A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites

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The interactions of numerous regulatory small RNAs (sRNAs) with target mRNAs have been characterized, but how sRNAs can regulate multiple, structurally unrelated mRNAs is less understood. Here we show that *Salmonella* GcvB sRNA directly acts on seven target mRNAs that commonly encode periplasmic substrate-binding proteins of ABC uptake systems for amino acids and peptides. Alignment of GcvB homologs of distantly related bacteria revealed a conserved G/U-rich element that is strictly required for GcvB target recognition. Analysis of target gene fusion regulation in vivo, and in vitro structure probing and translation assays showed that GcvB represses its target mRNAs by binding to extended C/A-rich regions, which may also serve as translational enhancer elements. In some cases (*oppA*, *dppA*), GcvB repression can be explained by masking the ribosome-binding site (RBS) to prevent 30S subunit binding. However, GcvB can also effectively repress translation by binding to target mRNAs at upstream sites, outside the RBS. Specifically, GcvB represses *gltI* mRNA translation at the C/A-rich target site located at positions –57 to –45 relative to the start codon. Taken together, our study suggests highly conserved regions in sRNAs and mRNA regions distant from Shine-Dalgarno sequences as important elements for the identification of sRNA targets.

[**Keywords:** Small RNA; riboregulator; post-transcriptional control; translation inhibition; ABC transporter; GcvB]

Supplemental material is available at <http://www.genesdev.org>.

Received July 2, 2007; revised version accepted September 3, 2007.

Small noncoding RNAs (sRNAs) that act as regulators of gene expression have been identified in all kingdoms of life. The enterobacterial sRNAs constitute a structurally diverse class of molecules that are typically 50–200 nucleotides (nt) in length and function as stress-response or virulence gene regulators (Wassarman 2002; Gottesman 2004; Romby et al. 2006; Vogel and Papenfort 2006). Recent systematic searches (Vogel and Sharma 2005) showed that *Escherichia coli* expresses >80 sRNAs; the total number of sRNAs in a typical enterobacterium may well range in the hundreds (Hershberg et al. 2003; Zhang et al. 2004).

The majority of the ~20 enterobacterial sRNAs of known function are antisense RNAs that repress *trans*-encoded target mRNAs (Majdalani et al. 2005; Storz et al. 2005). Many sRNAs mask the ribosome-binding sites (RBS) of their targets, thus inhibiting ribosome entry on

mRNA (Storz et al. 2004). Specifically, the *E. coli* MicA, MicC, and OxyS sRNAs were shown by in vitro toeprinting experiments to directly interfere with 30S ribosome binding of target mRNAs (Argaman and Altuvia 2000; Chen et al. 2004; Udekwu et al. 2005). Other well-characterized *E. coli* sRNA–target pairs that are likely to use the same mechanism include DsrA–*hns*, MicF–*ompF*, RyhB–*sodB*, SgrS–*ptsG*, and Spot42–*galK* (for review, see Wagner and Darfeuille 2006). Since the half-life of bacterial mRNA is strongly affected by the association with ribosomes (Deana and Belasco 2005), translation inhibition will promote the decay of the repressed target; e.g., by accelerating RNase E-mediated mRNA turnover (Massé et al. 2003; Morita et al. 2005). Besides RNase E, the bacterial Sm-like protein Hfq has been identified as a key player in this type of translational silencing (Valentin-Hansen et al. 2004). Hfq binds all of the aforementioned sRNAs with high affinity and is most often required for both their intracellular stability and their interaction with target mRNAs (Zhang et al. 2003; Aiba 2007; Urban and Vogel 2007, and references therein). Hfq and some sRNAs form ribonucleoprotein complexes

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.447207>.

with RNase E to mediate target mRNA destabilization (Morita et al. 2005).

The enterobacterial sRNAs exhibit short and/or imperfect complementarity to their target(s). For example, nine residues of RyhB sRNA interact with *sodB* mRNA (Geissmann and Touati 2004), and OxyS sRNA targets *fhlA* mRNA through the formation of two short kissing complexes of 9 and 7 base pairs (bp), respectively (Argaman and Altuvia 2000). Systematic mutational analysis of SgrS-*ptsG* RNA duplexes revealed that few (six) residues of this interaction are key to mediating SgrS repression of *ptsG* mRNA (Kawamoto et al. 2006). The limited sequence complementarity has rendered the identification of new sRNA targets difficult, and for the majority of the sRNAs studied to date, a single mRNA remains the only experimentally validated target. However, it was early recognized that some *E. coli* sRNAs could regulate multiple mRNAs. For example, DsrA was shown to directly act on the *hns* and *rpoS* mRNAs (Lease et al. 1998; Majdalani et al. 1998), and constitutive overexpression of OxyS altered the expression of >40 genes (Altuvia et al. 1997). Recent biocomputational and experimental approaches predicted more sRNAs to target multiple mRNAs. For example, pulse expression of several *E. coli* and *Salmonella* sRNAs (Massé et al. 2005; Guillier and Gottesman 2006; Johansen et al. 2006; Papenfort et al. 2006; Tjaden et al. 2006) followed by global transcriptome profiling showed the rapid depletion of many mRNAs. The kinetics of target decay observed in these experiments strongly argues that the regulated mRNAs are direct targets.

Multiple mRNA targeting by sRNAs could help bacteria to balance different transcriptional responses at the post-transcriptional level in response to stress or changes in nutrient availability. This additional layer of gene expression control could mediate the coregulation of mRNAs that belong to different transcriptional regulons. However, evidence for direct interactions of sRNAs with multiple mRNAs has been rare in enterobacteria (Lease et al. 1998) and has come primarily from investigations of RNAIII of the Gram-positive pathogen *Staphylococcus aureus* (Boisset et al. 2007).

In this work, we have studied GcvB sRNA of *Salmonella enterica* serovar Typhimurium (from here on *Salmonella*) and found that it directly binds multiple mRNAs that collectively encode periplasmic substrate-binding proteins of amino acid and peptide transporters. The *gcvB* gene was originally identified in *E. coli* and is controlled by GcvA/GcvR, the major transcription factors of the glycine cleavage system (Urbanowski et al. 2000). A *gcvB* deletion caused constitutive synthesis of OppA and DppA, periplasmic substrate-binding proteins of the two major peptide transport systems (Higgins and Hardie 1983; Abouhamad et al. 1991), which are normally repressed in nutrient-rich growth conditions. Gene fusion experiments indicated that GcvB repressed *dppA* and *oppA* at the post-transcriptional level, yet the molecular mechanism remained elusive (Urbanowski et al. 2000). Furthermore, the pleiotropic nature of *E. coli* and *Yersinia gcvB* mutants (Urbanowski et al. 2000;

McArthur et al. 2006), and biocomputational predictions (Tjaden et al. 2006) suggested that GcvB may have additional mRNA targets.

This study provides biochemical and genetic evidence that a conserved G/U-rich region within GcvB mediates translational repression of seven ABC transporter mRNAs. While GcvB represses some of these targets by covering their RBS, this sRNA can also inhibit mRNA translation by binding to regions upstream of the RBS. This latter mode of action may also apply to other regulatory sRNAs.

Results

Characterization of GcvB sRNA in Salmonella

The *Salmonella* and *E. coli gcvB* genes are ~95% identical in the GcvB RNA region (Urbanowski et al. 2000; Argaman et al. 2001). Expression of the ~200-nt *Salmonella* GcvB RNA (Fig. 1A) was confirmed on Northern blots of RNA extracted from different growth phases and media. GcvB is expressed in exponentially growing *Salmonella* in rich medium (L-broth) but not detectable in stationary phase or upon growth in minimal medium (Fig. 1B). This pattern is reminiscent of *E. coli gcvB* (Argaman et al. 2001) and in keeping with the postulated GcvB repressor function of peptide transporters under nutrient-rich conditions (Urbanowski et al. 2000).

Several observations suggested that GcvB is an Hfq-dependent sRNA. GcvB coimmunoprecipitates with Hfq in extracts of *E. coli* (Zhang et al. 2003) and *Salmonella* (A. Sittka and J. Vogel, in prep.) and is unstable in Δhfq strains of *E. coli* (Urban and Vogel 2007) and *Salmonella* (Supplementary Fig. S1). Since repression of the predicted GcvB target, *oppA*, is abrogated in *hfq* mutants of both *E. coli* and *Salmonella* (Ziolkowska et al. 2006; Sittka et al. 2007), we compared OppA protein levels in *Salmonella* strains deleted for *gcvB*, *hfq*, or both (Fig. 1C). Deletion of *gcvB* did not impair *Salmonella* growth in rich broth (data not shown), similar to what was reported for *E. coli* (Urbanowski et al. 2000). The single *gcvB* or *hfq* mutations each elevated OppA levels, whereas the double mutation had no pronounced cumulative effect. This predicts GcvB and Hfq to act in concert, and GcvB to be an Hfq-dependent antisense RNA that regulates *trans*-encoded mRNA(s).

GcvB targets *dppA* and *oppA* mRNAs in vivo and in vitro

To study *dppA* and *oppA* mRNA repression by GcvB, we constructed translational fusions to the N terminus of green fluorescent protein, GFP (Urban and Vogel 2007). The *Salmonella dppA* or *oppA* 5' regions spanning the entire 5' untranslated region (UTR) and 30 bp (*dppA*) or 50 bp (*oppA*) of the coding region were cloned into the low-copy *gfp* fusion vector, pXG10 (Fig. 2A). The cloning strategy used here ensures that the fusions are tran-

To map the GcvB interaction sites, we synthesized RNAs of the previously cloned *dppA* and *oppA* fragments in vitro and performed structural probing experiments. Gel mobility shift assays showed that GcvB

The reciprocal experiment—i.e., probing of labeled *dppA* or *oppA* RNAs in complex with GcvB—identified the GcvB-binding site on the mRNAs. Wild-type GcvB RNA yields a strong footprint on the *dppA* (Fig. 3B) and *oppA* (Supplementary Fig. S4) RNAs, which correspond to regions -31 to -14, and -8 to +16, respectively, relative to the AUG start codon (Fig. 3D). In contrast, no footprints were obtained with a mutant RNA, GcvB_{ΔR1}, which lacks the G/U-rich region (Fig. 3B; Supplementary Fig. S4).

The duplexes shown in Figure 3D were further supported by cleavage of the GcvB/*dppA* and GcvB/*oppA* complexes with the double-strand-specific nuclease, RNase III. While several weak RNase III-dependent bands were observed in GcvB RNA alone (Fig. 3A, lane 11), the enzyme cleaved GcvB strongly and specifically in the G/U-rich region in the presence of either *dppA* or *oppA* RNA (Fig. 3A, lanes 12–15). Reciprocally, the *dppA* and *oppA* RNAs were specifically cleaved in the proposed GcvB-binding site (Fig. 3C; Supplementary Fig. S4). Collectively, these results indicated the single-stranded, G/U-rich region of GcvB as an important determinant for target recognition.

A conserved G/U-rich region mediates GcvB repression in vivo

The importance of the G/U-rich element was also evident from its strong conservation in GcvB sequences of distantly related species. Computer-based searches predicted *gcvB* genes in many enterobacteria as well as in *Pasteurellaceae* and *Vibrionaceae* (Supplementary Fig. S5). The location of these candidate genes adjacent to and divergent from *gcvA* and the conservation of promoter and terminator elements argue that these are *gcvB* homologs. Although the corresponding RNA sequences proved of enormous sequence diversity, we find that much of the G/U-rich linker between stem-loops SL1 and SL2 is highly conserved in all identified *gcvB* sequences (Fig. 1D). We refer to this linker region as R1 (Conserved Region 1). The single-stranded region between SL3 and SL4 contains another highly conserved sequence (ACUUCUGUA) found immediately upstream of SL4; this sequence is referred to as R2.

To assess the role of these two regions in target regulation, we constructed mutant alleles, *gcvB*_{ΔR1}, *gcvB*_{ΔR2}, and *gcvB*_{5'Δ}, the latter being a 5'-truncated *gcvB* lacking SL1 and R1 (Fig. 2C). Plasmids carrying these alleles expressed distinct GcvB-derived RNAs at levels similar to wild-type *gcvB* (Fig. 2D). Next, these alleles were cloned under control of the P_{LacO-1} promoter to test their ability to regulate the *dppA*::*gfp* and *oppA*::*gfp* fusions. Figure 2B shows that loss of R1 (*gcvB*_{ΔR1} and *gcvB*_{5'Δ}) abrogated *dppA* and *oppA* fusion repression, whereas loss of R2 (*gcvB*_{ΔR2}) had no effect.

Is the 5' part of GcvB (including R1) sufficient to confer target repression in vivo? Urbanowski et al. (2000) postulated that SL3 of GcvB, which is followed by a U-stretch in many GcvB species (Fig. 1D), may serve as a ρ-independent transcription terminator, leading to expression of a shorter GcvB RNA. However, truncation of *gcvB* after residue T₁₃₄ (allele *gcvB*_{3'ΔT}, expected to yield an ~134-nt GcvB RNA) (Fig. 2C) did not result in such an RNA (Fig. 2D), whereas modification of SL3 created a functional terminator (allele *gcvB*_{3'ΔT}) (Fig. 2C) and led to detection of an ~130-nt RNA (Fig. 2D). Figure 2B shows that the 3'-truncated GcvB RNA of *gcvB*_{3'ΔT} was fully active in *dppA* and *oppA* fusion repression. Taken together, these in vivo experiments further support a key

role of the G/U-rich element, R1, of GcvB for *dppA* and *oppA* mRNA regulation.

More GcvB targets

The observation that additional periplasmic proteins accumulated in *gcvB* or *hfq* mutant strains (Urbanowski et al. 2000; Sittka et al. 2007) prompted us to screen for more GcvB targets. Analysis of periplasmic proteins from a *Salmonella gcvB* overexpression strain predicted *gltI*, *livJ*, *argT*, and STM4351 as further candidate targets (Fig. 4A). In an independent approach, we used the RNA-hybrid algorithm (Rehmsmeier et al. 2004) to search for stable RNA duplexes of the R1 sequence of GcvB with the 5' regions of all *Salmonella* genes. This biocomputational search supported *gltI*, *livJ*, *argT*, and STM4351 and further predicted *livK* as a GcvB target (Fig. 5A). Intriguingly, the predicted GcvB-binding sites on these mRNAs do not match in sequence but are rich in C and A residues, which also holds true for *dppA* and *oppA* (Fig. 3D).

The predicted binding sites were subsequently confirmed by in vitro probing experiments as above (summarized in Fig. 5; shown in Supplementary Fig. S6). Although the footprint obtained for *gltI* mRNA is relatively weak, the predicted interaction is strongly supported by specific RNase III cleavage in the presence of GcvB (Supplementary Fig. S6A). Conversely, R1 is the GcvB region that is most strongly protected upon incubation with the target RNA fragments (Supplementary Fig. S7). When in complex with target RNAs, GcvB was cleaved by RNase III exclusively in the R1 sequence, except with a *livK*-derived fragment that also promotes cleavage in the GcvB 3' part (Supplementary Fig. S7). We constructed translational *gfp* fusions to all of these targets, of which the *gltI* and *livJ* fusions showed fluorescence on agar plates (Fig. 4B). Both fusions were strongly repressed by *gcvB* alleles with an intact R1 sequence (*gcvB*, *gcvB*_{ΔR2}, and *gcvB*_{3'ΔT}), whereas the *gcvB*_{5'Δ} and *gcvB*_{ΔR1} alleles lacking R1 failed to repress the fusions (Fig. 4B). Western blot analysis of all five target fusions as well as the *dppA*::*gfp* and *oppA*::*gfp* fusions further confirmed that regulation required an intact R1 sequence (Fig. 4C). These results increased the number of GcvB targets to seven mRNAs.

GcvB inhibits translation initiation in vitro

GcvB binds near the Shine-Dalgarno (SD) sequence of *dppA* and *oppA* (Fig. 3D) and was thus predicted to prevent ribosome binding to these mRNAs. To test this, we performed 30S ribosome toeprinting assays (Hartz et al. 1988). A *dppA* mRNA fragment was annealed to an end-labeled primer complementary to the *dppA* coding region (+58 to +73) and incubated with 30S subunits in the presence or absence of uncharged tRNA^{fMet}, followed by cDNA synthesis. Analysis of the extension products (Fig. 6A) revealed one ribosome-induced, tRNA^{fMet}-dependent termination site at the characteristic +15/+16 posi-

Figure 4. GcvB targets additional periplasmic transporter mRNAs. (A) *Salmonella* Δ *gcvB* carrying control plasmid pTP11 (left) or *gcvB* mid-copy plasmid pTP05 (right) were grown to early stationary phase, and periplasmic protein fractions of these bacteria were resolved on two-dimensional gels. Relevant subsections of the gels are shown. Protein spots indicating repression by GcvB are labeled. (B) Agar plate-based assay of colony fluorescence of *E. coli* strains carrying either *gltI::gfp* (left; plasmid pJL24-1) or *livJ::gfp* (right; plasmid pJL20-1). Each fusion plasmid was combined with control plasmid pTP11, or plasmids expressing *Salmonella* GcvB and mutant RNAs as in Figure 2B. (C) Western blots of target::GFP fusion proteins prepared from *E. coli* Δ *gcvB* *recA*⁻ carrying the indicated fusion plasmids in combination with control plasmid pTP11, or plasmids expressing wild-type *gcvB* or the *gcvB*_{ΔR1} allele. GroEL was probed as the loading control. Fold changes of GFP fusion protein levels (upon normalization to GroEL levels) by *gcvB* or *gcvB*_{ΔR1} coexpression relative to the control plasmid were OppA::GFP, -2.8/-1.2; DppA::GFP, -3.6/-1.3; GltI::GFP, -1.8/+2.2; LivJ::GFP, -5.3/-1.4; LivK::GFP, -2.1/-1.0; ArgT::GFP, -1.7/-1.1; STM4351::GFP, -1.8/-1.3; and GFP alone, -1.1/-1.2.

tions (start codon A is +1). This “toeprint” signal was decreased when wild-type GcvB RNA was added prior to incubation with 30S/fMet (Fig. 6A, lanes 4–7), suggesting inhibition of 30S binding. Inhibition was also observed with the GcvB_{ΔR2} and GcvB_{3'Δ} mutant RNAs, which have an intact R1 sequence (Fig. 6A, lanes 11,12). In contrast, the GcvB_{ΔR1} mutant RNA (Fig. 6A, lane 10) did not inhibit ternary complex formation.

The same assay performed on *oppA* mRNA confirmed that GcvB inhibited ternary complex formation, and that it required an intact R1 sequence (data not shown). Since GcvB did not inhibit 30S binding to the unrelated *ompA* mRNA (data not shown), we conclude that it acts as a sequence-specific translation initiation inhibitor by masking the *dppA* and *oppA* RBS.

GcvB inhibits *gltI* translation by binding far upstream of the start codon

GcvB binds to its other five target mRNAs further upstream of SD and the start codon as compared with *dppA* and *oppA* (Fig. 5). Notably, GcvB binds to region -57 to -45 of *gltI*, and -57 to -42 of *argT*. These interaction sites lie outside the -35 to +19 mRNA region covered by 30S ribosomes (Hüttenhofer and Noller 1994). Toeprinting of 30S on *gltI* mRNA (Fig. 6B) gave a single toeprint at +15 relative to the *gltI* AUG in the presence of 30S/fMet (Fig. 6B, lane 3), confirming the *gltI* start codon

shown in Figure 6C (note that the *gltI* coding region is misannotated in *Salmonella* and likely in several additional bacteria). Strikingly, GcvB dramatically reduced 30S binding on the *gltI* mRNA (Fig. 6C, lane 4–6), dependent on an intact R1 sequence (Fig. 6C, lane 7). Thus, GcvB can inhibit mRNA translation by binding upstream of a 30S-binding site.

The C/A-rich target site enhances *gltI* translation

To further evaluate GcvB regulation at upstream mRNA sites, the C/A-rich target region (-71 to -44) was deleted in *gltI::gfp*, yielding fusion *gltI*_{ΔCA}::*gfp*. This deletion abolished fusion repression in the presence of pP_L*gcvB* (Fig. 7A), further supporting that GcvB pairs to this region. Intriguingly, loss of this region also resulted in lower GFP fluorescence as compared with the parental *gltI::gfp* fusion. Since C/A-rich stretches were reported to enhance mRNA translation (Martin-Farmer and Jansen 1999), we determined whether the GcvB target site acts as an enhancer of *gltI* translation. In vitro synthesized, full-length *gltI::gfp* and *gltI*_{ΔCA}::*gfp* fusion mRNAs were translated with reconstituted 70S ribosomes, and GltI::GFP fusion protein synthesis was monitored over time. Figure 7B shows a linear increase in GltI::GFP synthesis from both mRNA templates within a 45-min assay. However, the *gltI::gfp* mRNA gave approximately threefold higher translation rates compared with that of

Figure 5. Summary of in vitro probing results of GcvB–target complexes. (A) Proposed RNA duplexes formed by GcvB with five periplasmic transporter mRNAs (*gltI*, *livK*, *livJ*, *argT*, *STM4351*). Positions in the target sequences are given as distance to the mRNA start codon. Vertical arrows denote in vitro RNase III cleavage of the GcvB–*gltI* complex, and of GcvB in complex with the four other targets (Supplementary Figs. S6A, S7B). Residues that showed protection in in vitro footprinting experiments (Supplementary Figs. S6, S7A) are set in bold and capitalized. Biocomputational prediction of target sites proposed the formation of longer duplexes around the interaction sites mapped by footprint analysis. (B) Location of GcvB-binding sites on target mRNAs. 5' UTRs of target genes are drawn to scale. Asterisks indicate promoter positions that were mapped by 5' RACE; “#” indicates promoters according to EcoCyc annotations. SD sequences are shadowed. Positions of the GcvB-binding sites on the mRNAs are marked by a horizontal bar.

*gltI*_{ΔCA}::*gfp*. Addition of GcvB (10-fold excess) to the reactions strongly inhibited protein synthesis from *gltI*::*gfp* but not *gltI*_{ΔCA}::*gfp* mRNA. None of these variations are due to RNA degradation since the *gltI*::*gfp* and GcvB RNAs were stable during the assay (Supplementary Fig. S8). In addition, the inhibitory activity of various GcvB mutants on *gltI*::*gfp* in vitro (Fig. 7C) perfectly correlated with their ability to inhibit the fusion mRNA in vivo (Fig. 4B).

Creation of an upstream C/A-rich target site permits translational control of an unrelated mRNA

Next, we inserted the C/A-rich GcvB-binding site of *gltI* at position –42 of an *E. coli ompR*::*gfp* fusion, yielding *ompR*_{CA}::*gfp* (Fig. 7D). The *Salmonella gltI* and *E. coli ompR* genes are unrelated in function, and their 5' mRNA regions have little sequence identity (Supplementary Fig. S9). Insertion of the C/A-rich element resulted in an approximately twofold increase in *OmpR*::GFP synthesis in the in vitro translation assay (Fig. 7E; cf. lanes 3 and 9), confirming the stimulatory effect of this element on mRNA translation. When GcvB was present in the reaction, it strongly inhibited translation of the *ompR*_{CA}::*gfp* but not of the parental *ompR*::*gfp* fusion mRNA (Fig. 7E). Intriguingly, GcvB inhibits *ompR*_{CA}::*gfp* translation ~10-fold; i.e., as

strongly as observed with *gltI*::*gfp*. In other words, GcvB effectively represses a structurally unrelated mRNA upon insertion of a C/A-rich target site at an upstream position.

Discussion

ABC transporters constitute a major class of amino acid uptake systems and commonly have at least one periplasmic solute-binding protein to take up substrates upon their diffusion through outer membrane porins (Hosie and Poole 2001; Davidson and Chen 2004). Given the key roles of amino acids in nitrogen and carbon metabolism, regulation of transporter expression and activity is complex. Intriguingly, genes involved in amino acid uptake and metabolism also are the most strongly misregulated class in *hfq* mutants of *E. coli* and *Salmonella* (Guisbert et al. 2007; A. Sittka and J. Vogel, in prep.), suggesting that Hfq-dependent sRNAs are involved in the post-transcriptional control of these pathways. We have here established one such sRNA, GcvB, as a direct regulator of many ABC transporter mRNAs in *Salmonella*. The conservation of *gcvB* genes combined with the previous data from the Stauffer laboratory (Urbanowski et al. 2000; McArthur et al. 2006) as well as successful prediction of GcvB–target interactions in related organisms (Supplementary Table S10; Tjaden et al.

2006) strongly argue that the GcvB function in amino acid uptake is conserved in many other bacteria. Interestingly, some enterobacteria encode an additional sRNA, RydC, which regulates the expression of a putative ABC transport system, *yejABEF* (Antal et al. 2005).

GcvB directly interacts with seven *Salmonella* mRNAs that belong to various transcriptional regulons but collectively encode periplasmic substrate-binding proteins of ABC transporters (<http://www.tcd.org>; <http://www.kegg.org>). These proteins are known or predicted to bind di- and oligopeptides (DppA, OppA) and polar (GltI, STM4351, ArgT) and branched amino acids (LivK, LivJ). Since GcvB is specifically expressed in rich medium in fast-growing cells (Fig. 1B), its function appears to lie in the general repression of amino acid uptake when nutrients are plentiful. Consistent with this prediction, a *Salmonella gcvB* deletion strain exhibits elevated protein and mRNA levels of almost all established GcvB targets (Figs. 1C, 4A; Supplementary Fig. S10). The increased steady-state levels of nearly all target mRNAs in $\Delta gcvB$ (Supplementary Fig. S10) argue that by repressing mRNA translation, GcvB also promotes the decay of its targets. Western blot quantification of OppA protein levels revealed a sixfold increase in $\Delta gcvB$ as compared with wild type (Fig. 1C); the corresponding RT-PCR experiment

(Supplementary Fig. S10) suggests a similar increase in *oppA* mRNA levels. Comparison of the increases in target protein levels (spot intensity on Coomassie-stained 2D gels) (Fig. 4A) and mRNA levels (Supplementary Fig. S10) upon *gcvB* deletion suggests a similar correlation in mRNA/protein level changes for *dppA*, *gltI*, *livJ*, STM4351, and *argT* (LivK was not detected on the 2D gels). Whether RNase III, here used as a tool for structure probing in vitro, is the primary nucleolytic activity to degrade the GcvB target mRNA in vivo as shown previously for the IstR-1 sRNA (Vogel et al. 2004) and RNAIII (Huntzinger et al. 2005), remains to be determined. At least *dppA*, *livK*, *gltI*, and *oppA* each constitutes the first gene of a polycistronic transporter operon. Considering that the downstream cistrons of these polycistronic mRNAs are likely translationally coupled to the direct GcvB targets, the sRNA may, in fact, repress >20 genes. However, since GcvB is intrinsically unstable (half-life 2–3 min) (Vogel et al. 2003; Supplementary Fig. S1), ABC transporter repression will be quickly alleviated upon decreased availability of amino acids.

The sheer number of sRNA genes discovered by systematic searches of bacterial genomes over the last years led to an increasing recognition of the potential impact of these riboregulators on bacterial physiology. It is well

Figure 7. The C/A-rich upstream element functions both as GcvB target site and as translational enhancer element. (A) Agar plate-based assay of colony fluorescence of *E. coli* strains carrying either the parental *gltI::gfp* (left; plasmid pJL24-1) or the mutant *gltI_{ΔCA}::gfp* (right; plasmid pJL45-3) fusion. In the latter plasmid, the C/A-rich GcvB target site (–71 to –44) (cf. Fig. 6C) is deleted. The fusion plasmids were combined with control vector pTP11 or GcvB expression plasmid pP_L*gcvB*. (B) In vitro synthesized, full-length mRNAs (40 nM) of the *gltI::gfp* and *gltI_{ΔCA}::gfp* fusions were in vitro translated with reconstituted 70S ribosomes in the presence of 40 nM Hfq as described in Materials and Methods. Syntheses of GltI::GFP fusion protein levels were determined after incubation for 15 min (lanes 1,4,7,10,13,16), 30 min (lanes 2,5,8,11,14,17), and 45 min (lanes 3,6,9,12,15,18) by Western blot analysis. The reactions in lanes 4–9 and 13–18 contained a 10-fold excess of GcvB or GcvB_{ΔR1} mutant RNAs as indicated above the lanes. Fold regulation upon deletion of the C/A-rich target site or by addition of GcvB according to quantification of GltI::GFP fusion protein levels is given below the blot. The results of a representative experiment (out of three) are shown. (C) Full-length *gltI::gfp* mRNA was in vitro translated as above for 30 min. (Right two lanes) In addition, mutant RNAs GcvB_{ΔR2} and GcvB_{ΔR3} (10-fold excess) were included in the assay. (D) Insertion of the 27-nt C/A-rich element of *gltI* at position –42 relative to the start codon of an *E. coli* *ompR::gfp* fusion yields mutant *ompR_{ΔCA}::gfp* (plasmids pJU-063 and pJL50-11, respectively). (E) In vitro translation assay (reconstituted 70S ribosomes) as in B but with in vitro synthesized *ompR::gfp* and *ompR_{ΔCA}::gfp* fusion mRNAs (250 nM) and in the presence of 250 nM Hfq. Synthesis of OmpR::GFP fusion protein was determined on Western blots following incubation for 15 min (lanes 1,4,7,10), 30 min (lanes 2,5,8,11), and 45 min (lanes 3,6,9,12). A 10-fold excess of GcvB RNA over mRNA was added to the reactions where indicated. The results shown are representative of the experiment performed in triplicate.

established that sRNAs that act to modulate protein activity can control the expression of many genes; e.g., by binding to RNA polymerase (Wassarman and Storz 2000) or to CsrA-like proteins (Babitzke and Romeo 2007). sRNAs can also affect the expression of larger sets of genes by targeting the mRNAs of global transcriptional regulators; e.g., RpoS or FhlA (Lease et al. 1998; Majdalan et al. 1998; Argaman and Altuvia 2000). In contrast, our understanding of how sRNAs could directly control multiple mRNAs by an antisense mechanism has been limited by the low number of validated sRNA–target interactions and hence difficulty to reliably predict new targets. This notwithstanding, the results of several recent studies suggest that multiple targeting may be more common than previously thought, and common denomi-

nators are beginning to emerge. Tjaden et al. (2006) suggested that functional relationship of the proteins encoded by target candidates, as it is also seen among the GcvB targets, may add confidence to predictions. Similarly, the OmrA/B and RyhB sRNAs were predicted to directly target multiple mRNAs that collectively encode for outer membrane proteins (Guillier and Gottesman 2006; Johansen et al. 2006; Papenfort et al. 2006). By the same token, the iron stress-responding *E. coli* RyhB sRNA was shown to regulate multiple mRNAs that encode proteins involved in iron metabolism (Massé and Gottesman 2002), and many of the mRNAs demonstrated to be direct targets of *S. aureus* RNAIII encode bacterial virulence factors (Boisset et al. 2007).

The strong C/A bias of GcvB-binding sites may point

to yet another feature of multiple target regulation. CA multimers placed downstream from mRNA start codons were reported to stimulate translation *in vivo*, and to increase ribosome-binding affinity to mRNAs *in vitro* (Martin-Farmer and Janssen 1999). While the effect of CA-multimers far upstream of a start codon was not addressed by Martin-Farmer and Janssen (1999), we find that the C/A-rich element has a stimulatory effect on the *gltI* and *ompR* fusion mRNAs (Fig. 7). Thus, multiple mRNA targeting by GcvB may have evolved through hijacking a translational enhancer element shared by numerous mRNAs that encode periplasmic transporters. We are thus tempted to speculate that other evolutionarily constrained mRNA regions—e.g., those encoding signal peptides—may also constitute binding sites for sRNAs with multiple targets. However, we emphasize that the effects of GcvB as a translational repressor are clearly much greater than the effects of removing the C/A-rich target site (Fig. 7); thus GcvB does not primarily act by simply blocking any enhancer effect.

In eukaryotes, the recognition that a 5'-terminal seed sequence of the ~22-nt microRNAs provides the base-pairing specificity to target mRNAs greatly advanced target predictions. In contrast, the target interaction sites of the much longer bacterial sRNAs are not necessarily located at the 5' end. An alignment of GcvB RNAs (Fig. 1D) revealed two strongly conserved regions, R1 and R2. While R2 may constitute an interaction region for yet another target(s), R1 is a key determinant for base-pairing to seven target mRNAs. Although our structure probing of target mRNAs and GcvB indicates additional, weaker contacts, the C/A-rich motif in the targets and R1 in the sRNA clearly are the anchoring and essential motifs for interaction. Accordingly, giving higher weight to deeply conserved regions in sRNAs is expected to improve the currently available target search algorithms. In support of this, several other regulatory sRNAs—e.g., MicA, MicC, and SgrS—exhibit a higher degree of conservation in their target interaction regions (Chen et al. 2004; Vanderpool and Gottesman 2004; Rasmussen et al. 2005; Udekwu et al. 2005). Unlike these enterobacterial sRNAs, *S. aureus* RNAIII is not conserved in other species. However, it is still intriguing that a short (~40-nt) region within the 514-nt RNAIII facilitates base-pairing to multiple mRNAs (Huntzinger et al. 2005; Boisset et al. 2007). Different from GcvB, however, RNAIII typically covers the SD and/or the start codon of a target mRNA (Huntzinger et al. 2005; Boisset et al. 2007).

Competition with ribosome binding explains the inhibitory activity of sRNAs that bind mRNAs within RBS regions (Argaman and Altuvia 2000; Chen et al. 2004; Huntzinger et al. 2005; Udekwu et al. 2005). We show that the same applies to GcvB inhibition of *dppA* and *oppA*, whose GcvB-binding sites are very close to the SD (Fig. 3D). However, GcvB binds many of its targets further upstream of the two sequence elements, SD and start codon, that are the key determinants of 30S binding (Fig. 5B). *gltI* is the mRNA with the most upstream binding site; probing of GcvB/*gltI* RNA complexes as well as transfer of the GcvB site from *gltI* to the unrelated *ompR*

mRNA showed that GcvB effectively represses translation by forming a duplex >42 nt upstream of the AUG.

How is translational repression brought about at such upstream sites? Footprinting experiments revealed the maximal ribosome-binding region on mRNAs to range from -39 to +19 relative to the AUG (Hüttenhofer and Noller 1994). Since the GcvB-binding site on *gltI* lies outside this window, a simple interference model in which GcvB occludes mRNA residues required for base-pairing with 16S ribosomal RNA is unlikely. In eukaryotes, ribosomes generally enter mRNAs at their 5' ends to subsequently scan for downstream AUG triplets. A requirement for scanning is unknown in prokaryotes, which argues against the possibility that GcvB could act as a roadblock for ribosomes scanning from the 5' end of *gltI* mRNA.

Two other bacterial sRNAs, IstR-1 and RyhB, were recently reported to repress translation by binding upstream of the target RBS (Darfeuille et al. 2007; Vecerek et al. 2007), yet the underlying mechanisms do not seem to apply for GcvB either. IstR-1 targets the *tisAB* mRNA ~100 nt upstream of the *tisB* start codon (Vogel et al. 2004). The *tisAB* mRNA is highly structured, and the *tisB* SD is entrapped in a stable hairpin. Thus, *tisB* translation requires ribosomes to bind to an upstream "standby" site, which will be masked upon IstR-1 binding (Darfeuille et al. 2007). Unlike in *tisAB*, the *gltI* 5' UTR is not strongly structured (Supplementary Fig. S6A). Moreover, since GcvB also inhibits the unrelated *ompR::gfp_{CA}* mRNA, structure cannot be a primary cause of translational repression. RyhB inhibits *fur* translation by forming an imperfect duplex with the -96 to -48 region of *fur* mRNA. Translation of *fur* is coupled to that of an upstream located reading frame, the direct target of RyhB (Vecerek et al. 2007). We have not found evidence for an upstream reading frame in the *gltI* mRNA, nor for translation initiation upstream of the determined *gltI* start codon (Fig. 6B).

The *gltI* 5' UTR contains a putative hairpin motif (-40 to -7 region), which could bring the C/A-rich motif in closer proximity to the *gltI* SD to facilitate repression by GcvB (Supplementary Fig. S11). However, the following observations do not support such a model. Firstly, the SD of *ompR::gfp* is not preceded by a hairpin that would bring the GcvB target site and SD in closer proximity; yet transfer of the GcvB site to *ompR::gfp* yields the same degree of mRNA repression as observed with the *gltI::gfp* construct. Secondly, we disrupted the putative hairpin motif in the *gltI* 5' UTR by two point mutations. These mutations did not impair GcvB's ability to repress the *gltI* fusion mRNA *in vivo* (Supplementary Fig. S11).

Consequently, our present results suggest that either the first three GcvB stem-loops or the G/U:C/A-rich helix of the GcvB-target mRNA complex constitute an inhibitory signal for 30S entry on the *gltI* and *argT* mRNAs. However, the underlying mechanism may be a more general one, since we have found that *Salmonella* RybB sRNA also represses several targets by binding upstream of the RBS (F. Mika and J. Vogel, unpubl.). It might therefore be of interest to consider the possibility

that target searches are not yet exhausted for those sRNAs that do not show obvious complementarity to RBS sequences.

Materials and methods

Bacterial strains, oligonucleotides, and plasmids

DNA oligonucleotides used for cloning, PCR amplification of T7 templates, and toeprinting assays, and as hybridization probes are provided as Supplemental Material (Supplementary Table S1).

Bacterial strains are listed in Supplementary Table S2. The *gcvB* deletion strains of *Salmonella* (JVS-0236) and *E. coli* (JVS-6081) were constructed using the λ red protocol (Datsenko and Wanner 2000) by replacing residues 17–176 with a kanamycin marker gene, PCR-amplified with primer JVO-0133/-0134 and JVO-0131/-0132, respectively. Mutants were verified by PCR with primers JVO-0135/-0136 or JVO-0137/-0138, respectively. The *Salmonella* $\Delta gcvB/\Delta hfq$ strain (JVS-0617) was constructed by P22 transduction of $\Delta hfq::Cm^R$ (JVS-0255) into strain JVS-0236. Marker genes were removed with FLP recombinase (Datsenko and Wanner 2000).

Plasmids, details of their construction, and insert sequences are given in Supplementary Tables S3–S6. Control plasmid pTP11 carries a p15A origin and expresses an ~50-nt nonsense transcript derived from the *rrnB* terminator. Plasmid pgcvB (pTP05) carries the *Salmonella gcvB* locus (292 bp upstream of the +1 site to 116 bp downstream from the terminator), while plasmid pP_LgcvB (pTP09) expresses the gene from a constitutive promoter. *gfp* fusions were constructed as described (Urban and Vogel 2007). The *dppA*, *oppA*, *gltI*, *livI*, and *argT* fusions were cloned as 5'RACE cDNA fragments into fusion plasmid pXG-20. The *livK* and *STM4351* fusions were cloned in vector pXG-10. To construct fusion *gltI*_{ΔCA}::*gfp* (pJL45-3), base pairs –71 to –44 relative to *gltI* AUG were deleted from the *gltI*::*gfp* fusion plasmid. The deleted 27-bp C/A-rich element was inserted in the *ompR*::*gfp* fusion (pJU-63) at position –42 according to the *ompR* AUG to yield *ompR*_{CA}::*gfp* (pJL50-11). The insert sequences of all GFP fusions are given in Supplementary Table S7.

Media and growth conditions

Cells were grown aerobically at 37°C in Lennox broth or M9 minimal medium supplemented with 0.4% glucose. When required, antibiotics were added at 92 µg/µL streptomycin, 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 20 µg/mL chloramphenicol.

RNA and protein detection

RNA preparation and Northern analysis followed previously published protocols (Urban and Vogel 2007). GcvB RNAs were detected with 5'-end-labeled oligos (see figure legends), and 5S rRNA or *gfp* fusion mRNAs with oligos JVO-0322 or JVO-155, respectively.

OppA protein was detected on a Western blot (protocol in Sittka et al. 2007) using a polyclonal OppA antibody kindly provided by K. Igarashi (Chiba University, Chiba, Japan). GFP fusion and GroEL proteins were detected as described in Urban and Vogel (2007). Periplasmic proteins were prepared from *Salmonella* cultured to OD 2 as described in Sittka et al. (2007), and analyzed by high-resolution 2D electrophoresis, protein staining, and peptide mass fingerprinting at the MPI-IB protein analysis core facility according to previously published standard protocols (<http://info.mpiib-berlin.mpg.de/jungblut>).

Colony fluorescence imaging

E. coli carrying *gfp* fusion plasmids were grown on LB plates overnight. Colonies were photographed with a Fuji LAS-3000 image analyzer with a 510-nm emission filter and excitation at 460 nm.

T7 transcription, purification, and 5'-end labeling of RNA

DNA templates carrying a T7 promoter sequence for in vitro transcription were generated by PCR. Primers and sequences of the T7 transcripts are given in Supplementary Tables S8 and S9. T7 templates of *gfp* fusion mRNAs were amplified from plasmids using a sense primer that adds a T7 promoter to the +1 site of the 5' UTR, and antisense oligo pZE-T1, which binds 122 nt downstream from the *gfp* stop codon. These transcripts end with the *rrnB* terminator of the fusion plasmids. RNA was in vitro transcribed and quality-checked as described in Sittka et al. (2007). The protocol for 5'-end labeling of RNA is published in Papenfort et al. (2006).

In vitro structure mapping and footprinting

Secondary structure probing and mapping of RNA complexes were conducted on 5'-end-labeled RNA (~0.1 pmol) in 10-µL reactions. RNA was denatured for 1 min at 95°C and chilled on ice for 5 min, upon which 1 µg of yeast RNA and 10× structure buffer (0.1 M Tris at pH 7, 1 M KCl, 0.1 M MgCl₂; Ambion) were added. The concentrations of unlabeled sRNA/mRNA leader added to the reactions are given in the figure legends. Following incubation for 10 min at 37°C, 2 µL of a fresh solution of lead(II) acetate (25 mM; Fluka #15319), 2 µL of RNase T1 (0.01 U/µL; Ambion, #AM2283), or 2 µL of RNase T2 (0.02 U/µL; Invitrogen #18031-013) were added and incubated for 2, 3, or 5 min at 37°C, respectively. RNase III cleavage reactions contained 1 mM DTT and 1.3 U of enzyme (New England Biolabs #M0245S), and were incubated for 6 min at 37°C.

Reactions were stopped with 5 µL of 0.1 M EDTA, precipitated, and dissolved in loading buffer II (95% Formamide; 18 mM EDTA; 0.025% SDS, Xylene Cyanol, and Bromophenol Blue; Ambion), or by direct addition of 12 µL of loading buffer on ice. RNase T1 ladders were obtained by incubating labeled RNA (~0.2 pmol) in 1× sequencing buffer (Ambion) for 1 min at 95°C. Subsequently, 1 µL of RNase T1 (0.1 U/µL) was added, and incubation was continued for 5 min at 37°C. OH ladders were generated by 5 min of incubation of 0.2 pmol of labeled RNA in alkaline hydrolysis buffer (Ambion) at 95°C. Reactions were stopped with 12 µL of loading buffer. Samples were denatured for 3 min at 95°C prior to separation on 6% polyacrylamide/7 M urea sequencing gels in 1× TBE. Gels were dried and analyzed using a PhosphorImager (FLA-3000 Series; Fuji), and AIDA software.

30S ribosome toeprints

Toeprinting reactions were carried out as described (Hartz et al. 1988; Udekwa et al. 2005) with few modifications. An unlabeled *dppA* mRNA fragment (0.2 pmol; 236 nt; T7 template amplified with JVO-1034/-1035), and 0.6 pmol of 5'-end-labeled primer JVO-1035 complementary to the *dppA* coding region was annealed. For inhibition analysis, 0.2, 0.6, 1, and 2 pmol of GcvB RNA or 1 pmol of control RNA (MicA) or GcvB mutant RNAs were added. Nucleic acids were denatured in annealing buffer (10 mM Tris-acetate at pH 7.6, 1 mM DTT, 100 mM potassium acetate) for 1 min at 95°C and chilled for 5 min on ice, upon which Mg²⁺ acetate and all NTPs were added to final concentrations of 10 mM and 0.5 mM, respectively. All subsequent

incubations steps were at 37°C. After 5 min of incubation, 2 pmol of 30S ribosomal subunit (Knud Nierhaus, Max Planck Institute for Molecular Genetics, Berlin, Germany; preactivated for 20 min prior to the assay) were added. Following incubation for 5 min, uncharged tRNA^{fMet} (10 pmol) was added, and incubations continued for 15 min. Reverse transcription was carried by adding SuperScript II (100 U; Invitrogen) for 20 min. cDNA synthesis was terminated with 100 μ L of stop buffer (50 mM Tris-HCl at pH 7.5, 0.1% SDS, 10 mM EDTA). Following phenol-chloroform extraction, alkaline hydrolysis of template RNA at 90°C, and ethanol precipitation, cDNA was dissolved in 10 μ L of loading buffer II (Ambion). Toeprint analysis on *gltI* 5' RNA (161 nt; T7 template amplified with primers JVO-1039/-1040) was performed using 5'-end-labeled primer JVO-1775. See the figure legends for final concentrations of other components.

Sequencing ladders were generated with CycleReader DNA Sequencing Kit (Fermentas #K1711) according to the manufacturer's protocol on the same DNA template used for T7 transcription and the same 5'-end-labeled primer as in the toeprinting reactions. cDNAs and sequence ladders were separated on a 6% polyacrylamide/7 M urea gel. Autoradiograms of dried gels were obtained as above.

In vitro translation assays

Translation reactions were carried out using PureSystem (Cosmo Bio Co., Ltd, PGM-PURE2048C) according to the manufacturer's instructions. Ten-microliter (Fig. 7B,C) or 20- μ L (Fig. 7E) reactions contained, in addition to 70S ribosomes, mRNA template, Hfq, and—where applicable—GcvB RNAs (see figure legends for final concentrations). Supplementary Tables S8 and S9 list the details of DNA fragments used for T7 transcription. Before addition of PureSystem mix, RNA was denatured for 1 min at 90°C and chilled on ice for 5 min. Hfq was mixed with mRNA (and sRNA) and preincubated for 10 min at 37°C. PureSystem mix was added, and incubation continued at 37°C for the time given in the figure legends. Reactions were stopped with 4 vol of ice-cold acetone and kept on ice for 15 min, and proteins were collected by centrifugation (10,000g, 10 min, 4°C). Proteins were quantified by Western blot analysis with a monoclonal GFP antibody as described in Urban and Vogel (2007).

Prediction of sRNA–target mRNA duplexes

GcvB–target mRNA complexes were predicted with *RNAhybrid* (Rehmsmeier et al. 2004). 5' Regions of *Salmonella* LT2 genes (± 50 nt of annotated start codon) were extracted as a multifasta file and used as *target sequence* input. GcvB or shorter versions thereof (R1 or R2 ± 10 nt) were used as *miRNA* input. For predictions of GcvB/target RNA duplexes, internal and bulge loops were restricted to a size of 1 nt.

Acknowledgments

We thank K. Nierhaus for providing 30S ribosomes; M. Schmid and P. Jungblut for mass spectrometry; K. Igarashi for OppA serum; Marc Rehmsmeier for discussions on target predictions; L. Bui for technical assistance; and E.G. Wagner, K. Nierhaus, and members of our laboratory for critical comments on the manuscript and helpful discussions. C.M.S. was supported by grants from Deutsche Forschungsgemeinschaft, Germany (DFG; VO 875/1-1,3).

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Genes Dev. 2007, **21**:

Access the most recent version at doi:[10.1101/gad.447207](https://doi.org/10.1101/gad.447207)

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