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Silent mutations in the *Escherichia coli ompA* leader peptide region strongly affect transcription and translation *in vivo*

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ABSTRACT

In order to test the effect of silent mutations on the regulation of gene expression, we monitored several steps of transcription and translation of the *ompA* gene *in vivo*, in which some or all codons between codons 6 and 14, frequently used in *Escherichia coli*, had been exchanged for infrequent synonymous codons. Northern blot analysis revealed an up to 4-fold reduction in the half-life of the mutated messengers and a >10-fold reduction in their steady-state amounts. Western blot analysis showed a 10-fold reduction in the amount of OmpA protein. Use of a system expressing a Rho-specific anti-terminator allowed us to detect a strong transcription polarity effect in the silent mutants. These results demonstrate that silent mutations can severely inhibit several steps of gene expression in *E.coli* and that code degeneracy is efficiently exploited in this species for setting signals for gene control and regulation.

INTRODUCTION

Silent mutations have attracted less attention than they deserve with respect to their effect on overall gene expression. However, it was observed in several species that gene expression levels tend to correlate with the frequency of codon usage and that the latter tends also to correlate with the cellular amount of cognate tRNA (1). It was also observed in *Escherichia coli* that the cell makes optimal usage of its tRNA resources to face the codon demand of its most expressed (100 or so) genes, which produce close to 90% of the proteins present in the cell (2). Similar trends occur in other species as well, in spite of the fact that they use synonymous codons with quite different, sometimes opposite, selection rules.

These correlations led to the statement that a frequent codon is usually translated faster than a less frequent synonymous one, because its cognate tRNA is more abundant (3,4). The codon translation rate is indeed thought to be limited by cognate tRNA availability. It follows that traffic of ribosomes on mRNA could be controlled by codon usage. We propose as a rationale that rows of frequent (or rare) codons would give smooth fast (or slow) traffic of ribosomes on the mRNA. Accordingly, passage from a segment carrying frequent codons to one carrying rare codons would provide a bottleneck to ribosome traffic and could produce jamming, which in turn could reduce the level of gene expression and waste part of the cellular resources invested.

The *ompA* gene of *E.coli* exhibits a strong bias for major codons and is expressed at a high level (3% of total soluble protein). We focus here on the possible effects due to major to minor synonymous codon exchanges near the N-terminus of the *ompA* coding sequence. Introduction of slow codons at the very beginning of the coding sequence should hamper translation start, delay the formation of the next translation complex on the same mRNA and introduce perturbations of ribosome trafficking over the whole mRNA

We report that the exchange of as few as four frequent codons for synonymous infrequent ones strongly reduces the stability and amount of the mRNA *in vivo* and results in a drastic reduction in the amount of OmpA protein produced. We show that at least part of the reduction in mRNA amount is due to a strong transcription polarity associated with premature, Rho-dependent transcription termination. Our results show that the flexibility provided by the degeneracy of the genetic code can be an important means of establishing gene expression levels.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E.coli ompA* gene deleted strain BRE2413 (5) was used as host for plasmids carrying our wild-type and mutant *ompA* genes. The *E.coli* strain RZ1032 (*dut*, *ung*) was used as host to produce single-stranded phagemid DNA for oligonucleotide site-directed mutagenesis (6). Strain M15 (7) served to produce large amounts of a His_6–OmpA fusion protein. Plasmid pAD1 (8) carries the pSC101 and ori f1 replication origin and the entire *ompA* gene. The *ompA* 3′ riboprobe plasmid pTZTB is a pTZ19R derivative, constructed by insertion of the *Taq*I–*Bam*HI fragment from pAD1 into the *Acc*I–*Bam*HI sites of pTZ19R. The *ompA* 5′ riboprobe plasmid pBFA is a pBluescript KSII(+) (Stratagene) derivative, constructed by insertion of the *Fok*I–*Acc*I fragment from pAD1. Plasmid pNL3 (9) carries the *psu* gene (a bacteriophage P4

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transcription anti-termination factor for rho-dependent termination) under control of the λ P_L promoter. pRK2K is a kanamycin resistance plasmid derived from pRK248cIts (10) which carries the thermosensitive λ cI repressor gene. Plasmid pQE-10 (Qiagen) was digested with *Bam*HI, filled-in using Klenow fragment and redigested with *Pst*I. The fragment *Nru*I–*Pst*I from pAD1 (carrying the *ompA* coding sequence from codon 9 to the end) was introduced into the digested pQE-10, under *lac* operator/promoter control. The resulting plasmid pQEOmpA was used to produce a His_6–OmpA fusion protein appropriate for the production of anti-OmpA polyclonal antibodies in mice. pREP4 (Qiagen) carries the *lacI* gene. All the constructions described here were confirmed by dideoxy termination sequencing.

Oligonucleotide site-directed mutagenesis

Oligonucleotide-directed site-specific *in vitro* mutagenesis was performed by the procedure of Kunkel (6). Synthetic oligonucleotide primers were prepared on a Pharmacia Gene Assembler Plus DNA synthesizer (Pharmacia-LKB). Mutants were identified directly by DNA sequencing.

mRNA decay assays

Total RNA was recovered from BRE2413 cells harbouring plasmids pAD1 (or mutated *ompA* forms) grown to mid-logarithmic phase at 37°C (unless stated otherwise) in Luria broth and purified according to Hagen and Young (11). Cells were removed from the culture immediately before and at regular time intervals after addition of rifampicin (0.5 mg/ml), as indicated in the legends to the figures. Northern blotting was performed by standard procedures (12). The probe used for hybridization was an antisense RNA, obtained by transcribing the linearized plasmid pTZTB or pBFA *in vitro* with T7 RNA polymerase. mRNA half-lives as well as steady-state levels of *ompA* full-length mRNA were computed from autoradiogram band intensities.

OmpA protein detection

High amounts of His_6–OmpA fusion protein were obtained by inducing strain M15 (7), harbouring both the pQEOmpA and pREP4 plasmids, with IPTG to a final concentration of 2 mM. After 3 h induction, cells were lysed and a Ni^{2+}–NTA–agarose column was used to recover pure His_6–OmpA fusion protein, following the recommendations of the supplier (Qiagen). Mice were immunized with the fusion protein and high specific activities for anti-OmpA antibodies were obtained (dilution of sera 1:20 000). Western blotting and chemiluminescent detection of the OmpA protein were performed with a goat anti-mouse IgG secondary antibody–alkaline phosphatase conjugate, using CSPD [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.3,7]decan}-4-yl)phenyl phosphate] as the substrate for the alkaline phosphatase (Tropix)

RESULTS

mRNA stability dependence on growth temperature

Since our goal is to analyze the effect of silent mutations on gene expression, we chose to carry out experiments at physiological temperature. We decided therefore to determine the dependence of wild-type *ompA* mRNA stability on temperature, with the further goal to compare our data with those obtained previously at 30°C (13). We first analyzed the stability of wild-type chromosomal *ompA* mRNA of strain HB101. The probe used in these nothern blot experiments was complementary to the 3′-region of the *ompA* mRNA. We found that the stability decreases rapidly with increasing growth temperature (the corresponding northern blots are shown in Fig. 1): the *ompA* mRNA half-life is 15.0 min at 30°C, 9.5 min at 37°C and 4.0 min at 42°C (accuracy ±0.2 min; northern blot analysis at 30 and 42°C not shown). The value measured at 30°C agrees with that reported previously under similar conditions (13).

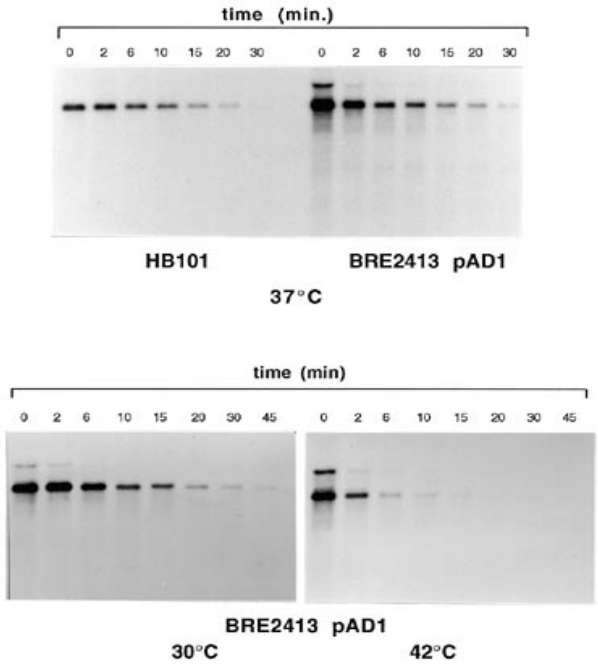

Figure 1. Temperature dependence of the stability of wild-type *ompA* mRNA by northern blot analysis. The time intervals (in min) at which the samples were removed following addition of rifampicin are indicated at the top of the corresponding lanes. The probe used for hybridization is an antisense RNA to the 3′-end of the *ompA* mRNA (Materials and Methods). Two bands are seen in the autoradiograms from experiments using strain BRE2413, in which the *ompA* gene is carried by plasmid pAD1. They correspond to two transcripts of the *ompA* gene, initiated respectively by the *lac* promoter (length 1470 nt) and by the normal *ompA* promoter (length 1220 nt), located 250 bp upstream in our construction (Materials and Methods). Only half-lives of transcripts initiated at the *ompA* promoter were measured, by plotting the band intensities measured by densitometry (Shimadzu CS-930) versus time. All half-lives given in the text are the averages from several independent experiments.

We also measured the stability of the same wild-type *ompA* mRNA, but transcribed from our low copy number plasmid pAD1 in strain BRE2413 (*ompA*$^-$) (Fig. 1). The half-life of *ompA* mRNA is now 7.8 min at 30°C, 4.5 min at 37°C and 1.8 min at 42°C (data accurate to ±0.2 min).

We observe that in both cases the half-life decreases substantially as growth temperature rises. At a given temperature, the stability of chromosomal *ompA* mRNA in HB101 is about twice that of the same mRNA transcribed from the low copy number plasmid in the *ompA*$^-$ strain BRE2413. The temperature dependence is similar in both cases, since the half-life is reduced ~1.6 times from

30 to 37°C, 2.4 times between 37 and 42°C and 4-fold between 30 and 42°C.

Synonymous codon choice affects mRNA stability

Genes *ompA*6–9, *ompA*6–11, *ompA*6–14 and *ompA*10–14 are derived from the wild-type *ompA* gene by silent mutations exchanging major for minor synonymous codons between codons 6 and 9, 6 and 11, 6 and 14 and 10 and 14 of the *ompA* coding sequence (Fig. 2A). These genes were inserted in plasmid pAD1 in place of the wild-type *ompA* gene and expressed in the *ompA*⁻ strain BRE2413 at 37°C. The stability of mRNA from these mutants and wild-type *ompA* genes was analyzed by northern blotting with the 3′ probe (Fig. 2B). The half-life of the wild-type *ompA* mRNA is 4.5 min, the mRNA from *ompA*6–14, 6–11 and 10–14 share a half-life of 1.3 min and that from *ompA*6–9 is 1.8 min (each value is the average from several experiments). The silent exchange of three or four codons at the beginning of the coding sequence and at the indicated places reduces mRNA stability >2- or 3-fold, respectively. Silent mutations at two different places (6–11 and 10–14) introduce the same reduction (within the precision of the measurement). The double mutant altered at both sites of 6–11 and 10–14 (mutant 6–14) does not further reduce the stability.

Synonymous codon choice affects steady-state amount of mRNA

We measured the steady-state amounts of mRNA of the wild-type and silent mutants of the *ompA* gene, transcribed from the pAD1 and derived plasmids in BRE2413 (Fig. 3, top). The probe used in these nothern blot experiments was complementary to the 5′-untranslated region of the *ompA* mRNA. Compared with the amount of wild-type *ompA* mRNA (lane 3), the relative amounts of mRNAs from *ompA*6–14 (lane 4), *ompA*6–9 (lane 5), *ompA*10–14 (lane 6) and *ompA*6–11 (not shown) were reduced to 3, 13, 18 and 16%, respectively. The silent mutations severely reduced the amount of mRNA produced, by >80% for the 6–9, 6–11 and 10–14 mutants and by an additional 80% for the 6–14 mutant. For the three former, the reduction seems independent of the number of codons mutated (three, four or five) and their place. The reduction in the steady-state mRNA levels is much larger than that expected from the reduced mRNA stabilities. Therefore, additional mechanisms should contribute to this effect.

We further analyzed the relative amounts of OmpA proteins in whole protein extracts, by western blotting using an anti-OmpA serum. As expected from the results obtained for the *ompA* mRNAs, OmpA protein levels were reduced in all mutants, as shown in Figure 3 (bottom).

The *ompA*6–14 mutant is subjected to strong transcription polarity

The reduced steady-state level of the mutated mRNAs, and that of *ompA*6–14 in particular, may be the consequence of transcription polarity. We reasoned that the infrequent codons might elicit a decrease in the translation rate at each of these codons. This would uncouple, to some extent, transcription from translation and thus increase the length of mRNA exposed free between RNA polymerase and the first translating ribosome. This in turn could favor premature transcription termination, by factors like Rho for instance (14).

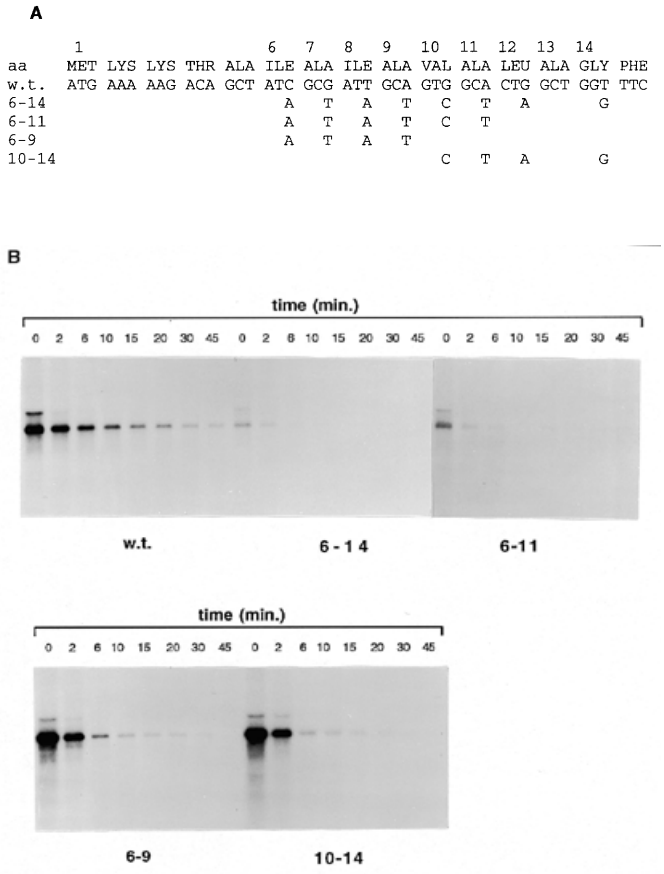

Figure 2. (**A**) Sequences of the wild-type and mutated *ompA* genes. The line (aa) indicates the N-terminal amino acid sequence (1–15) of the *ompA* gene product; the line (w.t.) shows the corresponding coding sequence for the wild-type gene; lines 6–14, 6–11, 6–9 and 10–14 display the silent mutations made in the wild-type gene to produce *ompA*6–14, *ompA*6–11, *ompA*6–9 and *ompA*10–14, respectively. (**B**) Northern blot autoradiogram used to measure mRNA half-life. Wild-type (w.t.), 6–14, 6–11, 6–9 and 10–14 correspond to the decay of *ompA*, *ompA*6–14, *ompA*6–11, *ompA*6–9 and *ompA*10–14, respectively. The 6–9 and 10–14 decay experiment was overexposed to show the differences in decay compared with the wild-type.

We selected the *ompA*6–14 gene to explore this possibility. Rho-dependent transcription termination is opposed by the anti-terminator factor Psu, the product of the *psu* gene of bacteriophage P4 (9). It was suggested that Psu acts by binding to Rho, not to mRNA (9). BRE2413 cells were co-transformed with plasmids pNL3 (bearing the *psu* gene under control of the λ P_L promoter/cI repressor system), pRK2K (which expresses the thermosensitive cI repressor) and pAD1 (or pAD6–14). Cells growing exponentially at 30°C (absence of Psu) were shifted to 42°C (synthesis of Psu) and the amounts of wild-type *ompA* and *ompA*6–14 mRNAs were measured at 10 min intervals following temperature shift. Measurements were performed by northern blotting, using a probe complementary to the 3′-end of the mRNA. Following a 2.5-fold drop, after 10 min the amount of wild-type mRNA steadily increased to the level prior to induction of *psu* (Fig. 4). This drop at 10 min in wild-type mRNA could be due to a very fast response in diminishing mRNA stability when the temperature is shifted from 30 to 42°C. After that, the increase in transcription initiation and elongation due to the temperature shift could re-establish normal steady-state levels (as from 20 min).

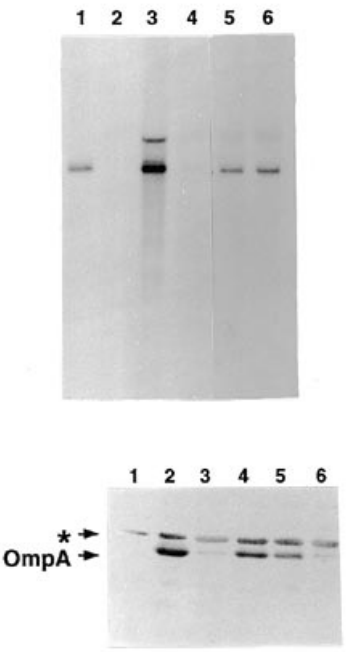

Figure 3. Steady-state amounts of mRNA measured by northern blot analysis. A labeled antisense RNA probe, complementary to the 5′-unstranlated region of *ompA* mRNA (probe FA) (Materials and Methods), was hybridized to equal amounts of total RNA from HB101 (lane 1), BRE2413 without any plasmid (lane 2), BRE2413 pAD1 (lane 3), BRE2413 pAD6–14 (lane 4), BRE2413 pAD6–9 (lane 5) and BRE2413 pAD10–14 (lane 6). At the bottom, a western blot (chemiluminiscent) displays the amount of OmpA protein present in equal amonts of total proteins recovered from: an *ompA*⁻ strain (BRE2413) not transformed with plasmid pAD1 (lane 1); BRE2413 transformed with plasmid pAD1, which carries the wild-type *ompA* gene (lane 2); with plasmid pAD6–14 (lane 3); with plasmid pAD6–9 (lane 4); with plasmid pAD10–14 (lane 5); with plasmid pAD6–11 (lane 6). *, a protein band present even in the *ompA*⁻ strain (lane 1), corresponding probably to another outer membrane protein presenting homologies to OmpA.

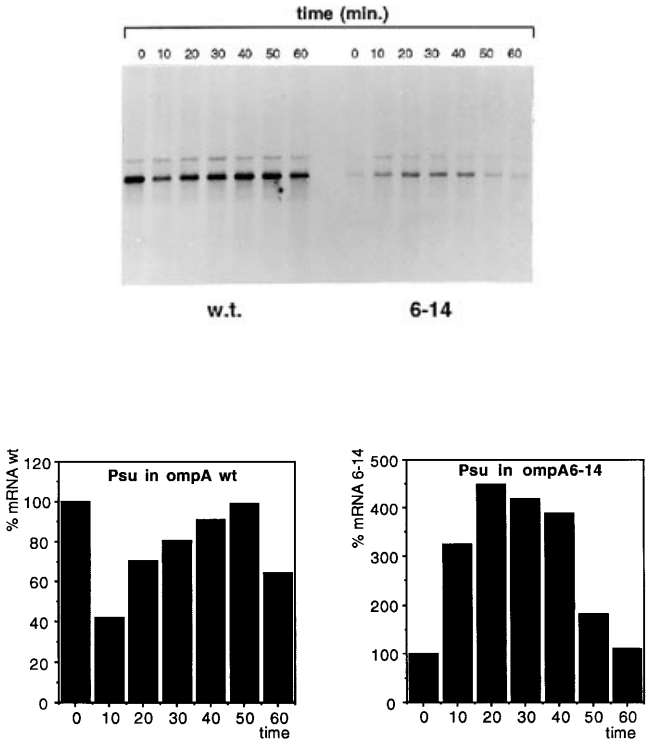

Figure 4. Effect of Psu (Rho factor anti-terminator) on the amount of mRNA. (**Top**) Northern blot autoradiogram of mRNA hybridized to an RNA probe complementary to the 3′-end of the *ompA* mRNA. Samples were removed at the time intervals (min) indicated at the top of the lanes following induction of expression of Psu (temperature shift from 30 to 42°C at time 0). w.t., *ompA* mRNA; 6–14, *ompA*6–14 mRNA. (**Bottom**) Plot of the amount of *ompA* mRNA, wild-type and 6–14 versus time (min). In both cases, the initial amount (time 0), taken as 100%, was measured just before the shift to 42°C. At time 0 the amount of the 6–14 mRNA was only 5% that of the wild-type mRNA. Values are means of two different experiments and for each lane they do not diverge by >15% from each other.

Instead of the initial decrease observed for the wild-type mRNA, the amount of *ompA*6–14 mRNA increases >3-fold during the first 10 min, levels off at 20 min (almost 5-fold increase), then decreases. Psu activity is known to be lethal to the cell (9), which may explain the decrease in mRNA levels in the 6–14 mutant observed from 20 min. The initial decrease (at 10 min) as observed for wild-type mRNA was not observed for *ompA*6–14 mRNA levels. One explanation could be that in spite of the different mRNA stabilities between wild-type and 6–14 mutant, the sudden suppression of polarity could produce immediate accumulation of *ompA*6–14 mRNA. The major conclusion is that, compared with wild-type, the *ompA* 6–14 mRNA is subject to a strong transcription polarity.

DISCUSSION

Synonymous codon selection is a potent determinant of *ompA* gene expression levels

The results presented here show that exchange of few frequent codons for synonymous infrequent ones in the *ompA* leader region strongly reduces the amounts of *ompA* protein and mRNA *in vivo*, lowers by several-fold the mRNA stability, and results in copious Rho-mediated transcription polarity. We showed elsewhere (8,15) that the same conclusion holds if silent mutations are made inside the coding sequence, although the effects are less dramatic. We conclude that in the examples studied, selection of synonymous codons contributes greatly to control and regulation of gene expression, at several crucial steps.

Can this conclusion be generalized?

The observations made with the *ompA* gene can be consistently understood as consequences of changes in ribosome traffic, to which individual codons contribute depending on codon-specific translation rates and their relative positions in the coding sequence. A previous work showed that premature Stop codons, which eliminate downstream ribosome traffic, cause rapid degradation of *ompA* mRNA (16). Other authors (17) observed that depriving *lacZ* transcript of the ribosomes required for translation makes the mRNA vulnerable to RNase E cleavage.

The effects of synonymous codon translation rates on gene expression observed in this report are not restricted to the *ompA* gene. A previous work of Rosenberg *et al*. (18) demonstrated that

insertion of rare AGG Arg codons in tandem in a test gene produced inhibitory effects on translation, depending on the location of these tandem inserts, the 5′-region being the most affected. One would expect, in particular, the effect of synonymous codon selection on gene expression to be larger for codons in the initially translated gene sequence, as these control ribosome trafficking over the whole mRNA. Here we explored the effect of synonymous exchange for minor codons. We have not yet analyzed the effects of opposite exchanges, but one should probably not expect exchange of minor for major synonymous codons, made at random, to systematically enhance gene expression. Indeed, it is only in the case that these silent mutations could ease ribosome trafficking throughout the coding sequence that enhanced expression would be expected. Ribosome traffic jamming should be considered as a critical factor in biotechnological applications, for instance when trying to express heterologous genes in *E.coli*. Ways to design smooth and fast ribosome traffic using synonymous codon selection are presented elsewhere (2; J.Solomovici and C.Reiss, manuscript in preparation).

How can silent mutations affect nucleases and Rho activities?

The results presented in this report provide at least a qualitative understanding of how RNases or Rho can achieve the effects observed. The smallest known RNase (RNase M, 26 kDa; 19), if assumed to be spherical, would have a diameter equivalent to a stretched mRNA sequence of ~25–30 nt or 8–10 codons. The relaxed trafficking on mRNA of silently mutated genes would increase both the average size of the free sequence and the time of base exposure between consecutive ribosomes. The same would hold for the average size and time of exposure of the mRNA sequence free between RNA polymerase and the leading ribosome. Indeed, a sequence of at least 70–80 nt would be required to accomodate the Rho factor on the mRNA (20). The mutated mRNA would therefore be an easily accessible substrate for all kinds of RNases as well as Rho.

What makes *ompA* mRNA stable?

For wild-type mRNA it was shown that RNase E cleaves at places in the 5′-untranslated region which might involve mRNA secondary structures (21–24). The mutations introduced between codons 6 and 14 do not affect these secondary structures. Furthermore, they do not affect the accessibility of the Shine-Dalgarno sequence or the initiation codon, which could in turn lower translation initiation events. The optimal secondary structures were computed for the wild-type and mutated mRNAs using the GCG package (25). This suggests that the wild-type as well as the silently mutated *ompA* mRNAs bear many sites for nuclease cleavage in their coding sequence, which are, however, efficiently hidden by ribosomes in the case of the wild-type mRNA. Control of wild-type mRNA stability would then be left primarily ‘by default’ to RNase E activity in the 5′-untranslated region.

Transcriptional polarity is the main factor responsible for the reduced expression of silently mutated genes

The steady-state amount of mRNA is a function of three independent parameters: transcription rate, transcription polarity and mRNA stability. The former, set mainly by the initiation rate, can be assumed to be identical for all genes tested. Our findings suggest that, compared with wild-type *ompA*, loose transcription–translation coupling on the *ompA*6–14 mRNA allows Rho to force premature termination of nine out of 10 transcripts, and loose ribosome spacing gives RNases (in addition to RNase E) the opportunity to reduce by 3-fold the stability of mature molecules.

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