Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA

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A novel 15-kD protein, RbfA, has been identified by virtue of its ability to act as a high copy suppressor of a previously characterized dominant cold-sensitive mutation (C23U) in 16S rRNA. RbfA is found associated with free 30S ribosomal subunits, but not with 70S ribosomes or polysomes, and is essential for maximal cell growth, particularly at low temperatures. Cells lacking RbfA in a wild-type rRNA background exhibit a cold-sensitive phenotype that is strikingly similar to that of the cold-sensitive C23U rRNA mutant. The observed patterns of allele specificity of suppression and synthetic lethality in cells containing an RbfA knockout in combination with various 16S rRNA mutations suggests that RbfA interacts with the 5′-terminal helix region of 16S rRNA, possibly during a late step of 30S maturation.

[*Key Words*: 16S rRNA; RbfA; ribosome assembly; cold sensitivity]

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In recent years methodological advances have made it possible to begin to dissect the functional complexity of the ribosome through the use of genetics (Dahlberg et al. 1986; Hui and De Boer 1987; Morgan et al. 1988; Triman et al. 1989; Ofengand et al. 1993). In a previous study, we screened for conditional dominant 16S rRNA mutants with the expectation that such mutants would be more likely to have defects in specific functional processes. We isolated and characterized a dominant cold-sensitive mutant containing a C → U transition at position 23 of 16S rRNA (Dammel and Noller 1993). It was unexpected that this mutation, which results in conversion of a G-C base pair in the 5′-terminal helix to a thermodynamically less stable G-U pair, should confer a cold-sensitive phenotype. Cells expressing the C23U mutation have decreased polysome levels and accumulate free 30S and 50S subunits. Additionally, we observed particles that resemble those seen previously in in vitro reconstitution of 30S subunits carried out at low temperature (Nomura and Held 1974) and in cold-sensitive alleles of ribosomal protein S5 (Guthrie et al. 1969; Traub and Nomura 1969; Feunteun et al. 1974), which is believed to interact with the 30S subunit in the neighborhood of the location of the C23U mutation (Osswald et al. 1987; Stern et al. 1988). The properties of the C23U mutant, and its suppression by second-site mutations in 16S rRNA as well as in its upstream leader, suggested that cold sensitivity was the result of destabilization of the 5′-terminal helix, relative to a competing precursor helix. According to this model, the rate-limiting step of an early phase of 30S subunit assembly would be conversion of the precursor helix to the 5′ mature helix, followed by its rapid incorporation into a more stable structure (Dammel and Noller 1993). Thus, at low temperature, 16S rRNA would be trapped in an immature conformation, but at elevated temperatures, an increased rate of conversion to the mature helical form would promote the rate of its subsequent incorporation into a stable structure. We chose to search for extragenic suppressors of the C23U allele, with the hope that such an approach might provide further insight into the functional basis of the cold-sensitive phenotype of the C23U mutation.

Here, we describe the identification and characterization of a novel protein, RbfA (formerly, P15B; Sands et al. 1988), which, when overexpressed, suppresses the cold-sensitive C23U phenotype. Cells lacking this protein in a wild-type rRNA background are cold sensitive for growth and are phenotypically very similar to the C23U rRNA mutant. Immunolocalization studies demonstrate that RbfA is associated with free 30S subunits, but not with 50S subunits, 70S ribosomes, or polysomes. Additionally, we find that free 30S subunits isolated either from a knockout strain for RbfA (CD28) or from C23U-containing cells contain an additional protein present at a stoichiometry comparable to that of the known 30S ribosomal proteins. We conclude that RbfA interacts, either directly or indirectly, with the 5′-terminal helix of 16S rRNA and may function as a late maturation or initiation factor.

Results

Isolation of high copy suppressors

To gain additional clues to the basis of the functional

defect of the cold-sensitive 16S rRNA C23U mutation, we searched for gene products that, when overexpressed, could suppress the dominant cold-sensitive phenotype of the C23U 16S rRNA mutant. An *Escherichia coli* genomic library was constructed by inserting *Sau*3A partially digested *E. coli* genomic DNA into the *Bam*HI site of pACYC184, disrupting the gene encoding tetracycline resistance (Tet^r) (Chang and Cohen 1978). This chloramphenicol-resistant (Cam^r) plasmid and its insert-containing derivatives are compatible with ampicillin-resistant (Amp^r) pSTL102-derived plasmids such as pU23 (which contains the C23U mutation in 16S rRNA). Cells containing pU23 (Dammel and Noller 1993) were secondarily transformed with the *Sau*3A genomic library. Twelve overexpressing suppressor candidates were obtained by screening double transformants directly for loss of the cold-sensitive phenotype at 26°C on $Cam_{40}Amp_{60}$ plates. Phenotypes were confirmed by isolating the plasmids from the revertant candidates and demonstrating that the Amp^r plasmid pU23 sustained its cold-sensitive phenotype upon retransformation. Similarly, the Cam^r plasmids containing specific cloned inserts were directly shown to suppress cold sensitivity by retransforming pU23-containing cells.

Identification of high copy suppressors

To identify the genes responsible for the high-copy extragenic suppression of the cold-sensitive C23U allele, the ends of the genomic plasmid inserts from six randomly chosen Cam^r suppressing plasmids were sequenced. Data base searches using this sequence information revealed that all six suppressor candidates could be localized to the *metY* operon at 67.5′ on the *E. coli* chromosome (Fig. 1A). This entire operon has been sequenced previously (Portier et al. 1981; Plumbridge et al. 1982; Kurihara et al. 1983; Ishii et al. 1984a,b; Portier and Regnier 1984; Sacerdot et al. 1984; Regnier et al. 1987; Sands et al. 1988), and a detailed restriction fragment map of the region could therefore be generated. Restriction fragment mapping of the inserts showed that all six clones contained a region in common at or near the *infB* gene. A sublibrary was constructed from a partial *Sau*3A digest of the smallest plasmid conferring suppression, and from this a plasmid containing a smaller insert was isolated, which conferred suppression of the C23U phenotype. Comparison of restriction fragments from this plasmid in common with the other larger suppressing plasmids identified a single open reading frame, immediately following the *infB* gene, in common to all of the suppressors. We term this gene *rbfA* (formerly, P15B; Sands et al. 1988), for ribosome-binding factor, in view of the binding of its gene product to ribosomal particles (see below).

To confirm that overexpression of RbfA was responsible for suppression of the cold-sensitive C23U allele, a PCR fragment containing the gene encoding RbfA, as well as a putative promoter region, was cloned into the *Bam*HI site of pACYC184 (p15B-3; Fig. 1B,b). Transformation with p15B-3 completely suppressed the cold-sensitive phenotype of pU23-containing cells. Similarly, the plasmid p15B (Fig. 1B,e), containing only the *rbfA*-coding region (lacking the promoter) in a protein expression vector, also conferred complete suppression. These results demonstrate that overexpression of the *rbfA* gene product is sufficient to completely suppress the cold-sensitive phenotype of C23U-containing cells. To determine whether the remaining suppressing plasmids contained

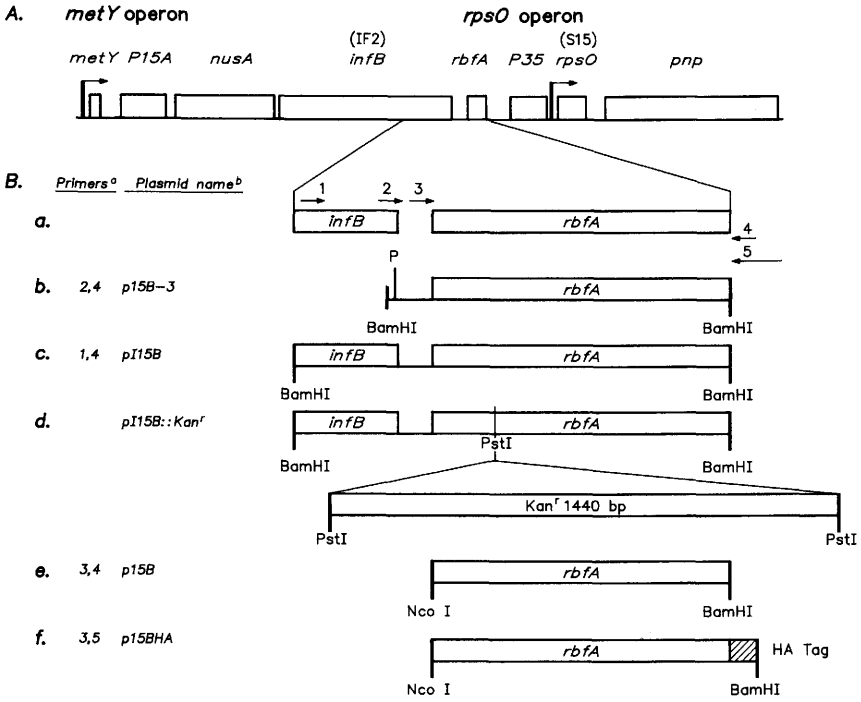


Figure 1. (*A*) The *metY/rpsO* operon. The location of the *rbfA* (formerly P15B) gene relative to the other genes in the *metY/rpsO* operon is shown. (*B*) Configuration of gene constructs used in this study. (*a*) Relative positions of PCR oligonucleotide primers used to generate each gene construct. ([a]) Oligonucleotide sequences are as follows; primer 1 (5′-GAT-CAT-CGG-ATC-CGG-CGT-AGG-3′), primer 2 (5′-GGC-GAT-GGG-ATC-CAA-GT-3′), primer 3 (5′-GAA-TTT-ACC-ATG-GCG- AAA-G-3′), primer 4 (5′-CGA-GGA-GGA-TCC-ATT-AGT-C-3′), and primer 5 (5′-GCT-CGA-GGA-TCC -TTA-CTA-GCT-CGA-AGC-GTA-ATC-TGG-AAC-ATC-ATA-TGG-GTA-GCT-CGA-GTC-CTC-CTT-GCT-GTC-GTC-CG-3′). ([b]) The host vectors for PCR-derived inserts are described in Materials and methods. (*b–f*) Construction of plasmids containing *rbfA* (see text for details).

the *rbfA* gene, all 12 suppressing plasmids were analyzed by Southern blot analysis using the *rbfA* PCR fragment from p15B-3 (Fig. 1B,b) as a source of random-primed DNA probe. This analysis indicated that all six of the suppressing clones contained the RbfA-coding region (data not shown).

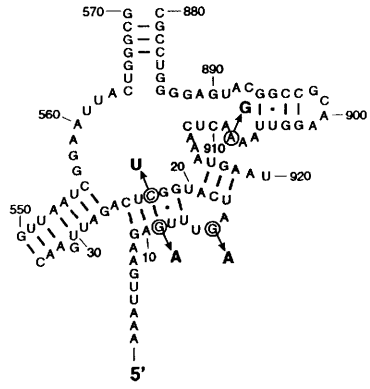
The *rbfA* gene encodes a 15-kD protein of unknown function and is part of a complex polycistronic operon (*metY/rpsO*) comprising the genes *metY, P15A, nusA, infB, rbfA* (*P15B*), and *P35*; some readthrough is also observed, connecting expression of the *metY* operon to the downstream *rpsO* and *pnp* genes (Fig. 1A, Sands et al. 1988). Among the gene products of this operon are a minor form of initiator tRNA (*metY*) as well as the transcription antitermination factor *nusA*, initiation factor 2 (*infB*), and polynucleotide phosphorylase (*pnp*), which are expressed in response to cold shock (Jones et al. 1987). Additionally, the gene encoding ribosomal protein S15 (*rpsO*) is found here. The predicted amino acid sequence for *E. coli* RbfA, as well as a putative RbfA homolog from *Bacillus subtilis* (K. Shazand, GenBank accession no. Z18631) are shown in Figure 2. The two proteins share 38% identity and 57% homology. A search of available data bases using BLAST failed to identify any sequence motifs bearing significant homology to any other proteins.

Characterization of suppression

The growth of doubly transformed cells containing pU23 (cold sensitive) or pSTL102 (wild type) and either pACYC184 (no insert) or p15B-3 (*rbfA*-containing insert) on $Cam_{40}Amp_{60}$ plates is summarized in Figure 3. Cells containing pU23 + pACYC184 are cold sensitive when compared with pSTL102 + pACYC184, as expected. However, cold sensitivity is suppressed in cells containing pU23 + p15B-3 and appear to grow as well as the wild-type control transformants, containing pSTL102 + p15B-3. It should be noted that overexpression of RbfA does not appear to affect the growth rate of wild-type pSTL102-containing cells (Fig. 3, cf. pSTL102 + pACYC184 with pSTL102 + p15B-3).

Ribosomes isolated from pU23-containing cells have unusual sedimentation profiles, characterized by an increase in free 30S and 50S subunits and a decrease in polysomes relative to 70S monosomes (Dammel and Noller 1993). Because overexpression of RbfA can suppress the C23U cold-sensitive growth phenotype, we asked whether it also affects the aberrant gradient profiles associated with the C23U allele. Extracts from cells containing pSTL102, pU23 or pU23 + p15B-3 grown at 37°C (an intermediate temperature where all defects are apparent) were analyzed by sucrose gradient centrifugation. The results (Fig. 4) indicate that the profile obtained from pU23 + p15B-3-containing cells resembles that of wild-type (pSTL102-containing) cells much more closely.

To learn more about the nature of the genetic interaction between C23U and RbfA, we asked whether the



plasmids	42°C	26°C
pSTL102 / pACYC184	++++	++++
pSTL102 / p15B-3	++++	++++
pU23 / pACYC184	++(+)	–
pU23 / p15B-3	++++	++++
pA11 / pACYC184	+	–
pA11 / p15B-3	++++	++
pU-5U23 / pACYC184	++++	+++
pU-5U23 / p15B-3	++++	++++
pA15U23 / pACYC184	+++	+
pA15U23 / p15B-3	++++	+++
pU-55U23 / pACYC184	++++	+++
pU-55U23 / p15B-3	++++	+++
pG908 / pACYC184	++++	+
pG908 / p15B-3	++++	+(+)

Figure 3. Growth phenotypes of doubly transformed cells containing wild-type plasmid pSTL102 or 16S rRNA mutant plasmids and plasmid pACYC184 (no insert, wild-type control) or p15B-3 (containing *rbfA*) at 42°C and 26°C. The 5′-terminal helix pseudoknot region of 16S rRNA and the locations of C23U, G11A, G15A, and A908G mutations are shown. (++++) Full sized growth; (+++, ++, +) isolated colonies that are less than full size; (–) no detectable growth.

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                1          11         21         31         41         51         61
E.coli      MAKEFGRPQRVAQEMQKEIALILQREIKDPRLGMMTTVSGVEMSRDLAYAKVYVTFLNDKDEDAVKAGIK 70
B.subtilis  ---LSMRANRVGEQMKKELGDIISRKLKDPRIGFLT-VTDVRVSGDLQIAKVYISVLG--DEKKREEALK 64
Consensus   ......R..RV...M.KE...I..R..KDPR.G..T.V..V..S.DL..AKVY...L...DE.......K

                71         81         91        101        111        121        131
E.coli      ALQEASGFIRSLLGKAMRLRIVPELTFFYDNSLVEGMRMSNLVTSVVKHDEERRVNPDDSKED 133
B.subtilis  GLAKAKGFIRSEIGSRIRLRKTPEIEFEFDESIDYGNRIETLIHELHSEKPSE---------- 117
Consensus   .L..A.GFIRS..G...RLR..PE..F..D.S...G.R...L.....................
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Figure 2. Predicted amino acid sequence for *E. coli* RbfA protein and a putative *B. subtilis* RbfA homolog (K. Shazand, GenBank accession no. Z18631). The two proteins are 38% identical and 57% homologous.

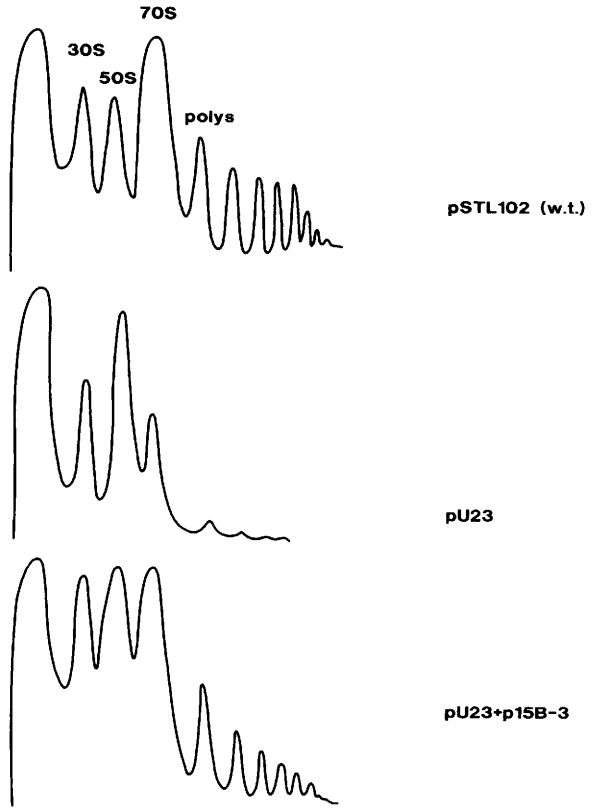

Figure 4. Sedimentation profiles of extracts from cells containing wild-type pSTL102, mutant pU23, or pU23 + p15B-3 plasmids. Positions of 30S and 50S ribosomal subunits, 70S monosomes, and polysomes are indicated. Sedimentation is from *left* to *right*.

suppression of cold sensitivity by RbfA is allele specific. Second-site RNA partial suppressors of C23U isolated previously (Dammel and Noller 1993) and another cold-sensitive dominant 16S rRNA mutation A908G (pG908; Allen 1992) were tested for suppression by RbfA overexpression. As indicated in Figure 3, the cold-sensitive phenotype of G11A (pA11) was partially suppressed, and the phenotypes of the double mutants U-5U23 (pU-5U23) and A15U23 (pA15U23), were strongly suppressed by expression of plasmid-encoded RbfA. Interestingly, the strongest suppression observed was for mutants containing the U23 alteration. Weaker suppression was observed for the alleles G11A (pA11) and A908G (pG908), which lack the U23 mutation but contain alterations in bases that are located very close to U23 in the 16S rRNA secondary structure.

In addition to the 16S rRNA mutants, four unrelated cold-sensitive strains were examined for suppression by overexpression of RbfA. Two of these strains, MM122 and MM123 (Ferro-Novick et al. 1984), were isolated as cold-sensitive suppressors of the temperature-sensitive SecA secretion mutant MM52 (Oliver and Beckwith 1981). The mutation in MM123 has been mapped to the ribosomal protein S15 (*rpsO*) gene, in the *metY/rpsO* operon, and the mutation in MM122 responsible for cold sensitivity has not been mapped precisely but has also been localized to the *rpsO* region of the chromosome. Strain AJ120 (A. Jacq, unpubl.) contains a cold-sensitive *nusB* mutation (*nusB* is involved in a transcription antitermination event involving *nusA*) and EE583 (Lee and Beckwith 1986) maps near the *rpmE* (large subunit ribosomal protein L31) operon at 89 min. Transformation with p15B-3 failed to confer suppression to the cold-sensitive phenotypes of AJ120, EE583 or MM123. However, complete suppression of cold sensitivity was observed for MM122 (Table 1). This was confirmed by curing MM122 of the *rbfA*-containing plasmid by treatment with acridine orange whereupon the strain recovered its cold sensitivity; p15B-3 isolated from MM122 again could be demonstrated to suppress the cold-sensitive phenotype upon retransformation of MM122 (data not shown). It is possible that MM122, like MM123, contains a mutation in ribosomal protein S15; however, the gene encoding S15 (*rpsO*) is very near the gene encoding RbfA (Fig. 1A), therefore raising the possibility that it contains a cold-sensitive mutation in RbfA that can be complemented by plasmid-encoded RbfA (p15B-3). In addition, the combination of pU23 and MM122 is lethal, providing further evidence that MM122 is a *rbfA* allele. These results, although not conclusive, indicate that suppression of cold sensitivity by RbfA overexpression is likely to be allele or at least gene specific.

Construction of a rbfA *knockout strain*

To test the physiological requirement for RbfA we disrupted the gene by inserting a 1.4-kb fragment containing kanamycin resistance into a plasmid-borne copy of *rbfA*. The disrupted *rbfA* gene was then cloned into *Bam*HI-digested pMAK705 (Fig. 1B,d); this plasmid is temperature sensitive for replication and specifically designed for homologous recombination-promoted insertion into the *E. coli* chromosome (Hamilton et al. 1989). The resulting plasmid pI15B::Kan was used to replace the chromosomal copy of *rbfA* in strain CSH142 with the *rbfA::kan* sequences. Kanamycin-resistant (Kan^r) re-

Table 1. *Growth phenotypes resulting from overexpression of RbfA in different cold-sensitive strains*

Strain/plasmid	42°C[a]	26°C[a]
EE583/pACYC184	+ + +	+
EE583/p15B-3	+ + +	+
MM122/pACYC184	+ + +	−
MM122/p15B-3	+ + + +	+ + + +
MM123/pACYC184	+ + +	−
MM123/p15B-3	+ + +	−
AJ120/pACYC184	+ + +	+ +
AJ120/p15B-3	+ + +	+ +

All cold-sensitive strains were a generous gift of Jon Beckwith (Harvard Medical School, Cambridge, MA) and are described in Materials and methods. Cam_{50}-containing plates were used.
[a](+ + + +) Full-sized growth; (+ + +, + +, +) isolated colonies that are less than full size; (−) no detectable growth.

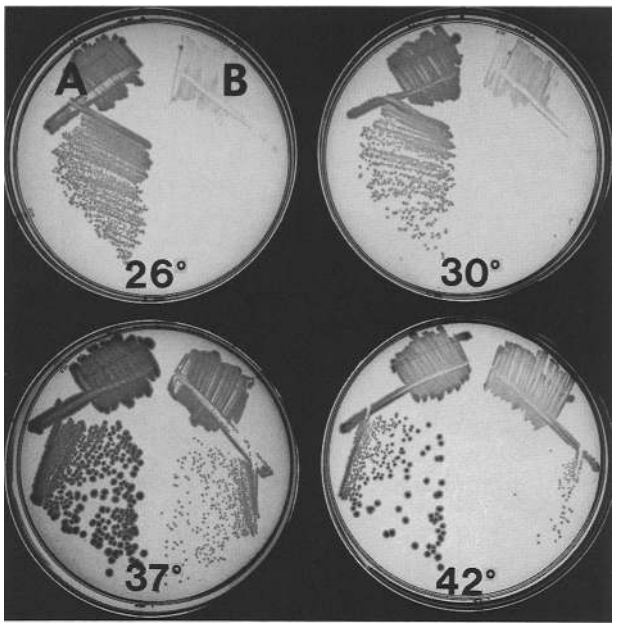

Figure 5. Growth of CD28 (RbfA knockout strain) cells (*B*) compared with that of the wild-type parent strain CSH142 (*A*), at 42°C, 37°C, 30°C, and 26°C on Luria broth media.

combinants CSH142:rbfA::kan (CD28), were shown to contain a disrupted *rbfA* gene by PCR analysis of chromosomal DNA using primers specific for *rbfA* (primers 3 and 4, Fig. 1B,a). The only detectable PCR product derived from recombinant chromosomal DNA (CD28) is larger by 1440 bp than the corresponding PCR product derived from wild-type chromosomal DNA, consistent with insertion of the kan^r resistance gene (data not shown).

Characterization of CD28

Growth of the *rbfA* knockout strain (CD28) on plates at 26°C, 30°C, 37°C, and 42°C is impaired severely, compared with growth of the wild-type parent strain CSH142 (Fig. 5). However, the growth defect is most severe at low temperatures (i.e., 26°C and 30°C). These results indicate that RbfA is required for maximal cell growth, particularly at low temperatures. CD28 cells were grown in Kan_{40} broth at 26°C and 42°C, and their growth curves were compared with those from CSH142 cells (data not shown). The doubling time for CD28 at 42°C and 26°C is 51 and 168 min, respectively, significantly slower than that of wild-type CSH142 at 42°C (27 min) and 26°C (66 min). Consistent with the plate phenotypes, this growth rate difference is more severe at low temperature. The cold-sensitive phenotype of CD28 can be completely complemented by transformation with the RbfA expression plasmids p15B-3 (Fig. 6) or p15B (data not shown). This result indicates that the cold-sensitive phenotype of CD28 is most likely attributable to absence of the *rbfA* gene product, rather than the result of transcription polarity effects caused by disruption of the *rbfA*-coding region by the Kan^r gene.

The cold-sensitive growth phenotype of CD28 is virtually identical to the cold-sensitive phenotype associated with the original 16S rRNA C23U mutation. To determine whether the phenotypic similarities between the C23U 16S rRNA mutant and CD28 extend to the molecular level, extracts from CD28 and wild-type CSH142 cells grown at 30°C or 42°C (data not shown) were analyzed by sucrose gradient sedimentation (Fig. 7). The resulting profiles were very similar to those observed for cold-sensitive C23U-containing cells (Fig. 4), showing a decreased level of polysomes and an increase in 30S and 50S subunits relative to 70S monosomes. These effects were observed for cells grown at high and low temperatures but were most pronounced at 30°C, as for C23U-containing cells. These sedimentation anomalies are completely reversed when ribosomes are isolated from CD28 cells containing p15B-3 or p15B (data not shown).

Suppression of C23U-induced cold sensitivity by overexpression of RbfA has been demonstrated. To further explore the genetic interaction between C23U and RbfA, we performed a synthetic enhancement experiment. We asked whether the cold-sensitive phenotype of CD28 is exacerbated by the presence of various 16S rRNA (or precursor 16S rRNA) mutations (Dammel and Noller 1993). Plasmids pSTL102, pU-5, pU-55 and pA11U23 can be transformed into CD29 [CD28 (*recA*$^-$)] cells at the per-

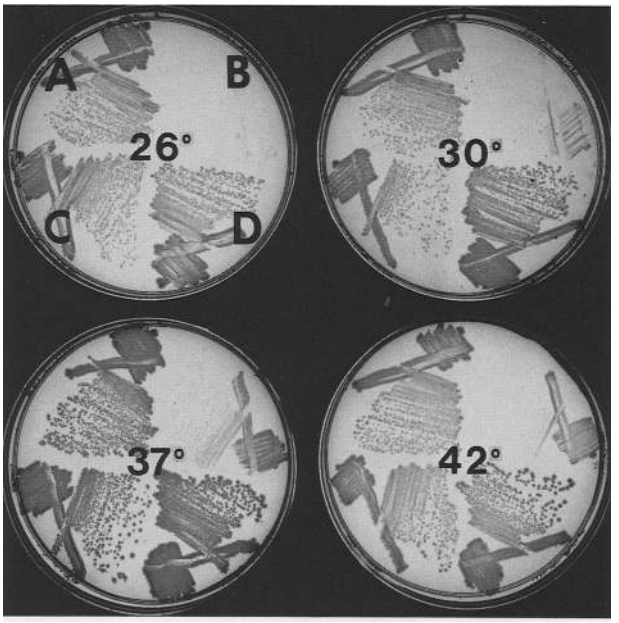

Figure 6. Complementation of the CD28 (RbfA knockout strain) cold-sensitive phenotype by the *rbfA*-containing plasmid p15B-3 (*A*) compared with CD28 carrying the parent vector pACYC184 (*B*), at 42°C, 37°C, 30°C, and 26°C. The wild-type parent strain, CSH142, containing p15B-3 (*C*) and pACYC184 (*D*) plasmids is also shown, demonstrating that overexpression of RbfA does not affect growth of wild-type CSH142.

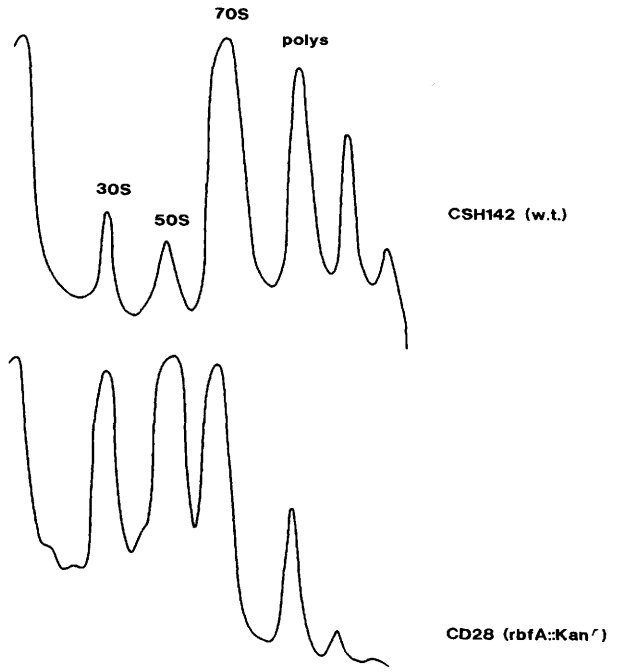

Figure 7. Sedimentation profiles of extracts from cold-sensitive CD28 cells compared with wild-type CSH142 cells grown at 30°C.

missive temperature (42°C). However, we failed to recover any transformants of CD29 at 42°C with plasmids pU23, pA15U23, pU-5U23, pA11, or pU-55U23 (Table 2). Although both wild-type cells (DH1) containing these plasmids and CD29 cells containing no plasmid are normally viable at 42°C, the combination of the CD29 knockout and these plasmids, which encode 16S rRNA in which the 5′-terminal helix is predicted to be destabilized, appears to confer a synthetic lethal phenotype. The growth phenotype of pA11U23 in a wild-type strain (DH1) is nearly that of the wild-type pSTL102 in DH1 (Dammel and Noller 1993). In contrast, in the CD29 background, although viable, the phenotype of pA11U23-containing cells is very severe, compared with that of pSTL102-containing cells. CD29 containing pA11U23 is inviable at 26°C, as expected, but is also barely viable at 42°C, at what in a wild-type background (DH1) would otherwise be a permissive temperature (Table 2). Whereas an A11–U23 base pair functions nearly as well as the wild-type G11–C23 in a RbfA$^+$ background, the behavior of pA11U23 in a RbfA$^-$ background indicates that the base sequence itself may be of functional importance, as it relates to RbfA function. This result is consistent with the high phylogenetic conservation of the G11–C23 base pair (Larsen at al. 1993). The results of genetic suppression, combined with genetic enhancement of mutations located in the central pseudoknot of 16S rRNA in a strain lacking RbfA, indicate interaction (direct or indirect) between RbfA and the 5′-terminal helix.

Cellular localization of RbfA

The genetic results presented thus far provide evidence for interaction between the RbfA protein and 30S ribosomal subunits. This interaction was tested directly by immunochemical localization. The 9-amino-acid epitope tag, HA (Wilson et al. 1984), recognized by the 12CA5 antibody, was placed at the carboxyl terminus of *rbfA* using oligonucleotide-directed PCR mutagenesis. The *rbfA::HA*-containing PCR fragment was ligated to pSE420, resulting in plasmid p15BHA (Fig. 1B,f). The plasmid-encoded, epitope-tagged, RbfA protein (RbfA–HA) was shown to be functional by demonstrating complete complementation of cold-sensitive CD28 cells containing a disrupted *rbfA* gene (data not shown), in which RbfA–HA is the sole source of functional RbfA protein.

Extracts prepared from CD28 cells grown at 37°C, containing p15BHA, were fractionated by sucrose gradient centrifugation. Proteins from fractions across the gradient were analyzed by gel electrophoresis. RbfA–HA was then localized by Western blot analysis using the anti-HA (12CA5) antibody to the HA epitope tag. RbfA–HA protein was found at the top of the gradient and in fractions containing free 30S subunits, but was undetectable in 50S, 70S, or polysome fractions (Fig. 8). The RbfA–HA/30S interaction was shown to be stable (rather than the result of contamination from the top of the gradient or from pre-30s fractions) by diluting, then pelleting the RbfA–HA-containing 30S peak followed by reisolation

Table 2. *Synthetic enhancement of the CD29 mutant phenotype by 16S rRNA mutants*

A. Viability of transformants

Plasmid name[a]	42°C DH1[b]	42°C CD29[b]
pSTL102	+	+
pU23	+	−
pA11U23	+	+
pA15U23	+	−
pU-5	+	+
pU-5U23	+	−
pA11	+	−
pU-55U23	+	−
pU-55	+	+

B. Growth phenotypes

Plasmid name[a]	42°C DH1[c]	42°C CD29[c]	26°C DH1[c]	26°C CD29[c]
pSTL102	+ + + +	+ + + +	+ + + +	−
pA11U23	+ + +	+	+ + +	−

Amp_{100} plates were used for all experiments.
[a]Plasmids, pU23, pA11U23, pA15U23, and pA11, which contain 16S rRNA mutations; pU-5, pU-5U23, pU-55U23, and pU-55, which contain precursor 16S rRNA mutations; and pSTL102, have been described previously (Triman et al. 1989; Dammel and Noller 1993).
[b](+) Transformants obtained after 12 hr growth; (−) no transformants obtained after 2 days growth.
[c](+ + + +) Full-sized colonies; (+ + +, +) isolated colonies that are less than full size, where + + + is the largest; (−) no detectable growth.

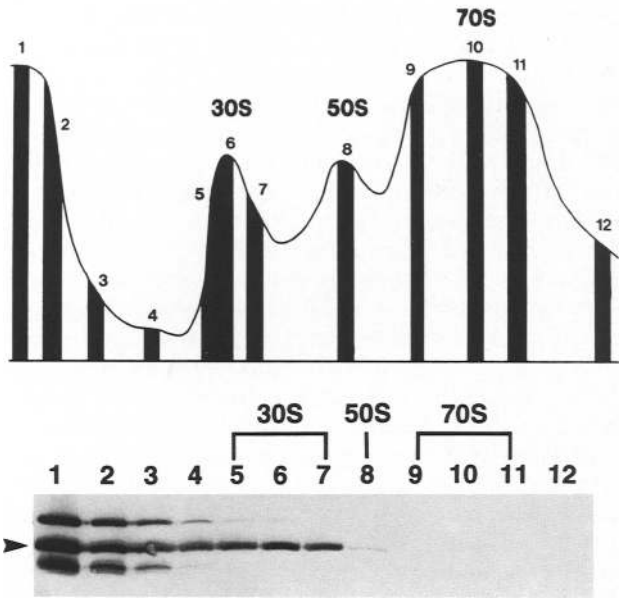

Figure 8. Western blot analysis of sucrose gradient fractions, using the 12CA5 antibody, showing the presence of RbfA–HA protein in free 30S and more slowly sedimenting fractions, but not in 50S or 70S fractions. The fractions probed (1–12) are indicated on the sucrose gradient profile. Equal volumes of gradient fractions were analyzed by 15% SDS/urea gel electrophoresis, transferred to nitrocellulose membrane, and probed using the anti-HA 12CA5 antibody (see Materials and methods). The arrowhead shows the position of RbfA–HA.

on sucrose gradients containing increasing concentrations of NH_4Cl. RbfA–HA was again found associated with 30S subunits, even at 1 M NH_4Cl (data not shown). The large amount of free RbfA–HA seen at the top of the gradient may be attributable to overexpression. Possible cross-reactivity of the 12CA5 antibody with nontagged cellular protein was excluded by Western blot analysis of the equivalent fractions shown in Figure 8, using extracts from nontransformed CSH142 cells. No significant cross-reactivity was observed. Two other 12CA5-reactive bands are evident in the Western blot (not a result of cross reactivity) and are likely to be shorter or longer versions of the RbfA translation product.

Protein composition of free 30S subunits

The protein composition of free 30S subunits from CD28 (cold-sensitive) cells was compared with that from CD28 transformed with p15BHA (phenotypically wild type), using two-dimensional gel electrophoresis (Geyl et al. 1981). The location of the RbfA–HA protein was identified by examining total 30S ribosomal proteins alone and in the presence of excess partially purified RbfA–HA (data not shown). As expected, RbfA–HA was absent in native 30S subunits isolated from CD28 and present in native 30S subunits isolated from CD28 containing p15BHA (Fig. 9A, arrowhead). Estimation of the levels of RbfA–HA by staining with Coomassie blue indicates that RbfA–HA is present at substochiometric amounts relative to 30S ribosomal proteins in free 30S subunits from CD28 + p15BHA. This result was confirmed by semiquantitative Western blotting using the 12CA5 antibody (data not shown). Comparison of the two gel patterns also revealed the presence of an unidentified protein (Fig. 9B, arrowhead), in apparently stoichiometric amounts in free 30S subunits from CD28, but not from CD28 + p15BHA. Interestingly, this unidentified protein also appears in C23U containing free 30S subunits at stoichiometric levels similar to that seen in free 30S subunits from CD28 (data not shown). This protein was not detectable in 70S ribosomes from CD28, CD28 + p15BHA or C23U-containing cells (data not shown). These results indicate the presence of an additional unidentified protein associated with free 30S subunits in cold-sensitive CD28 cells.

Discussion

Identification of a novel 30S-associated factor

We have identified a protein factor, RbfA, that is associated with free 30S ribosomal subunits. Overexpression of this protein completely suppresses the rapidly reversible, cold-sensitive dominant phenotype of the 16S rRNA C23U mutation (Dammel and Noller 1993). Conversely, a gene knockout of *rbfA* in a wild-type rRNA background confers a cold-sensitive phenotype that is strikingly similar to that of the C23U mutation itself and shows that RbfA is required for maximal cell growth.

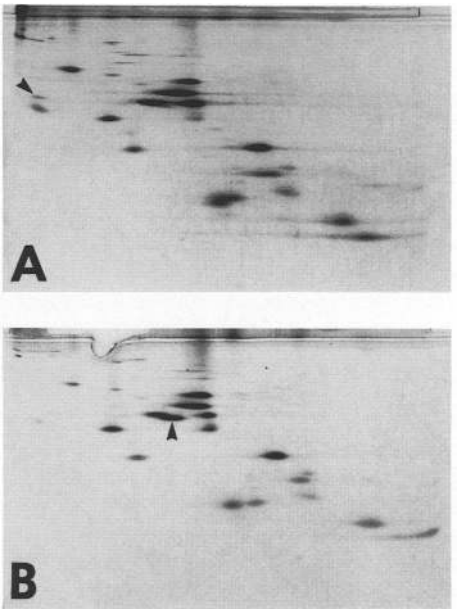

Figure 9. Two-dimensional gels of proteins from free 30S ribosomal subunits from CD28 + p15BHA (*A*) and CD28 (*B*) grown at 37°C. The location of RbfA–HA is shown in *A* (arrowhead). The location of an unknown protein present in CD28 free 30S subunits is shown in *B* (arrowhead).

Immunolocalization studies reveal that this factor interacts with free 30S subunits but not with 30S subunits that are part of 70S ribosomes or polysomes; this finding is consistent with the involvement of RbfA in a late step of 30S maturation, or in translational initiation.

Does RbfA interact with the 5′-terminal helix of 16S rRNA?

Of the 16S rRNA mutants tested, suppression of the C23U allele was by far the strongest. However, other mutations located nearby in the 16S rRNA secondary structure can also be suppressed, albeit to lesser extents (Table 1). This allele specificity of suppression suggests that the RbfA protein interacts with the 5′-terminal helix of 16S rRNA.

Synthetic enhancement experiments are also consistent with interaction of RbfA with the 5′ helix. For instance, a strain lacking RbfA (CD29) cannot be transformed with any plasmid that codes for 16S rRNA, containing a disrupted base pair at positions 11 and 23 of the 5′ mature helix, at what would otherwise be permissive temperatures (e.g., 42°C), in a wild-type background. Furthermore, the degrees of severity of base pair disruptions at 11–23 in a wild-type RbfA background correlate with their effects on cell growth: A11C23 (nearly dead)>G11U23 (cold sensitive)>A11U23 (nearly wild type)>G11–C23 (wild type). The effect on cell growth is magnified in the absence of RbfA: A11C23 (dead)= G11U23 (dead)>A11–U23 (nearly dead)>G11–C23 (cold sensitive). These results support the idea that RbfA requires a stable base pair at this position to function. The phenotype of A11-U23 in a wild-type background is nearly wild type on ampicillin-containing media. However, on spectinomycin-containing media where presumably only plasmid encoded ribosomes are functional, the A11–U23 phenotype is more severe (Dammel and Noller 1993). These results suggest that although base-pairing at this position is of primary importance for cell viability, there does appear to be a preference for the G-C over A-U pair. These results are consistent with our synthetic enhancement experiments, where the nearly wild-type behavior of the 16S rRNA A11U23 double mutant in a wild-type background, compared with its severe phenotype in the RbfA knockout strain implies that RbfA has a specific preference for a G-C base pair at the 11–23 positions.

What is the role of RbfA in translation?

In a previous study (Dammel and Noller 1993) the locations of several intragenic second-site suppressors of the C23U mutation led us to propose that creation of a G11U23 base pair, in place of a G11C23 pair, causes a shift in an equilibrium between the 5′ mature helix of 16S rRNA and a competing helix formed by base-pairing of the upstream precursor sequence with one strand of the mature helix. Our findings showed a correlation between cold sensitivity and the relative stabilities of the precursor versus mature helices; The cold sensitivity conferred by apparent weakening of the mature helix in the C23U mutant was compensated for by destabilization of the precursor helix in the second-site suppressors.

A simple physical interpretation would be that the phenotype is caused by a shift in the equilibrium between the two competing helices in the precursor RNA population and that restoration of the equilibrium suppresses cold sensitivity. At low temperature, the rate of conversion of helix A (precursor helix) into helix B (mature helix) would be rate limiting, and an increase in temperature would accelerate the rate of interconversion of the two forms. However, the rate of conversion of B into A would also be expected to increase at high temperature, and so an increased rate of equilibration in itself would not account for relief of cold sensitivity at elevated temperatures. But if the rate of conversion of B to a further assembly product, C, were much faster than the rate of conversion of B to A, rapid recruitment of B to form product C would drive the conversion of A to B forward, thus overcoming the imbalance in the equilibrium caused by the C23U mutation.

The striking similarities between the 16S rRNA C23U and CD28 (*rbfA* knockout) mutant phenotypes, together with the allele-specific suppression of the C23U mutation by RbfA and the synthetic lethal phenotypes that result from combining the *rbfA* knockout with various 16S rRNA alleles, provide evidence for interaction (direct or indirect) between RbfA and the 5′ helix of 16S rRNA. We conclude that the RbfA–30S subunit interaction is somehow perturbed in the C23U mutant as a result of disruption of a single base pair in the 5′ helix. Binding of RbfA to the 30S subunit may act to stabilize the 5′-terminal helix, enabling assembly to procede productively.

Based on this reasoning, two distinct models could account for the observed dominance of the C23U phenotype. In one model, C23U-containing ribosomes (which account for ~70% of cellular ribosomes), would bind and sequester RbfA at the expense of wild-type (chromosomally encoded) ribosomes. Sequestration of RbfA could directly result in the observed dominant phenotype if RbfA were a factor required for a late maturation or initiation event. Overexpression would then supply wild-type ribosomes with the necessary RbfA to overcome their functional impairment. One shortcoming of this model is that wild-type ribosomes (which account for only ~30% of total cellular ribosomes), would have to support the completely wild-type phenotype observed upon overexpression of RbfA in C23U-containing cells. A more serious inconsistency is that if C23U-containing cells sequester RbfA nonproductively, then this model predicts that a complete absence of RbfA in C23U-containing cells should not drastically affect the C23U phenotype. This model is eliminated by the synthetic lethal phenotype conferred by combining the C23U mutation with the RbfA knockout.

A contrasting model, and one that we favor, is that C23U-containing ribosomes are impaired in their ability to bind RbfA. In this case, a functional block would result from sequestration of some limiting factor, other

than RbfA, by C23U-containing 30S subunits. The unidentified protein that we observe in free 30S subunits from CD28 and C23U containing cells, but not from CD28 + p15BHA cells (Fig. 9B), might be a candidate for such a sequestered factor. Increasing the intracellular concentrations of RbfA by overexpression would overcome its weak binding to C23U-containing ribosomes, which then proceed into the translational cycle, thereby releasing the sequestered limiting factor. According to this model, one would predict that a complete absence of RbfA would contribute to a more severe phenotype, consistent with the results of our synthetic lethality experiments.

The finding that RbfA interacts with free 30S subunits but not with 70S ribosomes or polysomes, together with the observation that the C23U and RbfA knockout mutants have increased levels of 30S and 50S subunits and a decrease in polysomes relative to 70S ribosomes, strongly suggests that RbfA acts as a late maturation or initiation factor. The strongest argument in favor of a possible role for RbfA as a late maturation factor comes from genetic evidence indicating that mutations in precursor 16S rRNA suppress the observed C23U phenotype. The dominant phenotype of the C23U mutation could be explained if immature, but partially functional, 30S precursor particles accumulate at the restrictive temperature and compete with normal 30S subunits for a limiting cellular component, as described above. The differences in rate and temperature dependence between in vivo and in vitro assembly of ribosomes has led to the suggestion that assembly factors may assist in the in vivo assembly pathway. To date, both an eIF-4A-like protein, SrmB (Nishi et al. 1988), and the molecular chaperone, DnaK (Alix and Guerin 1993), have been implicated in 30S and 50S ribosomal subunit assembly in *E. coli*. If RbfA is an assembly factor, it could function by binding the 5′ helix, thereby assisting selfassembly by allowing correct assembly to predominate over incorrect assembly.

The distinction between a role for RbfA late in 30S maturation versus translational initiation could be subtle. Evidence implies that final maturation of 30S particles requires their participation in at least some partial reaction of protein synthesis (Schlessinger et al. 1974; Hayes and Vasseur 1976). If immature ribosomes are able to form an initiation complex and must bind mRNA to complete assembly, then an impairment in a step following complex formation (perhaps involving RbfA) would block the movement of mature ribosomes on mRNA, consistent with the dominant phenotype of the C23U mutant. However, we cannot exclude the alternative possibility that RbfA is directly involved in initiation, a hypothesis consistent with its apparent preference for the 5′ mature helix, rather than the precursor helix.

The finding that an inhibitor of translational initiation is able to induce the cold shock response, together with the known inherent cold sensitivity of translational initiation (Friedman et al. 1971; Broeza et al. 1978) has led to the proposal that the ribosome may be the physiological sensor for the induction of the cold shock response in *E. coli* (VanBogelen and Neidhardt 1990; Jones and Inouye 1994). Three known cold shock genes, *infB nusA*, and *pnp* are found within the *metY/rpsO* operon (Jones et al. 1987). The presence of the *rbfA* gene in this same operon raises the possibility that RbfA itself might participate in signalling the cold shock response by binding free 30S subunits at low temperatures.

Materials and methods

Restriction enzymes and T4 DNA ligase were from New England Biolabs. Shrimp alkaline phosphatase and Sequenase were from U.S. Biochemical, and *Taq* DNA polymerase was from Promega. 12CA5 anti-HA antibody was obtained from Boerhinger Mannheim, and the Western blotting detection kit (streptavidin–alkaline phosphatase conjugate as the signal-generating system) was from Amersham. Deoxy- and dideoxynucleotides were from Pharmacia, and radiolabeled nucleotides were from New England Nuclear. Standard procedures for plasmid and genomic DNA isolation, partial *Sau*3A1 DNA digestion, agarose gel electrophoresis, Western blotting, ligations, transformations, and sequencing were performed using standard procedures as described by Ausubel et al. (1987). Standard PCR amplifications were performed as described in Saiki et al. (1988).

The National Center for Biotechnology Information BLAST electronic mail server was used to identify sequences related to *rbfA* in the GenBank, EMBL, PIR, and SWISS-PROT data bases as recently as August 1994, using the BLASTP program.

Plasmids

Amp^r plasmid pU23 containing a C → U change at position 23 of 16S rRNA conferring a cold-sensitive dominant phenotype, as well as plasmids pA11, pU-5U23, pA15U23, pU-55U23, and pG908, are derivatives of wild-type plasmid pSTL102 (Triman et al. 1989) and have been described previously (Dammel and Noller 1993). Plasmid pACYC184 (Chang and Cohen 1978) carries the p15A origin of replication, enabling it to coexist with plasmids that carry the colE1 origin such as pSTL102 and pU23. pACYC184 carries genes encoding Cam^r and Tet^r. Amp^r plasmid pSE420 (Brosius 1989) was designed for protein overexpression and contains the p^{trc} (*trp–lac* fusion) promoter and the *lac* repressor gene, *lac^{Iq}*. Additionally, it contains many useful cloning sites, including a unique *Nco*I site allowing it to be used for the direct expression of genes carrying an *Nco*I site at their start codon. The Cam^r plasmid pMAK705 (Hamilton et al. 1989) is temperature sensitive for replication and specifically designed for homologous recombination-promoted insertion of DNA sequences into the *E. coli* chromosome. The Kan^r plasmid pUC4K (Vieira and Messing 1982) contains a 1440-bp *Pst*I restriction fragment containing the gene encoding Kan^r.

Plasmid constructions

An *E. coli* genomic library was constructed in pACYC184. *E. coli* genomic DNA was partially digested with *Sau*3AI and bound to glass powder for recovery using standard procedures (Geneclean Glassmilk, Bio 101, Inc.). This step should select against DNA fragments of 200 bp or smaller as these fragments do not efficiently elute from glass powder. Genomic fragments ranging in size from ~200 to 9000 bp were then isolated by excising the appropriate region on a 1% agarose gel. DNA was again isolated by binding to glass powder. The *E. coli Sau*3AI fragments were ligated into pACYC184 that had been digested

with *Bam*HI, which cuts in the Tet^r gene, and treated with phosphatase using standard procedures. This ligation mixture was then used to transform DHI cells. Cam^r transformants were scraped from Cam_{50} plates, resuspended in Luria broth and used to inoculate 50 ml of Luria broth–Cam_{50} cultures that were grown for ~3 hr (to early log) at 37°C. Cells were harvested and the isolated plasmid DNA was used as a source for the *Sau* AI library.

Plasmid p15B-3 was generated by PCR amplification of the *rbfA* gene, including a putative promoter region, from CSH142 genomic DNA using primers 2 (5′-GGC-GAT-GGG-ATC-CAA-GT-3′) and 4 (5′-CGA-GGA-GGA-TCC-ATT-AGT-C-3′) (Fig. 1B,b). Both primers included an engineered *Bam*HI (GGATCC) site to facilitate cloning. *Bam*HI-digested PCR products were cloned into the Tet gene of pACYC184 as described above. A clone was chosen in which the *rbfA* gene was oriented in the same direction as the Tet gene.

Plasmid p15B was generated by PCR amplification of the *rbfA* gene from CSH142 genomic DNA. Primers 3 (5′-GAA-TTT-ACC-ATG-GCG-AAA-G-3′) and 4 were used for PCR amplification (Fig. 1B,e). Primer 3 contains an engineered in-frame *Nco*I (CCATGGC) site at its *rbfA* ATG start codon. An *Nco*I- and *Bam*HI-digested PCR product was ligated to *Nco*/*Bam*HI-digested pSE420. Even in the absence of IPTG, genes cloned into pSE420 are expressed at low levels. As discussed later, this low level of RbfA expression from p15B is enough to complement the cold-sensitive C23U and CD28 mutant phenotypes discussed in this paper.

Plasmid p15BHA was generated by PCR amplification of the *rbfA* gene from genomic DNA (CSH142 source) using primers 3 and 5 (5′-GCT-CGA-GGA-TCC-TTA-CTA-GCT-CGA-AGC-GTA-ATC-TGG-AAC-ATC-ATA-TGG-GTA-GCT-CGA-GTC-CTC-CTT-GCT-GTC-GTC-CG-3′). Primer 5 includes codons for double translational stops, and for the amino acids SSAYDPVDYPYSS, including the 9-amino-acid epitope tag HA (Wilson et al. 1984), derived from the influenza hemagglutinin protein and recognized by monoclonal antibody 12CA5. A *Bam*HI site is also included downstream of the stop codons. As with p15B, *Nco*I- and *Bam*HI-digested *rbfA–HA* containing PCR product was cloned into *Nco*I- and *Bam*HI-digested pSE420. The function of HA-tagged *rbfA* (RbfA–HA) was assessed by its ability to completely complement the cold-sensitive phenotype of a strain in which the *rbfA* gene is disrupted (CD28; see materials and methods).

Plasmid pI15B::Kan^r was generated by PCR amplification using primers 1 (5′-GAT-CAT-CGG-ATC-CGG-CGT-AGG-3′) and 4 (Fig. 1B,c and d) both including engineered *Bam*HI sites. *Bam*HI-digested PCR product was ligated to *Bam*HI digested pMAK705. The resulting clone, pI15B was then digested with *Pst*I, which cuts once in the *rbfA*-coding region, and a 1.4-kb *Pst*I fragment containing the gene encoding Kan^r (Vieira and Messing 1982) was inserted by ligation. The resulting plasmid pI15B::Kan^r contains an insertion mutation disrupting the open reading frame corresponding to *rbfA*.

Strains

All physiological experiments were performed with Luria broth media containing ampicillin at a concentration of 100 mg/liter (Amp_{100}) for solid media and 50 mg/liter for liquid culture, kanamycin at a concentration of 40 mg/liter (Kan_{40}), chloramphenicol at a concentration of 50 mg/liter (Cm_{50}), and chloramphenicol/ampicillin at a concentration of 40 mg/liter and 60 mg/liter, respectively ($Cam_{40}Amp_{60}$).

E. coli strains: DH1(*recA*$^-$) used as host for plasmid constructions, CSH142 (Miller 1992) parent strain to cold-sensitive strain CD28 (CSH142:*rbfA::kan*, this paper), and CD29 [CSH142:*rbfA::kan*(*recA*$^-$), this paper]. Strains MM122 and MM123 (Ferro-Novick et al. 1984), EE583 (Lee and Beckwith 1986), and AJ120 (gift from A. Jacq, unpubl.) are all cold sensitive. Protease-deficient strain W3110 (ATCC 39936) was used as host for overexpression of RbfA and RbfA–HA proteins.

Plasmid p15B::kan was used to replace the chromosomal copy of *rbfA* in CSH142 by homologous recombination with the plasmid-encoded *rbfA::kan* sequences in strain CSH142 as described by Hamilton et al. (1989). Kan^r recombinants CSH142:*rbfA::kan* (CD28) were confirmed by PCR analysis of their chromosomal DNA using primers specific for *rbfA* (primers 3 and 4). PCR product derived from recombinant genomic *rbfA::kan* DNA is larger (2040 bp) than wild-type *rbfA* (600 bp) because of insertion of the 1440-bp Kan^r gene. The PCR product derived from resolved plasmid is the size of wild-type *rbfA* (600 bp) as expected, attributable to the resolution of genomic wild-type *rbfA* with the pMAK plasmid. A *recA*$^-$ version of CD28 [CD29; CSH142:*rbfA::kan*^{r}(*recA*$^-$)] was constructed using interrupted mating as described by Miller (1992). Hfr *recA1* donor MS367 (*thi-1 drm3 recA1 relA1* λ$^-$ *srl*::Tn*10*) was a generous gift from Stanley Brown (National Cancer Institute, Frederick, MD).

Protein purification and analysis

Ribosomal proteins were prepared for two-dimensional gel electrophoresis by concentrating ribosome-containing fractions from sucrose gradients using centricon 3 units from Amicon, which have a molecular weight cutoff of 3000. Sucrose-containing buffer was exchanged for buffer containing 20 mM Tris-HCl, 10 mM $MgCl_2$, and 100 mM NH_4Cl, and ribosomal proteins were extracted and precipitated as described in Siegmann and Thomas (1987). Two-dimensional gel electrophoresis was performed as described in Geyl et al. (1981).

Partially purified RbfA and RbfA–HA proteins were obtained by overexpressing the proteins using IPTG induction in W3110 containing either p15B (RbfA) or p15BHA (RbfA–HA), as described by Amann et al. (1988). Cells were lysed using freeze–thaw lysis and cellular debris was cleared by spinning extracts in a SS34 rotor at 6000 rpm for 30 min. Supernatants were then cleared of ribosomes by spinning in a Ti-50 rotor at 40,000 rpm for 4 hr. The resulting supernate was dialyzed against buffer 1 containing 6 M urea, 20 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, and 6 mM β-mercaptoethanol overnight. The dialyzed supernate was then loaded onto a phosphocellulose column prepared in dialysis buffer and washed extensively with the same buffer. A salt gradient from 25 mM NaCl to 1 M NaCl in 6 M urea, 20 mM Tris-HCl, was passed over the column. The desired proteins eluted at low salt concentrations (~40 mM NaCl). RbfA- and RbfA–HA-containing fractions were dialyzed overnight against four changes of buffer containing 20 mM Tris-HCl and 300 mM KCl. Purified proteins were analyzed by SDS and two-dimensional electrophoresis. The difference in migration of RbfA–HA compared with RbfA was consistent with both its larger size (146 vs. 133 amino acids), and its increased acidity attributable to the presence of two additional aspartic acid residues in the HA epitope tag.

Preparation of ribosomes

The preparation of ribosomes was essentially as described in Dammel and Noller (1993). To prepare ribosomal particles from

DH1 containing pSTL102, pU23 or pU23 + p15B-3 and CSH142, CD28 or CD28 + p15BHA, Luria broth cultures, containing appropriate selective drugs, if necessary, were grown at 37°C (an intermediate temperature, where most defects are apparent in cold-sensitive mutants) to an A_{650} of 0.4. Hygromycin B (p15B-3 is Cam^r) was added to DH1 containing pSTL102, pU23, or pU23 + p15B-3 cultures at a concentration of 0.5 mg/ml during the last 5 min of growth to stabilize polysomes, for the sedimentation profiles shown in Figure 4. Ribosomal particles from CD28 and CSH142 were obtained from Luria broth cultures grown at 30°C or 42°C to an A_{650} of 0.4 followed by addition of chloramphenicol to 0.1 mg/ml for stabilization of polysomes (Brow and Noller 1983). Cultures were chilled rapidly, followed by freeze–thaw lysis of cell pellets in buffer 1 containing 20 mM Tris-HCl at pH 7.6, 10 mM $MgCl_2$, 100 mM NH_4Cl_2, and 6 mM β-mercaptoethanol. Ribosomal particles were isolated by sucrose gradient centrifugation as described by Powers and Noller (1990) except that buffer 1 was used in place of referenced buffer components.

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