Role of the AGA/AGG codons, the rarest codons in global gene expression in *Escherichia coli*

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AGA and AGG codons for arginine are the least used codons in *Escherichia coli*. Previous findings have shown that these codons are used preferentially within the first 25 codons in *E. coli* genes. More than 100 genes having a single AGA/AGG codon within the first 25 codons were identified to be associated with various essential cellular functions. The *lacZ* gene, containing 5 AGG codons after the tenth codon from the initiation codon, was constructed as a model system. The production of β -galactosidase was inhibited almost completely during the stationary phase, whereas the production of the control β -galactosidase without AGG codons was not. The inhibitory effect by the 5 AGG codons was substantially suppressed either by coexpressing the *argU* gene for tRNA^{Arg}_{UCU/CCU} or by moving the 5 AGG codons by >50 codons away from the initiation codon. In addition, the production of a number of proteins resolved by two-dimensional gel electrophoresis was enhanced significantly during the stationary phase in the cells harboring a plasmid containing *argU*. At least one of them was identified as the *hns* product encoded by an ORF having an AGA codon at the nineteenth position. On the basis of these results, it is proposed that the expression of a group of essential genes for various cellular functions that have a single AGA/AGG codon very close to the initiation codon are globally regulated by the availability of the least abundant tRNA^{Arg}_{UCU/CCU}. A model for this regulation is proposed.

[Key Words: Codon usage; ribosome; translation growth rate; tRNA]

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In various organisms, some synonymous codons are used more frequently than others. Such nonrandom usage of synonymous codons has been well documented and shown to correlate with the relative quantities of individual tRNAs (Ikemura 1981a,b; Maruyama et al. 1986). It has been suggested that genes encoding abundant protein species selectively use the "optimal codon" or major codons as determined by the abundance of isoaccepting tRNA (Ikemura 1981a). However, it has not been established whether the codon usage determines the rate of protein synthesis or whether the rate of protein synthesis under normal growth conditions is simply determined by the rate of translation initiation and not affected by different rates of translation at different codons (Sørensen and Pedersen 1989; Sørensen et al. 1990; for review, see Anderson and Kurland 1990).

To elucidate the role of minor codons in gene expression in *Escherichia coli*, we had previously examined the distribution of minor codons within a gene and found that minor codons such as AGA, AGG, CUA, UCA, AGU, ACA, GGA, CCC, and AUA are used preferentially within the first 25 codons (Chen and Inouye 1990). Among the genes containing a single AGA/AGG codon, the least used codon in *E. coli* within the first 25 codons, we found a number of key genes essential for various

cellular functions, such as genes for single-stranded DNA-binding protein, ribosomal S10 protein, some tRNA synthetases, adenylate cyclase, LexA, integration host factor (IHF), SecY, FtsA, and ribonuclease P (Inouye and Chen 1990). On the basis of these analyses, we proposed that the rate of translation at the minor codons decreases as a result of limited availability of charged tRNAs for the minor codons when cell growth slows down. This causes ribosome stalling at a minor codon. If such a translational pause is located closer to the initiation codon, then a queue of ribosomes may form after the minor codon, thereby inhibiting the effective entry of a ribosome at the initiation site.

This hypothesis, the minor codon modulator hypothesis, may explain the preferential localization of minor codons within the first 25 codons in *E. coli* genes, which may globally regulate the expression of a number of key genes and by cellular functions under growth rate-limiting conditions (Inouye and Chen 1990). We have experimentally explored the validity of this hypothesis (Chen and Inouye 1990). Five AGG codons were inserted after the tenth codon downstream of the initiation codon of the *lacZ* gene. In this case, production of β -galactosidase was ceased completely after a mid-log phase of cell growth; in contrast, the *lacZ* gene with five CGU

codons, a major codon for arginine, was expressed during this growth phase. However, β -galactosidase production increased linearly, almost to the level of the control β -galactosidase, as a function of increased distance between the site of the AGG codons and the initiation codon (Chen and Inouye 1990). This result is consistent with the minor codon modulator hypothesis.

In this paper we first reexamined the distribution of the AGA/AGG codons in *E. coli* genes using a much larger data bank—3540 polypeptides in contrast to the earlier 678 polypeptides (Chen and Inouye 1990)—and confirmed the previous conclusion concerning the preferential AGA/AGG usage within the first 25 codons. Then, to prove unambiguously the role of the minor codons, we measured the synthesis of β -galactosidases from various genes with and without the AGG codons by [35 S]methionine incorporation. We demonstrate that the inhibitory effect of the five AGG codons in the *lacZ* gene during the stationary phase can be suppressed either by overproduction of tRNA for the minor codon or by moving the AGG site away from the initiation codon. In addition, the production of a number of proteins was enhanced significantly during the stationary phase in the cells with a plasmid harboring the gene for tRNA^{Arg}_{UCU/CCU}. These results strongly support the minor codon modulator hypothesis and the important role of minor codons in global regulation of cellular functions in *E. coli*.

It has been shown from the analysis of the codon adaptation index of *E. coli* genes that codon usage bias is weaker at the start of the gene than elsewhere (Bulmer 1988; Eyre-Walker and Bulmer 1993). This tendency is particularly significant for those genes that are highly expressed. Although these results are consistent with our conclusion (Chen and Inouye 1990; this paper), these investigators proposed that preferential minor codon usage near the start of the gene is to avoid mRNA secondary structures around the initiation codon but not to play a role in gene regulation, as we have proposed earlier (Chen and Inouye 1990; Inouye and Chen 1990). On the basis of our previous and present experimental results, we argue that their hypothesis is unlikely and that minor codons play more active roles in the regulation of gene expression.

Results

Location-dependent usage of the AGA/AGG codon in E. coli genes

Previously, we have examined 678 polypeptides of *E. coli* available in GenBank at that time for the usage of AGA/AGG codons (Chen and Inouye 1990). Since then, sequences of many more polypeptides have been determined. In this study we reexamined the usage of AGA/AGG codons using 3540 polypeptides currently available in the data base (GenBank, Release 80; EMBL, Release 37, December 1993). Of these, 2105 have no AGA/AGG codons. In the remaining 1435 polypeptides, 1363 are larger than 50 residues. Of the 1363 polypeptides, 620

contain only one AGA or AGG codon and 743 contain more than one AGA/AGG codon. As shown previously (Chen and Inouye 1990), the frequency of (AGA + AGG) codon usage for every 25 codons from the initiation codon up to the 250th codon was calculated in two different ways. First, the frequency of the AGA/AGG usage was expressed as a fraction of the total arginine codons (CGU, CGC, CGA, CGG, AGA, and AGG) within a unit of 25 codons as shown by stippled bars in Figure 1. This fraction is defined as (n/N) , where n is the total number of (AGA + AGG) codons within a unit of 25 amino acid residues and N is the total number of arginine codons within the same unit. Second, the frequency of the AGA/AGG usage for each 25 codons was calculated as a fraction of the total AGA/AGG codons used within the first 250 codons from the initiation codon. These frequencies are expressed with hatched bars in Figure 1.

As shown in Figure 1A, by both stippled and hatched bars, AGA and AGG codons are used more frequently within the first 25 codons. This high frequency within the first 25 codons becomes much more significant when those proteins that have only one AGA or AGG codon are analyzed, as shown in Figure 1B. The minor codon is used three to four times more frequently within the first 25 codons than any other parts of the polypeptides. Of 620 polypeptides containing only one AGA or AGG codon, 129 (20.8%) have the minor codon within the first 25 codons. In contrast to these polypeptides, those that have more than one AGA/AGG codon were found to contain the minor codons quite randomly throughout the entire polypeptide (Fig. 1C). This is particularly evident with the stippled bars. These results confirmed our previous conclusions, obtained from a smaller sample size; whenever the AGA/AGG codon is used only once in an *E. coli* gene, it is preferentially used within the first 25 codons from the initiation codon (Chen and Inouye 1990; Inouye and Chen 1990). All genes having a single AGA/AGG codon within the first 25 codons are classified by their functions and listed in Table 1, where the total number of amino acid residues for each protein, the total number of arginine residues used in the protein, and the position of the single AGA/AGG codon from the amino-terminal end are indicated. The analysis of Table 1 will be discussed later, in the Discussion, on the basis of our previous hypothesis designated Minor codon modulator hypothesis (Inouye and Chen 1990).

Suppression of the inhibitory effect of five AGG codons in lacZ by tRNA overproduction

Previously, we have shown that the insertion of five AGG codons at the eleventh codon substantially reduced the production of β -galactosidase during the stationary growth phase (Chen and Inouye 1990). *E. coli* SB4288 cells harboring pIT-lacI or pIT-lacI-AGG5 grew almost identically as shown in Figure 2A. In both cases, cell growth stopped after 5 hr and very little increase in the optical density was observed during the next 17.5 hr of incubation. However, during the stationary phase, the β -galactosidase activity steadily increased to ~95,000

Figure 1. Frequency of AGA and AGG codons usage in *E. coli*. The positions of AGA/AGG codons in 2557 different *E. coli* genes available in GenBank (Release 80), EMBL (Release 37), (December 1993) were analyzed as described previously (Chen and Inouye 1990). Of 3540 polypeptides encoded by these loci, 2105 proteins lack both AGA and AGG codons. Among the remaining 1435, 1363 polypeptides consist of >50 amino acid residues, and of these 1363 polypeptides (A), 620 have either a single AGA or AGG codon (B); and the other 743 have more than one AGA/AGG codon (C). Each group of proteins was analyzed for the frequency of (AGA+AGG) codon usage for every 25 amino acid residues from the amino-terminal ends. The frequencies indicated by stippled bars represent those defined as (n/N) , where n is the total number of (AGA+AGG) codons within a unit of 25 codons and N is the total number of arginine codons within the same unit. The frequencies indicated by hatched bars represent those calculated as a fraction of the total AGA/AGG codons used within the first 250 codons.

units in the case of the cells harboring pIT-lacI. On the other hand, the cells harboring pIT-lacI-AGG5 were un-

able to produce β -galactosidase at all during the stationary phase. However, cells harboring the construct pIT-lacI-CGU5, in which five AGG codons on pIT-lacI-AGG5 were substituted with five CGU codons (a major codon for arginine) (Chen and Inouye 1990), became capable of producing the enzyme again during the stationary phase (Fig. 2B).

Previously, the dramatic effect of the insertion of five AGG codons at the eleventh codon of the *lacZ* gene was postulated to arise from the severe limitation of tRNA for the AGG codon, which causes ribosomes to stall at the minor codons (Inouye and Chen 1990). A queue of stalling ribosomes was proposed to form nearby the initiation codon so that the entry of a new ribosome is blocked. On the basis of this model, one can predict that this blockage can be eliminated by overproducing the minor tRNA. This is the case as shown in Figure 2B: Whereas *lacZ*-AGG5 expression was inhibited almost completely in the cells with pIT-lacI-AGG5, recovery from this inhibition was obtained using pIT-lacI-AGG5 [(*lpp*^P)*argU*-AGA], which contains the *argU* gene in conjunction with *lacZ*-AGG5. The *argU* gene decodes tRNA^{Arg}_{UCU} (Garcia et al. 1986; see Fig. 3A), expressed under the constitutive *lpp* promoter. Cells carrying pIT-lacI-AGG5 [(*lpp*^P)*argU*-AGA] (Fig. 3B) grew as well as control cells (Fig. 2A), and the production of β -galactosidase was found to continue during the stationary phase, although the rate of the production was approximately one third of that with pIT-lacI-CGU5 (Fig. 2B). As a control, pIT-lacI-CGU5 [(*lpp*^P)*argU*-AGA] was constructed. It was found that the *argU* gene had no effect on the expression of the *lacZ* gene with five CGU codons in any growth phases (data not shown).

Biosynthesis of β -galactosidase

To demonstrate unambiguously that β -galactosidase was synthesized de novo during the stationary phase by the overproduction of tRNA^{Arg}_{UCU}, cells were grown at 37°C to the stationary phase. Figure 4 shows the protein patterns of cells at 22 hr after the addition of 2 mM IPTG. β -Galactosidase from *lacZ*-AGG5 is barely detected in the cells carrying pIT-lacI-AGG5 (lane 1), whereas its level increased substantially when the *argU* gene was cloned into the same plasmid (lane 2). Notably, we also confirmed our previous observation (lane 3) that the inhibitory effect of the five-AGG insertion in the *lacZ* gene was suppressed simply by adding linkers between the initiation codon and the AGG insertion site (Chen and Inouye 1990). The *lacZ* gene used in lane 3 contained eight linkers, each of which consisted of 18 nucleotides in size (equivalent to 6 amino acid residues). Therefore, a total of 48 extra amino acid residues were added to β -galactosidase between the initiator methionine and the consecutive arginine residues. Note that as a result of the insertion, the β -galactosidase band migrates a little slower in the gel, indicating that there is no abnormal translational event at the five-AGG site (lane 3). Importantly, the insertion of the linkers resulted in the pro-

Table 1. Genes containing only one AGA or AGG codon that exists within the first 25 codons

Name of protein	Gene	Total amino acid residues	Total arginine residues	Position of AGG/A ^a
A. DNA synthesis and repair				
Gyrase A	<i>gyrA</i>	875	66	6 (AGA)
Inhibitor of <i>oriC</i> replication	<i>iciA</i>	298	22	7 (AGA)
DNA mismatch repair protein	<i>mutS</i>	854	66	19 (AGG)
RadC	<i>radC</i>	100	9	15 (AGG)
Holliday junction-specific endonuclease	<i>ruvC</i>	247	14	11 (AGA)
Single-stranded DNA-binding protein	<i>ssb</i>	179	10	4 (AGA)
B. Protein synthesis				
FRR	<i>frr</i>	186	17	6 (AGA)
Glycyl-tRNA synthetase α -subunit	<i>glyS</i>	304	16	7 (AGG)
Phenylalanyl-tRNA synthetase β -subunit	<i>pheT</i>	332	24	2 (AGG)
Peptide chain release factor 2	<i>rf2X</i>	366	23	22 (AGG)
Ribonuclease P-protein component	<i>rnpA</i>	120	18	8 (AGG)
σ -Factor	<i>rpoN</i>	164	10	16 (AGG)
Ribosomal protein S11	<i>rpsK</i>	129	14	13 (AGA)
Ribosomal protein S10	<i>rpsJ</i>	103	12	5 (AGA)
Ribosomal protein S4	<i>rpsD</i>	206	22	3 (AGA)
Protein export protein PrlA	<i>rplN</i>	444	21	22 (AGA)
Ribosomal protein L34	<i>rpmH</i>	72	10	8 (AGG)
Ribosomal protein	<i>rpsfri</i>	104	6	13 (AGG)
Ribosome releasing factor	<i>rrfX</i>	186	17	6 (AGA)
C. Regulation/cell division				
Ams protein	<i>ams</i>	1026	101	3 (AGA)
Crl gene product	<i>crl</i>	134	9	11 (AGA)
Adenylate cyclase	<i>cyaA</i>	849	56	11 (AGA)
FtsA protein	<i>ftsA</i>	421	22	7 (AGA)
FtsE protein	<i>ftsE</i>	222	18	15 (AGA)
IHF	<i>himD</i>	95	8	9 (AGA)
Inhibitor of chromosome initiation	<i>iciA</i>	298	22	7 (AGA)
LclR repressor protein	<i>iclR</i>	275	16	11 (AGA)
Repressor of the <i>aceBAK</i> operon	<i>iclRA</i>	288	16	24 (AGA)
SOS function regulatory protein	<i>lexA</i>	203	15	7 (AGG)
Multiple antibiotic resistance protein	<i>marA</i>	130	10	5 (AGA)
Mannitol repressor	<i>mtlR</i>	196	11	14 (AGA)
Multicopy suppressor	<i>mysA</i>	138	11	19 (AGA)
DNA-binding protein OsmZ	<i>osmZ</i>	138	11	19 (AGA)
ParC protein	<i>parc</i>	98	9	13 (AGG)
Plasmid copy number protein	<i>pcnB</i>	411	40	3 (AGG)
PhoR protein	<i>phoRG</i>	432	37	9 (AGG)
Attenuator	<i>pnd</i>	51	4	25 (AGG)
RcsF gene product	<i>rscF</i>	134	8	21 (AGA)
ATP:GTP 3'-pyrophosphotransferase	<i>relA</i>	745	55	5 (AGA)
SpoT protein	<i>spoT</i>	230	15	15 (AGG)
DNA-binding protein	<i>uvra</i>	59	8	2 (AGA)
D. Metabolic enzymes and others				
Fimbriae 987p subunit precursor	<i>987P</i>	195	2	2 (AGA)
Acriflavine resistance protein	<i>acrA</i>	398	17	5 (AGA)
Positive regulator of Int transcription	<i>alpA</i>	71	7	8 (AGA)
3-Dehydroquinate synthase	<i>aroB</i>	363	20	3 (AGG)
Shikimic acid kinase I	<i>aroK</i>	145	13	2 (AGG)
Aspartase	<i>aspA</i>	365	8	18 (AGA)
BetT gene product	<i>bet</i>	677	32	8 (AGG)
BglC (transport protein)	<i>bglC</i>	626	15	6 (AGA)
Cal protein precursor	<i>cal</i>	52	1	25 (AGG)
Lysine-specific permease	<i>cir</i>	125	8	3 (AGG)
Colicin I receptor	<i>ciriR</i>	664	38	3 (AGG)
ClpA protein	<i>clpA</i>	759	50	16 (AGA)
CopA gene product	<i>copA</i>	61	5	5 (AGG)
GDP-mannose pyrophosphorylase	<i>cpsB</i>	322	21	20 (AGG)

Table 1. (Continued)

Name of protein	Gene	Total amino acid residues	Total arginine residues	Position of AGG/A ^a
CscB protein	<i>cscB</i>	416	11	8 (AGG)
CssA	<i>cssA</i>	155	3	19 (AGA)
CysT	<i>cys</i>	278	12	7 (AGA)
Serine acetyltransferase	<i>cysE</i>	274	12	18 (AGA)
DacB	<i>dacB</i>	478	26	5 (AGA)
Deoxycytidine triphosphate deaminase	<i>dcd</i>	200	17	2 (AGA)
Presumed ATP-dependent RNA helicase	<i>deaD</i>	572	61	13 (AGG)
DNA damage-inducible protein	<i>dinF</i>	460	26	15 (AGG)
EBG repressor	<i>ebgR</i>	328	20	20 (AGG)
EnvA protein	<i>envA</i>	306	18	5 (AGG)
EnvZ protein	<i>envZ</i>	451	34	2 (AGG)
Fimbrial subunit F107	<i>f107</i>	171	1	3 (AGA)
Fdv protein	<i>fdv</i>	854	66	19 (AGG)
FhuE precursor protein	<i>fhuE</i>	729	34	8 (AGG)
1-Phosphofructokinase	<i>fruK</i>	313	18	3 (AGA)
1,2-Propanediol oxidoreductase	<i>fucO</i>	384	17	5 (AGA)
L-Fucose utilization protein	<i>fucP</i>	438	12	22 (AGA)
Glutamate decarboxylase	<i>gadB</i>	467	26	10 (AGG)
UDP galactose-4-epimerase	<i>galE</i>	338	16	2 (AGA)
Gall	<i>gall</i>	121	8	11 (AGA)
α -D-Glucosyl-1-phosphate uridylyltransferase	<i>galU</i>	303	8	21 (AGG)
2-Keto-3-deoxy-6-phosphogluconate adolase	<i>garA</i>	123	7	8 (AGA)
Glyoxylate carboligase	<i>gcl</i>	594	32	5 (AGA)
Branching enzyme	<i>glgB</i>	729	53	7 (AGA)
Glycerol-3-phosphate dehydrogenase	<i>glpda</i>	94	3	4 (AGA)
Succinate dehydrogenase (large subunit)	<i>gltA</i>	589	40	6 (AGA)
Succinate dehydrogenase (small subunit)	<i>gltA</i>	239	17	2 (AGA)
Glutamate synthase	<i>gltBA</i>	272	30	9 (AGG)
Glutamate-1-semialdehyde 2,1-aminomutase	<i>gsaG</i>	427	19	2 (AGG)
Porphobilinogen synthase	<i>hemB</i>	324	24	23 (AGA)
Porphobilinogen deaminase	<i>hemC</i>	313	27	7 (AGA)
Urogen III methylase	<i>hemX</i>	393	18	15 (AGG)
DNA-binding protein H-NS	<i>hnS</i>	138	11	19 (AGA)
Hsn-dependent expression ORF-D	<i>hsnD</i>	191	7	22 (AGA)
IncC	<i>incC</i>	79	7	21 (AGG)
InsA peptide	<i>is1F</i>	91	9	7 (AGA)
KpsM product	<i>kpsM</i>	259	12	3 (AGA)
LuxH	<i>luxH</i>	387	16	3 (AGA)
1,4- α -D-Glucan glucanohydrolase	<i>malS</i>	159	6	19 (AGA)
α -Galactosidase	<i>melA</i>	80	3	13 (AGA)
OSB synthase	<i>menC</i>	319	24	22 (AGG)
Cell-wall hydrolase	<i>mutI</i>	446	30	6 (AGA)
MysA:ORF1	<i>MysA:ORF1</i>	303	8	21 (AGG)
N-acetylglucosamine transport protein	<i>nagBE</i>	649	18	12 (AGG)
NarK protein	<i>narK</i>	463	17	9 (AGG)
NADH-dependent nitrite reductase	<i>nirBC</i>	839	55	5 (AGA)
Nitrite reductase	<i>nrjA</i>	479	21	3 (AGG)
O-6-alkylguanine-DNA alkyltransferase	<i>ogt</i>	172	14	3 (AGA)
Oligopeptide-binding protein precursor	<i>oppA</i>	544	16	7 (AGA)
OrfA	<i>orfA</i>	235	24	23 (AGA)
Oxacillin-resistance protein	<i>oxa2</i>	134	16	8 (AGA)
Prepropenicillin acylase	<i>pal</i>	350	15	4 (AGA)
L-Isoaspartyl-protein carboxyl-methyltransferase	<i>pcm</i>	209	17	4 (AGA)
Periplasmic protein	<i>perpI</i>	202	4	5 (AGA)
PhnE protein	<i>phnAQ</i>	277	11	13 (AGA)
Membrane protein	<i>phoE</i>	133	9	11 (AGA)
Pyridine nucleotide transhydrogenase	<i>pntA</i>	511	18	7 (AGA)
Phosphoenolpyruvate synthase	<i>pps</i>	793	48	23 (AGG)
Proline-betaine transporter	<i>probe</i>	501	19	4 (AGG)
5'-Phosphoribosyl-5-amino-imidazole synthetase	<i>purM</i>	346	11	25 (AGA)
RhlE gene product	<i>rhlE</i>	161	14	7 (AGG)

Table 1. (Continued)

Name of protein	Gene	Total amino acid residues	Total arginine residues	Position of AGG/A ^a
Thioredoxin precursor	<i>rhoB</i>	128	3	11 (AGG)
Rhs repetitive element	<i>rhcC</i>	69	6	5 (AGA)
Ribonuclease E	<i>rne</i>	816	84	3 (AGA)
REPA3 reading frame	<i>rsc1</i>	61	5	5 (AGG)
Succinate dehydrogenase flavoprotein subunit	<i>sdhA</i>	588	40	6 (AGA)
Succinate dehydrogenase iron-sulfur protein subunit	<i>sdhB</i>	238	17	2 (AGA)
PrlA polypeptide	<i>secY</i>	443	21	22 (AGA)
Slp gene product	<i>slp</i>	165	6	7 (AGG)
SpdE protein	<i>spdE</i>	116	5	6 (AGA)
TolC product	<i>tolC</i>	86	9	13 (AGG)
Uracil permease	<i>uraA</i>	430	10	11 (AGA)
Hemed:ORFX	<i>Uro</i>	393	18	15 (AGG)

From 2557 genes available in GenBank (Release 80), EMBL (Release 37, December 1993), those proteins that have a size larger than 50 residues contain either a single AGA or AGG codon that exists within the first 25 codons were screened. They are listed under the following subdivisions. (A) DNA synthesis and repair; (B) protein synthesis; (C) regulation/cell division; and (D) metabolic enzymes and others.

^aFrom the initiation codon.

duction of β -galactosidase almost as high as in the control cells with the *lacZ* gene without any insertion (pIT-lac1; lane 5) and with the *lacZ* gene with five CGU codons (pIT-lac1-CGU5; lane 4). The amount of proteins applied to the gel were equivalent in all cases as verified by the levels of β' and β RNA polymerase subunits (duplex bands) on the top of the gel.

Cells harboring pIT-lac1-AGG5 [(*lpp*^P)*argU*-AGA] overproduced tRNA^{Arg}_{UCU} as shown in Figure 5. Cells with pIT-lac1-AGG5 [(*lpp*^P) *argU*-AGA] appear to contain ~50 times more tRNA^{Arg}_{UCU} than the control cells harboring pIT-lac1-AGG5 (cf. lanes 2 and 1, respectively).

Production of β -galactosidase during the stationary phase

On the basis of the assay of the β -galactosidase activity during cell growth shown in Figure 2B, the production of β -galactosidase in the cells harboring pIT-lac1 is fairly constant through the exponential phase to the stationary phase, whereas it stops almost completely at the late exponential phase in the cells harboring pIT-lac1-AGG5. This was confirmed by pulse-labeling experiments as shown in Figure 6A. In the cells with pIT-lac1, the rates of the β -galactosidase production are almost the same at 1, 2, 4, and 6 hr of cell growth (lanes 1, 2, 3, and 4, respectively). In contrast, the rates of the β -galactosidase production in the cells with pIT-lac1-AGG5 (lanes 5–8) were reduced remarkably at 4 and 6 hr (lanes 7 and 8, respectively), which agrees with the activity assay shown in Figure 2B.

We again tested the effect of the overproduction of tRNA^{Arg} on the rate of the β -galactosidase production at 3 and 5 hr of cell growth. In these experiments we also constructed the mutated *argU* gene in which the anticodon sequence was altered from UCU to CCU (see Fig. 3A) so that the mutated tRNA is able to decode the AGG

codon directly. This tRNA gene was constructed because the tRNA from the *argU* may be specific for AGA and may not be able to translate the AGG codon efficiently in view of the fact that there is another minor tRNA species for AGG in *E. coli* (Kiesewetter et al. 1987).

As can be seen in Figure 6B, the inhibitory effect of five AGG codons was clearly suppressed by the overproduction of either tRNA^{Arg}_{UCU} or tRNA^{Arg}_{CCU}. In particular, at 5 hr, no production of β -galactosidase with five AGG codons was observed (Fig. 6B, lane 5), whereas remarkable resumption of the β -galactosidase synthesis occurred as a result of the overproduction of tRNA^{Arg} (Fig. 6B, lanes 6,7). It should be noted that no significant difference was observed between tRNA^{Arg}_{UCU} (lane 6) and tRNA^{Arg}_{CCU} (lane 7), indicating that the anticodon for AGA is able to decode the AGG codon effectively.

Effects of *argU* on protein production during the stationary phase

The results described above suggest that the overproduction of tRNA^{Arg}_{UCU} during the stationary phase may affect the expression of chromosomal genes containing AGA/AGG codons within the first 25 codons. To examine whether the overproduction of tRNA^{Arg}_{UCU} affects protein patterns during the stationary phase, *E. coli* JM83 with or without the *argU* gene was pulse-labeled with [³⁵S]methionine during the stationary phase and proteins were separated by two-dimensional gel electrophoresis.

As shown in Figure 7, there are no significant differences in the protein patterns during the exponential phase between the cells without and with the overproduction of the *argU* gene (A and B, respectively). However, when cells were labeled during the stationary phase, a large number of proteins were labeled much more strongly in the cells with the *argU* gene (D) than in the cells without the gene (C). Some of the proteins

Figure 2. Growth curves and β -galactosidase production. Growth curves and β -galactosidase production of cells harboring pIT-lac1-AGG5, pIT-lac1-AGG5 [*lpp^P*argU], pIT-lac1-CGU5, and pIT-lac1 were compared. *E. coli* cell SB4288 harboring the following plasmids was grown as described in Materials and methods: pIT-lac1-AGG5, five tandem repeats of the minor arginine codon; pIT-lac1-AGG5 [*lpp^P*argU], the minor codon plus the *argU* gene (decodes minor tRNA^{Arg}_{UCC}); pIT-lac1-CGU5, five tandem repeats of major arginine codon (CGU) (Chen and Inouye 1990); or pIT-lac1, a control wild-type plasmid. After induction of *lacZ* expression with 2 mM IPTG, aliquots were taken at the times indicated. (A) Growth curves after the addition of 2 mM IPTG at 0 time; cell growth was measured by a Klett-Summerson colorimeter. (B) β -Galactosidase activity after the addition of 2 mM IPTG at 0 time was assayed by the procedure of Miller (1972). (□) Cells with pIT-lac1-AGG5; (●) pIT-lac1-AGG5 [*lpp^P*argU]; (■) pIT-lac1-CGU5; and (○) pIT-lac1.

whose production was significantly enhanced are circled in Figure 7D. On the basis of well-established two-dimensional gel coordinates of *E. coli* proteins (VanBogelen et al. 1990), the protein at spot 7 was identified as the *hns* product. Interestingly, *hns* contains a single AGA codon at the nineteenth codon (see Table 1), indicating that the overproduction of tRNA^{Arg}_{UCC} in the stationary phase not only affects the expression of genes in plasmids but also of chromosomal genes. However, it is not known at present whether the enhanced production of all the proteins observed in Figure 7D is the result of the direct effect of the overproduction of tRNA^{Arg}_{UCC} at the level of translation of their mRNAs.

Discussion

Analysis of genes with a single AGA/AGG codon

Of 620 *E. coli* genes containing only one AGA/AGG codon and encoding a polypeptide of >50 residues, there are 129 that have the codon within the first 25 codons from the initiation codon. All of these genes are listed in Table 1. The positions of the AGA/AGG codons in these genes are plotted in Figure 8. Interestingly, 55% of these codons are located between the second and eighth codon, which is much higher than a random distribution of these codons ($7/24 = 29\%$). The genes in Table 1 are classified into four groups, depending on their functions; and one can see that among the proteins containing a single AGA/AGG codon within the first 25 codons, there are various essential genes in wide varieties of functions such as (A) a protein required for DNA synthesis (single-stranded DNA-binding protein), (B) many proteins associated with protein synthesis including some aminoacyl-tRNA synthetases and ribosomal proteins, (C) proteins associated with gene regulation and cell division, such as LexA, IHF, and adenylate cyclase, and (D) a large number of proteins with various metabolic functions.

Minor codon modulator hypothesis

This study, together with previous results (Chen and Inouye 1990), demonstrates the following points: (1) The insertion of five AGG codons at the eleventh codon of *lacZ* inhibited its expression severely at the late log and stationary phases of *E. coli* cell growth. (2) The inhibitory effect is decreased when the five AGG codons are moved farther away from the initiation codon. When 48 extra codons (thus, a total of 58 codons from the initiation codon) are added upstream of the five AGG codons, the inhibitory effect can be suppressed almost completely. (3) The inhibitory effect of the five AGG codons is the result of unavailability of tRNA for the minor codons. The inhibitory effect can be suppressed by the overproduction of the tRNA for the minor codons for arginine during the stationary phase. (4) There are a large number of *E. coli* genes that possess a single AGA/AGG codon preferentially within the first 25 codons from the initiation codon. (5) A number of cellular proteins encoded by chromosomal genes are abnormally overproduced only in the stationary phase in the cells carrying a plasmid with the *argU* gene for tRNA^{Arg}_{UCC}. One of the proteins was identified as Hns, whose gene contains a single AGA codon at the nineteenth position.

We designate these genes described above as primary target genes, which are defined as those genes having AGA/AGG codons within the first 25 codons. On the basis of our experimental evidence, we can safely assume that the expression of the primary target genes is specifically regulated by the concentration of tRNA for minor AGA/AGG codons. The tRNA for the minor codons is known to exist in a very limited amount even in exponentially growing cells (Ikemura 1981). We propose that the expression of the *argU* gene is tightly associated with cell growth in such a way that the concentration of the

Figure 3. Structure of pIT-lac1-AGG5, [(*lpp*^P)*argU*]. (A) Oligonucleotides used for the construction of wild-type and mutated *argU* genes. The DNA sequence of tRNA^{Arg}_{UCC} for arginine is from Garcia et al. (1986). The 5'-end G residue and the 3'-end A residue are indicated by solid dots, respectively. The arrows indicate the junction sites between the synthetic oligonucleotides used. Both the 5' end and the 3' end of the DNA fragment correspond to the *Hind*III site. The anticodon sequence TCT of the wild-type *argU* gene for tRNA^{Arg}_{UCC} is underlined. For the mutated *argU* gene, the same oligonucleotides were used except for two central pieces that contain the different anticodon sequence, CCT for tRNA^{Arg}_{UCC}. (B) Structure of pIT-lac1-AGG5 [(*lpp*^P)*argU*]. The plasmid pIT-lac1-AGG5 was constructed from pIT-lac1 as described previously (Chen and Inouye 1990). At the unique *Sal*I site of pIT-lac1-AGG5, the DNA fragment containing the synthetic *argU* gene (Garcia et al. 1986) was inserted as described in Materials and methods. The resulting plasmid was designated pIT-lac1-AGG5 [(*lpp*^P)*argU*]. The synthetic *argU* gene is flanked by the synthetic *lpp* promoter at the 5' end and the synthetic *rrnC* transcriptional termination signal derived from pGFIB1 (Normanly et al. 1986). The insertion site of the five AGG codons within the *lacZ* gene is indicated.

tRNA becomes extremely limited as cell growth reaches the stationary phase. As a result, the expression of primary target genes becomes inhibited by having the AGA/AGG codon very close to the initiation codon. This hypothesis, as designated the minor codon modulator hypothesis (Inouye and Chen 1990), is supported by the fact that the overexpression of tRNA for the minor

codons is able to overcome the inhibitory effect by the minor codons.

The genes listed in Table 1 are likely to be the primary

Figure 4. Detection of β-galactosidase produced under different conditions by SDS-PAGE. *E. coli* cells harboring different plasmids were grown for 22 hr after the addition of 2 mM IPTG. SDS-PAGE was carried out as described in Materials and methods. (Lane 1) Cells harboring pIT-lac1-AGG5; (lane 2) pIT-lac1-AGG5 [(*lpp*^P)*argU*]; (lane 3) pIT-lac1-AGG5 with eight linkers (Chen and Inouye 1990); (lane 4) pIT-lac1-CGU5; (lane 5) pIT-lac1 as a control. The arrowhead indicates the position of β-galactosidase production by pIT-lac1 (lane 5). Note that β-galactosidase in other lanes migrated slower than that in lane 5 because of its larger sizes.

Figure 5. Production of tRNA^{Arg}_{UCC}. The tRNA fraction was isolated, electrophoresed, and hybridized as described in Materials and methods. (Lane 1) The tRNA preparation was from cells harboring pIT-lac1-AGG5; (lane 2) tRNA from cells harboring pIT-lac1-AGG5 [(*lpp*^P)*argU*-AGA]. After washing off the probe for tRNA^{Arg}_{UCC} from the filter, 5'-TTTTCAATCCCCTGCTC-3', which is complementary to the anticodon loop of tRNA^{Phe}, was used for the same filter as a control to detect tRNA^{Phe}. Both lanes 1 and 2 showed almost identical hybridization signals (data not shown), indicating that both RNA preparations contained the same amount of tRNA.

Figure 6. Incorporation of [³⁵S]methionine into β -galactosidase during different growth phases. (A) *E. coli* cells harboring either pIT-lacI or pIT-lacI-AGG₅ were labeled with [³⁵S]methionine for 10 min under various growth phases as described in Materials and methods. Times when the labeling was carried out are indicated at the top of the lanes in hours. (B) The effects of tRNA^{Arg}_{CCU} and tRNA^{Arg}_{CCU} on the production of β -galactosidase with five AGG codons were examined at 3 and 5 hr of cell growth. Cells were labeled as described in A. Plasmids used were pIT-lacI-AGG₅ for LacZ(AGG)₅, pIT-lacI for LacZ (control), pIT-lacI-AGG₅ [(lpp^p)argU-AGA] for tRNA(AGA), and pIT-lacI-AGG₅ [(lpp^p)argU-AGG] for tRNA(AGG). The total cell extracts were analyzed by SDS-PAGE as described in Materials and methods. Arrows in both A and B indicate the position of β -galactosidase.

target genes regulated by the proposed model. Interestingly, these genes are highly diverse in their functions, including DNA synthesis, protein synthesis, gene regulation, and metabolic activities. Although individual genes are considered to be regulated by different mechanisms at various levels such as transcription, translation, and protein stability, their expression may also be regulated at the level of translation during the stationary phase by the mechanism proposed in this paper. Thus, the proposed regulatory mechanism by the AGA/AGG minor codons plays an important role in global gene expression of *E. coli* by regulating a number of key genes at the same time when cellular growth becomes limited.

Possible molecular mechanisms

The molecular mechanism of the minor codon modulator hypothesis is postulated in Figure 9. The rate of the synthesis of a protein is determined by the rate of translation initiation (R_i) and the rate of the speed of a ribosome moving along mRNA. The speeds of decoding of major codons (r_a) have been shown not to be significantly different from those of minor codons (r_b) in exponentially growing cells of *E. coli* so that a queue of ribosomes is unlikely to be formed at minor codons (Sørensen et al. 1990). However, when tRNA^{Arg}_{CCU} becomes limited

under certain conditions, r_b for an AGA/AGG codon becomes much slower than r_a , causing ribosome stalling at the minor codon. As a result, a queue of ribosomes is formed in front of the minor codon (see Fig. 9, II). The number of ribosomes in the queue is determined by how slow r_b is in comparison with r_a and, in turn, by the availability of the tRNA. If the position of the minor codon is far away from the initiation codon, the queue formation does not reach to the initiation codon (Fig. 9, II) so that the rate of protein synthesis from the gene is not affected by the limited amounts of the tRNA and is determined by the rate of initiation, R_i in both cases, I and II (Fig. 9). However, as the position of the minor codon is nearby the initiation codon as shown in Figure 9, III, the queue formation blocks the entry of a ribosome, and thus the rate of protein synthesis in this case is determined by r_b .

Our results indicate that if the site of the minor codons is ~60 codons away from the initiation codon, which corresponds to a size of five ribosomes (about 12 codons per ribosome; Gold et al. 1981), the inhibitory effect by the queue formation is eliminated quite effectively (Chen and Inouye 1990). This suggests that under the condition used in this study, the queue was not formed by more than four ribosomes. The overproduction of tRNA^{Arg}_{CCU} did not fully resume the β -galactosidase production to the control level in the experiment shown in Figure 2. The reason for this result is unknown at present. However, it is possible that the tRNA produced under the present condition was not fully active because of incomplete processing and/or modification of the tRNA molecule.

The closer the AGA/AGG codon is to the initiation codon, the more effective the translation blockage is likely to be. Most of the primary target genes listed in Table 1 contain the minor codon within the first 10 codons (see also Fig. 8). The fact that the blockage can be released gradually as the minor codons are positioned farther away from the initiation codon (Chen and Inouye 1990) suggests that the regulations of primary target genes are subtly different for individual genes, depending on the position of the minor codon. It should be noted that in our experimental system, we exaggerated the effect of the minor codons by inserting five AGG codons. However, we have shown earlier that the insertion of even a single AGG codon caused significant reduction in β -galactosidase activity (Chen and Inouye 1990). In addition to the AGA/AGG codons for arginine, other minor codons for other amino acids are also preferentially used near the initiation codon (Chen and Inouye 1990; Eyre-Walker and Bulmer 1993). Therefore, even if there is only one AGA/AGG codon within the first 25 codons in a given primary target gene, it may have other additional minor codons in this region. There are numerous examples for the existence of many minor codons within the first 25 codons, such as, IHF [Ser-4 (TCA), Ile-7 (ATA), Arg-9 (AGA), Pro-18 (CCC)] (Flamm and Weisberg 1985), phenylalanyl-tRNA synthetase β -subunit [Arg-2 (AGG), Ser-6 (TCA), Ser-14 (AGT), Ser-23 (TCA)] (Mechulam et al. 1985), adenylate cyclase [Arg-11 (AGA),

Figure 7. Two-dimensional gel electrophoresis of proteins from *E. coli* JM83 without or with the *argU* gene. Cells were grown and labeled at 1 hr (mid-exponential phase) and 10 hr (late stationary phase) with [35 S]methionine as described in Materials and methods. Two-dimensional gel electrophoresis was carried out using 2% (pH 3.5–10) ampholines for the first dimension and 11.5% acrylamide for the second dimension (Van-Bogelen et al. 1990). (A) *E. coli* JM83 with pIT-lacI-AGG5 at 1 hr; (B) *E. coli* JM83 with pIT-lacI-AGG5 [(*lpp*^P)*argU*-AGA] at 1 hr; (C) *E. coli* JM83 with pIT-lacI-AGG5 at 10 hr; and (D) *E. coli* JM83 with pIT-lacI-AGG5 [(*lpp*^P)*argU*-AGA] at 10 hr. Those proteins that were overproduced in D are circled.

Ile-15 (ATA)] (Aiba et al. 1984), succinate dehydrogenase C [Ile-2 (ATA), Arg-3 (AGA), Arg-9 (AGA), Leu-15 (CTA), Pro-21 (CCC), Ile-25 (ATA)] (Wood et al. 1984), ribonuclease P [Pro-7 (CCC), Arg-8 (AGG), Pro-15 (CCC), Ser-16 (AGT), Thr-19 (ACA)] (Hansen et al. 1985), and EnvZ [Arg-2 (AGG), Ser-10 (AGT), Ser-11 (TCA)] (Mizuno et al. 1982). Therefore, the minor codons for arginine as well as for other amino acids existing in various key functional genes play an important role in global gene regulation in *E. coli* under stress conditions such as nutrient limitation.

It has been shown that synonymous codon usage is less biased at the start of *E. coli* genes than elsewhere (Bulmer 1988; Eyre-Walker and Bulmer 1993). In particular, those genes highly expressed use minor codons preferentially near the initiation codon. These investigators proposed that preferential selection of minor codons at the start region is to avoid or minimize the amount of secondary structures at the start of the mRNA for more efficient translation initiation. However, these investigators did not present any examples in which a substitution of a minor codon near the start site of a mRNA

with its synonymous major codon creates a stable secondary structure. We argue that their proposal is highly unlikely for the following reasons, although in some

Figure 8. Distribution of AGA/AGG codons within the first 25 codons in the 129 *E. coli* genes that contain a single AGA/AGG codon within the first 25 codons.

Figure 9. Effect of the positions of an AGA/AGG codon in an mRNA on its translational efficiency. (I) Under a normal growth condition. A ribosome initiates translation entering at the ribosome binding site (rbs) at a rate of R_i . The ribosomes are then translated in the mRNA (solid line) by decoding major codons at a rate of r_a and minor codons at a rate of r_b . (II) Under a growth-limiting condition, r_b becomes much slower than r_a , resulting in the formation of a queue of ribosomes at the AGA/AGG minor codon. Note that because the minor codon is away from the AUG initiation codon, the queue does not block the entry of a ribosome. (III) In the case of the minor AGA/AGG codon locating very close to the initiation codon, the queue reaches the initiation codon, which then inhibits the entry of a ribosome.

cases the usage of minor codons accidentally eliminates the formation of a secondary structure. First, our experimental results demonstrate clearly that the existence of minor codons near the start site reduces the gene expression. Note that as discussed above, the existence of a single AGG codon is enough for the effect (Chen and Inouye 1990). Second, this effect is not the result of the formation of a secondary structure, because it is growth-phase dependent and, most importantly, can be overcome by the overproduction of tRNA (this paper). The effect of the tRNA overproduction described here cannot be explained by the secondary structure hypothesis by Eyre-Walker and Bulmer (1993). Third, introduction of extensive tandem repeat sequences near the start site by inserting tandem repeat sequences does not reduce gene expression (Chen and Inouye 1990). On the contrary, these insertions dramatically reduce the negative effect of minor codons on gene expression by further moving the minor codons away from the initiation codon (Chen and Inouye 1990).

Materials and methods

Bacterial strains and plasmids

E. coli strain SB4288 [K-12 F⁻ *recA thi-1 relA mal-24 spc12 supE-50 DE5 Δ[lac-proB]*] (Green and Inouye 1984) and JM83 [*ara Δ[lac-proAB] rpsL Φ80 lacZ ΔM15 relA⁺*] (Yanisch-Perron et al. 1985) were used. The plasmid pIT-lac1-AGG5 was constructed from pIT-lac1 as described previously (Chen and Inouye 1990). The *argU* gene was constructed as follows: The entire Arg-tRNA coding region was synthesized according to the sequence of tRNA for arginine (Garcia et al. 1986) by six oligonucleotides (see Fig. 3A). The 83-bp fragment thus constructed contained *Hind*III sites at both the 5' and 3' ends, which was inserted into the *Hind*III site of pIN III-*lpp*^{P5}-A3 (Inouye and Inouye 1985). The plasmid with the right orientation and sequence was then screened by DNA sequencing; subsequently, the 90-bp *Eco*RI-*Bam*HI was inserted into the unique *Eco*RI and *Bam*HI sites of pGFIB1 (Normanly et al. 1986). The plasmid thus obtained was designated pGF [(*lpp*^P)*argU*], in which the synthetic *argU* gene is flanked by the synthetic *lpp* promoter at the 5' end and the synthetic *rrnC*

transcriptional termination signal at the 3' end. By *Pvu*II digestion of pGF [(*lpp*^P)*argU*], the 377-bp fragment containing the *argU* gene with the *lpp* promoter and the *rrnC* terminator was inserted into the unique *Sal*I site of pIT-lac1 (Chen and Inouye 1990), which was filled into blunt ends by the Klenow fragment of DNA polymerase I. This resulted in pIT-lac1-AGG5 [(*lpp*^P)*argU*-AGA] and pIT-lac1-AGG5 [(*lpp*^P)*argU*-AGG] (see Fig. 3A,B).

Protein analysis

Cells harboring various plasmids were grown at 37°C in M9 medium supplemented with casamino acids (Miller 1972). At a Klett unit of 25, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM. Aliquots were taken at time intervals after the addition of IPTG, and β-galactosidase activity was assayed by the procedure of Miller (1972). The production of β-galactosidase was analyzed by SDS-PAGE as described previously (Chen and Inouye 1990). The gels were stained by Coomassie brilliant blue.

Labeling experiments

E. coli strain JM83 containing different plasmids was grown at 37°C in M9 medium supplemented with glucose (0.2%), proline (20 μg/ml), and ampicillin (50 μg/ml). The overnight culture was diluted 30-fold with fresh M9 medium supplemented, as described above, except that the concentration of glucose was 0.4%. The diluted culture was incubated at 37°C until the Klett reading reached 40; IPTG was then added to a final concentration of 2 mM. At a certain time point after the addition of IPTG, 1 ml of the cell culture was taken out and [³⁵S]methionine (Trans³⁵S-label, ICN) was added to a final concentration of 50 μCi/ml. After 10 min, nonradioactive methionine was added to a final concentration of 200 μg/ml. The cells were collected by centrifugation and washed once with 20 mM phosphate buffer (pH 7.0). The pelleted cells were then heated at 100°C for 3 min in 100 μl of 10 mM sodium phosphate buffer containing 1% SDS and 5% glycerol; solubilized proteins were analyzed by 10% SDS-polyacrylamide gel (SDS-PAGE). SDS-PAGE was performed according to the procedure described by Laemmli (1970). Two-dimensional gel electrophoresis was carried out according to VanBogelen et al. (1990). After electrophoresis, the gels were dried and autoradiographed at -70°C with Kodak X-Omat AR films.

Isolation and Northern analysis of tRNA

Isolation of tRNA was carried out as described (Wittwer and Stadtman 1986). Briefly, *E. coli* cells were harvested from a 10-ml culture by centrifugation, washed once with buffer A [20 mM sodium acetate, 10 mM MgCl₂, 1 mM dithiothreitol (DTT) at pH 5.0], and resuspended in 1 ml of buffer A. RNA was then extracted with 1 ml of 88% phenol for 1 hr with occasional vortex. After addition of 0.2 ml of 5 M NaCl, the mixture was centrifuged to separate phases. Two volumes of ethanol were added to the aqueous phase. After standing overnight at -20°C, the precipitate was collected by centrifugation, dissolved in 1 ml of buffer A, and applied to a small (0.3 ml) column of DEAE-cellulose (DE-52, Whatman) equilibrated with buffer A. The column was washed with 1 ml of buffer A containing 0.3 M NaCl, and the tRNA fraction was eluted with 1 ml of 1 M NaCl in buffer A. The tRNA fraction thus prepared was separated on a 20% polyacrylamide gel. The ratio of acrylamide to bisacrylamide was 19:1. Electrophoresis was carried out at 145 V for 20 hr at 4°C in TBE (26.7 mM Tris-HCl, 26.7 mM boric acid, and 0.752 mM EDTA at pH 8.3). After electrophoresis, the gels were soaked in 250 ml of transfer buffer (80 mM Tris-HCl, 118 mM boric acid, and 2.4 mM EDTA at pH 8.3) for 15 min. MagnaGRAPH nylon membranes (ISS-Enprotech, Natick, MA) were used in a semidry electroblotter for the transfer. The tRNA-specific probe used was a 16-mer synthetic DNA oligonucleotide complementary to the anticodon loop of tRNA^{Arg}_{UCU}; 5'-CCCACGACTTAGAAGG-3'.

Enzymes and chemicals

Restriction enzymes were obtained from New England Biolabs. IPTG was purchased from Sigma Chemical Co. DNA oligomers were synthesized on an Applied Biosystems 380B DNA synthesizer.

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Role of the AGA/AGG codons, the rarest codons in global gene expression in *Escherichia coli*.

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