

Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon

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The physiological role of *Escherichia coli* Spot 42 RNA has remained obscure, even though the 109-nucleotide RNA was discovered almost three decades ago. Structural features of Spot 42 RNA and previous work suggested to us that the RNA might be a regulator of discoordinate gene expression of the galactose operon, a control that is only understood at the phenomenological level. The effects of controlled expression of Spot 42 RNA or deleting the gene (*spf*) encoding the RNA supported this hypothesis. Down-regulation of *galk* expression, the third gene in the *gal* operon, was only observed in the presence of Spot 42 RNA and required growth conditions that caused derepression of the *spf* gene. Subsequent biochemical studies showed that Spot 42 RNA specifically bound at the *galk* Shine-Dalgarno region of the *galETKM* mRNA, thereby blocking ribosome binding. We conclude that Spot 42 RNA is an antisense RNA that acts to differentially regulate genes that are expressed from the same transcription unit. Our results reveal an interesting mechanism by which the expression of a promoter distal gene in an operon can be modulated and underline the importance of antisense control in bacterial gene regulation.

[Key Words: Antisense RNA; galactose operon; riboregulation; small RNAs; Spot 42 RNA; translational regulation]

Received March 22, 2002; revised version accepted May 13, 2002.

Over the past two decades it has become clear that cells contain a variety of small untranslated RNA molecules (sRNAs). These RNAs play important or essential roles and regulate diverse cellular functions such as RNA processing and splicing, RNA editing and modification, protein stability and secretion, mRNA stability, and translation. Posttranscriptional control by sRNAs has been documented in bacteria and in a variety of eukaryotic cells including nematodes, plants, and mammals (Jorgensen et al. 1998; Panning and Jaenisch 1998; Eddy 1999; Wassarman et al. 1999; Adoutte 2000). The majority of these riboregulators act as antisense RNAs that pair to complementary regions on target RNAs. Antisense control was first discovered in plasmids, bacteriophages, and transposable elements. In all these cases, the antisense and target RNAs are transcribed from opposite DNA strands of the same region (i.e., *cis*-encoded; Wagner and Simons 1994; Zeiler and Simon 1996). In a growing number of cases, however, the antisense and target RNAs are expressed from unlinked genes, and complementarity is incomplete and relatively modest. Such *trans*-encoded antisense RNAs can carry separate determinants for binding/regulation, function by more

than one mechanism, and affect more than one target gene (for review, see Altuvia and Wagner 2000).

Until recently only a dozen sRNAs in addition to tRNAs and 5S RNA were known to be encoded by the *E. coli* genome (for review, see Wassarman et al. 1999; Urbanowski et al. 2000; Majdalani et al. 2001). However, novel approaches including genomic mining have increased the number to more than 40 (Argaman et al. 2001; Rivas et al. 2001; Wassarman et al. 2001). Thus, sRNAs are much more widespread than previously imagined. At present three of the sRNAs are known to be antisense regulators (i.e., OxyS, DsrA, and MicF) that act at the level of translation initiation by base-pairing to the 5' end of target mRNAs. They either repress translation by interfering with ribosome binding or activate translation by interfering with an inhibitory intramolecularly base-paired structure of the target RNA (Mizuno et al. 1984; Altuvia et al. 1998; Lease et al. 1998; Majdalani et al. 1998; Delihias and Forst 2001). Also, unique functions have been discovered for most of the other well-known sRNAs (Romeo 1998; Wassarman and Storz 2000; for review, see Wassarman et al. 1999); however, the role of the Spot 42 RNA has not yet been established.

Spot 42 RNA was discovered nearly three decades ago by two-dimensional (2D) gel electrophoresis as the most apparent RNA of *E. coli* when cells were labeled briefly with [³²P]phosphate (Ikemura and Dahlberg 1973; Sahagan and Dahlberg 1979). The RNA is transcribed from

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.231702>.

the *spf* (spot forty-two) gene, whose expression is negatively regulated by the cAMP-CRP complex (Joyce and Grindley 1982; Rice and Dahlberg 1982; Polayes et al. 1988b). The gene includes a ρ -independent terminator site, and the mature RNA is unmodified and identical to the primary transcript. Spot 42 RNA has a half-life of 12 to 13 min at 37°C and is present in ~200 copies per cell when cells are growing in media supplemented with glucose (Sahagan and Dahlberg 1979). This number is reduced three- to fivefold when cells are grown on nonglucose carbon sources or when cAMP is added to a glucose-grown culture (Polayes et al. 1988b). The *spf* gene is dispensable, and no major defects are observed in Spot 42 RNA null mutants (Hatfull and Joyce 1986; Polayes et al. 1988a). A 10-fold overproduction of Spot 42 RNA resulted in pronounced growth defects under a variety of conditions (e.g., small colony size and slow growth, inability to grow on succinate as carbon source and to respond normally to nutritional up-shift; Rice and Dahlberg 1982). However, in spite of this knowledge, the function of Spot 42 RNA has remained elusive.

Here we show that the *spf* gene is absolutely required for discoordinate expression of the *gal* operon cistrons. Moreover, *in vitro* studies show that Spot 42 RNA specifically binds at the *galK* Shine-Dalgarno region of the *gal* mRNA and that the RNA interferes with the formation of *galK*-30S initiation complexes. The data are discussed in terms of a model wherein Spot 42 RNA acts as an antisense RNA to differentially regulate the *gal* genes by competing with 30S ribosomes for binding to the *galK* translational initiation region.

Results

What is the function of Spot 42 RNA?

The *E. coli* cAMP receptor protein (CRP) is a global transcriptional regulator that can serve as an activator, repressor, coactivator, and corepressor (Kallipolitis et al. 1997; Busby and Ebright 1999). Moreover, work on the galactose operon has revealed that the cAMP-CRP complex, directly or indirectly, influences synthesis of the products of this operon differentially (Ullmann et al. 1979; Joseph et al. 1981; Adhya 1987). The *E. coli galETKM* operon is unusual in two respects. First, it possesses two overlapping promoters (Fig. 1). The cAMP-CRP complex stimulates transcription from the downstream promoter P1 and inhibits transcription from the upstream promoter P2. Second, although the genes are translated from a polycistronic mRNA, the relative synthesis of the enzymes UDP-galactose-4-epimerase (GalE), galactose-1-phosphate uridyl transferase (GalT), and galactokinase (GalK) has been found to vary under different metabolic conditions, a phenomenon that has been termed discoordinate expression. When cAMP levels are reduced by mutations (*cya*⁻) or catabolite repression (glucose as carbon source), the levels of GalK and GalT decline (the GalK level, in particular), but the GalE level remains high, resulting in an approximately fourfold increase in the GalE-to-GalK ratio. We note, how-

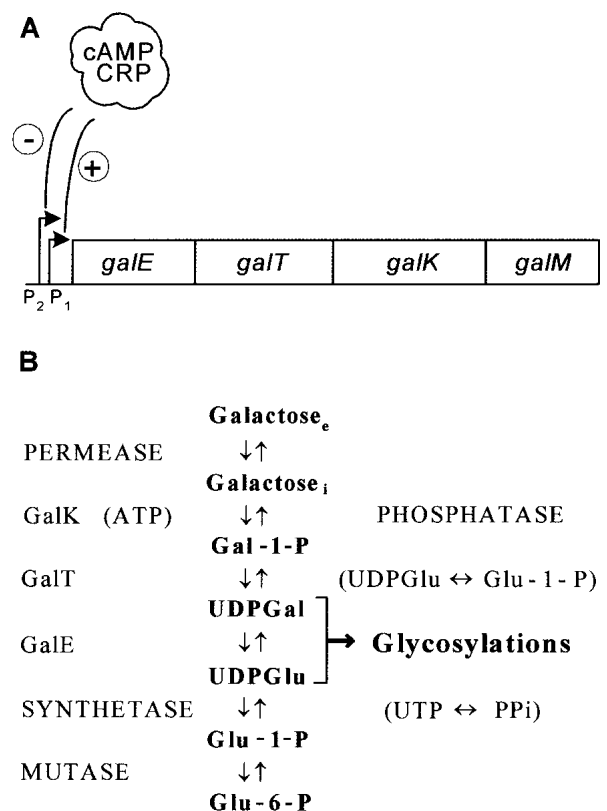


Figure 1. The *E. coli* galactose operon. (A) Schematic diagram of the *E. coli* galactose operon. Relative positions of the four genes are shown. The genes encode: *galE*, epimerase (GalE); *galT*, transferase (GalT); *galK*, kinase (GalK); and *galM*, mutarotase. The two promoters (P1 and P2) are modulated negatively by Gal repressor, and the cAMP-CRP complex abolishes transcription from P2 and is required for P1 transcription. (B) The pathway for galactose metabolism. Note that the GalE, GalT, and GalK enzymes are part of an amphibolic pathway that produces substrates for biosynthetic glycosylations (UDP-glucose and UDP-galactose). Moreover, the pathway generates galactose from UDP-galactose when cells are growing in other carbon sources. Subscripts: (e) extracellular; (i) intracellular.

ever, that there are minor disagreements as to how cAMP levels or carbon source affect *galE* expression (Ullmann et al. 1979; Joseph et al. 1981; Adhya 1987). The discoordinate expression is more severe in the absence of a functional cAMP-CRP complex and can be suppressed in glucose-grown cells or in *cya*⁻ strains by the addition of cAMP to the growth medium. It has been proposed that transcription termination at intercistronic regions, preferential degradation of the promoter distal portion of the mRNA, or different translational efficiencies of the two *gal* transcripts could account for the regulation (Ullmann et al. 1979; Joseph et al. 1981; Queen and Rosenberg 1981; Adhya 1987).

Several observations suggested to us that Spot 42 RNA is involved in mediating discoordinate expression of the *gal* operon. First, BLAST searches revealed that Spot 42 RNA has significant complementarity to the region surrounding the translation initiation region of *galK*. Sec-

ond, Spot 42 RNA was predicted to form three stem-loop structures in which the first hairpin contains a seven-nucleotide single-stranded loop presented by a loosely paired top stem of interrupted helicity. Such features are often observed in antisense RNA regulatory units (Wagner and Brantl 1998). Third, the opposing regulation of *spf* and *galK* by the cAMP-CRP complex was striking.

Effects of Spot 42 RNA on plasmid-encoded *GalE*, *GalT*, and *GalK* proteins

To assess the role of Spot 42 RNA on *gal* operon regulation, we first investigated the effect of controlled Spot 42 RNA expression on the *GalE*, *GalT*, *GalK*, and *GalM* protein synthesis pattern by 2D gel electrophoresis. To this end we constructed a low-copy-number plasmid (pSpf33) that expressed *spf* under the control of the inducible *araBAD* promoter. Moreover, we constructed a Δ *spf* strain (SØ928 Δ *spf*) carrying the entire *gal* operon on plasmid pBR322 (pGal4). This strain was transformed with pSpf33 and as a control with pBAD33. Uninduced and induced cultures of the transformed strains were pulse-labeled with [³⁵S]methionine, and their proteins were analyzed by standard 2D gel electrophoresis and autoradiography (Fig. 2). Induction of *spf* expression had little effect on the net synthesis rate of the *GalE*, T, and M polypeptides (~10% increase of *GalE*, ~10% decrease of *GalT*, and ~15% decrease of *GalM* synthesis), whereas a significant reduction of the net rate of synthesis (~two-fold) was observed for *GalK* (Fig. 2, cf. panel A, uninduced culture, with panel B, induced culture; see legend to Fig. 2). Addition of arabinose to the control strain (SØ928 Δ *spf*/pGal4, pBAD33) had no effect on the net synthesis rates of the four *Gal* polypeptides (data not shown). These results showed that expression of *spf* specifically affected *GalK* synthesis, supporting the idea that Spot 42 RNA might be responsible for discoordinate expression of the *gal* operon.

Effect of deleting the *spf* gene on *gal* operon expression

We next examined the consequences of deleting the *spf* gene. For these studies, we constructed isogenic *spf*⁺ and Δ *spf* strains in *gal* constitutive [i.e., *galR*⁻] *crp*⁺ and Δ *crp* backgrounds. The various strains were grown in minimal medium with glucose or glycerol as carbon and energy source to an OD₆₀₀ of 0.4. Following this, Spot 42 RNA levels and *GalE*, *GalT*, and *GalK* levels were determined by Northern blot and Western blot analysis, respectively. The results of the experiments are presented in Figure 3. We emphasize the following observations. Expression of *galE* and *galT* was not significantly altered by deleting *spf* (Fig. 3, cf. lanes 1–2 with lanes 3–4). In accordance with previous work, the expression of *spf* was enhanced (Sahagan and Dahlberg 1979; Polayes et al. 1988b) and the expression of *galK* was reduced in cells lacking the cAMP-CRP complex (Fig. 3, lanes 5,6) or deficient in cAMP and CRP (Fig. 3, lane 2, glucose grown; Ullmann et al. 1979; Joseph et al. 1981; Queen

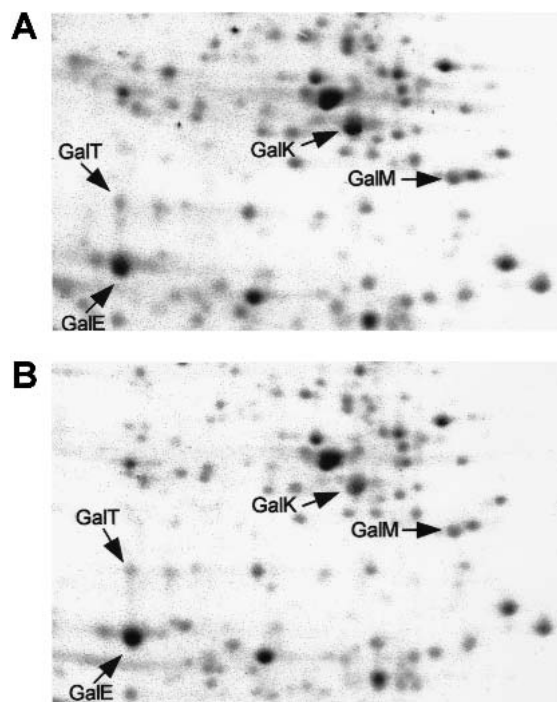


Figure 2. Effects of controlled *spf* expression on the *Gal* enzyme pattern. The figure shows the relevant part of two-dimensional gels of SØ928 Δ *spf* cells harboring pGal4 (a derivative of pBR322 carrying the *galETKM* genes) and pSpf33 (a derivative of pBAD33 carrying the *spf* gene under the control of the inducible *araBAD* promoter). Uninduced and induced cultures (induced with 1 mM arabinose for 20 min) were labeled with [³⁵S]methionine for 1 min and concentrated. Following this, their proteins were analyzed by two-dimensional gel electrophoresis and autoradiography. (A) Uninduced cells. (B) Induced cells. Arrows mark the positions of the four *Gal* enzymes. After quantification of the *Gal* protein spots from uninduced cells (A) and induced cells (B), the following ratios were obtained: $GalE_B/GalE_A = 1.09$, $GalT_B/GalT_A = 0.92$, $GalK_B/GalK_A = 0.47$, and $GalM_B/GalM_A = 0.83$ (all normalized to standard spots that were not affected by Spot 42 RNA).

and Rosenberg 1981; Adhya 1987). Strikingly, this down-regulation of *galK* was eliminated in the Δ *spf* strains (Fig. 3, cf. lane 2 with lane 4, and lanes 5–6 with lanes 7–8). For the *spf* mutant, expression of the three *gal* genes was slightly higher in glycerol-grown cells (Fig. 3, lane 3) than in glucose-grown cells (Fig. 3, lane 4), and a similar expression was observed for *galE* and *galT* in the wild-type strain (Fig. 3, cf. lanes 1–2 with lanes 3–4). Accordingly, previous work has shown that the addition of cAMP to a Δ *cya*, *galO*^{c81} mutant stimulated *galE* expression 40%–70% (Joseph et al. 1981), and that *galT* expression in *gal* constitutive strains (*galR*⁻ or *galO*^{c81}) was 40%–70% higher in glycerol-grown cells than in glucose-grown cells (Adhya and Echols 1966). On this basis we conclude that cAMP-CRP also stimulates *galE* expression. Furthermore, the levels of the three *Gal* enzymes increased coordinately in the *spf* mutant when glycerol was used as carbon source (Fig. 3, lanes 3,4; see legend), indicating that transcription initiation is more

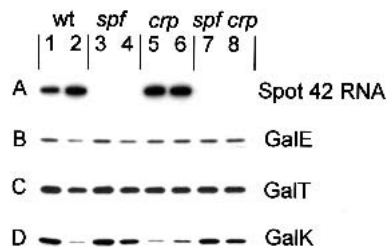


Figure 3. Effects of Spot 42 RNA on the cellular levels of the Gal enzymes. (A) Northern blot analysis of Spot 42 RNA levels and (B–D) immunoblots of GalE, GalT, and GalK levels in isogenic *gal* constitutive *spf*⁺ and Δ *spf* strains. The RNA and the proteins from equal amounts of cells growing to early-exponential phase were electrophoretically separated on a urea–8% polyacrylamide gel and on an SDS–10% polyacrylamide gel, respectively, and probed as described in Materials and Methods. (Lanes 1,3,5,7) Glycerol-grown cells; (lanes 2,4,6,8) glucose-grown cells. In all experiments casamino acids (0.05%) were included in the growth medium. After quantification of the Gal proteins from glycerol- and glucose-grown Δ *galR* and Δ *galR*, Δ *spf* cells, the following ratios were obtained for GalE, GalT, and GalK, respectively: Δ *galR* strain (lane 1/lane 2) 1.5, 1.4, and 4.9; Δ *galR*, Δ *spf* strain (lane 3/lane 4) 1.4, 1.4, and 1.4. The results are the average of three independent experiments.

efficient from the cAMP–CRP-dependent P1 promoter than from the cAMP–CRP-repressed P2 promoter (Fig. 1). Finally, we note that a similar effect of Spot 42 RNA and carbon source on *gal* operon expression was observed with *galR*⁺ strains (data not shown). Taken together our results corroborate the hypothesis that discoordinate expression of the *gal* operon is dependent on the presence of Spot 42 RNA. Moreover, the data suggest that the cAMP effect on *galET* expression is transcriptional.

The secondary structure of Spot 42 RNA

To learn more about the mechanism of Spot 42 RNA action, we carried out structural studies. The secondary structure of the RNA, predicted by the folding program Mfold, contains three stem–loop structures (Fig. 4B). To evaluate this structure, the conformation of Spot 42 RNA in solution was deduced by structural probing using RNase T₂, RNase V₁ and lead (II) acetate, and 5'-end-labeled Spot 42 RNA. The results are presented in Figure 4A, and the structural information derived from these analyses is summarized in Figure 4B. Partial cleavage with the single-strand-specific endonuclease RNase T₂ confirmed the presence of three loop regions and an unstructured segment between the first and second hairpins (Fig. 4A, lanes 7,8). Strikingly, the predicted double-stranded region of hairpin I was also accessible to cleavage by RNase T₂ (e.g., positions 12 and 16–17), indicating that nucleotides in this region of Spot 42 RNA are only loosely paired. Probing with lead (II) acetate, which preferentially cleaves in single-stranded regions, supported this interpretation (Fig. 4A, lanes 4,5).

We further characterized the secondary structure of Spot 42 RNA by treatment with RNase V₁, which can

cleave double-stranded RNA of 4–6 bp and may also cleave stacked nucleotides in single-stranded regions (Fig. 4A, lanes 10,11). The cleavage pattern suggested the existence of several regions of double-stranded nature within Spot 42 RNA (stems I and II). The nucleotides (52A, 55G, 56G, and 57A) that are accessible to RNase V₁, Pb²⁺, and RNase T₂ cleavage are likely to be stacked. Taken together, the probing data are consistent with the conformation predicted for Spot 42 RNA. Furthermore, the data suggest a dynamic or loosely paired character of hairpin I.

Support for the predicted structure was also obtained by comparative studies. Spot 42 RNA is highly conserved in *Klebsiella*, *Shigella*, *Salmonella*, *Yersinia*, and *Vibrio* species (Fig. 4C). Sequence variations mostly occur in regions predicted to be single-stranded (i.e., loops II and III and the linker region between hairpins I and II) or, when in regions predicted to be double-stranded, are compensated by alternative pairing or by second-site mutations that restore base-pairing. Hence, the phylogenetic studies support the structural analyses.

Spot 42 RNA binds to the translational initiation region of *galK*

Spot 42 RNA has significant sequence complementarity to the *gal* mRNA in the region surrounding the translational initiation region of *galK* (Fig. 5A), and, strikingly, the *galK* complementary regions reside within exposed regions of Spot 42 RNA (Fig. 6B). To test whether Spot 42 RNA might bind the complementary regions, we performed binding experiments with 5'-end-labeled Spot 42 RNA and an unlabeled *galK'* RNA substrate that carried the *galK* Shine–Dalgarno sequence in the central portion of the molecule. Samples containing a fixed amount of Spot 42 RNA, a 500-fold excess of tRNA, and increasing amounts of *galK'* RNA were incubated on ice; formation of complexes was assayed in gel-retardation assays. The results, presented in Figure 5B, show that Spot 42 RNA and the *galK'* RNA formed a single retarded complex. A similar complex was observed by adding unlabeled Spot 42 RNA to incubations containing a fixed amount of 5'-end-labeled *galK'* RNA. Addition of excess unlabeled *galT'* RNA substrate, which carried the distal part of *galE* and the proximal part of *galT* (i.e., the *galT* translational initiation region), did not affect formation of the retarded complex (data not shown). We infer that Spot 42 RNA specifically binds the *galK'* RNA, supporting the idea that regulation of *galK* expression occurs via RNA–RNA interaction.

We used structural probing for further characterization of *galK*–Spot 42 RNA interaction. Here, 5'-end-labeled Spot 42 RNA was mixed with increasing concentrations of unlabeled *galK'* RNA, and the incubations were treated with RNase T₂. The structural probing is presented in Figure 6 and shows that additions of *galK'* RNA reduced or abolished cleavage by RNase T₂ in the 5' tail, loop I, and at positions 47–48 and 53–58. Thus, three short antisense sequences exposed in single-stranded regions of Spot 42 RNA appear to interact with

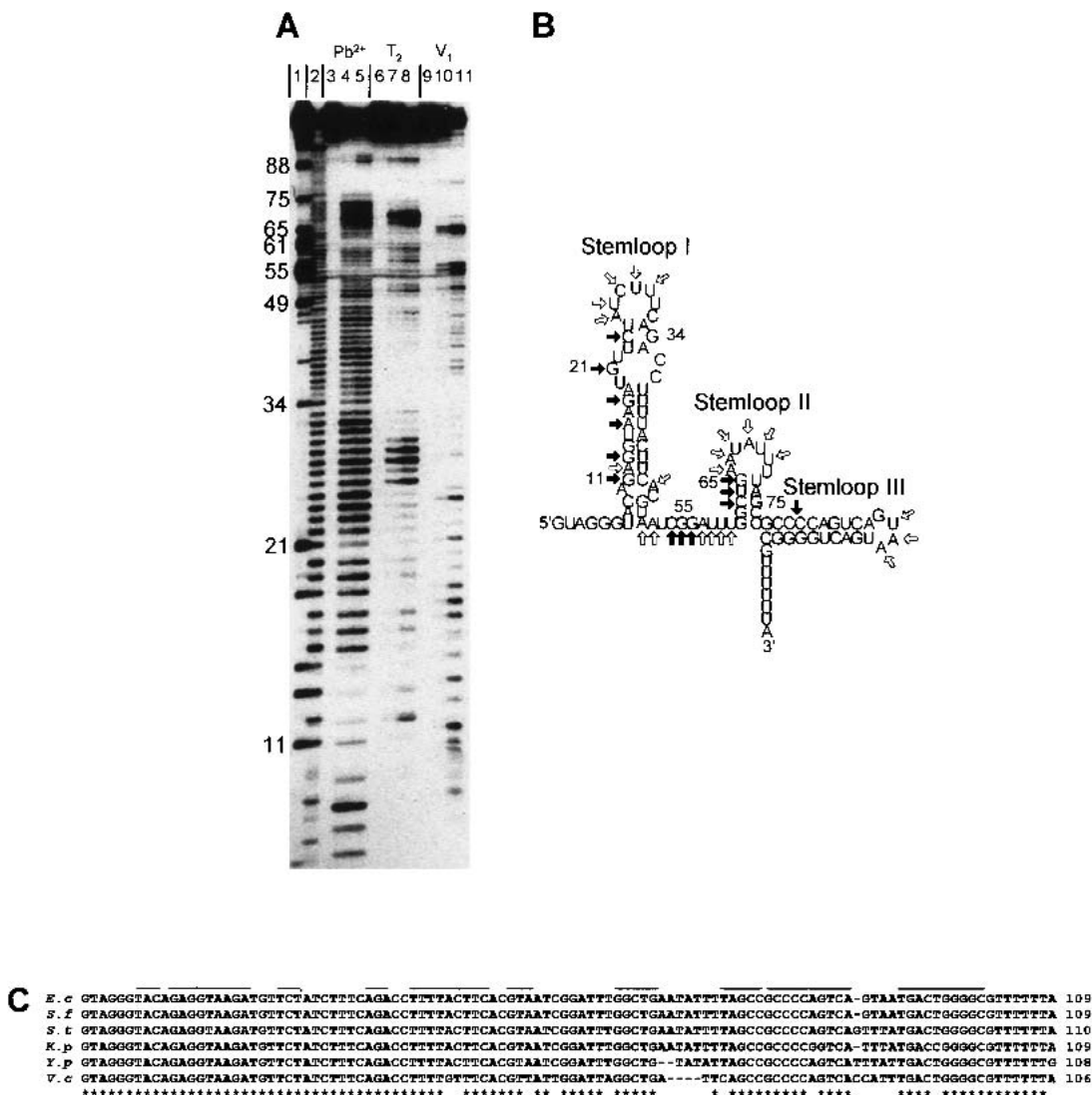


Figure 4. Secondary structure of Spot 42 RNA. (A) Nuclease and lead (II) acetate probing of 5'-end-labeled Spot 42 RNA. (Lane 1) RNase T₁ cleavage of Spot 42 RNA under denaturing condition (G-specific cleavage); (lane 2) alkaline hydrolysis ladder; (lanes 3,6,9) control (untreated Spot 42 RNA); (lanes 4,5), partial cleavage by lead (II) acetate; (lanes 7,8) partial cleavage by RNase T₂; (lanes 10,11) partial cleavage by RNase V₁. (B) Structure predictions of Spot 42 RNA based on thermodynamic criteria and summary of the structural probing. Single-stranded-specific and double-stranded-specific nuclease susceptibility are indicated by open and filled arrows, respectively. (C) Phylogenetic structure analysis of Spot 42 RNA. The DNA sequences of Spot 42 RNA from *E. coli*, *Shigella flexneri*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Yersinia pestis* and *Vibrio cholerae* were aligned. A star indicates identity. The inverted repeats predicted to fold into stem structures are overlined. Sequences for *Shigella*, *Salmonella*, *Klebsiella*, and *Yersinia* were obtained from a BLAST search of the *E. coli* sequence with the unfinished microbial genome database at the Institute for Genomic Research Web site at <http://www.tigr.org>. *Shigella* and *Yersinia* sequencing data were from the University of Wisconsin–Madison Genome Project. The Washington University Consortium sequencing project obtained the data for the *Salmonella* and *Klebsiella* sequences.

separate target sites located at or near the translational initiation region of *galk* (Fig. 5A).

Spot 42 RNA prevents ribosome binding

To test whether Spot 42 RNA binding to *galk* mRNA might interfere with ribosome binding, we used toeprinting assays (Hartz et al. 1989). Briefly, in the presence of initiator tRNA (*fMet*-tRNA), the ribosomal 30S subunits

bind to the Shine–Dalgarno sequence of mRNAs and block for reverse transcription primed downstream. The inhibition of primer extension generates a stop signal at the 3' end of the bound 30S subunit (usually 15–17 nt downstream of the start codon). The strength of this stop signal provides a measure for the formation of the ternary complexes.

For the toeprint analysis we used the *galT'* and *galk'* RNA substrates. The probes were annealed with a 5'-

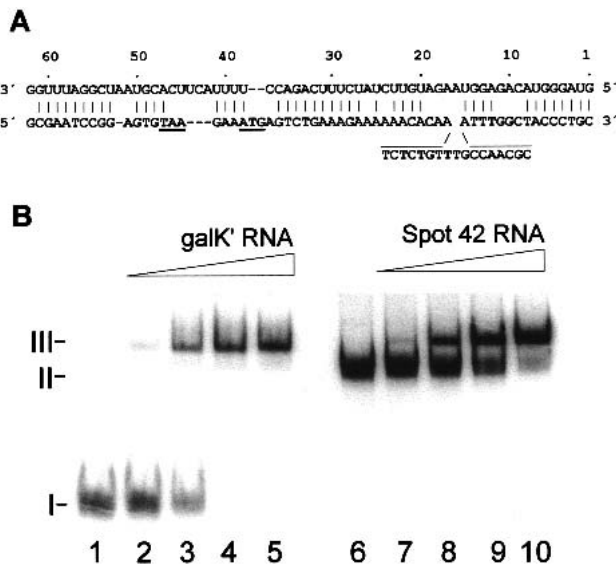


Figure 5. Spot 42 RNA–*galK* mRNA interaction. (A) The complementarity between Spot 42 RNA (top sequence) and the *galK* translation initiation region (bottom sequence). The stop codon of *galT* and the start codon of *galK* are underlined. (B) Gel mobility shift assays of Spot 42 RNA binding to *galK'* RNA. (Lanes 1–5) 5'-End-labeled transcript of Spot 42 RNA (0.05 pmole) and 500-fold molar excess tRNA were incubated with increasing amounts of unlabeled *galK'* RNA (0, 1, 5, 10, and 20 pmole) to allow complex formation and then resolved on a native polyacrylamide gel. (Lanes 6–10) 5'-End-labeled *galK'* RNA (0.05 pmole) and 500-fold molar excess of tRNA were incubated with increasing amounts of unlabeled Spot 42 RNA (0, 1, 5, 10, and 20 pmole) to allow complex formation and then resolved on a native polyacrylamide gel.

end-labeled primer complementary to a region downstream of the translation start site and incubated with 30S subunits in the presence or absence of fMet-tRNA. As shown in Figure 7A, the addition of Spot 42 RNA at different molar ratios to *galT'* RNA before the addition of 30S subunits and fMet-tRNA did not interfere with ternary complex formation. In contrast, when the experiments were performed with the *galK'* RNA (Fig. 7B), the presence of Spot 42 RNA resulted in a diminished toeprint signal, and an almost complete inhibition of ternary complex formation was observed at a molar excess of 5:1 of Spot 42 RNA to *galK'* RNA (Fig. 7B, lane 8). A diminished toeprint signal, however, was not detected if 30S subunits and fMet-tRNA were added prior to Spot 42 RNA to incubations containing *galK'* RNA (data not shown). These assays show that Spot 42 RNA binding interferes directly with the formation of *galK*–30S initiation complexes.

Discussion

In this work we have provided insight into two longstanding questions concerning a physiological function of Spot 42 RNA and the regulation of discoordinate gene expression of the *E. coli* galactose operon. Our results

establish that Spot 42 RNA is a riboregulator that acts to regulate the *gal* operon genes differentially under certain physiological conditions. Our results further show that Spot 42 RNA inhibits expression of *galK*, the third gene in the operon, by an antisense mechanism. Three short antisense sequences exposed in single-stranded regions of Spot 42 RNA appear to interact with three separate target sites located at or near the translational initiation region. Finally, toeprinting experiments with 30S ribosomal subunits revealed that *galK*–Spot 42 RNA interactions interfere with the formation of translational initiation complexes. Taken together, our results suggest that Spot 42 RNA inhibits *galK* translation by sterically

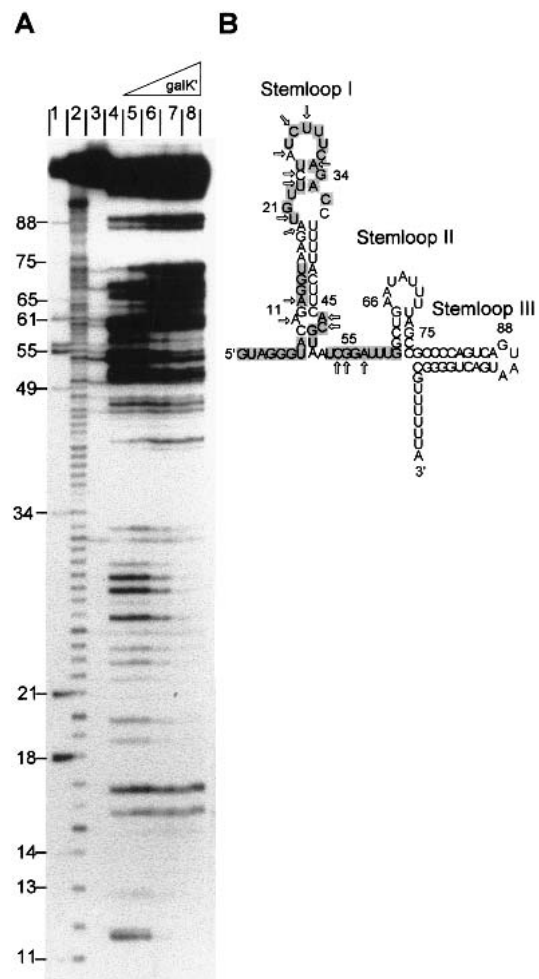


Figure 6. Nuclease probing of Spot 42 RNA–*galK'* RNA complexes. (A) In vitro synthesized 5'-end-labeled Spot 42 RNA (0.05 pmole) was mixed with increasing concentrations of unlabeled *galK'* mRNA (1, 5, 10, and 20 pmole), and the incubations were treated with RNase T₂. (Lane 1) RNase T₁ cleavage of Spot 42 RNA under denaturing condition (G-specific cleavage); (lane 2) alkaline hydrolysis ladder; (lane 3) control (untreated Spot 42 RNA); (lanes 4–8) RNase T₂ footprinting reactions in absence (lane 4) and in presence (lanes 5–8) of *galK'* RNA. (B) Summary of changes in the cleavage pattern induced by *galK'* RNA. The *galK* complementary regions are shown on a gray background. Arrows indicate reduced cleavage.

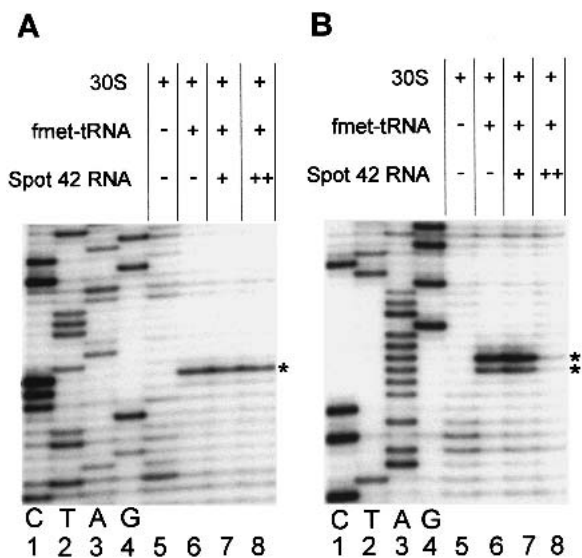


Figure 7. Toeprinting analysis of 30S ribosomal subunit binding to *galT'* and *galK'* RNA. (A) Ternary complex formation on *galT'* RNA. The toeprint signal is at position +15 relative to A of the start codon (marked by a star). (B) Ternary complex formation on *galK'* RNA. The toeprint signal is at positions +16 and +17 relative to A of the start codon (marked by stars). In all reactions the concentration of mRNA and 30S subunits was 0.04 μ M. fMet-tRNA was in molar excess over 30S subunits. When present, Spot 42 RNA was added prior to the addition of 30S subunits and fMet-tRNA. Spot 42 RNA was added at the molar ratios to mRNA, 1:2 (lane 7) and 5:1 (lane 8). (Lanes 1–4) The DNA sequence reactions (G A T C) were carried out with the same end-labeled oligonucleotide used in the toeprinting assays.

hindering ribosome binding. As Spot 42 RNA regulation only interfered weakly with the expression of the two proximal genes and the distal gene of the *gal* operon (Figs. 2, 3), it seems reasonable to suppose that the stability of the *gal* message is not grossly altered by Spot 42 RNA interaction. This view is further supported by gene array analysis of the expression of the individual genes of the *gal* operon in *spf⁺* and Δ *spf* cells (T. Møller, unpubl.). Therefore, we favor the idea that Spot 42 RNA acts primarily at the level of translation initiation.

Previously, the stimulatory effect of cAMP on the GalT levels (~1.5) and on the GalK levels (~fourfold) led to the idea that cAMP–CRP acts, directly or indirectly, at the transcriptional level by modulating polarity (e.g., by interfering with the transcription terminator ρ ; Ullmann et al. 1979). However, the hypothesis was based on the assumptions that the *gal* operon carried a single cAMP–CRP-independent promoter and that expression of the promoter proximal gene (*galE*) was unaffected by carbon source and/or cAMP. Moreover, the authors did not take into account the fact that *galE* expression was stimulated (40%–70%) by cAMP in a Δ *cya*, *galO^c81* strain (Joseph et al. 1981). Also, when the amount of *gal* mRNA corresponding to promoter proximal and distal genes was measured in a Δ *cya* strain, grown in the absence or in presence of cAMP, it was found that cAMP could only

partly restore coordinate expression (Guidi-Rontani et al. 1984). Therefore, it was concluded that a translational control was probably involved in the discoordinate regulation of the *gal* operon (Guidi-Rontani et al. 1984). The present results on *gal* operon expression, consistent with data for *gal* operator constitutive mutants (Adhya and Echols 1966; Joseph et al. 1981), suggest that the cAMP effect on *galE* and *galT* expression is transcriptional. Thus, we showed that the expression of *galE* and *galT* in a Δ *galR* strain and the expression of *galE*, *galT*, and *galK* in a Δ *galR* Δ *spf* strain was affected to a very similar extent by carbon source (i.e., 40%–50% higher in glycerol-grown cells than in glucose-grown cells; see Fig. 3), indicating that transcription initiation is more efficient from the cAMP–CRP-dependent P1 promoter than from the cAMP–CRP-repressed P2 promoter (Fig. 1).

A second interesting aspect of Spot 42 RNA regulation concerns ongoing translation. Because sequences encoding the end of the *galT* gene overlap with sequences involved in ribosome binding and initiation of *galK* translation as well as with sequences involved in Spot 42 RNA binding (Fig. 5), a terminating ribosome would be expected to interfere with Spot 42 RNA binding. We speculate that the proposed interaction between Spot 42 RNA and separated target regions could be crucial for an efficient control. Thus, the three binding regions of Spot 42 RNA are all exposed, and each of these may possibly facilitate bimolecular RNA–RNA interaction. Moreover, the separated target regions within the coding region of *galK* may allow formation of initial duplex formation in the presence of a terminating ribosome. Such binding features could lead to an increase in the local concentration of Spot 42 RNA, and facilitate rapid formation of repression complexes.

Finally, we note that recent work has shown that Spot 42 RNA regulation of *gal* operon expression relies on the global posttranscriptional regulator Hfq, which shares functional and structural features with the eukaryotic Sm and Sm-like proteins (Møller et al. 2002; Zhang et al. 2002). In *hfq⁻* strains, the half-life of Spot 42 RNA is strongly reduced and Hfq binds specifically to the RNA. Moreover, in vitro Hfq strongly cooperates in complex formation between Spot 42 RNA and *galK* mRNA (Møller et al. 2002). Because Hfq also increases OxyS RNA interaction with target mRNAs (Zhang et al. 2002), is required for riboregulation of *rpoS* by DsrA and RprA RNA (Majdalani et al. 2001; Sledjeski et al. 2001), and binds to several other sRNAs (Wassarman et al. 2001), it has been proposed that Hfq acts as a general cofactor for antisense RNAs that rely on short stretches of base pairing (Wassarman et al. 2001; Møller et al. 2002; Zhang et al. 2002).

Physiological significance of Spot 42 RNA regulation

Despite the great understanding of D-galactose metabolism in *E. coli*, the physiological significance of the uncoordinated expression of the *gal* operon genes is still unclear. It is intriguing, however, that the GalE, T, and K enzymes form part of an amphibolic pathway that pro-

duces substrates for biosynthesis of lipopolysaccharides (i.e., UDP-glucose and UDP-galactose; see Fig. 1; for review, see Adhya 1987). When the cell derives its energy from galactose, all three enzymes are required. The net result is the conversion of galactose to the glycolytic intermediate glucose-1-phosphate. When the cell derives its energy from other carbon sources, a relatively high basal level of epimerase (GalE) is required to produce UDP-galactose for biosynthetic glycosylations. Under such metabolic conditions, the amphibolic pathway, together with a galactose-1-phosphatase activity, generates galactose from UDP-galactose (i.e., GalT is also required). However, the galactose generated intracellularly does not accumulate to a level that is high enough to induce the *gal* operon because it is either excreted or recycled. Excretion takes place in the presence of glucose, the preferred carbon source, whereas the intracellular galactose is recycled when the cell uses less favorable carbon sources. In accordance with this metabolism, discoordinate expression of the *gal* operon is operative when there is no need for GalK (glucose-grown cells), and we suggest that Spot 42 RNA repression of *galK* expression may help facilitate excretion of galactose. It is also possible that Spot 42 RNA regulation may be important for optimal utilization of carbon sources. These explanations assign a physiological significance to Spot 42 RNA regulation and reveal a form of cellular regulation that may play a decisive role in the fine-tuning of gene expression. We expect that additional operons required for appropriate carbon-source utilization are regulated in a similar fashion and note that one likely candidate is the *sucABCD* operon encoding subunits for two enzymes of the tricarboxylic acid cycle—ketoglutarate dehydrogenase (*sucAB*) and succinylcoenzyme A synthetase (*sucCD*). Thus, Spot 42 RNA has extended complementarity to sequences near the start of *sucC*, and one of the reported phenotypes associated with overproducing the sRNA is the inability to grow on succinate as energy and carbon source.

Materials and methods

Bacterial strains

All strains used were *E. coli* K-12 derivatives: SØ928 (Δdeo , Δlac ; Valentin-Hansen et al. 1978); SØ2928 (Δdeo , Δlac , Δcrp -96zdh-732:Tn10; Sogaard-Andersen et al. 1991); MW130 (Δgal -R::Cm^R; Weickert and Adhya 1993); KM22 [$\Delta recC$, *ptr*, *recD*::P_{lac}-bet, *exo*, *kan*] (Murphy 1998), and MC1000 Δ ssr [F⁻, *araD139*, Δ (*argF-lac*)U169, Δ ssr::apha]. The Δgal derivatives of SØ928 and SØ2928 were constructed by P1-transduction from MW130. The $\Delta spf4$ derivatives of SØ928 Δgal R and SØ2928 Δgal R strains were constructed by P1-transduction from KM22 $\Delta spf4$. This strain was constructed by transforming strain KM22 with a *Sma*I-linearized pSpf Δ 4 plasmid as described in Murphy (1998). PCR and Northern blot analysis verified gene replacement and the absence of Spot 42 RNA.

Plasmid construction

To construct pSpf Δ 4, the DNA regions flanking the *spf* gene were amplified from SØ928 chromosomal DNA using primers

ospfdel1 (5'-CCCGGATCCGCCGGGTGGCGCGCCGTC-3') plus ospfdel2 (5'-GCCGGAATCCCGGACTCGAGTTGTTCA GTTCAGAAAGCAC-3'); and Ospfdel3 (5'-GCCGCTCGAGG GTACCTCACGAGCTCTAGCCGCCAGTCAATG-3') plus ospfdel4 (5'-GCCGGAATTCGTTAGCGTCCTCGCTCT ATGG-3'), respectively. The upstream DNA fragment was digested with *Eco*RI and *Bam*HI and cloned into *Eco*RI/*Bam*HI-restricted pUC19, generating pUspf. Subsequently, pUDspf was constructed by replacing the *Xho*I-*Eco*RI segment of pUspf with the downstream DNA fragment (digested with *Xho*I and *Eco*RI). The *aphA* gene was amplified from chromosomal DNA of strain MC1000 Δ ssr using *ossr1* (5'-GCCGGAGCTCCAGTTGGTC TTCATTGCCGATTG-3') and *ossr2* (5'-CGGCGAGCTCAC GGGTACCGGGATTTACGATGGCAGGCCAGC-3') as primers. The *aphA* fragment was digested with *Kpn*I and cloned into the unique *Kpn*I site of pUDspf, generating pSpf Δ 1 and pSpf Δ 4 (with *aphA* in the opposite or the same orientation as the original *spf* gene, respectively). Concerning construction of *spf* knockout strains, we did not observe any importance of the orientation of the kanamycin cassette.

To create pSpf33, two PCR products were generated using (1) pBAD33 as template and *ospfara* (5'-CATCTTACCTCTGTAC CCTACATGGAGAAACAGTAGAGAGTTG-3') and o33mluI (5'-CAAAAACGCGTAACAAAAGTG-3') as primers; and (2) SØ928 chromosomal DNA as template and *oarasf* (5'-CAAC TCTCTACTGTTTCTCCATGTAGGGTACAGAGGTAAGA TG-3') and *ospfpstI* (5'-CGCCTGCAGCGTTGGCCTGATA AACATAGCGC-3') as primers. A third round of amplification with these two products as template and o33mluI and *ospfpstI* as primers generated a DNA fragment carrying the *spf* gene fused to the arabinose promoter. Finally, pSpf33 was constructed by replacing the *Mlu*I-*Pst*I fragment of pBAD33 with the restricted *araP*-*spf* fragment.

Plasmid pSpf3 was constructed by replacing the *Eco*RI-*Bam*HI fragment of pUC18 with a PCR-generated fragment, prepared using oT7spf (5'-CCGGATCCTGTAATACGACTCAC TATAGTAGGGTACAGAGGTAAG-3') and o3spf (5'-CCGAA TTCGGATGTTCCGCAATAAAAAACGC-3') as primers and SØ928 chromosomal DNA as template, followed by digestion with *Eco*RI and *Bam*HI.

Plasmid pFps was constructed by replacing the *Eco*RI-*Bam*HI fragment of pGem4 (Promega) with a PCR-generated fragment, prepared using ospf1 (5'-GTTAGAATTCGCCTGAACATGCC 3') and ospf2 (5'-CCGGATCCGTTGGCCTGATAAACATA GCGC-3') as primers and SØ928 chromosomal DNA as template, followed by digestion with *Eco*RI and *Bam*HI.

Plasmid pGal, containing *galETK* cloned between the *Eco*RI and *Pvu*II sites of pBR322, was constructed as described (Busby and Dreyfus 1983). Plasmid pGal4 encoding the entire *gal* operon was generated by replacing the *Sap*I-*Sap*I fragment of pGal with a PCR-generated fragment, prepared using ogalM1 (5'-CGTCGCGCTGATCCCGGAAGAGCTGG-3') and ogalM2 (5'-GCGCGCAGCGGAAGAGCAAAAAGCCCTCATGACGA GGGCGT-3') as primers and SØ928 chromosomal DNA as template, followed by digestion with *Sap*I.

Plasmids pH8-E, pH8-T, and pH8-K, which encode N-terminally 8xhistidine-tagged GalE, GalT, and GalK proteins from the IPTG-inducible phage T7-promoter derivative P_{A1/O4/O3}, were constructed by replacing the *Eco*RI-*Bam*HI fragment of pUHE24-2 (obtained from Hermann Bujard, University of Heidelberg, Germany) with PCR-generated fragments using pGal as template. The primers used were *galE*, oh8E (5'-CGC CCGAATTCATAAGGAGTTTTATAAATGCATCACCATCA CCATCACCATCACAGAGTTCTGGTTACCGGTGGTAGC-3') and oE (5'-GCCCGGATCCGGCGATGTGGATGATCA ACGGG-3'); *galT*, oh8T (5'-CGCCCGAATTCATAAGAGT

TTTATAAATGCATCACCATCACCATCACCATCACCATCACACGC AATTTAATCCCCTTGGATCATC-3') and oT (5'-GCCCCG GATCCCCGGCCAGGCGCCTGAATGGTG-3'); *galK*, oh8K (5'-CGCCCCAATTCATAAAGGAGTTTTATAAATGCATCACC ATCACCATCACCATCACCAGTCTGAAAAGAAAAACACAA TCTC-3') and oK (5'-GCCCCGGATCCCAGCGACAGCTTG CTGTACGGC-3').

Growth media

Strains were grown at 37°C in AB minimal medium (Clark and Maaløe 1967) containing 1 µg/mL thiamine, 0.2% carbon source (glucose or glycerol), and 0.05% casamino acids. For the growth media used for [³⁵S]methionine labeling, casamino acids were replaced by a 1/20 volume defined amino acid mixture (concentrations: L-alanine, 1.75 g/L; L-arginine, 1.75 g/L; L-histidine, 0.78 g/L; L-lysine, 1.5 g/L; L-proline, 1.15 g/L; L-threonine, 1.20 g/L; glycine, 1.50 g/L; L-asparagine, 1.33 g/L; L-glutamine, 2.18 g/L; L-isoleucine, 1.30 g/L; L-phenylalanine, 0.60 g/L; L-tyrosine, 0.90 g/L; L-valine, 1.75 g/L; L-aspartic acid, 1.33 g/L; L-glutamic acid, 2.2 g/L; L-cysteine, 0.30 g/L; L-serine, 25.0 g/L; L-tryptophan, 0.50 g/L). Ampicillin (100 µg/mL), spectinomycin (100 µg/mL), and chloramphenicol (50 µg/mL) were added where appropriate.

2D gel electrophoresis

Samples (1 mL) of exponentially grown cells were labeled with 10 µCi of carrier-free [³⁵S]methionine for 1 min, followed by a chase with unlabeled methionine (final concentration 200 µg/mL) for 1 min. The chase was terminated by the addition of 5 µL of chloramphenicol (50 mg/mL). Cells were collected by centrifugation at 0°C, resuspended in 50 µL of loading buffer (10% sucrose, 3% SDS, 0.16 M Tris-HCl at pH 6.8, with freshly added β-mercaptoethanol), boiled for 3 min, and mixed with 25 µL of 10% NP-40. A 30-µL sample was loaded onto the first dimensional gel. Gel electrophoresis was carried out as described by O'Farrell (1975). Quantification of protein spots was done by PhosphorImager and the Imagequant software package (Molecular Dynamics).

Protein purification, antibody production, and Western blotting

The N-terminally histidine-tagged Gal enzymes were expressed from pH8-E, pH8-T, or pH8-K, respectively, in strain SØ928 harboring pMS421 (pSC101 derivative that encodes the *lacI^q* allele; Grana et al. 1988). Exponentially grown cells were induced for 2 h by addition of IPTG to 500 µM. Protein purification was carried out using Talon Metal Affinity Resin (Clontech) according to the manufacturer's specifications (denaturing procedure with 8 M urea). Antisera were generated by immunizing rabbits with the purified proteins. For Western blot analysis, the strains were grown to an OD₆₀₀ of 0.4. Equal amounts of total cellular extracts were electrophoretically separated on SDS–10% polyacrylamide gels, and blotted to an Immobilon-P membrane (Millipore). The membrane was probed with rabbit anti-GalK, anti-Gal E, or anti-GalK polyclonal antisera, and the antibody–antigene complexes were visualized with the ECL plus system (Amersham Pharmacia Biotech).

Synthesis, purification, and labeling of RNA

In vitro synthesis of RNA was carried out with T7 RNA polymerase (Promega) as described (Franch and Gerdes 1996). *FokI*-digested pSpf3 was used as the DNA template for synthesis of

Spot 42 RNA. Two DNA templates carrying a T7 promoter were generated by PCR for synthesis of *galK'* RNA and *galT'* RNA, prepared using oT7ktp (5'-TGTAATACGACTCACTATA GGGCTCTGCTGCGTCCGCCACCG-3') plus oktp (5'-GGTGCACAACTGATCACGGTTTG-3'); and oT7ttp (5'-TG TAATACGACTCACTATAGGGCGCGTTCGCGAGGGCGAC CTTCCGG-3') plus otpp (5'-GAGGAAGCAATCTGGATCGT GCGC-3') as primers and plasmid pGal as template. The RNAs were purified and quantified as described (Thisted et al. 1994). Transcripts were radioactively labeled at the 5' end with T4 kinase (Roche Molecular Biochemicals) and [γ-³²P]ATP.

Northern blot analysis

Preparation of total RNA from *E. coli* and Northern blot analyses were carried out as described elsewhere (Franch et al. 1997). The ³²P-labeled RNA probe used for detection of Spot 42 RNA was generated by in vitro transcription of plasmid pFps (linearized with *EcoRI*) with T7 RNA polymerase.

Gel mobility shift assay

The binding assays were performed as follows: 5'-end-labeled RNA (0.05 pmole of Spot 42 RNA or *galK'* RNA), 1 µg of tRNA (Boehringer Mannheim), and unlabeled RNA (1, 5, 10, and 20 pmole of Spot 42 RNA or *galK'* RNA) were mixed in binding buffer (20 mM Na-HEPES at pH 8, 100 mM KCl, 1 mM DTT, 1 mM MgCl₂) and incubated on ice for 20 min. The binding reactions (10 µL) were mixed with 5 µL of loading dye (10% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) and analyzed on a 5% nondenaturing polyacrylamide gel in TBE buffer at 200 V at 4°C for 1.5 h. Subsequently, the gel was dried and subjected to autoradiography.

Toeprinting analysis

Purification of ribosomal 30S subunits and toeprinting assays were carried out as described previously (Franch et al. 1997). The 5'-end-labeled oligonucleotides otpt (5'-GCCTGAATG GTGTGAGTGG-3'), complementary to the *galK* gene, and otpp (5'-GAAACCAGAATCCATTGCC-3'), complementary to the *galT* gene, were used as primers for cDNA synthesis in the toeprinting reactions.

Enzymatic and chemical probing of Spot 42 RNA

RNase T₂ (Sigma) and RNase V₁ (Amersham Pharmacia Biotech) digestion was performed as described previously (Franch et al. 1997). 5'-End-labeled Spot 42 RNA (0.05 pmole) was incubated in RNase buffer (20 mM Tris-acetate at pH 7.8, 100 mM NH₄Cl, 10 mM Mg-acetate, 200 mM K-glutamate, and 5 µg of tRNA) at 37°C. Partial digestion was carried out with 0.001 units of RNase T₂ or 0.005 units of RNase V₁ for 3 min and 15 min. For lead (III) acetate probing, 5'-end-labeled Spot 42 RNA (0.05 pmole) was incubated in 50 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, and 50 mM KCl, at 37°C. Samples were treated with Pb(Ac)₂ (final concentration, 5 mM) for 3 min and 15 min. Alkaline hydrolysis and RNase T₁ cleavage of Spot 42 RNA were carried out as described in Franch et al. (1999).

Acknowledgments

This work was supported by grants from the Danish Natural Science Research Council (P.V.-H.) and the Carlsberg Foundation (T.F.).

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