The Deleterious Effect of an Insertion Sequence Removing the Last Twenty Percent of the Essential \textit{Escherichia coli} \textit{rpsA} Gene Is Due to mRNA Destabilization, Not Protein Truncation\textsuperscript{\textcopyright}

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Ribosomal protein S1, the product of the essential \textit{rpsA} gene, consists of six imperfect repeats of the same motif. Besides playing a critical role in translation initiation on most mRNAs, S1 also specifically autoregulates the translation of its own messenger. \textit{ssyF29} is a viable \textit{rpsA} allele that carries an IS10R insertion within the coding sequence, resulting in a protein lacking the last motif (S1\textsuperscript{AC}). The growth of \textit{ssyF29} cells is slower than that of wild-type cells. Moreover, translation of a reporter \textit{rpsA-lacZ} fusion is specifically stimulated, suggesting that the last motif is necessary for autoregulation. However, in \textit{ssyF29} cells the \textit{rpsA} mRNA is also strongly destabilized; this destabilization, by causing S1\textsuperscript{AC} shortage, might also explain the observed slow-growth and autoregulation defect. To fix this ambiguity, we have introduced an early stop codon in the \textit{rpsA} chromosomal gene, resulting in the synthesis of the S1\textsuperscript{AC} protein without an IS10R insertion (\textit{rpsA}AC\textsuperscript{C} allele). \textit{rpsA}AC\textsuperscript{C} cells grow much faster than their \textit{ssyF29} counterparts; moreover, in these cells S1 autoregulation and mRNA stability are normal. In vitro, the S1\textsuperscript{AC} protein binds mRNAs (including its own) almost as avidly as wild-type S1. These results demonstrate that the last S1 motif is dispensable for translation and autoregulation: the defects seen with \textit{ssyF29} cells reflect an IS10R-mediated destabilization of the \textit{rpsA} mRNA, probably due to facilitated exonucleolytic degradation.

In \textit{Escherichia coli}, the expression of many ribosomal proteins (r-proteins) is regulated at the translational level (25, 50). Most r-proteins are encoded by polycistronic operons and, according to classical models, one of them acts as a repressor that, when present in excess over rRNA, binds the polycistronic mRNA. In turn, this binding represses not only the translation of its own downstream cistron, but also that of other cistrons, via translational coupling or reorganization. In several cases, an obvious similarity has been found between the binding sites of the repressor on mRNA and rRNA, suggesting that repression simply exploits the same highly specific RNA-protein interactions that are used during ribosome assembly (25, 50).

Ribosomal protein S1—the largest r-protein—consists of six imperfect repeats of the S1 motif, an RNA-binding module found in many procaryotic and eucaryotic proteins involved in RNA metabolism (6, 36, 37, 42) (Fig. 1A). The corresponding gene (\textit{rpsA}) is well conserved in the bacterial kingdom, but curiously S1 is not found associated with the ribosome in bacilli (24), and it is dispensable for growth in these organisms (34). On the contrary, in \textit{E. coli}, r-protein S1 is essential for protein synthesis and for viability (33). Moreover, it is toxic when overexpressed (8), so that its expression must be tightly regulated. Indeed, S1 was shown to autoregulate its own synthesis at the translational level (3, 32). However, it differs from other r-protein repressors in an essential point: S1 is the only known r-protein that interacts tightly with mRNAs (36). The mRNA-binding capacity of S1, which is mediated by its four last repeats, is used to bind the translation initiation region (TIR) of mRNAs during translation initiation (29, 36, 43). Consistent with the lack of sequence conservation among TIRs, S1 binds RNA in vitro with relaxed specificity (36, 37). However, it must be able to recognize specifically its own TIR to achieve autoregulation (3, 32).

The features of the \textit{rpsA} TIR that make it sensitive to S1 autoregulation have been studied (3, 4, 41; S. Hermann-Le Denmat, unpublished data). This TIR lacks a Shine-Dalgarno (SD) element (31), and yet it is extremely efficient. Maximum translational efficiency requires an unusual long sequence (i.e., from ~91 to +15 with respect to the translational start) that folds into a complex, phylogenetically conserved, structure (4, 41). Chemical and enzymatic probing, as well as band-shift and toeprint assays, have suggested that several molecules of S1 bind cooperatively to the A/U-rich single-stranded regions within the \textit{rpsA} TIR (4). Insofar as this TIR lacks a typical SD element (4, 31), its interaction with 30S subunit may be particularly dependent upon ribosome-bound S1 (43). Autoregulation might then simply reflect the fact that ribosome-bound S1 is readily competed out by the cooperative binding of several free S1 molecules (4). The \textit{ssyF29} allele (30; hereafter referred to as \textit{ssyF}), is one of the few viable \textit{rpsA} mutants known. It bears an IS10R insertion toward the end of the \textit{rpsA} gene, resulting in the synthesis of a truncated S1 protein (S1\textsuperscript{AC}) that lacks most of the last S1 motif (3) (Fig. 1B). Although slow-growing, \textit{ssyF} cells are viable, showing that the S1\textsuperscript{AC} r-protein is functional for protein synthesis. Comparison of the translational activities of a variety of TIRs in wild-type and \textit{ssyF} strains confirmed that the last S1 motif plays no specific role for the recognition of individual
From these results, the authors concluded that the last S1 motif is required for autoregulation but dispensable for translation initiation. However, the rpsA mRNA is also strongly destabilized in ssyF cells (3; the present study). By causing S1\textsuperscript{AC} shortage, this destabilization might be indirectly responsible for the observed slow growth and rpsA deregulation in the ssyF mutant, a possibility that was considered but not ruled out (3). We present here experiments designed to fix this ambiguity and explain the ssyF-mediated destabilization of the rpsA mRNA.

MATERIALS AND METHODS

**Bacterial strains.** Strain DH5\textsuperscript{a} [supE44 ΔlacU169 (80dgalUZAM15) supE44 recA1 endA1 gyrA96 thi-1 relA1] was used for plasmid construction and propagation. To quantify the effect of various rpsA alleles on the translational efficiency of the rpsA TIR, we used derivatives of strain HfrG6 that carry an rpsA-lacZ translational fusion on their chromosome (3, 12). Briefly, the small chromosomal region encoding the genuine lacZ TIR (nt -16 to +21); in all numberings, the first base of the start codon is taken as +1) has been replaced by fragments encoding either the rpsA TIR (nt -91 to +57) or, as a control, the unrelated gacE L TIR (nt -32 to +30) (3, 12) (Fig. 2A). These fusions were initially constructed in a strain carrying the wild-type rpsA\textsuperscript{AC} allele (3). The ssyF or rpsA\textsuperscript{AC} mutations were introduced into these strains by P1 transduction, using a nearby tetracycline resistance marker (3; the present study).

Using a one-step inactivation procedure (9), a srrA::kan derivative of MG1655 was obtained and kindly provided to us by Pru levei (Biomedical Center, Uppsala, Sweden). The srrA::kan allele was transferred to DH5\textsuperscript{a} derivatives by selecting kanamycin resistance. Transductants were checked by PCR.

**Construction of the rpsA\textsuperscript{AC} mutant strain.** The rpsA\textsuperscript{AC} mutation was first introduced into plasmid pSP261 (pS1), a derivative of pACYC184 (pCtr) that contains the rpsA gene under the control of its own promoter (21). To this end, the 466th and 467th codons of the pS1-borne rpsA gene were changed to TAA stop codons (Fig. 1B) using DpnI mutagenesis (Stratagene) and the following complementary oligonucleotides: 5'-gtt gac gct aaa ggc TAa TAa gta gaa ctg gct 3' and 5'-gtg tgc acg ctc tac tta TTA gct ttt ggc ccc-3' (substituted nucleotides are indicated in uppercase). After verification by sequencing, the rpsA\textsuperscript{AC} allele was transferred from this plasmid (pS1\textsuperscript{AC}) to the genome of E. coli by P1 transduction, using a nearby tetracycline resistance marker.

**Purification of the S1 and S1\textsuperscript{AC} proteins and Western blot analyses.** Wild-type and truncated S1-protein were expressed from plasmids pET-rpsA\textsuperscript{AC}-6His (47) and pET-rpsA\textsuperscript{AC}-6His (the present study). The latter was constructed by replacing the NdeI-XhoI fragment of pET-rpsA\textsuperscript{AC}-6His by a fragment of pS1\textsuperscript{AC} amplifed with oligonucleotides 5'-tAAcTTTcAAgagGAGGATATcAtAATGACtGA AtcTTTTTtgCTCAcAC-3' and 5'-GtGtGtGtGGTGGGCtGACggGcCtcTtg 3'. Purification of the rpsA\textsuperscript{AC} protein was performed by overnight dialysis against 10 mM Tris-HCl (pH 7.4), 50 mM NH\textsubscript{4}Cl, 0.1 mM EDTA, and 7 mM β-mercaptoethanol concentrated on Centricon TIRs (3). A remarkable exception, however, was the rpsA TIR itself, whose activity was markedly stimulated in ssyF cells (3). From these results, the authors concluded that the last S1 motif is required for autoregulation but dispensable for translation initiation. However, the rpsA mRNA is also strongly destabilized in ssyF cells (3; the present study). By causing S1\textsuperscript{AC} shortage, this destabilization might be indirectly responsible for the observed slow growth and rpsA deregulation in the ssyF mutant, a possibility that was considered but not ruled out (3). We present here experiments designed to fix this ambiguity and explain the ssyF-mediated destabilization of the rpsA mRNA.

**FIG. 1.** Description of the ssyF and rpsA\textsuperscript{AC} alleles. (A) Schematic representation of ribosomal protein S1 (557 amino acids [aa]). S1 motifs are symbolized with closed boxes, and the numbers refer to the boundaries of the six motifs according to (6). The S1 motifs implicated in ribosome and mRNA binding are shown in gray and black, respectively. (B) Schematic representation of the ssyF and rpsA\textsuperscript{AC} alleles. Open boxes represent the wild-type rpsA\textsuperscript{AC} open reading frame. The initiation codon (ATG) and the genuine termination codon (TAA at +1671) are indicated. The IS1\textsubscript{OR} element present in the ssyF allele creates a premature termination codon (tga) at the insertion point (nucleotides from rpsA and IS1\textsubscript{OR} are shown in upper- and lowercase, respectively). Note that the IS1\textsubscript{OR} orientation is such that the integrase and rpsA genes are in opposite directions (3). Codons 466 (GCA) and 467 (ACC) that are both changed to the stop codon TAA in the rpsA\textsuperscript{AC} allele are indicated. The ssyF and rpsA\textsuperscript{AC} alleles are predicted to encode the same truncated protein S1\textsuperscript{AC} (465 aa). (C) Western blot analysis of 1 μg of total protein extracts from the indicated strains grown in rich medium at 37°C. Using a mixture of anti-S1 and anti-PNPase antibodies, the full-length (S1) and truncated protein (S1\textsuperscript{AC}) are visualized together with PNPase; the latter is used here as a control for equivalent loading. The molecular weight of each polypeptide is indicated in brackets. Note that the abundance of the S1\textsuperscript{AC} protein appears equivalent in ssyF and in rpsA\textsuperscript{AC} cells, in spite of the alleged shortage of this protein in ssyF cells: this paradox is explained in the Discussion.
Galactosidase activities from the indicated TIRs, in wild-type (rpsA of the S1 or S1 lacZ representation of the chromosome from the E. coli VOL. 189, 2007 LAST S1 MOTIF IS NOT REQUIRED FOR S1 FUNCTION 6207 pS1 the presence of the plasmids pCtr, pS1, or pS1/H18554 rpsA any respectively, are represented in light gray to emphasize the fact that they addition, in panel B, the (hatched and closed boxes) are shown, along with the wild-type (rpsA/C plasmids expressing full-length and truncated S1 proteins, re- /H11001 ssyF and genes used. The pS1 or rpsA/C proteins in a final volume of 10 µl, chilled on ice, and separated on a 6% native polyacrylamide gel at 4°C. Gels were dried and quantified with a FLA3000 Phos- /H9252 hoFluoImager (Fuji), using the Image Gauge V4.2 software (Fuji). β-Galactosidase assays. Cells were grown in LB medium containing IPTG (isopropyl-β-D-thiogalactopyranoside; 500 µM), as well as chloramphenicol (20 µg/ml) when plasmids pCtr, pS1, or pS1/C were present (Fig. 2C). β-Galactosi- dase activity was assayed as described previously (3). Each measurement was done at least in triplicate.

Northern blot analyses. Cells were grown in LB medium and harvested at an optical density at 600 nm of 0.5 to 0.7. Total RNA was extracted with the hot phenol procedure as described previously (49). RNA (5 µg) was separated on a 0.8% Tris-borate-EDTA-agarose gel and blotted on nylon N + (Amersham), and the membrane was stained with methylene blue (in 0.3 M sodium acetate [pH 5.2]) to visualize 23S and 16S rRNAs. Hybridization was then performed at 65°C under sealed conditions (the T7 RNA polymerase promoter is interrupted by an IS10 element in the 3′-end of the rpsA gene), resulting in the introduction of a translational start codon at positions +1396 to +1401 (Fig. 1B, see Materials and Methods), resulting in the interruption of the rpsA open

RESULTS
The last S1 motif of r-protein S1 is dispensable for fast growth and for rpsA autoregulation. We have previously described the construction of a series of E. coli strains in which the chromosomal region of the genuine lacZ TIR has been replaced by DNA fragments encompassing TIRs from other genes (Fig. 2A) (12). In these strains, the lac promoter-operator sequence and most of the lacZ sequence are identical, so strain-to-strain differences in β-galactosidase expression reflect essentially the variable efficiencies of the inserted TIRs. The efficiency of the rpsA TIR is particularly high in this assay, i.e., >3-fold that of the genuine lacZ TIR when the rpsA gene is wild type; moreover, in the presence of the ssyF allele, this efficiency is further stimulated 2.5-fold, whereas that of other TIRs is not (3). This behavior is illustrated in Fig. 2B, where the rpsA TIR is compared to another very efficient TIR, that of the galE gene. As mentioned above, this specific stimulation reflects a relief of rpsA autoregulation in ssyF cells (3).

The ssyF mutation consists in an IS10 insertion that interrupts the rpsA coding sequence at codon 465, resulting in a truncated protein (S1/C; Fig. 1B and C). This mutation also markedly decreases the growth rate (Fig. 3). To determine whether truncation of the r-protein S1 is responsible for this slow growth and for the relief of rpsA autoregulation, we muta-
reading frame at the same position as in the ssyF allele but without IS insertion (\(rpsA^{AC}\) allele; Fig. 1B). As expected, \(rpsA^{AC}\) cells produced the same truncated S1\(^{AC}\) protein as ssyF cells (Fig. 1C). However, the growth defect of \(rpsA^{AC}\) cells was far less severe than that of ssyF cells (doubling times at 37°C were 36 and 60 min, respectively, versus 32 min for wild-type cells; Fig. 3). Moreover, in contrast to the ssyF allele, the \(rpsA^{AC}\) allele did not stimulate the activity of the \(rpsA\) TIR relatively to the \(rpsA^{+}\) allele. As expected, the activity of the noncognate \(galE\) TIR was also similar in \(rpsA^{+}\) and \(rpsA^{AC}\) cells (Fig. 2B).

Altogether, these results indicate that the absence of the last S1 motif is not responsible for the relief of \(rpsA\) autoregulation in ssyF cells or for the slow growth of these cells.

The S1 and S1\(^{AC}\) proteins behave similarly for repressing the activity of the \(rpsA\) TIR in vivo and for binding RNA in vitro. Plasmid pSP261 is a pACYC184 derivative carrying the \(rpsA\) operon (21). Hereafter, it is referred to as pS1, whereas pACYC184 is named pCtr for control (Fig. 2A). In \(rpsA^{+}\) or ssyF cells, the activity of \(rpsA\) TIR is specifically and markedly repressed by the presence of pS1, reflecting the autoregulation of the \(rpsA\) gene (3) (Fig. 2C). To further demonstrate that the last S1 motif is not required for autoregulation, we constructed a pS1 derivative, named pS1\(^{AC}\), in which the wild-type \(rpsA\) gene was replaced by the \(rpsA^{AC}\) allele (see Materials and Methods). In \(rpsA^{+}\) cells, the presence of the pS1\(^{AC}\) plasmid repressed the activity of the \(rpsA\) TIR 15-fold, much as with the pS1 plasmid itself (compare the open bars marked pCtr, pS1, and pS1\(^{AC}\) in Fig. 2C). In ssyF cells, the repression factor brought by pS1 or pS1\(^{AC}\) was even larger (50-fold), reflecting the genuine relief of \(rpsA\) autoregulation in these cells, but again repression was equivalent for the two plasmids (Fig. 2C, closed bars). In contrast, in \(rpsA^{+}\) or ssyF cells, neither pS1 nor pS1\(^{AC}\) had any effect on activity of noncognate TIRs such as the \(galE\) TIR (data not shown). Thus, in spite of the absence of the last S1 motif, the truncated S1\(^{AC}\) protein is equivalent to full-length S1 for repressing the translational activity of its own TIR.

The RNA-binding properties of the S1 and S1\(^{AC}\) proteins were then compared in vitro by electrophoretic mobility shift assays, using purified proteins carrying a His tag at their C-terminal ends (see Materials and Methods). As RNA fragments, we used in vitro transcripts of similar size encompassing the \(rpsA\) TIR or, as a control, the noncognate \(galE\) TIR (Fig. 4). It is noteworthy that for low protein concentrations the \(rpsA\) TIR RNA bound S1 less avidly than the \(galE\) TIR RNA (Fig. 4B; compare the percentage of complexed RNA for 100 nM S1). However, it responded more steeply to increasing S1 concentrations so that at 150 nM the binding was equivalent for both RNAs (Fig. 4B). Although the fragments we used extend beyond the \(rpsA\) and \(galE\) TIRs and may contain additional S1-binding sites, we regard this effect as supportive of a cooperative binding of S1 to the \(rpsA\) TIR but not to the noncognate \(galE\) TIR (see the introduction) (4). The truncated protein S1\(^{AC}\) bound both RNA molecules much like wild-type S1 (Fig. 4A), except that somewhat higher protein concentrations (~1.5-fold) were required to produce half-maximal binding (Fig. 4B). Thus, the loss of the last S1 motif did not strongly affect the RNA-binding properties of the S1 r-protein.

It is worth noting that the concentration of S1 or S1\(^{AC}\) required to saturate the \(rpsA\) TIR falls in the same range as for other repressor r-proteins and that it is similar to the concentration of 30S ribosomal subunits needed to saturate most TIRs (11). How r-protein repressors with a relatively modest affinity for their targets can efficiently challenge ribosome was the subject of several elaborated thermodynamic and kinetic models (see reference 28 and references therein).

The IS10R element present in the ssyF allele is responsible for the destabilization of \(rpsA\) mRNA. It was previously observed that the steady-state level of the \(rpsA\) mRNA is at least fivefold lower in ssyF cells than in \(rpsA^{+}\) cells (3). Since the IS10R insertion in the ssyF allele is located far downstream from the promoter, this lower steady-state level is likely to reflect mRNA destabilization rather than reduced transcription initiation. This destabilization, in turn, may be due to the IS10R insertion per se, or it may be related to the premature interruption of translation (Fig. 1B). To settle this point, we
compared the \( rpsA \) mRNA levels in \( ssyF \), \( rpsA^{\text{AC}} \), and \( rpsA^+ \) cells by using Northern blots. The probe used corresponds to the first five S1 motifs, i.e., to a region that is identical in three \( rpsA \) alleles. A well-defined \( rpsA \) mRNA species \( \sim 2,200 \text{ nt} \) in length was detected in \( rpsA^+ \) and \( rpsA^{\text{AC}} \) cells, whereas no such signal was observed in \( ssyF \) cells (Fig. 5). In the latter case, a smear of lower-molecular-weight RNA species, presumably corresponding to degradation intermediates, was detected on longer exposures (data not shown, see below). Normalization with the 23S and 16S rRNAs showed that the signals observed with \( rpsA^{\text{AC}} \) and \( rpsA^+ \) cells were indistinguishable, indicating that the presence of early stop codons had little or no effect on the stability of the \( rpsA \) mRNA. We conclude that the IS10R element is specifically responsible for the very low level of \( rpsA \) mRNA in \( ssyF \) cells.

One possibility is that the presence of the IS10R element results in an mRNA with an unprotected 3’ end, which could be sensitive to rapid 3’→5’ exonucleolytic degradation (see Discussion). This mode of degradation eventually produces decay intermediates that lack a stop codon and are thus targets for the \( ssrA \)-mediated \textit{trans}-translation system (1, 46). \textit{Trans}-Translation directs the resulting truncated polypeptides to degradation; conversely, in \( ssrA \) deficient strains, such truncated

![FIG. 4. Binding of purified S1 and S1^{\text{AC}} proteins to RNA, as assayed by mobility shift. (A) A 160-nt RNA fragment encompassing the \textit{galE} TIR (left) or a 157-nt RNA fragment encompassing the \textit{rpsA} TIR (right) was used as a probe (see Materials and Methods). The positions of free and protein-bound RNA are indicated. –, No protein. The concentrations of purified S1 or S1^{\text{AC}} proteins used are 50, 100, 150, 200, and 250 nM. (B) Quantification of the gels shown in panel A. Signals corresponding to free RNA and protein-RNA complexes were quantified with a FLA3000 imager (Fuji). Curves represent the percentages of the complexed RNA for the indicated concentrations of S1 (black diamonds) or S1^{\text{AC}} (gray triangles).](image-url)

![FIG. 5. Northern blots showing the steady-state level of \( rpsA \) mRNA in wild-type (\( rpsA^+ \)), \( ssyF \), and \( rpsA^{\text{AC}} \) cells. The strains used are the same as in Fig. 2B (\( rpsA^{\text{TIR}} \)). At the top is shown membrane hybridization with a \( ^{32}\text{P} \)-random-labeled \( rpsA \) specific probe (see Materials and Methods). At the bottom is shown methylene blue staining of the 23S and 16S rRNAs after blotting. The asterisk indicates the position of the \( rpsA \) mRNA signal.](image-url)
polypeptides can be stabilized and become detectable by Western blot analyses (1). To examine this possibility for the rpsA gene, we interrupted the ssrA gene from rpsA+ and ssyF cells with a kanamycin resistance cassette (ssrA::kan) tmRNA were analyzed by Western blotting with anti-S1 antibodies. The inactivation of the ssrA::kan strain are shown by arrows, and their estimated molecular masses are indicated in kilodaltons. The positions of size standards are shown on the left.

FIG. 6. Western blot analysis of truncated S1 polypeptides derived from rpsA+ and ssyF alleles. Equivalent amount of total proteins extracted from rpsA+ and ssyF strains containing (ssrA+) or lacking (ssrA::kan) tmRNA were analyzed by Western blotting with anti-S1 antibodies. Position of the full-length S1 and S1ΔC proteins are indicated (see Fig. 1C). Shorter S1 polypeptides detected in the ssyF::kan strain are shown by arrows, and their estimated molecular masses are indicated in kilodaltons. The positions of size standards are shown on the left.

DISCUSSION

The rpsA gene is essential to E. coli, presumably because its product, r-protein S1, is required for translation of most or all cellular mRNAs (33). Among the few viable rpsA mutants known, the ssyF mutant has been particularly characterized (3).

The ssyF mutation, which markedly depresses growth rate, consists of an IS10R insertion at the beginning of the last S1 motif, resulting in the synthesis of a truncated protein (S1ΔC). The mutation has no differential effect on the activity of individual TIRs, except for the rpsA TIR: the latter is strongly stimulated, due to the relief of the autoregulation that normally controls rpsA translation (3). By creating an rpsA allele (rpsAΔ10) that produces the same truncated protein as ssyF but without IS10R insertion, we show here that the autoregulation defect observed in ssyF cells owes nothing to the truncation of the S1 protein, as originally proposed (3). Moreover, most of the growth defect of ssyF cells is unrelated to S1 truncation. Both phenotypes actually reflect the presence of the IS10R element per se. This element results in a drastic reduction of the rpsA mRNA level (>5-fold) that, in turn, leads to the derepression of the rpsA autoregulation loop in order to meet the S1 demand. However, under these conditions, the efficiency of the rpsA TIR increases only 2.5-fold (Fig. 2B), indicating that although presumably maximal, derepression cannot match the drop in mRNA level so that the rate of S1 synthesis must be reduced. Shortage of this essential r-protein will then limit protein synthesis and growth. Thus, the reduction of mRNA level can explain both the autoregulation relief and the growth defect of ssyF cells. Incidentally, these considerations also explain the paradoxical observation that on Western blots, the S1ΔC protein appears as abundant in ssyF as in rpsAΔC cells in spite of its alleged reduced synthesis (Fig. 1C). Indeed, since S1 is essential for protein synthesis, its shortage shows itself by a decreased growth rate (Fig. 3), rather than as a decrease in its abundance versus that of total proteins.

The two main results of our study—the dispensability of the last S1 motif and the negative effect of IS10R insertion on rpsA mRNA level—are discussed separately.

What is the role of the last S1 motif? In vitro, removal of the last motif hardly affects the RNA binding properties of S1 (Fig. 4). In vivo, it does not compromise its function in protein synthesis, since the growth rate of rpsAΔC cells is only modestly reduced compared to that of wild-type rpsA cells (Fig. 3). Finally, this removal does not affect the specific interaction between S1 and its cognate TIR, since autoregulation is normal in rpsAΔC cells (Fig. 2). The first two observations are consistent with previous data showing that after removal of the last motif, S1 was still 75% functional for protein synthesis in vitro (38) and that even after removal of the last two motifs it could still bind a variety of RNAs (40). However, the six-motif organization of S1 is phylogenetically conserved in many gammaproteobacteria. It should be noted that beyond its role in translation initiation and in autoregulation, E. coli r-protein S1 presumably plays a variety of other functions in vivo. Thus, besides its well-documented role in the life cycle of several bacteriophages (5, 16, 26, 44, 45), it may also participate in important cellular processes, e.g., transcription termination (19), transcript release from RNA polymerase (39), or mRNA decay (13). In addition, the r-protein S1 specifically binds the transfer-mRNA (tmRNA, encoded by ssrA), even if its importance for trans-translation in vivo is still controversial (22, 27; see also below). Thus, it is possible that—in contrast to protein synthesis—one of these functions specifically requires the last S1 motif. This possibility is illustrated by in vitro observations on the stimulation of RegB, an endoribonuclease from bacte-
riophage T4, by protein S1 (2). Depending on the substrate cleaved by RegB, the last S1 motif is or is not dispensable for stimulation. The authors of that study considered the possibility that all of the four last S1 motifs contribute to the RNA binding site: some RNA targets would interact proficiently with a subset of these motifs, whereas others would require all of them (2). However, the possibility that isolated or combined S1 motifs differ in their substrate specificity remains largely uncharacterized.

**IS10-mediated mRNA decay.** The presence of an IS10/IR element within the ssY allele results in an almost complete disappearance of the rps4 mRNA (Fig. 5). This striking effect is not unprecedented: Yano and Yura have reported that the insertion of an IS10-like element in the 3’ untranslated region of the rpsO gene—encoding r-protein S15—causes a drastic reduction of the rpsO mRNA level and S15 synthesis (48). Although these effects mirror those observed with the ssyF allele, it is worth noting that in the case of the rpsO gene, the IS10 is inserted in the opposite direction, i.e., the rpsO and integrase genes are in the same orientation (see the legend of Fig. 1). Because in both cases the IS10 element is inserted far away from the promoter of the interrupted genes, the decrease in mRNA level is likely to reflect mRNA destabilization rather than reduced promoter activity. What can be the mechanism of this destabilization?

Whereas most mRNAs are stabilized by dedicated hairpins at their 3’ end (14, 35), the rpsA4 transcripts that would cross the lengthy integrase gene in the antisense direction should be sensitive to endonuclease attack or to Rho-dependent termination, as often observed with untranslated sequences (23, 49). The same may hold with the IS10-interrupted rpsO gene, because even if transcribed in the sense direction the transposase gene is very inefficiently translated (15). Such a situation would result in an unnatural 3’ end, favoring rapid mRNA degradation by 3’-5’ exonucleases. Consistent with this idea, the abolition of trans-translation in ssyF cells reveals the existence of polypeptides smaller than S112C (Fig. 6). trans-Translation relieves bacterial ribosome stalling at the end of mRNAs devoid of a stop codon (46). During this process, the tmRNA acts initially as a tRNA and then as an mRNA to rescue the ribosome and target the incomplete protein for degradation. In the absence of tmRNA, untagged polypeptides can be stabilized, and their detection becomes indicative of the presence of translatable truncated mRNAs, such as intermediates in 3’- to 5’ exonucleolytic degradation (1, 46). The degradation pathway of the genuine rpsA mRNA is unknown. However, the fact that in the absence of ssrA truncated polypeptides accumulate in ssyF but not in rpsA4 cells (Fig. 6) suggests that 3’-5’ degradation is specific to the IS10-interrupted mRNA. The nuclease(s) involved, which are presumably not unique (7), have not yet been identified. The observation of truncated polypeptides of discrete sizes (Fig. 6) suggests that these nucleases (and/or the ribosomes) may locally stall on the ssyF mRNA during degradation. However, we cannot formally exclude that these polypeptides are simply more efficiently recognized by the anti-S1 antibodies. It is interesting that the inactivation of ssrA does not rescue the growth defect of the ssyF cells (not shown), indicating that the polypeptides shorter than S112C that accumulate under these conditions are not functional for protein synthesis. Finally, the absence of these shorter polypeptides in ssyF ssrA cells (Fig. 6) strongly suggests that trans-translation is efficient in these cells, and thus the last S1 motif cannot be important for trans-translation in vivo. Consistently, this motif is not required for an efficient in vitro binding of S1 to tmRNA (18).

**Concluding remarks.** Whatever its exact mechanism, the IS10-mediated mRNA destabilization appears particularly efficient: for instance, the rpsA446 mutation fails to produce a similar effect (Fig. 5), even if it generates an additional 300 nt of naked mRNA sequence after the early stop codons (see Fig. 1B). This strong destabilization may have functional significance. Although in the case of the ssyF allele the IS10/IR insertion does not inactivate the product of the gene, in many other cases gene interruption by a mobile element results in a non-functional truncated polypeptide. Such products can be particularly toxic by blocking the machineries in which the normal protein participates, and mRNA destabilization may have evolved to protect the host against such “dominant-negative” effects of mobile element insertion. These elements are known to possess also sophisticated tools for protecting themselves against spurious host transcription which, if uncontrolled, would be harmful to the host by boosting transposition (10, 15). All of these mechanisms presumably contribute to the subtle compromise between transposition activity and host viability (20).

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**REFERENCES**


