INTRODUCTION

Organisms have evolved with two types of methionyl tRNAs, the initiators and the elongators. The initiators function at initiation, and the elongators decode the subsequent AUG codons in an mRNA. Both tRNAs are aminoacylated by the same methionyl tRNA synthetase. In eubacteria, while the elongator tRNAs (Met-tRNAMet) aminoacylated by the same methionyl tRNA synthetase.

In E. coli, while the elongator tRNAs (Met-tRNAMet) bind directly to EFTu, the initiators (Met-tRNAfMet) are modified to fMet-tRNAfMet by methionyl-tRNA (fMet) formyltransferase prior to their interaction with IF2 (Kozak, 1983; Guillón et al., 1996; Wu & RajBhandary, 1997). A striking feature of the eubacterial initiators, the presence of a mismatch at the 1:72 position, is responsible for at least three of their important properties: their recognition by methionyl-tRNA (fMet) formyltransferase; the prevention of their binding to EF-Tu; and their resistance to peptidyl-tRNA hydrolase, an enzyme which hydrolyses N-blocked aminoacyl and peptidyl moieties attached to tRNAs that possess a Watson–Crick base pair at this position (RajBhandary, 1994). Another highly conserved feature of most initiators is the presence of three consecutive G,C base pairs (G29G30G31 : C39C40C41) in the anticodon stem that preferentially direct the initiators to the ribosomal ‘P’ site (Seong & RajBhandary, 1987).

In E. coli, four genes encode initiator tRNAs. Three of these, metZ, metW and metV, are located in a single locus at 63·5 min, and the fourth one, metY, is located at 71·5 min (Berlyn, 1998). In E. coli K-12, the genes located at 63·5 min code for tRNAfMet and the one at 71·5 min encodes a variant, tRNAfMet. The two species differ by a single nucleotide at position 46 in the variable loop by which the tRNAfMet possesses 7mG and the tRNAfMet an A (RajBhandary & Chow, 1995). tRNAfMet represents the major form (~75–80%) of the tRNAfMet in the cell. On the other hand, tRNAfMet is a minor component (Mandal & RajBhandary, 1992), and a disruption of its gene (metY) with a kanamycin resistance gene produces a mutant strain that shows the same growth rate as the wild-type strain (Kenri et al., 1992). However, replacement of the tRNAfMet genes (metZWV) with a chloramphenicol resistance gene results in a mutant strain with a slow growth phenotype, the extent of which varies with the growth temperature (Kenri et al., 1991). We generated a T35A36 (termed U35A36) mutation in a plasmid-borne copy of metY to introduce a 34CUA36 anticodon in the encoded tRNAfMet. The tRNAfMet
(U35A36) thus produced initiates from a UAG initiation codon (Varshney & RajBhandary, 1990). Both in vitro and in vivo studies have shown that the initiator tRNA mutants containing U35A36 mutations are aminoacylated with Gln (Schulman & Pelka, 1985; Seong et al., 1989). Further, N-terminal sequence analysis of the translated products using such initiator tRNA mutants has confirmed that Gln is inserted in response to a UAG initiation codon, with no evidence of initiation with Met (O’Connor et al., 2001). However, when MetRS is overproduced in E. coli, a limited aminoacylation of tRNA2\textsuperscript{Met} (U35A36) by Met does take place, especially when its recognition by GlnRS is compromised by mutations in the acceptor stem (Varshney & RajBhandary, 1992).

Recently, Rothschild and co-workers demonstrated that N-terminal protein labelling efficiency could be drastically improved using such initiator ‘suppressors’ in vitro (Mamaev et al., 2004). Using the in vivo amber initiation assays, we have shown that the creation of a strong base pair at the 1:72 position (as in the G72 mutation, Fig. 1B) results in a loss of initiation activity, primarily because of a severe defect in formylation of the tRNA2\textsuperscript{Met} (U35A36/G72).

During the course of identifying suppressors of another formylation-defective initiator mutant, tRNA2\textsuperscript{Met} (U35A36/G72G73) (Fig. 1B), we have serendipitously isolated a stable strain of E. coli K-12 in which the metY locus encodes tRNA2\textsuperscript{Met} (U35A36). We demonstrate that a single copy of a chromosomally located gene for a minor form of initiator tRNA is sufficient for efficient initiation from a termination codon. The characterization of this strain is of considerable significance for various genetic and biotechnological applications.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids are listed in Table 1. E. coli strains were grown in LB broth or solid (1.5 % agar) media (Miller, 1972). Media were supplemented with ampicillin (Amp, 100 μg ml\(^{-1}\)), kanamycin (Kan, 25 μg ml\(^{-1}\)), tetracycline (Te, 15 μg ml\(^{-1}\)) or chloramphenicol (Cm, 50-200 μg ml\(^{-1}\)), as needed.

**Isolation of E. coli suppressor strains by spontaneous mutagenesis.** An overnight culture (0-2 ml) of E. coli CA274 harbouring pCAT\textsubscript{am1}metY\textsubscript{CUTA/G72G73} was spread on LB agar plates containing 100 μg Amp ml\(^{-1}\) and 50 μg Cm ml\(^{-1}\). Cm-resistant (CmR) colonies that appeared within 18 to 24 h, and which, upon subsequent culturing in antibiotic-free medium, lost resistance to Cm because of the loss of the resident plasmid, were selected for further characterization.

**Preparation of cell-free extracts.** The pCAT\textsubscript{am1} derivatives with or without pACQS were introduced into E. coli by transformation. The transformants were grown to exponential phase and the cells from 2 ml cultures were harvested. The cell pellet was thoroughly resuspended in 200 μl TME (25 mM Tris/HCl, pH 8.0, 2 mM β-mercaptoethanol, 1 mM Na\(_2\)EDTA), lysed by sonication and subjected to centrifugation at 10,000 r.p.m. at 4 °C for 30 min in a microfuge. The supernatant was transferred to a new tube, quantified for total proteins using Bradford’s method, mixed with an equal volume of 2× storage buffer (20 mM Tris/HCl, pH 8.0, 10 mM β-mercaptoethanol, 200 mM NaCl, 80 % glycerol, v/v) and stored at −20 °C.

**Immunoblot analysis.** Cell-free extracts (15 μg total protein) were resolved by 12 % SDS-PAGE and electroblotted onto a PVDF membrane (Amersham). The membrane was blocked with 1 % BSA in Tris/HCl buffered saline, TBS (20 mM Tris/HCl, pH 7.5, 0.9 % NaCl), overnight and then incubated for 4 h at room temperature with anti-chloramphenicol acetyl transferase and anti-β-lactamase rabbit antibodies (1:3000 dilutions). After three washings with TBS/Tween 20 (0-2 %, v/v), the blot was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution) for 4 h at room temperature. After three washings with TBS/Tween 20 (0-2 %, v/v), the blot was developed with p-nitro blue tetrazolium

[Fig. 1. (A) Diagrammatic sketches of the plasmids. pCAT\textsubscript{am1}metY\textsubscript{CUTA} harbours the CAT\textsubscript{am1} and mutant tRNA2\textsuperscript{Met} (U35A36) genes. pACQS harbours the E. coli glutaminyl-tRNA synthetase (GlnRS) gene. (B) Cloverleaf structure of the E. coli initiator tRNA2\textsuperscript{Met} indicating the sites of mutations.]

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chloride and 5-bromo-4-chloro-3-indolyl phosphate in 0-1 M Tris/HCl (pH 9-0) and 4 mM MgCl2 to visualize the bands (Sambrook et al., 1989).

**Assay for chloramphenicol acetyl transferase (CAT) activity.** The cell-free extracts (0-2-1 μg total protein) were assayed in 50 μl reaction volumes containing 150 μM Cm, 10 μM [14C]CoA (specific activity 12-5 mCi mmol-1, 463 MBq mmol-1), 500 mM Tris/HCl (pH 8-0) and 400 μM acetyl-CoA. The reactions were carried out at 37 °C for 20 min, extracted with 500 μl ethyl acetate and processed by TLC on silica gel plates (Merck) using CHCl3 and methanol (95:5, v/v) as the mobile phase (Shaw, 1983). The plates were dried, exposed to a phosphor-imaging screen and quantified using a BioImage analyser (BAS1800, Fuji Films). Enzyme activity was expressed as nanomoles of acetyl chloramphenicol (1 acetyl group) formed per minute per milligram of total protein.

**P1 transductional cross.** P1 phage lysate preparations and transductions were performed as described by Miller (1972).

**Northern blot analysis.** Total tRNA from various strains was isolated under acidic conditions, separated on 6-5% polyacrylamide acid urea (8 M) gels at 4 °C, and electroblotted onto a Nytran membrane (Varshney et al., 1991a). A 32P end-labelled oligodeoxyribonucleotide complementary to positions 29–47 of tRNA2ΔMet (U35A36) was used as probe. The probe possessed two mismatches from the tRNA2ΔMet, and three from the tRNA2ΔMet sequence complements. To generate the tRNA2ΔMet marker, the tRNA preparation was treated with 100 mM Tris/HCl (pH 9-0) (Sarin & Zamecnik, 1964).

### RESULTS

**In vivo assay system and initiator tRNA mutants**

A plasmid system used to carry out *in vivo* initiation assays is shown in Fig. 1A. The plasmid pCATam1metY_CUA carries the genes for the mutant initiator tRNA2ΔMet (U35A36) and the CATam1 reporter. Similarly, the plasmids pCATam1metY_CUA/G72 and pCATam1metY_CUA/G72G73 encode mutant tRNA2ΔMet (U35A36/G72) and tRNA2ΔMet (U35A36/G72G73), respectively, in addition to the reporter CATam1 (Table 1). The initiator tRNAs containing the U35A36 mutation (CUA anticodon) are aminoacylated with glutamine (Schulman & Pelka, 1985). To ensure efficient aminoacylation, a plasmid-borne copy of glutaminyl-tRNA synthetase (GlnRS) was also provided in some experiments from a compatible plasmid, pACQS. We have earlier shown that the tRNA2ΔMet (U35A36) initiates from the UAG initiation codon of a reporter CATam1 mRNA and confers chloramphenicol resistance (CmR) to the host. Mutations in the acceptor stem (A72 to G72, or A72A73 to G72G73) in tRNA2ΔMet result in a strong base pair at the top of the acceptor stem, severely affecting their formylation. In addition, they become substrates for peptidyl-tRNA hydrolase. Consequently, they are rendered inept in initiation from CATam1, leading to a CmR phenotype in the host (Thanedar et al., 2000; Mayer et al., 2001).

### Table 1. *E. coli* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/details</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>CA274</td>
<td><em>E. coli</em> K-12, Hfr, lacZ125am, trpA49am, relA1, spoT1</td>
<td>Brenner &amp; Beckwith (1965)</td>
</tr>
<tr>
<td>Su15</td>
<td>Derivative of CA274, metY_CUA</td>
<td>This work</td>
</tr>
<tr>
<td>Su31</td>
<td>Derivative of CA274, metY_CUA</td>
<td>This work</td>
</tr>
<tr>
<td>CAG12072</td>
<td>zhu-203::Tn10</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>CAG12152</td>
<td>zgi-3075::Tn10</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>CAG12153</td>
<td>zhc-6::Tn10</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>MA200</td>
<td><em>E. coli</em> K, F-, ΔlacX74 thy bgfR11(bglR::IS1) [Bgl+] srl::Tn10 recA56 [Bgl+] (bgfR7 bglC lacZ+ lacY+ φ[bgf-lac])</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCATam1</td>
<td>Renamed from pRSVCATam1.2.5. A pBR322 derivative harbouring the CAT reporter gene with UAG as an initiation codon</td>
<td>Varshney &amp; RajBhandary (1990)</td>
</tr>
<tr>
<td>pCATam1metY_CUA</td>
<td>Renamed from pRSVCATam1.2.5StrnMU35A36. A pBR322 derivative harbouring the CAT reporter gene with UAG as an initiation codon and expressing tRNA2ΔMet with a CUA anticodon (U35A36 mutation)</td>
<td>Varshney &amp; RajBhandary (1990)</td>
</tr>
<tr>
<td>pCATam1metY_CUA/G72</td>
<td>Derivative of pCATam1metY_CUA with additional mutations at positions 72 (A72 to G72)</td>
<td>Varshney et al. (1991b)</td>
</tr>
<tr>
<td>pCATam1metY_CUA/G72G73</td>
<td>Derivative of pCATam1metY_CUA with additional mutations at positions 72 and 73 (A72 to G72 and A73 to G73)</td>
<td>Varshney et al. (1991b)</td>
</tr>
<tr>
<td>pACQS</td>
<td>Derivative of pAC1 harbouring <em>E. coli</em> GlnRS gene</td>
<td>Varshney &amp; RajBhandary (1990)</td>
</tr>
</tbody>
</table>

Isolation and characterization of CmR strains from *E. coli* CA274

In our earlier studies, we used a tRNA\(^{\text{fMet}}\) (U35A36/G72) mutant to characterize suppressors that resulted in initiation from CATam1 mRNA (Varshney & RajBhandary, 1992; Thanedar *et al.*, 2000). In the present study, we used overnight cultures of *E. coli* CA274 harbouring pCAT\(_{\text{am1}}\)met\(_Y\)CUA/G72G73 and screened for a Cm\(^R\) phenotype. Of the several colonies that grew on the antibiotic plate, two, named Su15 and Su31, were selected for further studies. Multiple rounds of growth in antibiotic-free medium resulted in curing of the resident plasmid from these strains, and the cured strains showed a Cm\(^S\) phenotype (e.g. Fig. 4A, sectors 4 and 8).

In order to further characterize the suppressors, we reintroduced into these (Su15 and Su31) and the parent (CA274) strain, the pCAT\(_{\text{am1}}\) plasmids harbouring various mutant tRNA\(^{\text{fMet}}\) genes (Fig. 2). As tRNA\(^{\text{fMet}}\) (U35A36/G72) is a poor substrate for glutaminyl-tRNA synthetase (GlnRS), in this experiment, the pACQS plasmid was co-introduced into the strains to ensure efficient aminoacylation of the encoded tRNAs. The pCAT\(_{\text{am1}}\)met\(_Y\)CUA which encodes the formylation-proficient tRNA\(^{\text{fMet}}\) (U35A36), used here as a positive control, conferred Cm\(^R\) to the parent and Su15 and Su31 strains (Fig. 2A, right, sectors 10–12). As expected, the pCAT\(_{\text{am1}}\)met\(_Y\)CUA/G72G73 encoding tRNA\(^{\text{fMet}}\) (U35A36/G72G73) conferred Cm\(^R\) to Su15 and Su31 (sectors 8 and 9) but not to the parent CA274 strain (sector 7). Exactly the same results were obtained for the other formylation-defective mutant tRNA\(^{\text{fMet}}\) (U35A36/G72) (sectors 4–6). All transformants grew on Kan and Amp plates (Fig. 2A, left).

As a negative control, when the pCAT\(_{\text{am1}}\) plasmid lacking the tRNA gene was introduced into the parent strain CA274, as expected it did not confer Cm\(^R\) to the host (Fig. 2A, right, sector 1). However, it was surprising to note that it conferred Cm\(^R\) to the Su15 and Su31 strains (Fig. 2A, right, sectors 2 and 3). Immunoblot analysis (Fig. 2B) using anti-CAT antibodies revealed the presence of CAT protein in the transformants that grew on the Cm plates but not in the ones that did not (Fig. 2A, right, Fig. 2B) suggesting that Cm\(^R\) resulted from the presence of CAT protein. As an internal control, the blot was also probed for the presence of \(\beta\)-lactamase (Fig. 2B) to show the intactness and equivalence of amounts of total protein used in the analysis.

Mapping of mutation(s) in Su15 and Su31

The observation that the suppressors (Su15 and Su31) were able to grow on Cm in the presence of a CATam1 reporter, even in the absence of any plasmid-encoded tRNA\(^{\text{fMet}}\), led us to believe that one of the initiator tRNA genes (genomic
copy) in these strains might have been mutated to effect initiation from the CATam1 reporter. In order to genetically map the mutation by a candidate gene approach, we carried out P1 transductional crosses using various CAG strains marked with Tn10 (TcR) at 69-8 min, 71-7 min or 72-5 min in the vicinity of metY (71-8 min) encoding tRNA\textsubscript{2}\textsuperscript{fMet} (Fig. 3A). As shown in Table 2, replacement of the 71 min region of the suppressors (harbouring the plasmid pCATam1) with that from a CAG12072 (71-8 min) strain resulted in the loss of CmR by a co-transduction frequency \(\left(\frac{\text{TcR}^+ + \text{CmS}}{\text{TcR}^+}\right)\) of about 70%. The crosses with CAG12152 and CAG12153 strains (69-3 min and 72-5 min, respectively) did not result in any transductants that were CmS. These observations suggested that the mutation in Su15 and Su31, which conferred CmR, must be in the vicinity of 71-8 min. As metY is located at 71-5 min, to further narrow down the mutation, we amplified by PCR and sequenced the metY genes from both the suppressors. Each of them revealed two mutations corresponding to positions 35 and 36 of the anticodon resulting in a\textsubscript{34}CAU\textsubscript{36} to \textsubscript{34}CUA\textsubscript{36} change (Fig. 3B). Analysis of several other genes from this locus, including infB (which encodes IF2) at 71-4 min, revealed no mutations (data not shown). Further, back crosses onto the parent strain (CA274, harbouring pCATam1) using P1 phage raised from the Su15 and Su31 (TcR\textsuperscript{+} + CmS\textsuperscript{+}, marked at 71-8 min in the above experiment) resulted in an identical level of CmR in the presence of pCATam1; it was concluded that mutations in the metY gene alone resulted in the suppressor phenotype of Su15 and Su31 (data not shown).

**Abundance and mechanism of appearance of CmR colonies**

Since the mutations in both the suppressors and a large number of other CmR colonies that we obtained in the screen mapped to the metY gene, we reasoned that this mutation could have arisen via a mechanism that utilizes the plasmid-born copy of the metY gene bearing a U35A36 mutation. Since the plasmid-born copy of the metY gene contained additional mutations that were absent from the

**Table 2.** P1 transductional crosses with various CAG strains as donors and two independent mutants transformed with the pCATam1 construct

Transductants were selected on 15 µg Tc ml\(^{-1}\) and patched on 15 µg Tc ml\(^{-1}\) and 50 µg Cm ml\(^{-1}\). The number of Cm-sensitive transductants as a proportion of the total number of transductants patched (~50–100) is shown as a percentage.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Percentage of Cm-sensitive transductants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG12072 (71-8 min)</td>
<td>Su15</td>
<td>77</td>
</tr>
<tr>
<td>CAG12072 (71-8 min)</td>
<td>Su31</td>
<td>72</td>
</tr>
<tr>
<td>CAG12152 (69-5 min)</td>
<td>Su15</td>
<td>0</td>
</tr>
<tr>
<td>CAG12152 (69-5 min)</td>
<td>Su31</td>
<td>0</td>
</tr>
<tr>
<td>CAG12153 (72-5 min)</td>
<td>Su15</td>
<td>0</td>
</tr>
<tr>
<td>CAG12153 (72-5 min)</td>
<td>Su31</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 3.** (A) Genetic map (not to scale) depicting the *E. coli* chromosomal region at 71 min. The locations of metY, infB and Tn10 in CAG strains are as shown. (B) Comparison of the tDNA sequences of the metY genes from the wild-type, the suppressors (Su15/Su31) and the plasmid-borne copy of the mutant metY gene (U35A36/G72G73). The locations corresponding to positions 35, 36 and 72, 73 are boxed. While the wild-type tRNA\textsubscript{2}\textsuperscript{fMet} possesses nucleotides A35T36 resulting in a CAU anticodon, the Su15/Su31 and the U35A36/G72G73 mutants possess G35A36 (resulting in a CUA anticodon). Positions 72 and 73 are represented by A72A73 in the wild-type- and Su15/Su31-encoded tRNAs, and by G72G73 in the U35A36/G72G73 mutant tRNA. The alignment was obtained by using the PILEUP program.
chromosomal suppressors, it was of interest to further investigate the mechanism of the gene alteration. As depicted in Table 3, when the plasmid pCAT_ami1metY_CUA/G72 was introduced into E. coli CA274, CmR colonies arose with an abundance of approximately 10^7. This abundance was similar to the one obtained from the cells harbouring pCAT_ami1metY_CUA/G72, No spontaneous mutants (Cm^R) arose when the plasmid construct without any initiator tRNA (pCAT_ami1) was used. Similarly, no Cm^R colonies appeared when the cells harboured pCAT_ami1metY_CUA/3GC containing CATami1 and other initiation-defective tRNA^2^{^R}_{^\text{Met}} (U29C30A31/U35A36/U39G40A41, abbreviated as U35A36/3GC mutations) genes. Interestingly, in the latter instance, the mutations in the anticodon stem limited the sequence homology of the plasmid-borne copy with the chromosomal metY to only 2–3 nucleotides in the regions immediately upstream and downstream of the U35A36 mutation (Fig. 1B). These observations indicated that the Cm^R colonies that appeared with pCAT_ami1metY_CUA/G72 or pCAT_ami1metY_CUA/3GC as resident plasmids might have been a consequence of the chromosomal metY acquiring the U35A36 sequence from the plasmid-borne copy by homologous recombination, so as to encode a tRNA active in initiation from a CATami1 reporter. Any recombinants arising from recombination in the distal sequences (flanking both the anticodon and the other mutation sites in the tRNA genes) would encode a tRNA inactive in initiation and would not be selected in the screen.

To further probe the role of homologous recombination, we performed similar experiments using two strains of E. coli MA200 which are isogenic except for the recA allele, which encodes a protein involved in homologous pairing and strand exchange. We scored for spontaneously appearing Cm^R colonies to check if the event was mediated by RecA (Table 3). It was observed that the MA200 recA^+ strain produced results that were similar to those obtained with the CA274 strain. However, using the same assay, no Cm^R colonies appeared with the recA^− strain. These observations suggested that the acquisition of a U35A36 mutation in the chromosomal metY was via a RecA-dependent mechanism.

Table 3. Abundance of appearance of Cm-resistant colonies in transformants containing pCAT_ami1 with or without various tRNA mutants in E. coli CA274, E. coli MA200 recA^+ and E. coli MA200 recA^− backgrounds.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Abundance of Cm^R colonies appearing in strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA274</td>
</tr>
<tr>
<td>pCAT_ami1</td>
<td>0</td>
</tr>
<tr>
<td>pCAT_ami1metY_CUA/G72</td>
<td>3 x 10^−7</td>
</tr>
<tr>
<td>pCAT_ami1metY_CUA/G72</td>
<td>3 x 10^−7</td>
</tr>
<tr>
<td>pCAT_ami1metY_CUA/3GC</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4. (A) Growth of E. coli parent (CA274) and Su15 and Su31 strains on LB agar plates containing Amp (i) or Amp and Cm (ii–v) at 37 °C for 18 h (i–iv) or 24 h (v). Panels: (i) 100 µg Amp ml^−1, (ii) 100 µg Amp ml^−1 and 50 µg Cm ml^−1, (iii) 100 µg Amp ml^−1 and 100 µg Cm ml^−1, (iv) 100 µg Amp ml^−1 and 150 µg Cm ml^−1, (v) 100 µg Amp ml^−1 and 200 µg Cm ml^−1. Strains harboured pTrc99C (sectors 4 and 8), pCAT_ami1 (sectors 1–3) or pCAT_ami1 and pACQS (sectors 5–7), as indicated in (i). (B) Autoradiogram of the TLC plate showing CAT assays of cellular extracts prepared from various transformants of the parent (CA274) and the Su15 and Su31 strains. The amounts of acetyl-chloramphenicol (Ac-Cm) produced [nmol min^−1 (mg total protein)^−1] in the cell-free extracts are shown below the lanes. The various strains and the presence within them of pCAT_ami1, pCAT_ami1metY_CUA and pACQS (GlnRS) plasmids, and the total cell-free extracts used in assays are as indicated. (C) Northern blot analysis of rRNAs from the E. coli parent (CA274) and Su15 and Su31 strains. Total tRNA was isolated under acidic conditions (Methods), separated on acid urea gels, Northern-blotted using a DNA oligomer probe specific to the U35A36 mutation containing tRNA, imaged and quantified (shown below the lanes) using a Bioimage analyser (BAS1800, Fuji Films). The positions of formylated and deacylated forms of tRNA^2^{^R}_{^\text{Met}} are indicated. The presence of the pACQS plasmid (GlnRS) is indicated above the lanes.
system. As shown in Fig. 4B, in the absence of overproduced GlnRS, Su15 and Su31 produced a CAT activity which converted \( \sim 50-60 \text{ nmol Cm min}^{-1} (\text{mg total cell protein})^{-1} \) to acetyl-Cm (lanes 7 and 8). This activity was increased to \( \sim 90-100 \text{ nmol Cm min}^{-1} (\text{mg total cell protein})^{-1} \) in the presence of overproduced GlnRS (lanes 3 and 4). Under the same assay conditions, the CAT activity from the multicopy plasmid-based amber initiation system was \( \sim 1100 \text{ nmol Cm min}^{-1} (\text{mg total cell protein})^{-1} \) (lanes 2 and 6). Interestingly, the difference between the initiation efficiencies of Su15/Su31 and the multicopy plasmid system correlated well with the copy number of the ColE1 origin of replication (pBR322-derived pCAT<sub>am1</sub>-based plasmids) (Sambrook <i>et al</i>, 1989; Atlung <i>et al</i>, 1999). More importantly, these observations now allow us to choose the desired level of initiation from a UAG initiation
codon for regulated expression of genes, especially those that encode proteins toxic to the host.

To determine if the initiation activities correlated well with the availability of fGln-tRNA₂^{met} (U35A36) in the cell, we performed Northern blot analysis of the total tRNA prepared under acidic conditions. As shown in Fig. 4C, in the Su15 and Su31 strains, approximately 52% of the tRNA₂^{met} (U35A36) accumulated as fGln-tRNA₂^{met} (U35A36) (lanes 3 and 5). However, overproduction of GlnRS resulted in a slight increase in the steady-state accumulation of fGln-tRNA₂^{met} (U35A36) to ~61% (lanes 4 and 6). Such an increase in the steady-state accumulation of the fGln-tRNA₂^{met} (U35A36) agrees well with the increase in their initiation efficiency (Varshney et al., 1991a). The wild-type form of tRNA₂^{met} accumulated as fMet-tRNA₂^{met} quantitatively (lanes 1 and 2). It may be noted that the signals arising from the CA274 samples (lanes 1, 2 and 7) are less intense. This is merely because the hybridization probe [complementary to the tRNA₂^{met} (U35A36)] contains two mismatches from the wild-type sequence.

**DISCUSSION**

Both chromosomally and extra-chromosomally encoded elongator tRNA suppressors are important tools in various genetic applications (Steege & Soll, 1979). Because of their usefulness, *E. coli* strains (supD, supE, supF and supP) inserting serine, glutamine, tyrosine and leucine, respectively, in response to UAG codons have been generated. Creation of many more mutant tRNAs by *in vivo* and *in vitro* manipulations (Murgola et al., 1984; Normanly et al., 1990; Kleina et al., 1990) has resulted in the collection of a variety of useful suppressors. The availability of elongator tRNA-based suppressors has greatly facilitated the application of genetics to protein engineering (reviewed by Normanly & Abelson, 1989). Importantly, these systems have allowed the incorporation of modified amino acids into a polypeptide chain for use in various biophysical studies (Ellman et al., 1991; Bain et al., 1989; Cornish et al., 1994). The suppressors have also been instrumental in the propagation of lambda phages engineered to contain nonsense mutations in essential genes for their specialized use in molecular genetics as vehicles for transposon delivery.

Although the efficiency of termination at nonsense codons within the coding region can be minimized to some extent by placing them in an appropriate sequence context (Tate & Mannering, 1996), a drawback of the elongator tRNA-based suppressors is that suppression is not complete and results in the release of incomplete polypeptides due to competition from release factors. In another scenario, the suppressor tRNAs compete with the release factors for binding to the natural termination codons in the A site of the ribosome, resulting in translation of the cellular mRNAs beyond the termination codon. Both of these situations can be toxic to cells (Snyder & Champness, 1997). Furthermore, although not studied systematically, some of the elongator tRNA-based suppressors may also interfere with the autoregulatory mechanisms involving termination codons within ORFs as well as the mechanism that leads to the insertion of selenocysteine in proteins (Craigen & Caskey, 1986; Mansell et al., 2001). Notably, these drawbacks are essentially a consequence of the requirement of the elongator tRNA-based suppressors for recognition of the nonsense codons in the ribosomal A site.

The initiation of protein synthesis from an mRNA occurs from an initiation codon located in a specialized region, the translation initiation region (TIR). While the presence of the Shine–Dalgarno sequence (SD sequence) within the TIR is one of the most important elements that determines the efficiency of initiation, several other features, such as the sequence context within which an initiation codon is located and its spacing from the SD sequence, contribute to efficient utilization of the prokaryotic mRNA in translation (reviewed by Gold, 1988). The observation that the base pair between the initiating codon and the anticodon of the initiator tRNA, and not the AUG sequence per se, is responsible for initiation, allowed us to design a plasmid-based system for initiation from a UAG termination codon (Varshney & RajBhandary, 1990), and by virtue of their (initiator tRNA) binding at the ribosomal P site, they remedy the limitations of the elongator tRNA-based suppression system. In fact, in the plasmid-based system that we described, the initiator tRNA (with CUA anticodon) was overproduced from a multicopy plasmid without any detectable toxic consequences to bacterial growth. In the initiator tRNA-based systems, the issue of production of the incomplete peptide does not arise. Further, as the natural termination codons are generally not located in a sequence context (TIR) required to foster initiation, the chances of inappropriate initiation are also minimized.

Although the plasmid-based system using such initiator ‘suppressor’ tRNA was described some time ago (Varshney & RajBhandary, 1990), the fact that plasmid vectors are needed to introduce various test genes into the bacteria means that it was desirable to isolate/generate *E. coli* strains in which the initiator tRNA was altered in the chromosomal background. The characterization of the Su15 and Su31 strains in this report bridges this gap.

A question that the present study raises is whether or not such strains could also be generated by introducing mutations in any of the initiator tRNA genes located at 63-5 min. It is possible that the mechanism that led to the generation of Su15/Su31 or a directed approach (Datsenko & Wanner, 2000) could result in the isolation of a strain in which initiation from an amber codon would occur because of mutation in the metZ, metW or metV genes at 63-5 min. However, it may be noted that the tRNA sequences in this cluster have not diverged over the evolutionary time-scale. On the other hand, the tRNA sequence encoded by the metY locus (at 71-5 min), which is distantly located, has diverged, at least at position 46, from those located at
63.5 min. Recently, it was suggested that the multiple copies of rRNA gene sequences are prevented from diverging from each other by a gene-conversion process. In addition, genes located near to each other are corrected more efficiently than those located distantly (Hashimoto et al., 2003). Therefore, it is possible that the initiator tRNA genes located at 63.5 min are under selective pressure to maintain the intactness of their sequences. Also, the distantly located metY may be the only locus that can be allowed to accumulate mutations. Thus, at a first approximation, it would seem that the metY-based system for initiation from a termination codon would be a stable one. Hence, the characterization in this study of the E. coli strains that we isolated by serendipity is of notable significance. Interestingly, as we have shown (Fig. 4), the efficiency of synthesis (Mayer et al., 1991), it should be feasible to generate more strains for translation elongation factor Tu and translation initiation factor 2 in Escherichia coli. J Biol Chem 271, 22321–22325.


ACKNOWLEDGEMENTS

We thank Dr Mary Berlyn, E. coli Genetic Stock Centre, Yale, and Dr S. Mahadevan, IISc, Bangalore, for kindly providing us with the E. coli CAG strains and the E. coli MA200 strain, respectively, and our laboratory colleagues for their suggestions. This work was supported by grants from the Department of Science and Technology and the Indian Council of Medical Research, Government of India, New Delhi. G. D. is supported by a fellowship from the Council of Scientific and Industrial Research, New Delhi. A postdoctoral fellowship from the Department of Biotechnology, New Delhi, and a K. S. Krishnan Fellowship supported T. K. D. and S. T., respectively.

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