRNA was plotted vs. the time after transcription was blocked with rifampicin, with the amount of *proU* mRNA present in bacteria grown in LB with 0.3 M NaCl before addition of rifampicin was set to 100%. Dashed lines represent the part of degradation curve that was used for the estimation of RNA half-life in seconds.

immediate RNase III-mediated processing of the *proU* mRNA and its rapid degradation. In conclusion, post-transcriptional regulation by RNase III is thus an important means to shutdown

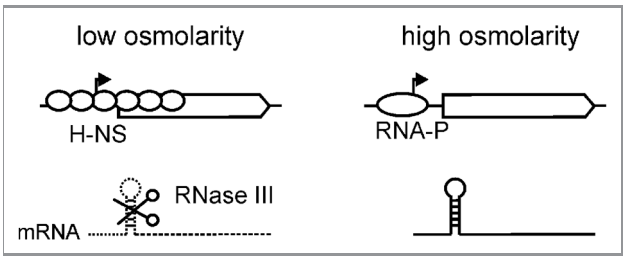

Figure 8. Model of *proU* osmoregulation. At low osmolarity, activity of the promoter (bent arrow) is low and transcription is blocked by H-NS (ovals). The minor amount of mRNA that is still transcribed is processed by RNase III and rapidly degraded. At high osmolarity, activity of the promoter is increased, H-NS does not repress transcription. This leads to the production of a large amount of mRNA. In addition, RNase III processing is inhibited resulting in stabilization of the mRNA. Hypo-osmotic stress leads to rapid processing by RNase III and consequently degradation of the *proU* mRNA. In addition, repression of transcription by H-NS is restored.

proU, and to maintain a very low steady-state expression level under hypo-osmotic conditions. As the RNase III processing site in *proU* is highly conserved in *Enterobacteriaceae*, RNase III is likely to be important for shutdown of *proU* in other species as well.

Materials and Methods

Media, strains and plasmids. *E. coli* K12 strains and plasmids are described in **Table 2**. Construction of strains by transduction using phage T4*GT7* and by integration of *lacZ* reporter constructs into the chromosomal λ*attB* site was performed as described.[30,33,34] Cloning of plasmids followed standard protocols.[35,36] Site specific mutations T279A and C282G were introduced by PCR-based mutagenesis of the plasmids. For bacterial growth standard LB medium with 0.85 M NaCl, or low salt LB with 0.01 M NaCl and high salt LB with 0.3 M NaCl were used. Antibiotics were added, where appropriate, to final concentration of 50 μg/ml ampicillin, 15 μg/ml chloramphenicol, 10 μg/ml or 25 μg/ml kanamycin, 100 μg/ml rifampicin, 50 μg/ml spectinomycin, and 12 μg/ml tetracycline.

β-galactosidase assays. For β-galactosidase assays[37] overnight cultures, which were grown at 37°C in low or high salt LB medium, were used to inoculate cultures in the same media to an OD_{600} of 0.1. These cultures were grown to an OD_{600} of 0.5 and harvested on ice. From every culture dilutions were set up in Z-buffer on ice, then cells were permeabilized by adding 15μl 0.1% (w/v) SDS and 30μl of chloroform. The samples were pre-incubate for 10 min at 28°C, followed by addition of 0.2 ml 4 mg/ml ONPG and further incubation at 28°C. Reactions were stopped by adding 0.5 ml 1M Na_2CO_3. The samples were cleared by centrifugation, the OD_{420} was measured, and the enzyme activity was calculated as described.[38] Each assay was performed at least 3 times independently and the standard error of the mean was calculated and less than 15%.

SDS-PAGE and protein gel blotting. Fresh overnight cultures grown at 37°C in low or high salt LB (0.01 M or 0.3M NaCl) were diluted to an OD_{600} of 0.1, grown at the same conditions

Table 2. *E. coli* K-12 strains and plasmids

Strain / Plasmid	genotype / features[a]	Reference / Construction[b]
N3433	*lacZ43*(Fs), *relA1, spoT1, thi-1*	[40] CGSC# 6976
IBPC633	N3433 rnc105 nadB51::Tn10 (tet) (stored as S3701)	[25]
BW30270	MG1655 rph^+	CGSC#: 7925
S541	CSH50 ΔbgI ΔlacZ	[30]
S3010	S541 Δhns::kan	[19]
S3066	S541 ΔproU::cm	x S696/S697, pKD3
S3077	S541 $\Delta proU_{FRT}$	S3066 x pCP20
S3460	N3433 ΔproU::cm	N3433 x T4GT7 (S3066)
S4007	N3433 ΔproU::cm Δhns::kan	S3460 x T4GT7 (S3010)
S4023	N3433 ΔproU::cm rnc105 nadB51::Tn10(tet)	S3460 x T4GT7 (IBPC633)
S4064	S3077 attB::[SpecR P_{lacUV5} $proV_{HA}$ lacZ]	S3077/pLDR8 x pKEKK5
S4066	S3077 attB::[SpecR P_{proU} $proV_{HA}$ lacZ]	S3077/pLDR8 x pKEKK6
S4068	S3077 attB::[SpecR P_{proU} Φ($proV_{HA}$-lacZ)]	S3077/pLDR8 x pKEKK7
S4070	S3077 attB::[SpecR P_{lacUV5} Φ($proV_{HA}$-lacZ)]	S3077/pLDR8 x pKEKK8
S4111	S3077 attB::[SpecR P_{lacUV5} $proV_{HA}$ lacZ] Δhns::kan	S4064 x T4GT7 (S3010)
S4113	S3077 attB::[SpecR P_{proU} $proV_{HA}$ lacZ] Δhns::kan	S4066 x T4GT7 (S3010)
S4115	S3077 attB::[SpecR P_{proU} Φ($proV_{HA}$ -lacZ)] Δhns::kan	S4068 x T4GT7 (S3010)
S4117	S3077 attB::[SpecR P_{lacUV5} Φ($proV_{HA}$ -lacZ)] Δhns::kan	S4070 x T4GT7 (S3010)
S4162	N3433 $\Delta proU_{FRT}$ Δhns_{FRT}	S4007 x pCP20
T100	S3077 attB::[SpecR P_{lacUV5} Φ($proV_{HA}$-lacZ)] rnc105 nadB51::Tn10(tet)	S4070 x x T4GT7 (IBPC633)
T811	S3077 attB::[SpecR P_{lacUV5} Φ(proV-C282G_{HA} lacZ)]	S3077/pLDR8 x pKEKK34
T825	S3077 attB::[SpecR P_{lacUV5} Φ(proV-C282G_{HA} lacZ)] rnc105 nadB51::Tn10(tet)	T811 x x T4GT7 (IBPC633)
T833	S3077 attB::[SpecR P_{lacUV5} Φ(proV-C282G_{HA} lacZ)] Δhns::kan	T811 x T4GT7 (S3010)
T879	S3077 attB::[SpecR P_{proU} Φ(proV-T279A_{HA} lacZ)]	S3077/pLDR8 x pKEKK39
T881	S3077 attB::[SpecR P_{proU} Φ(proV-C282G_{HA} lacZ)]	S3077/pLDR8 x pKEKK40
T887	S3077 attB::[SpecR P_{lacUV5} Φ(proV-T279A_{HA} lacZ)]	S3077/pLDR8 x pKEKK33
T891	S3077 attB::[SpecR P_{proU} Φ(proV-T279A_{HA} lacZ)] Δhns::kan	T879 x T4GT7 (S3010)
T893	S3077 attB::[SpecR P_{proU} Φ(proV-C282G_{HA} lacZ)] Δhns::kan	T881 x T4GT7 (S3010)
T909	S3077 attB::[SpecR P_{lacUV5} Φ(proV-T279A_{HA}-lacZ)] Δhns::kan	T887 x T4GT7(S3010)
T1000	BW30270 proU::C282G I-SceI Frt-neo-Frt C282G with duplication of 37bp (from +264 to +300) flanking the I-SceI Frt-neo-Frt cassette	BW30270/pKD46 x T524/T525
T1001	BW30270 proU-C282G	x pACBSCE
Plasmid		
pACBSCE	araC P_{ara} controlled λ-Red recombinase and endonuclease I-SceI, I-SceI site, CmR	[31]
pCP20	cI_{857} λ-P_R flp in pSC101 rep^{ts} bla	[32]
pKD46	araC P_{ara} γ-β-exo recombinase in pSC101-ori rep^{ts}	[32]
pLDR8	cI_{857} P_R λ-int in ori-pSC101 rep^{ts} neo	[33]
pKEKK5	P_{lacUV5} $proV_{HA}$ $lacZ^c$	This study
pKEKK6	P_{proU} $proV_{HA}$ $lacZ^c$	This study
pKEKK7	P_{proU} Φ($proV_{HA}$-lacZ)[c]	This study
pKEKK8	P_{lacUV5} Φ($proV_{HA}$-lacZ)[c]	This study
pKEKK11	P_{proU} $proV_{HA}$[d]	This study
pKEKK12	P_{lacUV5} $proV_{HA}$[d]	This study
pKEKK33	P_{lacUV5} Φ(proV-T279A_{HA}-lacZ)[c, e]	This study
pKEKK34	P_{lacUV5} Φ(proV-C282G_{HA}-lacZ)[c, e]	This study

Table 2. *E. coli* K-12 strains and plasmids (cont.)

Strain / Plasmid	genotype / features[a]	Reference / Construction[b]
pKEKK36	P_{lacUV5} proV-T279A_{HA}[d, e]	This study
pKEKK38	P_{lacUV5} proV-C282G_{HA}[d, e]	This study
pKEKK39	P_{proU} Φ(proV-T279A_{HA}-lacZ)[c, e]	This study
pKEKK40	P_{proU} Φ(proV-C282G-lacZ)[c, e]	This study

[a]All strains are derivatives of N3433 or S541.[30] The relevant genotype of the strains refers to the *lac*, *hns*, *rnc* and *proU* loci. For plasmids the relevant features are given. [b]Construction of strains by transduction was performed using T4*GT7* lysates grown on IBPC633 for transduction of *rnc105* and S3010 for transduction of Δ*hns*::kan, respectively. Transduction of the Δ*hns* allele was verified by PCR and transduction of the *rnc105* allele was verified by PCR and sequencing. In allele *rnc105* the *rnc* gene carries a G to A substitution at position +131 relative to the ATG start codon and encodes an inactive RNase III mutant with a G44D exchange.[24] Integration of plasmids into the attB site of chromosome was performed as described, using the integrase-expressing temperature-sensitive plasmid pLDR8 and circularized origin-less fragments of the *proU-lacZ* reporter fusions.[30,33] Integrants were analyzed by PCR and for loss of the helper plasmid pLDR8. Two independent integrants were selected for use in further experiments. [c]The vector backbone is P15A kan^R $spec^R$ λattP [d]The vector backbone is P15A kan^R. [e]The mutations T279A and C282G were introduced by PCR-based mutagenesis using oligonucleotides T376 and T378.

to OD_{600} of 0.5, and samples were collected. To perform salt upshift, cultures were grown in LB with 0.01 M NaCl to an OD_{600} of 0.5, then NaCl was added to a final concentration of 0.3M, and samples were taken just before as well as 10 min after addition of NaCl. The cells were harvested by centrifugation and re-suspended in sample buffer (10% Glycerol, 62.5mM TRIS-HCl pH6.8, 2% SDS, 0.05% Bromophenol blue) to a concentration of 0.05 OD_{600} per 10 µl sample buffer. Five µl (corresponding to OD_{600} of 0.025) were separated on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). The gel was blotted onto a 0.45 µm pore size poly vinylidene difluoride (PVDF) or nitrocellulose transfer membrane. The blot was handled using standard protein gel blotting protocol. Monoclonal rat antibody directed against the HA-tag (Roche Diagnostics, Cat. No. 11867423001) were used as the primary antibodies. Alexa Fluor 680 goat anti-rat immunoglobulin G (Molecular Probes/ Invitrogen, Cat. No. A21096) was used as the secondary antibodies. An Odyssey Imaging System (Li-Cor Biosciences) was used for visualization and quantification according to the instructions of the manufacturer.

RACE for mapping of 5' RNA ends. RACE (rapid amplification of RNA ends) for mapping of 5' ends of RNA was performed as described.[23] Fresh overnight bacterial cultures grown at 37°C in LB supplemented with 0.01 M or 0.3 M NaCl and with appropriate antibiotics were diluted to OD_{600} of 0.1 and grown to OD_{600} of 0.5. Samples were collected, mixed with RNAprotect Bacteria Reagent (Qiagen, Cat. No. 76506), and total RNA was isolated using the RNAeasy Mini kit (Qiagen, Cat. No. 74104) with on-column DNase digestion (RNase-free DNase Set, Qiagen, Cat. No. 79254). To distinguish between primary 5' ends which usually carry a triphosphate and 5' ends generated by RNase cleavage which do not carry a terminal triphosphate residue, 6 µg of each RNA sample were treated with Tobacco Acid Pyrophosphatase (TAP, Epicenter Biotechnologies, Cat. No. T19100) for 30 min at 37°C to remove 5' triphosphates. Another 6 µg of the RNA were not treated with TAP. The RNA was then purified by phenol-chloroform-isopropanol extraction followed by ethanol precipitation. Purified RNA was ligated to RNA adaptor T268 (**Table S1**) by overnight incubation with T4 RNA ligase (NEB, Cat. No. M0204S) at 17°C. T4 RNA ligase ligates the RNA adaptor only to transcripts that carry 5' monophosphate but not 5' triphosphate. RNA ligation products were then purified by phenol-chloroform-isopropanol extraction followed by ethanol precipitation, and the purified RNA was used as a template for reverse transcription with gene-specific DNA oligonucleotide T267 (annealing to *proU* +486 to +467) with ThermoScript Reverse Transcriptase (Invitrogen, Cat. No. 12236–014) for 60 min at 55°C. After termination of the reaction, RNase H (Fermentas, Cat. No. EN0201) was added and the mixture was incubated at 37°C for 20 min to destroy RNA template. The resulting cDNA was amplified by PCR with Platinum Taq DNA Polymerase Kit (Invitrogen, Cat. No. 10966–018) using the gene-specific primer T266 and 5'-adaptor-specific primer T265. The PCR products were analyzed by 2% agarose gel electrophoresis. Each band on a gel image represents either a primary transcript (expected to be enriched for TAP treated samples) or a product of mRNA processing. To further analyze the RACE-PCR products, bands of interest were excised, cloned and sequenced (**Fig. S1**). Sequences of five to six inserts per candidate were analyzed since both primary and processed 5' ends may vary by a few nucleotides. For primary transcript, the most upstream 5' nucleotide was regarded as the transcription initiation site.

Determination of proU transcript level and stability by qRT-PCR. The level of the *proU* transcript and the half-life of the transcript were determined by quantitative reverse transcriptase PCR (qRT-PCR). To determine the level of the *proU* transcript, fresh overnight bacterial cultures were grown at 37°C in LB supplemented with either 0.01M or 0.3M NaCl and kanamycin. The overnight culture was used to inoculate a culture to OD_{600} of 0.1 in the same medium and grown to OD_{600} of 0.5. Samples were collected and used to isolate total RNA as described below. To determine the stability of *proU* transcript fresh overnight bacterial cultures grown at 37°C in LB with 0.3M NaCl were used to inoculate a culture to OD_{600} of 0.1. This culture was grown to OD_{600} of 0.5 and the cells were harvested by centrifugation (4000 rpm, 5 min, 4°C). The cell pellet was resuspended in 1 ml of 0.3 M NaCl LB. Then 400 µl of the suspension were transferred to 11.6 ml of LB without NaCl, so that the final concentration of NaCl in the culture reached 0.01M. Another 400 µl of the suspension were transferred to 11.6 ml of LB with 0.3M NaCl. Immediately after transfer samples were collected followed by addition of rifampicin (100 µg/ml) to block

transcription. Further samples were collected 30 sec, 1 min, 2.5 min, 5 min and 10 min after rifampicin addition. The samples were mixed with RNAprotect Bacteria Reagent (Qiagen, Cat. No. 76506), and total RNA was isolated with RNAeasy Mini kit (Qiagen, Cat. No. 74104) with on-column DNase digestion (Qiagen, Cat. No. 79254). One microgram of RNA samples was used to synthesize cDNA with SuperScript III First-Strand Synthesis System for RT-PCR and random hexamer primers (Invitrogen, Cat. No. 18080–051). The resulting cDNA was used for PCR reactions with SYBR Green for quantification. PCR reactions for each pair of *proU* primers were set up in parallel to 16S rRNA primers as standard to allow direct comparison of the amounts of the *proU* mRNA under different osmolarity conditions. All the cDNA samples were analyzed in triplicate. In addition, equal volumes of all cDNA samples used in the PCR reactions on one plate were mixed together and used as series of dilutions to build the standard curves. These standard curves were then used to determine the relative quantity of *proU* +171 to +338 mRNA and *proU* +1071 to +1212 mRNA, respectively, normalized to 16S rRNA. To construct the decay curves, the relative amount of *proU* mRNA present in bacteria grown in LB with 0.3M NaCl before addition of rifampicin was taken as 100%, and the relative amount of *proU* mRNA present in the samples was plotted against the time. The part of the decay curve corresponding to exponential decay was used to estimate the RNA half-life according to the formula $t1/2 = t^*\ln(2)/\ln(N_0/N_t)$, where N_0 is the initial quantity of RNA, and N_t is the quantity of RNA present at time (t).

Construction of the single point mutation C282 to G in the native proU locus. The introduction of the single point mutation C282G into the chromosomal *proU* locus was achieved in two steps following described protocols.[31,39] Briefly, using plasmid pKD4 as template and oligonucleotides T524 and T525 (**Table S1**) a PCR fragment was amplified which carries a short *proU* segment from +246 to +300 encompassing the C282G mutation, followed by a I-SceI site, the Frt-*neo*-Frt cassette from pKD4,[32] and a second *proU* segment from +264 to +318 again encompassing the C282G mutation. This PCR fragment was used to transform strain BW30270 carrying the λ-Red-Gam recombinase expressing plasmid pKD46, as described.[32] This promoted the integration of the mutated *proU* sequences, together with the I-SceI site and the Frt-KanR-Frt cassette into the chromosomal *proU* locus. The resulting colonies were analyzed by PCR and sequencing. One clone, which carried the C282G mutation on both sites of the I-SceI Frt-KanR-Frt insert, was stored as strain T1000. In a second step, strain T1000 was transformed with plasmid pASBSCE carrying the arabinose inducible λ-Red-Gam-recombinase and I-SceI endonuclease genes.[31] I-SceI cuts the chromosome within the *proU* locus next to the kanamycin marker and λ-Red-Gam carries out the recombination between the chromosomal *proU*-C282G sequences resulting in the loss of the cassette. Plasmid pACBSCE is simultaneously lost due to I-SceI cleavage. All clones, which were kanamycin and ampicillin sensitive, carried the *proU*-C282G mutation, as verified by sequencing, and one of them was stored as strain T1001.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

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Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/rnabiology/article/18228

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SUPPLEMENTARY INFORMATION

RNase III initiates rapid degradation of *proU* mRNA upon hypo-osmotic stress in *Escherichia coli*

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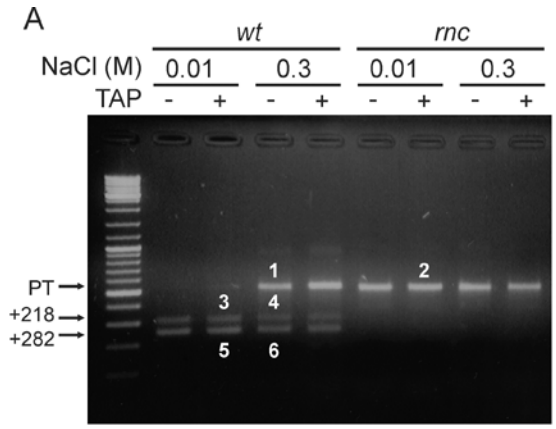

B

Band	Clones sequenced	Position of 5´ end (number of clones)
1	6	+1, +7, +15, +22, +24, +26
2	6	+1, +15 (3), +22, +25
3	5	+218 (4), +229
4	6	+218 (6)
5	5	+281 (5), +291
6	5	+281 (5)

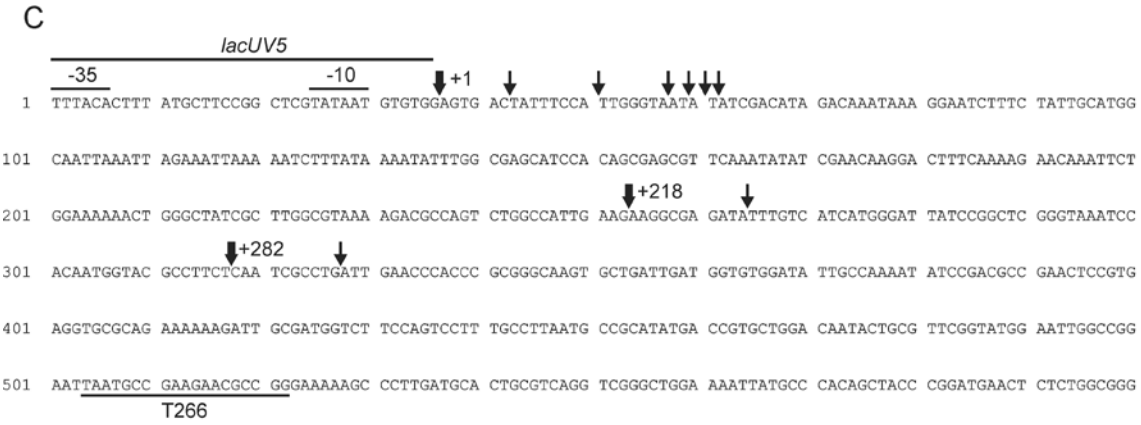

Fig. S1 5´ RACE analysis of *proU* mRNA processing by RNase III. (A) Gel electrophoresis of amplification products of cDNA. Analysis of processing of *proU* mRNA in wild type cells and in *rnc105* mutant cells is shown. Each DNA band on a gel corresponds to the separate RNA species, such as primary *proU* transcript (PT) or processing product. Expression of *proU* (+1 to +1260) encoded by low-copy-number plasmid pKEKK12 was directed by the *lacUV5* promoter. Transformants of the wild-type (S3460) and *rnc* mutant (S4023) with pKEKK12 were grown in LB supplemented with either 0.01 M or 0.3 M NaCl. RNA was extracted and further analyzed by 5' RACE. (+) and (-) refers to TAP treatment prior to ligation of the RNA adapter. Numbers indicate the DNA bands that were subsequently purified from the gel, cloned into pUC12, and analyzed by sequencing. (B) and (C) mapping of primary transcripts and RNase III processing sites by sequencing. (B) The band numbers in the table correspond to the band numbers in (A). Most DNA bands are a mixture of fragments with different 5' ends. Therefore, five to six clones were sequenced per cloning of each band. The number of clones sequenced, the positions of 5' ends

determined by sequencing, and the number of clones with corresponding 5' ends (in brackets) are shown. (C) Sequence of the plasmid pKEKK12 used for 5´ RACE analysis with RNase III processing sites. Sequence includes the *lacUV5* promoter and upstream part of *proU* analyzed by 5´ RACE. Primer T266 was used for PCR amplification of cDNA, and therefore corresponds to the 3´ end of the analyzed *proU* sequence. The location of 5´ ends of clones determined by sequencing are taken from Fig. S1B and are indicated by arrows. For each cloning, the most upstream position was depicted as a bold arrow.

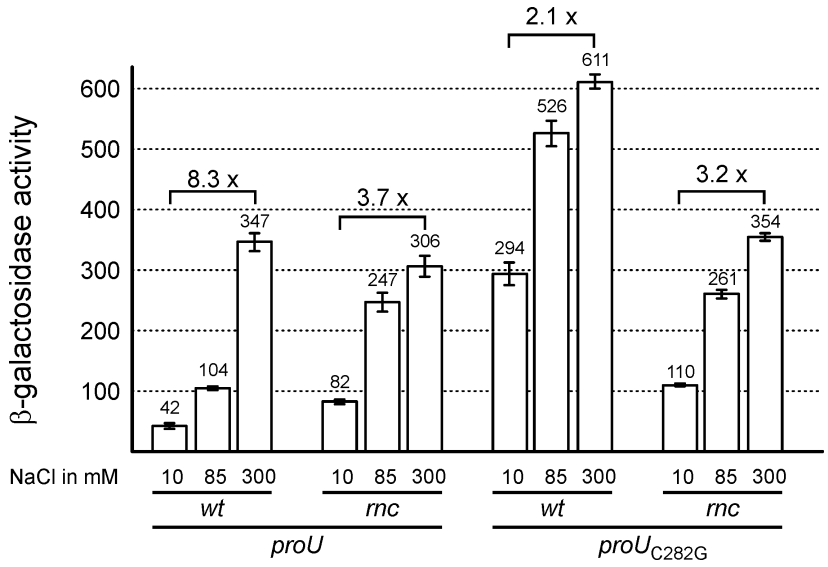

Fig. S2 Osmoregulation of *proU* in an *rnc* mutant. Steady-state expression levels of the translational *PlacUV5* Φ*proU-lacZ* (*proU*) and its C282G mutant (*proU*$_{C282G}$) were determined in the wild-type (wt) and *rnc* mutant. β-galactosidase values are indicated as bars, and the fold osmoregulation is indicated above the horizontal brackets. Strains used: *PlacUV5* Φ*proU-lacZ* in the wt (strain S4070), *PlacUV5* Φ*proU*$_{C282G}$*-lacZ* fusion in wt (strain T811), *PlacUV5* Φ*proU-lacZ* in the *rnc* mutant (strain T100), *PlacUV5* Φ*proU*$_{C282G}$*-lacZ* in the *rnc* mutant (strain T825).

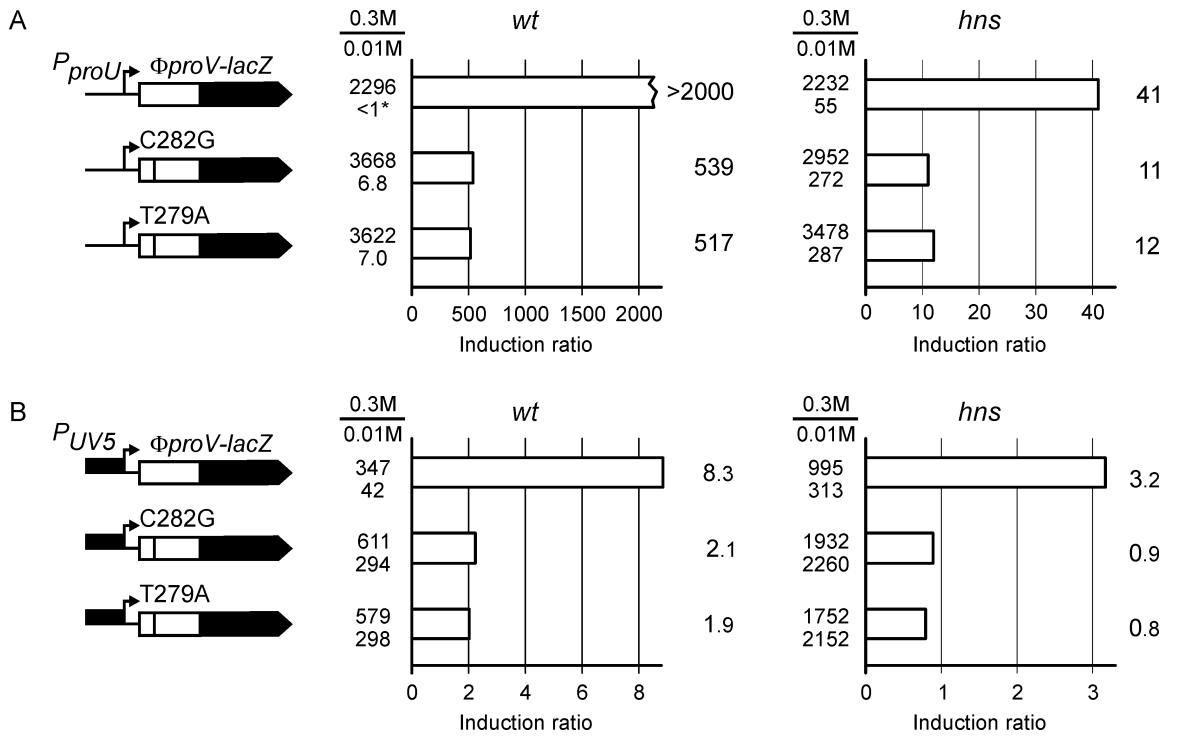

Fig. S3 Effect of mutation of the RNase III processing site on osmoregulation of *proU*. Steady-state expression levels of translational *proU-lacZ* reporter fusions were determined of cultures grown in low and high osmolarity LB medium. Expression levels of fusions whose expression is directed by the *proU* promoter (A) and the *lacUV5* promoter (B) were determined in the wild-type (*wt*) (A and B left panel) and *hns* mutants (A and B right panel). The values of the expression levels are given as numbers in Miller units, and the fold difference in expression at high and low osmolarity conditions is indicated by bars. Note that different scales are used. * indicates that the β-galactosidase activity was below the detection threshold of 1 unit. Mutation of the RNase III processing sites results in the same reduction of osmoregulation, independent of the promoter and of H-NS. Strains used: A: wild-type *proU* (strain S4068), $proU_{C282G}$ (T881), $proU_{T279A}$ (T879); B: *proU* (S4115), $proU_{C282G}$ (T893), $proU_{T279A}$ (T891); C, *PlacUV5-proU* (S4070), $proU_{C282G}$ (T811), $proU_{T279A}$ (T887); and D: *proU* (S4117), $proU_{C282G}$ (T833), and $proU_{T279A}$ (T909).

Table S1: Oligonucleotides

primer	Sequence	Description
S372	cggaattcAGTGACTATTTCCATTGGGTAATATATCG	Cloning of proU(+1 to +1260) EcoRI-site underlined, proU(+1 to +29) in capital letters
S779	ttccggctcgtataatgtgtggAGTGACTATTTCCATTGGGTAATATATCG	Cloning of PlacUV5 proU(+1 to +1260) PlacUV5 sequence in lower case letters, proU in upper case letters
S696	AATATCACTACCCGCAGCAGGGAAATAATTCCCGCCAAATAGCgtgtaggctggagctgcttcg	deletion of proU proU(-277 to -235) in upper case letters, sequence matching pKD3 in lower case letters
S697	GGATGCGGCAAAAACGCCTTATCCGCCCGAATAAAAATTAcatatgaatatcctccttagttcctattcc	deletion of proU proU sequence in upper case letters with proX stop codon underlined, sequence matching pKD3 in lower case letters
T005	cgagctcgagAGATGAAGACTGGAATTTCTGAGGG	Cloning of proU(-315 to +1260) XhoI site underlined, proU(-315 to -291) in upper case letters
T008	cgactctagaTTAAGCGTAATCTGGAACATCGTATGGGTAGCCATTATTTACCCCCTCACGA	Cloning of proU(-315 to +1260) with HA-tag proU(+1239 to +1260) in capital letters, HA-coding sequence in capital letters underlined, XbaI site underlined
T265	gcgcgaattcCTGTAGAACGA	Forward primer for RACE homologous to RNA adapter T268, EcoRI-site underlined
T266	gactgtcgacCCGGCGTTCTTCGGCATTA	Reverse primer for RACE homologous to proU(+468 to +486), SalI site underlined
T267	TCCGGGTAGCTGTGGGCATAA	Primer for reverse transcription of the proU mRNA for 5´RACE, homologous to proU(+528 to +548)
T268	AUAUGCGCGAAUUCCUGUAGAACGAACACUAGAAGAAA	RNA adapter for 5´RACE
T376	GGTAAATCCACAATGGTACGCCTACTCAATCGCCTGA	Mutation of T279 to A, proU(+262 to +299)
T378	GGTAAATCCACAATGGTACGCCTTCTGAATCGCCTGATT	Mutation of C282 to G, proU(+262 to +301)
T520	AACTGGGCTATCGCTTGGCGTA	qRT-PCR proU(+171 to +192)
T521	GCAATATCCACACCATCAATCAGCA	qRT-PCR proU(+314 to +338)
T522	CGAAATGATGCCGACATACTGTTGG	qRT-PCR proU(+1188 to +1212)
T523	AGGTCTTGATGCGGCGCTGA	qRT-PCR proU(+1071 to +1090)
T524	ATCCGGCTCGGGTAAATCCACAATGGTACGCCTTCTGAATCGCCTGATTGAACCCTAGGGATAACAGGGTAATgtgtaggctggagctgcttcg	Chromosomal mutagenesis of proU C282 to G proU(+246 to +300) in capital letters,I-SceI site underlined, sequence matching pKD4 in lower case letters
T525	CAGCACTTGCCCGCGGGTGGGTTCAATCAGGCGATTCAGAAGGCGTACCATTGTGcatatgaatatcctccttagttcctattcc	Reverse primer for chromosomal mutagenesis of proU C282 to G proU(+264 to +318) in capital letters, sequence matching pKD4 in lower case letters
T528	GGTGTAGCGGTGAAATGCGTAGAG	qRT-PCR, for amplication of 16S rRNA from position 682nt to 846nt (rrsA, -B, -C, -E, -H) or 697nt to 861nt (rrsG, -D)
T529	CTCAAGGGCACAACCTCCAAGTC	qRT-PCR, for amplication of 16S rRNA from position 682nt to 846nt (rrsA, -B, -C, -E, -H) or 697nt to 861nt (rrsG, -D)