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Eukaryotic selenocysteine inserting tRNA species support selenoprotein synthesis in *Escherichia coli*

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ABSTRACT

Although the tRNA species directing selenocysteine insertion in prokaryotes differ greatly in their primary structure from that of their eukaryotic homologues they share very similar three-dimensional structures. To analyse whether this conservation of the overall shape of the molecules reflects a conservation of their functional interactions it was tested whether the selenocysteine inserting tRNA species from *Homo sapiens* supports selenoprotein synthesis in *E.coli*. It was found that the expression of the human tRNA^{Sec} gene in *E.coli* can complement a lesion in the tRNA^{Sec} gene of this organism. Transcripts of the *Homo sapiens* and *Xenopus laevis* tRNA^{Sec} genes synthesised *in vitro* were amino-acylated by the *E.coli* seryl-tRNA ligase although at a very low rate and the resulting seryl-tRNA^{Sec} was bound to and converted into selenocysteyl-tRNA^{Sec} by the selenocysteine synthase of this organism. Selenocysteyl-tRNA^{Sec} from both eukaryotes was able to form a complex with translation factor SELB from *E.coli*. Although the mechanism of selenocysteine incorporation into seleno-proteins appears to be rather different in *E.coli* and in vertebrates, we observe here a surprising conservation of functions over an anourmous evolutionary distance.

INTRODUCTION

The UGA-directed insertion of the amino acid selenocysteine into selenopolypeptides appears to differ in its mechanism between bacteria and eukaryotes. In bacteria, mRNAs coding for selenopolypeptides contain a stem-loop structure immediately downstream of the UGA codon which functions as a recognition element for a specialized translation factor, the SELB protein (1, 2). SELB which forms a complex with selenocysteyl-tRNA^{Sec} and GTP binds to this secondary structure and donates the cognate aminoacyl-tRNA in an apparently localized manner to the ribosomal A-site occupied by the UGA codon (3). The positioning of the UGA codon relative to the secondary structure of the mRNA is crucial for the correct reading of the codon (4, 5).

A different situation is met in eukaryotic selenoprotein synthesis. mRNAs determining selenoprotein formation contain a structural motif in their 3′-untranslated region responsible for selenocysteine insertion (6, 7). When this 3′-untranslated region is present, UGA codons within the reading frame are translated as selenocysteine. In its absence, the codons are recognized as termination signals (8, 9). A model has been proposed which implicates that a SECIS (selenocysteine inserting sequence) motif in this 3′-untranslated region acts as the target site for a translation factor homologous to the prokaryotic SELB protein (3, 9). If this model is correct the eukaryotic SELB homologue would exert its influence on the reading of the UGA codon over a distance of 500 to 1000 bases, but with a principally similar mechanism.

A possible approach for the elucidation of this unique translational control mechanism in eukaryotes resides in the analysis of the components required for selenoprotein formation in eukaryotes and in the question whether they can functionally substitute for any homologues in the prokaryotic system. Particularly interesting in this respect is the selenocysteine inserting tRNA species (tRNA^{Sec}) since it possesses the structural determinants for the differentiation of four macromolecules: tRNA^{Sec} is charged with L-serine by seryl-tRNA synthetase (10), seryl-tRNA^{Sec} is converted into selenocysteyl-tRNA^{Sec} which is then bound by translation factor SELB (for review see ref. 11). tRNA^{Sec}, in addition, contains anti-determinants for interaction with elongation factor Tu (12). Fig. 1 shows that the primary structures of the *E.coli* and the human tRNA^{Sec} species differ grossly from each other. On the other hand, a recent analysis of the solution structure of tRNA^{Sec}

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from *E.coli* (13) and from *Xenopus laevis* (which is almost identical to the human species) (14) revealed that they share a number of identical and in part also novel tertiary structural properties. In view of these unexpected similarities of the three-dimensional structure of the molecules it seemed justified to investigate whether eukaryotic $tRNA^{Sec}$ species contain the structural determinants for interaction with the components of the prokaryotic selenylation system.

MATERIAL AND METHODS

Complementation of $tRNA^{Sec}$ function *in vivo*

Activities of formate dehydrogenase H in *Escherichia coli* strains FM433 (wild type), WL81460 (Δ*selC*) and WL81460 carrying plasmids pUC18, pCB2013 (*Escherichia coli-selC* gene) and p*selC*hum (human *selC* gene), respectively, were assessed by overlay of anaerobically grown cells with agarose containing formate and benzyl viologen (15), and by testing for gas formation. Labelling experiments with [^{75}Se]selenite were performed as described (4).

Preparation of RNA

$tRNA^{Sec}$ and $tRNA_1^{Ser}$ were overproduced and purified from *Escherichia coli* strain FM460 as described (12). RNA transcripts were synthesized *in vitro* from plasmid templates carrying the corresponding genes coding for $tRNA^{Sec}$ from *E.coli*, for human $tRNA^{Sec}$ (16), *Xenopus* $tRNA^{Sec}$ (14) and the *fdhF*-mRNA according to (ref. 17). T7 RNA polymerase was purified as described (18).

Charging with seryl-tRNA synthetase

50 μg tRNAs and tRNA-transcripts, respectively, were charged at 37°C for 30 min with L-[^{14}C]serine (14 μM, specific radioactivity 152 mCi/mmol, Amersham) or non-radioactive L-serine (100 μM) employing 10 μg purified seryl-tRNA synthetase in the presence of 1 unit pyrophosphatase (Sigma) and 0.5 units RNase inhibitor (Boehringer) in 250 μl 100 mM Tris−HCl pH 7.5, 10 mM $Mg(OAc)_2$, 10 mM KCl, 10 mM ATP and 1 mM dithiothreitol and further processed as described (12).

Interaction with selenocysteine synthase

The formation of a complex between selenocysteine synthase and [^{14}C]-labelled seryl-tRNAs was measured by binding to nitrocellulose filters as described by (12). The conversion of [^{14}C]-labelled seryl-tRNAs to [^{14}C]selenocysteyl-tRNAs, or seryl-tRNAs to [^{75}Se]selenocysteyl-tRNAs, respectively, was achieved employing purified selenocysteine synthase and selenophosphate synthetase protein under anaerobic conditions (5% H_2, 95% N_2); the course of the conversion reaction was monitored by thin layer chromatography after alkaline deacylation of reaction products and autoradiography (12).

Interaction with elongation factor SELB

Binding of [^{14}C]selenocysteyl-tRNAs to elongation factor SELB was measured either via protection against RNase degradation or protection against alkaline hydrolysis (12). The binding of translation factor SELB alone, or SELB in a complex with [^{14}C]selenocysteyl-tRNAs to [^{32}P]-labelled *fdhF*-mRNA transcript was analyzed in a gel shift assay as described (3).

The experiments involving human $tRNA^{Sec}$ transcripts were performed twice and repeated with *Xenopus* $tRNA^{Sec}$ which differs from the human species in 1 position. Identical results were obtained in the three sets of experiments.

RESULTS

Human $tRNA^{Sec}$ promotes selenocysteine and selenoprotein synthesis in *E.coli*

To evaluate whether $tRNA^{Sec}$ from *Homo sapiens* can substitute for its *E.coli* counterpart in selenoprotein synthesis the gene coding for human $tRNA^{Sec}$ was cloned from plasmid pHtU (16, 19) into plasmid pUC18 and transformed into *E.coli* strain WL81460 which carries a deletion in the *selC* gene coding for the $tRNA^{Sec}$ from *E.coli*. The expression of the gene, therefore, occurred from the lac promoter of plasmid pUC18. The transformants were tested for formate dehydrogenase H activity (a selenium dependent enzyme) by the benzylviologen dye overlay technique (15) and for overall formate hydrogenlyase activity by gas formation. It was found that all transformants were able to complement the defect of the *E.coli selC* lesion indicating that the human $tRNA^{Sec}$ species is functional in *E.coli*. The efficiency of complementation—as measured by gas production—was about 1/3 of a $selC^+$ wild-type strain (data not shown). The actual value, however, may be much lower since the gene for the human tRNA is expressed from the pUC18 multicopy plasmid in these experiments.

Direct proof for the complementation was then brought about by measurement of [^{75}Se] incorporation into selenopolypeptides in labelling experiments with strain WL81460 carrying either the *E.coli* or the human $tRNA^{Sec}$ genes, respectively, on a multicopy plasmid (Fig. 2). Overproduction of the human $tRNA^{Sec}$ species restored the capacity for formation of the selenopolypeptide of formate dehydrogenase H of the *selC*

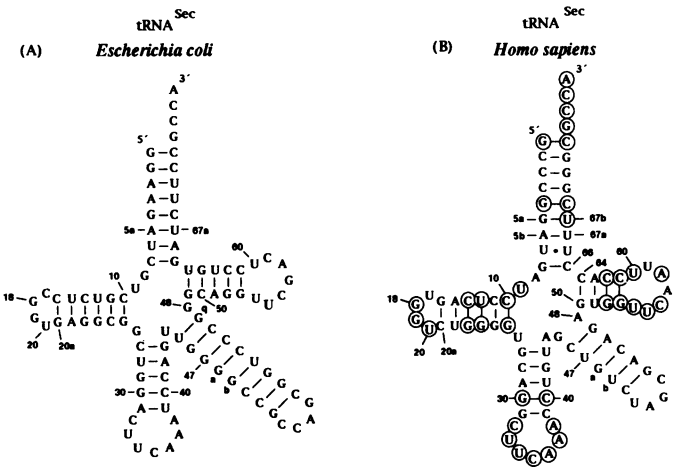

Figure 1. Cloverleaf structures of T7 RNA polymerase transcripts of the selenocysteine inserting tRNA species originating from the respective genes from (**A**) *Escherichia coli* ($tRNA^{Sec}$-tc), and (**B**) of $tRNA^{Sec}$ from *Homo sapiens*, ($tRNA^{Sec}$hum-tc). Nucleotides identical in both tRNAs are circled in the cloverleaf structure of the latter one. The numbering is according to 14 and 27. The sequence of $tRNA^{Sec}$ from *Xenopus laevis* differs from the human species at position 9 (C instead of U). *E.coli* $tRNA^{Sec}$ possess a dihydrouridine at position 20, a ribothymidine at position 54, a pseudouridine at position 55 and a i^6A in position 37 (28). The modified bases in eukaryotic $tRNA^{Sec}$ are: mcm^5U at position 34, i^6A at position 37, pseudouridine at position 55 and m^1A at position 58 (29, 30).

mutant, although at a lower extent (10–20%) when compared with the wild type. Only minute amounts of the selenopolypeptide of formate dehydrogenase N were made which is in accord with previous results indicating that the two selenoproteins are synthesised with different efficiencies in strains harboring mutations in $tRNA^{Sec}$ (20).

In order to analyze the proficiency of the human $tRNA^{Sec}$ species in *E.coli* selenoprotein synthesis in more detail its interaction with seryl-tRNA synthetase, selenocysteine synthase and translation factor SELB from *E.coli* was studied *in vitro*. Most of these studies were conducted with transcripts synthesized by T7 RNA polymerase *in vitro* (designated $tRNA^{Sec}$-tc); the results were compared with those obtained with 'native' $tRNA^{Sec}$ and $tRNA_1^{Ser}$, a serine isoacceptor species purified from *E.coli*, as controls.

Eukaryotic $tRNA^{Sec}$ is less efficiently charged by seryl-tRNA synthetase from *E.coli*

The efficiency of the interaction of seryl-tRNA synthetase with the five serine isoacceptor species and with $tRNA^{Sec}$ determines the relative flux with which L-serine is used directly for incorporation into protein or distributed into the pathway for selenocysteine biosynthesis (21). The sequence features that define serine identity in tRNA were elucidated both for *E.coli* (22), human $tRNA^{Ser}$ ($tRNA^{Ser}$-hum) (23) and human $tRNA^{Sec}$ ($tRNA^{Sec}$-hum) (19). From these studies the human $tRNA^{Sec}$ species was expected to be a very inefficient substrate for charging by *E.coli* seryl-tRNA synthetase. Under the experimental conditions employed (0.1 nmole seryl-tRNA synthetase, 1.7 nmole tRNA as substrate) the *E.coli* $tRNA^{Sec}$ was charged to a level of 75 % within 3 min. Charging levels of only 50% and 25% were achieved with the *E.coli* and human $tRNA^{Sec}$ transcripts, respectively, within a 60 min incubation period (data not shown). It was sufficient, however, to prepare the substrates for the analysis of the interaction with selenocysteine synthase and elongation factor SELB.

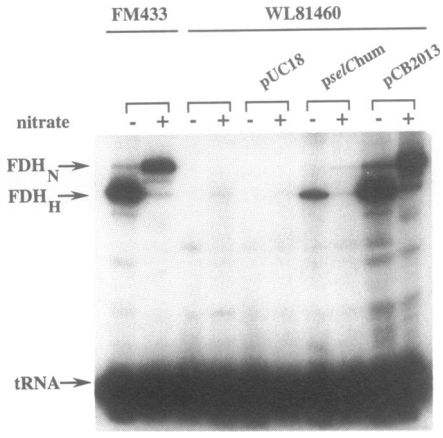

Figure 2. Synthesis of [^{75}Se]selenocysteine labelled formate dehydrogenase isoenzymes. Autoradiograph of a 10% SDS–polyacrylamide gel after separation of whole cell extracts. Strains FM433 (wild type) and WL81460 (deletion in *selC*), transformed with plasmids pUC18, p*selC* hum (human *selC* gene) and pCB2013 (carrying the *Escherichia coli selC* gene) as indicated were grown in the presence of [^{75}Se]selenite under anaerobic conditions in TGYEP medium. The migration positions of the selenopolypeptides of formate dehydrogenases H (80 kDa; growth without nitrate) and N (110 kDa; with nitrate), and of tRNAs are indicated. Quantitation of the radioactivity in the FDH_H bands was assessed with the aid of a laser densitometer (Molecular Dynamics, Düsseldorf). An appropriate stretch of the autoradiograph of the vector control (lanes pUC18) was taken as background value.

Eukaryotic $tRNA^{Sec}$ is a substrate for *E.coli* selenocysteine synthase

The first specific step of selenoprotein synthesis consists of the conversion of seryl-$tRNA^{Sec}$ into selenocysteyl-$tRNA^{Sec}$ which is catalyzed by selenocysteine synthase (24). The enzyme is highly specific for $tRNA^{Sec}$ (25) and the length of he aminoacyl acceptor helix of the tRNA is one of the antideterminants against serine inserting tRNAs (12).

At first, the binding of seryl-$tRNA^{Ser}$ and seryl-$tRNA^{Sec}$ species to selenocysteine synthase was measured. To this end, the tRNA species charged with L-[^{14}C]serine were incubated with increasing amounts of enzyme and the radioactivity bound to protein was determined in a nitrocellulose filter binding assay (12). As expected, no radioactivity remained bound to the filter when the enzyme was incubated with seryl-$tRNA_1^{Ser}$ (Fig. 3). In contrast, increasing amounts of *E.coli* seryl-$tRNA^{Sec}$, seryl-$tRNA^{Sec}$-tc and human seryl-$tRNA^{Sec}$-tc remained bound indicating the formation of a specific complex with the enzyme. Although an ideal enzyme–substrate equilibrium may not have been attained, the slopes of the binding curves may reflect the differential affinities for the tRNA species; they show that the human $tRNA^{Sec}$-tc displays a lower affinity to selenocysteine synthase than the $tRNA^{Sec}$-tc from *E.coli*. 'Native' $tRNA^{Sec}$ is bound with the highest affinity and a charged $tRNA^{Ser}$ species is not recognized at all.

Next, the synthesis of selenocysteyl-tRNAs from [^{14}C]seryl-$tRNA^{Sec}$ (Fig. 4A) and [^{14}C]seryl-$tRNA^{Sec}$-tc (Fig. 4B) from *E.coli* and [^{14}C]seryl-$tRNA^{Sec}$-tc from *Homo sapiens* was studied (Fig. 4C). A comparison of the intensity of the serine and selenocysteine spots shows that the rate of selenocysteine synthesis was much slower when the heterologous transcript was a substrate, but it was clearly detectable when compared to the control reaction with [^{14}C]seryl-$tRNA_1^{Ser}$, which is not a substrate in the conversion reaction (Fig. 4D). Final confirmation for the synthesis of selenocysteine was brought about in conversion reactions performed on seryl-tRNAs with

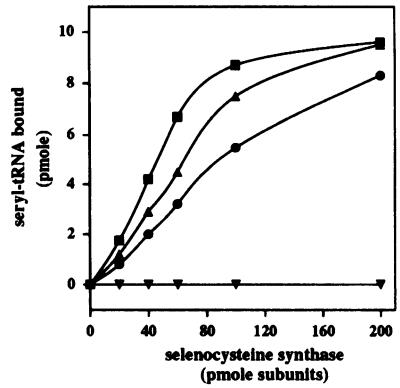

Figure 3. Binding of [^{14}C]seryl-tRNAs to selenocysteine synthase. Formation of a complex as measured by the amount of radioactivity that remained bound to a nitrocellulose filter after extensive washing. ■, $tRNA^{Sec}$; ▲, $tRNA^{Sec}$-tc; ●, $tRNA^{Sec}$hum-tc and $tRNA^{Sec}$Xen-tc, respectively; ▼, $tRNA_1^{Ser}$.

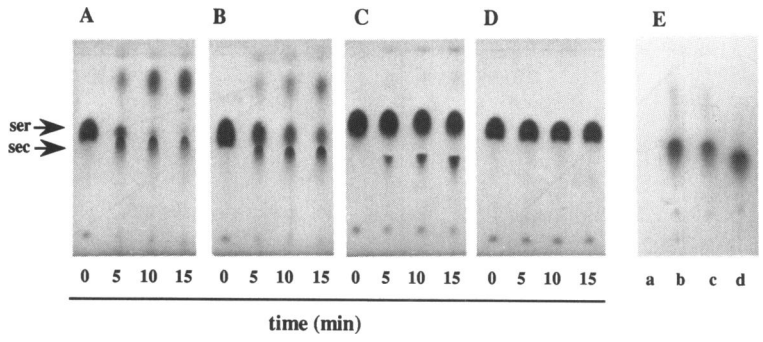

Figure 4. Selenocysteyl-tRNA synthesis by selenocysteine synthase and SELD. The incubation of tRNAs with the biosynthetic enzymes was stopped at the times indicated, the reaction products deacylated by incubation under alkaline conditions followed by separation via thin layer chromatography and autoradiography. Substrates: (**A**) [^{14}C]seryl-tRNA^{Sec}; (**B**) [^{14}C]seryl-tRNA^{Sec}-tc; (**C**) [^{14}C]seryl-tRNA^{Sec}hum-tc and (**D**) [^{14}C]seryl-tRNA$_1^{Ser}$. *Xenopus* seryl-tRNA^{Sec}-tc delivered results identical to those presented for the human tRNA^{Sec} transcript. The conversion reactions in (**E**) were performed for 30 min on seryl-tRNAs (a: seryl-tRNA$_1$Ser; b: seryl-tRNA^{Sec}; c: seryl-tRNA^{Sec}-tc and d: seryl-tRNA^{Sec}hum-tc) in the presence of [^{75}Se]selenite, and further processed as above. The migration position of serine (ser) and selenocystin (sec) was confirmed by co-chromatography of non-radioactive amino acids followed by staining with ninhydrin.

radioactively labelled selenite as a substrate. Fig. 4E shows the formation of [^{75}Se]selenocysteine from seryl-tRNA^{Sec} and seryl-tRNA^{Sec}-tc of *E.coli* and of human seryl-tRNA^{Sec}-tc; again, seryl-tRNA$_1^{Ser}$ was not a substrate.

In conclusion, selenocysteine synthase specifically binds to and catalyzes selenocysteine formation on *E.coli* seryl-tRNA^{Sec}, and also on serine-charged transcripts of *E.coli* and human tRNA^{Sec}. However, the interaction with transcripts is somewhat weaker than with native tRNA^{Sec} derived from *E.coli*.

The *E.coli* translation factor SELB interacts weakly with eukaryotic tRNA^{Sec}

SELB, an elongation factor alternate in its function to EF-Tu forms a specific complex with selenocysteyl-tRNA^{Sec} and GTP (1, 2). The ternary complex binds to a secondary structure element immediately 3′-adjacent to the UGA codon in the selenopolypeptide mRNA (3) and apparently locates the charged tRNA into the proximity of the ribosomal A site. In the case of mammalian selenoprotein formation a similar complex has been postulated to form with some recognition element in the 3′-untranslated region of the mRNA (3, 9), but experimental evidence for the existence of such a protein factor is still lacking.

Therefore, it was of particular interest to analyze whether the human tRNA^{Sec} possesses the structural determinants for the interaction with the alternative elongation factor. For this purpose, [^{14}C]seryl-tRNAs were converted to [^{14}C]selenocysteyl-tRNAs by incubation with selenocysteine synthase and seleno-phosphate synthetase (the *selD* gene product) and the interaction with the elongation factor SELB was assessed in three different test systems. First, it was analyzed whether the co-incubation with SELB confers protection of selenocysteyl-tRNAs against degradation by pancreatic RNase. The results displayed in Fig. 5 clearly show that selenocysteyl-tRNA^{Sec} and selenocysteyl-tRNA^{Sec}-tc from *E.coli* and to a weaker extent selenocysteyl-tRNA^{Sec}-tc from *Homo sapiens* were protected against degradation when SELB was present in the assay. Seryl-tRNA$_1$Ser or seryl-tRNA^{Sec} from *E.coli* were quickly degraded irrespective of the presence of SELB (12). Second, we analyzed the rate of alkaline hydrolysis (at pH 7.5) of the aminoacyl ester bond in the presence of SELB or—as a control—of bovine serum albumin. The deacylation of the seryl-tRNA$_1^{Ser}$ or seryl-tRNA^{Sec} (not shown) proceeded with the same rate in the presence of SELB or bovine serum albumin (Fig. 6A). However, incubation in the presence of SELB led to a slower rate of deacylation in the case of selenocysteyl-tRNA^{Sec} (Fig. 6B), selenocysteyl-tRNA^{Sec}-tc from *E.coli* (Fig. 6C) and human selenocysteyl-tRNA^{Sec}-tc (Fig. 6D), thus indicating a specific interaction with SELB. In a third test system, a crucial feature of SELB function *in vivo* was analyzed, namely that the binding of selenocysteyl-tRNA^{Sec} induces an increase in the affinity of SELB for the *fdhF*-mRNA (3). For this aim, transcripts identical to the *fdhF*-mRNA stem-loop structure were 5′-labelled with [γ-^{32}P]ATP, and their interaction with elongation factor SELB alone or in the presence of selenocysteyl-tRNAs was assessed in a gel retardation assay. A shift in the migration position of *fdhF*-mRNA transcript occurred after incubation with SELB in the presence of GTP. Incubation of mRNA transcripts with SELB and selenocysteyl-tRNA^{Sec} or selenocysteyl-tRNA^{Sec}-tc from *E.coli* led both to a faster migration of the complex and to an

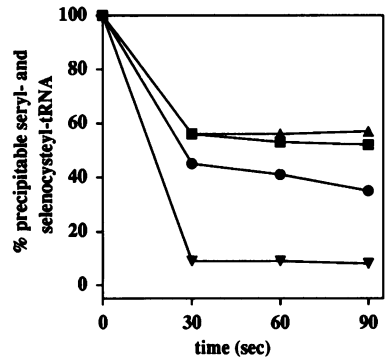

Figure 5. SELB-dependent protection of tRNAs against RNase degradation. [^{14}C]seryl- and [^{14}C]selenocysteyl-tRNAs were incubated with SELB followed by the addition of pancreatic RNase. At the times indicated, aliquots were spotted on Whatman paper and the TCA-precipitable radioactivity was determined by liquid scintillation counting. ■, *E.coli* selenocysteyl-tRNA^{Sec}; ▲, *E.coli* selenocysteyl-tRNA^{Sec}-tc; ●, selenocysteyl-tRNA^{Sec}hum-tc and selenocysteyl-tRNA^{Sec}Xen-tc; ▼, seryl-tRNA$_1^{Ser}$.

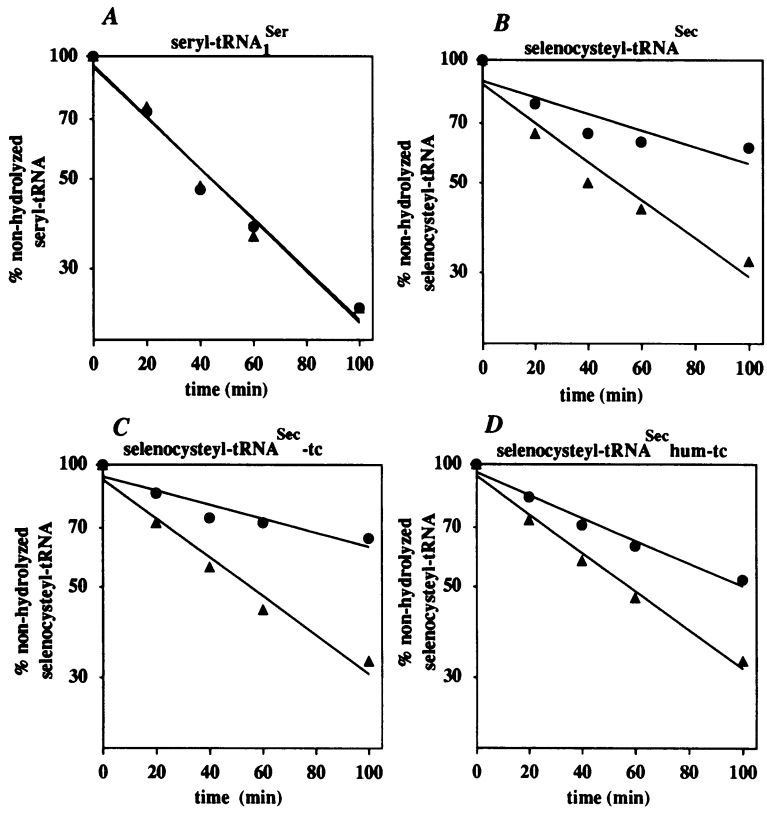

Fig. 6. SELB-dependent protection of the aminoacyl-ester bond against akaline hydrolysis. [^{14}C]seryl- and [^{14}C]selenocysteyl-tRNAs were incubated with SELB (●) or bovine serum albumin (▲) at pH 7.5. At the times indicated, aliquots were spotted on Whatman paper and the remaining TCA-precipitable radioactivity determined by liquid scintillation counting. (**A**) seryl-tRNA$_1^{Ser}$; (**B**) selenocysteyl-tRNA^{Sec}; (**C**) selenocysteyl-tRNA^{Sec}-tc; (**D**) selenocysteyl-tRNA^{Sec}hum-tc.

increase of the affinity for the mRNA, indicating the formation of a quaternary complex between SELB, selenocysteyl-tRNA, mRNA and presumably GTP (see also ref. 12). However, after incubation with seryl-tRNA^{Sec} from *E.coli* or selenocysteyl-tRNA^{Sec}-tc from *Homo sapiens* only a minute amount of the latter tRNA was entering the faster migrating complex (data not shown).

These results demonstrate that the eukaryotic tRNA^{Sec}-tc species are recognized by the selenocysteine-specific translation factor SELB from *E.coli*, a result which is in accord with our *in vivo* observations. As measured in an RNase protection assay or by deacylation kinetics, the binding was reduced in comparison to the homologous tRNA species. The reduced affinity of SELB for the heterologous tRNA species was even more pronounced in the gel shift assay which may be a consequence of the more stringent conditions.

DISCUSSION

In conclusion, the tRNA^{Sec} species from *Homo sapiens* and from *Xenopus* possess the structural features for interaction with the *E.coli* seryl-tRNA synthetase, with selenocysteine synthase and with translation factor SELB. The specificity with which they insert selenocysteine *in vivo* also indicates that they maintained the antideterminants against interaction with EF-Tu: no detectable incorporation occurred at UGA termination codons by EF-Tu-mediated suppression (see Fig. 2).

This strong conservation of functional properties over a wide evolutionary distance promotes the conclusion that the specific mechanisms involved are also conserved. This notion is supported by the recent demonstration that the overall tertiary structures of the *E.coli* and the *Xenopus laevis* tRNA^{Sec} species are very similar despite of their grossly different primary structures (13, 14). This, however, is true for all tRNAs despite their different identities.

In accordance with the lack of some of the prokaryotic identity elements (19, 22) tRNA^{Sec} from *Xenopus* and *Homo sapiens* are quite inefficient substrates for seryl-tRNA synthetase from *E.coli*. Their charging, however, still appears to suffice at least partially the demand of selenocysteine biosynthesis which is required in only trace amounts. In contrast, selenocysteine synthase, the first specific enzyme in selenocysteine biosynthesis in *E.coli*, quite efficiently binds seryl-tRNA^{Sec} from the two higher organisms studied and converts the seryl- into an aminoacrylyl-residue. Since this reaction does not depend on prior activation of the hydroxyl of serine, e.g. by phosphorylation (25) the role of the kinase present in eukaryotic tissues and converting seryl-tRNA^{Sec} into O-phosphoseryl-tRNA^{Sec} (16, 26) is not obvious in this reaction.

Most interesting, however, is the finding that the eukaryotic tRNA^{Sec} species are complexed by the *E.coli* SELB protein. Binding to SELB requires the presence of the selenocysteyl-residue at the tRNA and a number of structural properties not present in ordinary tRNA species (12). The conservation of all these features from *E.coli* to *Homo sapiens* suggests that they

are essential also in their homologous environment. If this conclusion holds true the SECIS elements in the 3'-untranslated regions of the mRNAs coding for selenoproteins in eukaryotes may indeed constitute binding sites for a eukaryotic homologue of the prokaryotic SELB (3, 8, 9). It would then function in polypeptide synthesis over a very long distance.

The function of the eukaryotic $tRNA^{Sec}$ in *E.coli* also provides the experimental basis for characterization of the unknown additional components involved, either by conventional purification using the established *E.coli in vitro* system or even by complementation of lesions in the *E.coli sel* genes. It may even be feasible to establish the complete eukaryotic selenoprotein synthesis system in the prokaryotic background.

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