Multiple layers of control govern expression of the *Escherichia coli* *ibpAB* heat-shock operon

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The *Escherichia coli* *ibpAB* operon encodes two small heat-shock proteins, the inclusion-body-binding proteins IbpA and IbpB. Here, we report that expression of *ibpAB* is a complex process involving at least four different layers of control, namely transcriptional control, RNA processing, translation control and protein stability. As a typical member of the heat-shock regulon, transcription of the *ibpAB* operon is controlled by the alternative sigma factor σ^{32} (RpoH). Heat-induced transcription of the bicistronic operon is followed by RNase E-mediated processing events, resulting in monocistronic *ibpA* and *ibpB* transcripts and short 3'-terminal *ibpB* fragments. Translation of *ibpA* is controlled by an RNA thermometer in its 5' untranslated region, forming a secondary structure that blocks entry of the ribosome at low temperatures. A similar structure upstream of *ibpB* is functional *in vitro* but not *in vivo*, suggesting downregulation of *ibpB* expression in the presence of IbpA. The recently reported degradation of IbpA and IbpB by the Lon protease and differential regulation of IbpA and IbpB levels in *E. coli* are discussed.

INTRODUCTION

Bacteria are continuously exposed to changes in environmental conditions. To respond to stress conditions in a timely fashion, they have developed numerous protective strategies that are tightly regulated. Global stress responses are primarily regulated at the level of transcription initiation (Campbell *et al.*, 2008). The induction of stress genes in many bacteria is orchestrated by alternative sigma factors, subunits of the RNA polymerase that direct the holoenzyme to specific promoter sequences. In addition to the housekeeping sigma factor σ^{70} , the *Escherichia coli* genome encodes six alternative sigma factors. The most prominent examples are σ^S (RpoS), responsible for induction of the general stress response, and σ^{32} (RpoH), responsible for initiating heat-shock gene expression (Gross *et al.*, 1998; Guisbert *et al.*, 2008; Hengge-Aronis, 2002).

mRNA degradation and maturation also play important roles in the regulation of gene expression. The inherent stability of an mRNA molecule determines how long it is available as template for translation (Rauhut & Klug, 1999). The half-life of many mRNAs varies with environmental changes; this is consistent with mRNA stability playing a role in the control of differential gene expression (Newbury *et al.*, 1987).

Regulatory RNAs have recently been recognized as important post-transcriptional gene control elements, often being involved in the coordination of stress responses (Narberhaus & Vogel, 2009; Waters & Storz, 2009). Small regulatory RNAs and riboswitches, functioning as *trans*- and *cis*-acting regulators, respectively, influence gene expression using various strategies, in most cases depending on RNA–RNA interactions. Riboswitches are structured RNA elements in the 5' untranslated region (5' UTR) of certain transcripts. Conformational changes upon the binding of a cellular metabolite alter gene expression, which can be controlled at the level of transcription elongation, translation initiation or RNA processing (Winkler & Breaker, 2005). Likewise, RNA thermometers are structured mRNA regions in the 5' UTR of temperature-controlled bacterial genes (Narberhaus *et al.*, 2006). They supervise translation initiation by folding into a structure that blocks the Shine–Dalgarno (SD) sequence at low temperatures. A temperature upshift induces melting of the structure, giving access to the ribosome and permitting translation of heat-shock or virulence genes (Narberhaus, 2010).

Numerous cellular processes, including mRNA translation and protein folding, are sensitive to temperature up- and downshifts (Klinkert & Narberhaus, 2009; Schumann, 2009). Therefore, bacteria precisely monitor changes in their ambient temperature and react accordingly with heat-shock or cold-shock responses. The *E. coli* heat-shock response is well characterized (Guisbert *et al.*, 2008). A combination of transcriptional and post-transcriptional mechanisms culminate in the synthesis of σ^{32} under heat-stress conditions. The sigma factor initiates transcription of heat-shock genes, most of which encode molecular chaperones or proteases.

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Abbreviations: RACE, rapid amplification of cDNA ends; ROSE, repression of heat-shock gene expression; SD, Shine–Dalgarno; UTR, untranslated region; WT, wild-type.

The *ibpAB* operon belongs to the σ^{32} regulon. The open reading frames are separated by 111 bp and are the most dramatically induced heat-shock genes in *E. coli* (Richmond *et al.*, 1999). The encoded small heat-shock proteins IbpA and IbpB (inclusion body-associated protein A and B) have been found associated with recombinant proteins in inclusion bodies (Allen *et al.*, 1992). They also recognize endogenous *E. coli* proteins that aggregate in response to heat shock (Laskowska *et al.*, 1996). IbpA and IbpB proteins share 57.5 % sequence identity and exhibit chaperone activity under *in vitro* conditions (Kitagawa *et al.*, 2000, 2002). IbpB was shown to stabilize stress-denatured proteins for subsequent refolding by a multichaperone network (Veinger *et al.*, 1998). IbpA cooperates with IbpB in this process (Matuszewska *et al.*, 2005). Regulation of the *ibpAB* operon is complex and not yet fully understood. As well as the transcriptional control of *ibpAB*, translational control of *ibpA* by an RNA thermometer in its upstream region has also been reported (Waldminghaus *et al.*, 2009). In this study, we closely examined the regulation of the *ibpAB* operon and discovered additional layers of control.

METHODS

Bacterial strains, plasmids, oligonucleotides and growth conditions. All strains, plasmids and oligonucleotides used in this study are listed in Table 1. *E. coli* cells were grown at 30 or 37 °C in Luria-Bertani (LB) medium supplemented with ampicillin (Amp, 200 µg ml⁻¹) and/or kanamycin (Km, 50 µg ml⁻¹), as needed.

Plasmid construction. Recombinant DNA work was performed according to standard protocols (Sambrook *et al.*, 1989). Site-directed mutagenesis to generate pBO668 was performed according to the instruction manual of the QuikChange mutagenesis kit (Stratagene). The correct nucleotide sequence of all plasmids was confirmed by automated sequencing. Detailed information on plasmid construction and characteristics is shown in Table 1.

Construction of an *ibpAB* mutation in *E. coli* C600. *E. coli* C600 Δ *ibpAB* was constructed by P1 transduction according to standard protocols (Sambrook *et al.*, 1989). *E. coli* JGT14 served as donor (Shearstone & Baneyx, 1999).

RNA isolation and Northern blot analysis. Cells were grown at 30 °C to exponential phase and an aliquot of the culture was heat-shocked at 42 °C for 1 min. To measure RNA stability, 250 µg rifampicin ml⁻¹ was added 1 min after heat shock. Samples were then taken at 0, 3, 6, 10 and 15 min after the addition of rifampicin. Isolation of total RNA and Northern blotting were performed as described previously (Waldminghaus *et al.*, 2005). Digoxigenin-labelled RNA probes were produced according to the instruction manual (Roche).

5' Rapid amplification of cDNA ends (RACE). 5' RACE experiments were conducted as described previously (Willkomm *et al.*, 2005) with minor modifications. DNase treatment was performed with 2 U DNase I (Promega) and 40 U RiboLock (Fermentas). Reverse transcription was carried out with SuperScript III (Invitrogen), according to the manufacturer's instructions. After PCR amplification with the adaptor primer and a gene-specific primer (primers EC1bpBP_{Erv} for the 5' end of the monocistronic *ibpB* transcript and 5RACE-*ibpB*_k for the 5' end of short *ibpB* fragments; Table 1), prominent bands were excised from 2 % agarose

gels, eluted and blunt-end cloned into pUC18 restricted with *Sma*I. The inserts of several plasmids from each experiment were sequenced.

***In vitro* transcription of RNA.** RNA for toeprint analyses and structure probing was synthesized *in vitro* by run-off transcription with T7 RNA polymerase, from linearized plasmids or PCR-generated DNA templates (Table 1).

Primer extension inhibition (toeprinting). Toeprinting experiments were carried out using 30S ribosomal subunits, target mRNA and tRNA^{fMet} basically according to Hartz *et al.* (1988). The 5'-³²P-labelled *ibpA*- and *ibpB*-specific oligonucleotides *ibpACDSrv* and T7*ibpBrv* (Table 1), complementary to nucleotides +66 to +43 and +69 to +50 of the *ipbA* and *ipbB* coding sequences, respectively, were used as primers for cDNA synthesis. A 0.08 pmol aliquot of *ibpA* or *ibpB* mRNA annealed to the appropriate oligonucleotide was incubated for 10 min at 30 or 42 °C together with 16 pmol uncharged tRNA^{fMet} (Sigma-Aldrich). 30S subunits (6 pmol) or Watanabe buffer (60 mM HEPES/KOH; 10.5 mM Mg(COO)₂; 690 mM NH₄COO; 12 mM β -mercaptoethanol; 10 mM spermidine; 0.25 mM spermine; negative control) were added, and incubated for another 10 min at 30 or 42 °C. Then 2 µl MMLV-Mix (VD+Mg²⁺ buffer, BSA, dNTPs and MMLV reverse transcriptase; USB) was added. cDNA synthesis was performed at the respective temperature. Reactions were stopped after 10 min by adding formamide loading dye and aliquots were separated on a denaturing 8 % polyacrylamide gel.

***In vitro* RNA structure probing.** RNAs were 5' end-labelled as described previously (Brantl & Wagner, 1994). Digestions were carried out with RNase T1 (Ambion). In a total volume of 4 µl, RNA corresponding to 30 000 c.p.m. was mixed with 1 µl 5 × TMN buffer (100 mM Tris/acetate; 10 mM MgCl₂; 500 mM NaCl; pH 7.5) and 0.4 µg tRNA. One microlitre of RNase T1 was added after 5 min of incubation at the respective temperature. After an additional incubation of 5 min, the reaction was stopped by adding 5 µl formamide loading dye. Samples were denatured at 95 °C for 5 min and an aliquot of each digest was loaded on an 8 % polyacrylamide gel. Alkaline ladders were generated as described by Brantl & Wagner (1994).

β -Galactosidase assay. β -Galactosidase activity of *E. coli* strains carrying *bgaB* fusions was measured as described by Miller (1972) with some modifications. For cell disruption, 0.5 µg lysozyme µl⁻¹ and 25 µl 4 × Z-buffer (with 14 µl β -mercaptoethanol ml⁻¹) were added to 400 µl of cell suspension and incubated at 55 °C for 15 min. The enzymic reaction was started at 55 °C by adding ONPG (2 mg ml⁻¹ in 1 × Z-buffer with 2.7 µl β -mercaptoethanol ml⁻¹). Standard deviations (SD) were calculated from at least three independent experiments.

Computer-based methods. Sequences for computer-based analysis were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>). RNA secondary structure was predicted by using the mfold server (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi>) running version 3.2 (Zuker, 2003). The AIDA image analyser software v. 4.03 was used for toeprint and structure-probing gel analysis.

RESULTS

Four transcript species derive from the *ibpAB* operon

To analyse transcriptional and post-transcriptional events involved in *ibpAB* regulation, we synthesized RNA probes against the individual genes (Fig. 1a). The *ibpA* probe reacted with both *ibpA* and *ibpB* whereas the *ibpB* probe

Table 1. Strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant characteristic(s) or sequence*	Source, reference or application
<i>E. coli</i> strains		
C600	F ⁻ <i>tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ⁻</i>	Laboratory collection
JGT14	BL21(DE3) Δ <i>ibp1::kan</i>	Shearstone & Baneyx (1999)
C600 Δ <i>ibpAB</i>	C600 Δ <i>ibpAB</i>	This study
N3431	Rne ⁻	Goldblum & Apririon (1981)
N3433	Rne ⁺	Goldblum & Apririon (1981)
BL321	Rnc ⁻	Studier (1975)
BL322	Rnc ⁺	Studier (1975)
Plasmids		
pK18	Cloning vector, Km ^R	Pridmore (1987)
pUC18	Cloning vector, Amp ^R	Norlander <i>et al.</i> (1983)
pBAD- <i>bgaB</i> (pBO415)	Translational <i>bgaB</i> fusion vector (<i>bgaB</i> : heat-stable β -galactosidase), Amp ^R	Waldminghaus <i>et al.</i> (2007)
pUC- <i>ibpA</i> (pBO403)	<i>E. coli</i> <i>ibpA</i> fragment in pK18	Waldminghaus <i>et al.</i> (2009)
pUC- <i>ibpB</i> (pBO1059)	<i>E. coli</i> <i>ibpB</i> fragment in pUC18	This study
pK- <i>ibpAB</i> (pBO1001)	<i>E. coli</i> <i>ibpAB</i> fragment in pK18	This study
pK- <i>ibpABstop</i> (pBO668)	<i>E. coli</i> <i>ibpAB</i> stop fragment in pK18	This study
pUC-run-off <i>ibpAB</i> -toe	<i>E. coli</i> <i>ibpAB</i> toeprint fragment in pUC18	This study
pBAD- <i>ibpA-bgaB</i> (pBO417)	<i>E. coli</i> <i>ibpA</i> fusion in pBAD- <i>bgaB</i> ; <i>NheI</i> - <i>EcoRI</i> fragment of pBO403	Waldminghaus <i>et al.</i> (2009)
pBAD- <i>ibpB-bgaB</i> (pBO1060)	<i>E. coli</i> <i>ibpB</i> fusion in pBAD- <i>bgaB</i> ; <i>NheI</i> - <i>EcoRI</i> fragment of pBO1059	This study
pBAD- <i>ibpAB-bgaB</i> (pBO629)	<i>E. coli</i> <i>ibpAB</i> fusion in pBAD- <i>bgaB</i> ; <i>NheI</i> - <i>EcoRI</i> fragment of pBO1001	This study
pBAD- <i>ibpABstop-bgaB</i> (pBO669)	<i>E. coli</i> <i>ibpABstop</i> fusion in pBAD- <i>bgaB</i> ; <i>NheI</i> - <i>EcoRI</i> fragment of pBO668	This study
Oligonucleotides		
<i>ibpA</i> proberv	TAATACGACTCACTATAGttgatttcgatacggcgcg	<i>ibpA</i> RNA probe
<i>ibpA</i> probefw	caggataatctgctgggtgtg	<i>ibpA</i> RNA probe
<i>ibpB</i> proberv	TAATACGACTCACTATAGctatttaacgcgggacgtt	<i>ibpB</i> RNA probe
<i>ibpB</i> probefw	acgcgcctgagcgtaaaagg	<i>ibpB</i> RNA probe
Adaptor primer	ctcgttagggattgctgac	5' RACE
ECIbpBPerv	gatccattgacgcatcagtg	5' RACE
5RACE- <i>ibpB</i> _k	ttagctatttaacgcgggacgt	5' RACE
T7ibpAfw	GAAATTAATACGACTCACTATAGGccgatgaggacgcgcctga	Run-off vector <i>in vitro</i> transcription
T7ibpBrv	cagtgcgttgccagtttgt	Run-off vector <i>in vitro</i> transcription
<i>ibpB</i> _SP2_fw	GAAATTAATACGACTCACTATAGGGcatctccatgctgccgtca	Structure probing <i>ibpB</i>
<i>ibpB</i> _SP_rv	tacgcatagtcatttctcttct	Structure probing <i>ibpB</i>
<i>ibpACDSrv</i>	gtgggttaacaacgggtcaaatcc	Toeprinting <i>ibpA</i>
<i>ibpB</i> _5UTR_lang	gctagcattccctaaggccgctg	Cloning of pBO1059
<i>ibpA1</i>	tctagctagccgatgaggacgcgc	Cloning of pBO403
<i>IbpA2</i>	ctagaattcacgcataatcaatagctcctg	(Waldminghaus <i>et al.</i> , 2009)
<i>IbpB2neu</i>	ccggaattcacgcatagtcatttctcttctaa	Cloning of pBO403, 1001
<i>ibpAstopfw</i>	gatttatccccgtttaaccgttctgctattg	Cloning of pBO1059, 1001
<i>ibpAstoprv</i>	atccaatagcagaacgggttaaacgcggggataa	Cloning of pBO668

*T7 promoter in capital letters.

was highly specific to the *ibpB* transcript (data not shown). Several transcripts of the *ibpAB* operon were detected by Northern blot analysis (Fig. 1b, c). Due to the σ^{32} dependency of the operon, previously described by Allen *et al.* (1992), the *ibpAB* transcripts only appeared after heat shock from 30 to 42 °C and were not detectable in an *rpoH* mutant (data not shown). Only 2 min after heat shock at 42 °C, the bicistronic transcript was readily detectable by

the *ibpA* (Fig. 1b) and *ibpB* (Fig. 1c) probes. The massive induction was transient, reached a maximum within a few minutes and declined to almost pre-shock levels after another 5–10 minutes. The concomitant appearance of a band between 500 and 600 nt suggested that the bicistronic message was processed into monocistronic *ibpA* and *ibpB* transcripts. Much shorter fragments of approx. 120 nt were detected between 8 and 15 min after heatshock with the

Fig. 1. Multiple RNA species derive from the *E. coli* *ibpAB* operon. (a) Schematic representation of the *E. coli* *ibpAB* operon. Transcription is known to be initiated from a σ^{32} -dependent promoter (Allen *et al.*, 1992). (b, c) Northern blot analysis of RNA isolated from heat-shocked *E. coli* C600 cells at the time points indicated and detected using *ibpA* (b) and *ibpB* (c) RNA probes. The O_1 sample was taken before half of the culture was heat shocked. The O_2 sample was taken after 30 min from the culture kept at 30 °C. M, RiboRuler high-range molecular marker (Fermentas).

ibpB RNA probe only (Fig. 1c), suggesting that further processing of the *ibpB* transcript occurred.

5'-end determination

The transcription start site upstream of *ibpA* has been reported previously (Chuang *et al.*, 1993; Nonaka *et al.*, 2006). To determine the 5' ends of the monocistronic and the short *ibpB* transcripts we used 5' RACE analysis. Total RNA was isolated from at least two independent cultures harvested after heat shock from 30 °C to 42 °C for 8 min. cDNA was produced by reverse transcription using appropriate oligonucleotides for the *ibpB* coding region. Six clones revealed a uniform 5' end of *ibpB* starting at the

second adenine of the *ibpA* stop codon (Fig. 2a). Hence, the monocistronic *ibpA* transcript has a size of 509 nt, whereas the monocistronic *ibpB* transcript consists of 584 nt. The 5' end of the short *ibpB* fragment was less well defined (Fig. 2b); eight clones contained seven different 5' ends, all between 134 and 94 nt from the presumed 3' end upstream of a typical factor-independent transcription terminator. To validate the 5' RACE data, total RNA of heat-shocked *E. coli* C600 cells was separated on a 10 % polyacrylamide gel and *ibpAB* fragments were detected with *ibpA* and *ibpB* probes (Fig. 2c). As expected, the monocistronic *ibpA* transcript migrated faster than the *ibpB* transcript. Various short *ibpB* fragments are consistent with the 5' RACE results.

RNases involved in *ibpAB* processing

To reveal the identity of the RNase(s) responsible for the processing of the *ibpAB* transcript, we measured the stability of the bicistronic mRNA in a temperature-sensitive RNase E mutant and an RNase III-negative background. RNase E is essential in *E. coli*. A shift from 30 to 42 °C induced transcription of the *ibpAB* operon and inactivated RNase E in the mutant strain. Decay of the bicistronic transcript after addition of rifampicin in the presence of RNase E and its stability in the absence of the RNase showed that degradation of the *ibpAB* transcript depends on RNase E (Fig. 3a). Furthermore, RNase E is responsible for the processing of the bicistronic *ibpAB* RNA, as the monocistronic *ibpB* transcript and the short *ibpB* products were detectable in the wild type (WT), whereas they did not appear in the RNase E mutant strain. Note that the monocistronic transcript in the RNase E mutant at time point zero was generated before the temperature-sensitive enzyme was inactivated. If at all, RNase III contributes only marginally to the processing and degradation of the *ibpAB* transcript because it had comparable half-lives in the wild-type and the RNase III mutant (Fig. 3b).

Heat-stimulated ribosome binding to the *ibpB* transcript *in vitro*

A potential secondary repression of heat-shock gene expression (ROSE)-like RNA structure in the 5' UTR of *ibpB* suggested that translation might be controlled by an RNA thermometer as is the case for *ibpA* (Waldminghaus *et al.*, 2009). The putative *ibpB* thermometer consists of four hairpins in the intergenic region between *ibpA* and *ibpB* (see Fig. 5a). The SD sequence is located in hairpin IV and is imperfectly paired with the anti-SD sequence, which exhibits the conserved U(U/C)GCU motif typical of ROSE-like elements (Waldminghaus *et al.*, 2005). To evaluate whether the 5' UTR of *ibpB* is able to control ribosome access, we tested the temperature-dependent binding of the 30S ribosomal subunits to the SD sequence *in vitro* by toeprinting experiments (Fig. 4). Two *in vitro*-synthesized RNAs were used (Fig. 4a). They contained either the

Fig. 2. Determination of the 5' ends of *ibpB* transcripts determined by 5' RACE. The positions of the 5' ends of the monocistronic *ibpB* transcript (a) and of short *ibpB* fragments (b) are indicated below a schematic presentation of the *ibpAB* operon. UAA and AUG in (a) represent the stop codon of *ibpA* and the start codon of *ibpB*, respectively. UAA and UUUUUUUU in (b) depict the stop codon of *ibpB* and the predicted 3' end of the transcript, respectively. (c) Northern blot analysis with RNA isolated from *E. coli* C600 cells heat-shocked for 8 min. The RNA was separated on a 10% polyacrylamide gel and *ibpAB* fragments were detected with the indicated probes.

bicistronic *ibpAB* region ranging from the *ibpA* 5' UTR to 69 nt into the coding region of *ibpB* or a shorter fragment containing only the *ibpB* 5' UTR up to 69 nt into the coding region of *ibpB*. Temperature-dependent binding of the 30S ribosomes to the *ibpA* SD sequence has already been demonstrated by using a short fragment containing only the *ibpA* 5' UTR (Waldminghaus *et al.*, 2009). Inefficient binding of the 30S ribosome to the *ibpA* SD

sequence at 30 °C and enhanced formation of the RNA-ribosome complex at 42 °C was also observed with the bicistronic *ibpAB* fragment (Fig. 4b). Binding of the ribosome to the *ibpB* leader region was equally temperature-sensitive. The absence of a prominent toeprint signal at 30 °C despite the presence of 30S ribosomes suggests that access of the ribosome to the *ibpB* SD sequence is blocked by the predicted RNA thermometer structure in

Fig. 3. Processing of *ibpAB* in RNase-deficient *E. coli* strains. An *E. coli* RNase E (a) and an *E. coli* RNase III (b) mutant strain and their isogenic wild-type strains were grown at 30 °C to exponential phase and heat-shocked at 42 °C for 1 min prior to the addition of rifampicin (250 µg ml⁻¹). Samples were taken at 0, 3, 6, 10 and 15 min after the addition of rifampicin. Transcripts were detected using the *ibpB* RNA probe. Arrowheads in (a) point at RNA fragments appearing after rifampicin addition that are absent in the heat-inactivated mutant strain.

Fig. 4. Temperature-dependent binding of the 30S ribosome to the 5' UTR of *ibpB* *in vitro*. (a) Schematic representation of *in vitro*-synthesized RNAs and oligonucleotides used for toeprinting analyses. (b–d) Toeprinting experiments demonstrating temperature-dependent binding of the 30S ribosomal subunit to the 5' UTR of *ibpA* (b) and *ibpB* (c, d), using the long and short RNA, respectively. Reaction components and conditions are shown above the gels. The corresponding DNA sequencing ladder was generated with the same end-labelled oligonucleotide as used in the toeprinting experiment. Ribosome-binding site (SD), translational start site (ATG) and the toeprinting signals are indicated.

both the long and the short transcript (Fig. 4c and d, respectively). Prematurely terminated reverse transcripts, at 42 °C and at the typical toeprint position around 15 nt downstream from the AUG start codon, showed that melting of the structured RNA under heat-shock conditions permits translation initiation of *ibpB* *in vitro*.

Structural features of the *ibpB* 5' UTR

To gain structural insights into the putative *ibpB* thermometer and two *ibpB* 5' UTR variants, structure-probing experiments were carried out with RNase T1 (cleaves 3' of single-stranded guanines) over a temperature range from 20 to 50 °C (Fig. 5). To reduce complexity, constructs analogous to the previously studied *ibpA* thermometer, containing the predicted hairpins II, III and IV of the *ibpB* 5' UTR, were chosen (Fig. 5a). The overall cleavage pattern of the *ibpB* WT UTR supports the predicted structure (Fig. 5b). Cleavage at positions G50, G51 and G52 at low temperatures suggests that the top loop is wider as predicted. The structure of hairpin IV was temperature-responsive. G101, G102 and G104 were barely cleaved at low temperatures but showed increased accessibility to RNase T1 at temperatures above 40 °C, suggesting liberation of the SD sequence according to the melting model. Probing of the C91A and

ΔG90 variants (Fig. 5c, d) provided structural evidence for their derepressed ('Derep') and repressed ('Rep') phenotypes, respectively, in reporter-gene assays (data not shown). In both RNAs, hairpins II and III behaved like the corresponding hairpins of the WT RNA. Hairpin IV of the 'Derep' RNA was more sensitive to RNase T1 cleavage compared to that of the WT (Fig. 5c); prominent cleavage of the guanines in the SD region already occurred at low temperatures. In the 'Rep' RNA, the SD sequence did not become accessible in the physiological temperature range (Fig. 5d).

IbpA-mediated regulation of *ibpB* expression

To further analyse the putative RNA thermometer of the *ibpB* 5' UTR we constructed translational *ibpB* and *ibpAB* reporter-gene fusions (Fig. 6a) by using our previously described *bgaB*-reporter system taking advantage of the thermostable β -galactosidase BgaB from *Bacillus stearothermophilus* (Waldminghaus *et al.*, 2007). At 30 °C, expression of the *ibpB* fusion measured in *E. coli* C600 was about 110 MU and increased only slightly to 160 MU at 42 °C (Fig. 6b). Expression of the *ibpAB* fusion was comparable, whereas the *ibpA* fusion was heat-inducible, as reported previously (Waldminghaus *et al.*, 2009). Hence, in

Fig. 5. Structure probing of the *ibpB* 5' UTR. (a) Secondary structure prediction of the *ibpB* 5' UTR made with the mfold program (Zuker, 2003). Nucleotide numbers were arbitrarily chosen starting from the second A of the UAA stop codon of *ibpA*. The sequence shown in black represents the *in vitro*-synthesized RNA used for structure probing. The SD sequence and AUG start codon of *ibpB* are indicated as well as single-point mutations of repressed ('Rep') and derepressed ('Derep') variants. (b–d) Structure probing of the *ibpB* WT 5' UTR (b), the predicted derepressed (c) and the predicted repressed (d) variants. L, alkaline ladder; K, water-treated control RNA.

Fig. 6. Expression of *ibpB* in vivo. (a) Schematic view of the constructed *ibp*–*bgaB* fusions. (b) Expression of translational *ibpA*, *ibpB* and *ibpAB* fusions in *E. coli* C600 and the isogenic Δ *ibpAB* strain. After transcription induction with 0.01 % L-arabinose, cells were heat-shocked for 30 min at 42 °C before β -galactosidase activity was measured. Activities are shown in Miller units (MU). Cells were grown at 30 °C to exponential phase.

contrast to the *in vitro* results, which showed temperature-dependent melting of, and ribosome binding to, the *ibpB* RNA, the *in vivo* data argue against the temperature-regulated translation of *ibpB*.

Measurements of the same fusions in an *E. coli* C600 Δ *ibpAB* strain suggested a solution for this apparent puzzle. In the absence of any IbpA and IbpB protein, the *ibpB* fusion showed heat induction like the *ibpA* fusion (Fig. 6b) (Waldminghaus *et al.*, 2009). Interestingly, expression was reduced in the *ibpAB* fusion, which is able to produce IbpA from the reporter plasmid. The possible influence of the IbpA protein on *ibpB* expression was determined by testing the introduction of a nonsense mutation at the beginning of the coding sequence of *ibpA* in the *ibpAB* fusion so that no IbpA protein could be synthesized from this plasmid. The temperature response of this fusion in the Δ *ibpAB* background was restored, suggesting that IbpA plays a role in controlling *ibpB* expression.

DISCUSSION

Regulation of the heat-shock response in *E. coli* is complex and involves several mechanisms, mostly controlling the cellular level of the key regulator σ^{32} in response to the physiological need (Guisbert *et al.*, 2008). Here, we describe novel mechanisms that control expression of the *ibpAB* operon at regulatory levels downstream of σ^{32} -mediated transcriptional control (Fig. 7).

Transcriptional regulation and RNA processing

Transcription of the *ibpAB* operon has been reported to depend on the alternative sigma factor σ^{32} (Allen *et al.*, 1992). In certain nutrient-poor media, the distal *ibpB* gene is transcribed from a σ^{54} -dependent promoter (Kuczyńska-Wisnik *et al.*, 2001). However, this does not play a role under the conditions used in the present study because corresponding 5' ends were not found by 5' RACE experiments. The σ^{32} promoter upstream of *ibpA* is responsible for the massive, several hundred-fold induction of the *ibpAB* operon after heat shock (Rasouly *et al.*, 2009; Wade *et al.*, 2006). The requirement for the heat-shock sigma factor was recapitulated in our experiments. Further, we discovered several RNA processing events that might result in differential expression of *ibpA* and *ibpB*. Cleavage of the bicistronic *ibpAB* transcript results in monocistronic *ibpA* and *ibpB* transcripts, the latter of which is further processed into short 3' ends. The 5' end of the monocistronic *ibpB* transcript was mapped within the stop codon of *ibpA*. There are no apparent promoter sequences upstream of this site. Instead, the sequence around the mapped 5' end (A $\downarrow$ AUUC) closely resembles the consensus sequence of an RNase E cleavage site RAUW (R=A/U; W=A/U), in which the cleavage usually occurs in the central AUU sequence (Ehretsmann *et al.*, 1992). The g55/ α gt RNA from the T4 phage was demonstrated to be cleaved by RNase E in *E. coli* at exactly the same nucleotide sequence (A $\downarrow$ AUUC) as in the *ibpAB* transcript (Loayza *et al.*, 1991). Fully

Fig. 7. Mechanisms involved in regulation of the *E. coli* *ibpAB* operon. See text for details.

consistent with the putative RNase E cleavage site, the *ibpB* transcript was only detectable in the WT strain, whereas it did not appear in the temperature-sensitive RNase E mutant after heat inactivation of the enzyme.

Subsequent decay of the *ibpB* RNA in the wild-type strain led to the transient accumulation of short degradation products containing the 3' end of *ibpB*. The 5' fragment was not detected, suggesting that it is rapidly degraded. In contrast to the well-defined initial cut in the *ibpAB* transcript, the *ibpB* fragments carry diverse 5' ends. RNase E-dependent cleavage is known to be the rate-limiting step in mRNA degradation. It processes polyribosomal mRNA by cleaving in the translation-initiation region or in the intercistronic regions of polycistronic messages. The mRNA fragments generated are further cleaved at RNase E-sensitive sites located downstream (Carpousis, 2007; Jain, 2002). Several of the mapped 5' ends within the *ibpB* mRNA are characterized by AU-rich sequences suggesting that RNase E is also responsible for this process.

Translational and post-translational regulation

Transcriptional induction of the *ibpAB* operon and mRNA processing are complemented by further control mechanisms (Fig. 7). Translation of the *ibpA* gene is temperature-controlled by an RNA thermometer (Waldminghaus *et al.*, 2009). The 5' UTR of the *ibpB* gene also exhibits characteristics of a ROSE-like element (Narberhaus *et al.*,

2006). Toeprinting experiments indeed showed heat-dependent binding of the 30S ribosomal subunit to the *ibpB* SD sequence in the monocistronic as well as in the bicistronic context. Structure-probing experiments suggested an opening of the RNA structure in hairpin IV, which harbours the *ibpB* SD sequence. These *in vitro* results demonstrated that the structural properties of the *ibpB* 5' UTR allow thermoregulation of *ibpB* translation. However, the temperature response *in vitro* was not reflected by translational fusions *in vivo*. Heat induction of an *ibpB*–*bgaB* fusion was only detected when no IbpA protein was present in the cells. The two homologous small heat-shock genes *ibpA* and *ibpB* are believed to originate from a duplication event and were initially discovered as inclusion-body-binding proteins during the production of heterologous proteins in *E. coli* (Allen *et al.*, 1992; Vaillancourt, 2003). In other studies it was shown that IbpA and IbpB levels increase almost 10-fold during overexpression of recombinant proteins while other heat-shock proteins like DnaK and GroEL increase only 2-fold (Han *et al.*, 2004). This suggests that IbpA and IbpB play an important role during recombinant protein production. Nevertheless, their cooperation and exact function *in vivo* are still not fully understood (Han *et al.*, 2004; Matuszewska *et al.*, 2008). The pool of substrates interacting with IbpA is much larger than that of IbpB (Butland *et al.*, 2005). The presence of IbpA alone during substrate denaturation did not increase the efficiency of reactivation but rather inhibited this process. IbpA-mediated recruitment of IbpB reversed this inhibitory effect (Ratajczak *et al.*,

2009). Although IbpA and IbpB seem to have distinct activities, they depend on an intricate interplay between the two proteins.

For reasons not yet fully understood, IbpA accumulation is stronger than IbpB accumulation during *ibpAB* overexpression and in cells exposed to copper (Lethanh *et al.*, 2005; Matuszewska *et al.*, 2008). Our results suggest two possible reasons for differential expression of *ibpA* and *ibpB* (Fig. 7). First, processing of the *ibpAB* transcript between *ibpA* and *ibpB* and subsequent cleavage within *ibpB* might result in preferential accumulation of *ibpA* transcripts in the cell. In addition, regulation of *ibpB* expression by IbpA might constitute a second mechanism involved in reducing *ibpB* expression. At present, we can only speculate on the putative mechanism of IbpA-mediated control. It is conceivable that IbpA interacts with the *ibpB* mRNA to prevent translation and/or stimulate ribonucleolytic decay. In plants, small heat-shock proteins are associated with mRNAs in heat-shock granules (Nover *et al.*, 1989). Other reports have shown that small heat-shock proteins are able to interact with single-stranded and double-stranded DNA (Singh *et al.*, 1998). Owing to its polydispersity and its tendency to precipitate, purification of IbpA for biochemical characterization is notoriously difficult (Shearstone & Baneyx, 1999). Hence, all our attempts to demonstrate a direct interaction between the *ibpB* 5' UTR and IbpA failed. The recently discovered degradation of IbpA and IbpB by the Lon protease provides another explanation for differing cellular levels of the two proteins (Bissonnette *et al.*, 2010). Proteolysis of IbpB was 15-fold faster than IbpA degradation. Interestingly, decay of IbpA was accelerated 7-fold in the presence of IbpB. Taking these findings together with our results, it is tempting to speculate an antagonistic mechanism, in which the IbpA protein represses its own degradation by inhibiting the translation of *ibpB*. It is going to be a challenging task to obtain deeper mechanistic insights into the control of the *ibpAB* operon.

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