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# Mammalian polypeptide chain release factor and tryptophanyl-tRNA synthetase are distinct proteins

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**A very high (~90%) structural similarity exists between the bovine, human and murine tryptophanyl-tRNA synthetases (WRS), and quite unexpectedly the rabbit polypeptide chain release factor (eRF). This similarity may point to a very close resemblance or identity between these proteins involved in distinct steps of protein synthesis, or inadvertently to an incorrect assignment of the clone reported to encode eRF, since the structure of clones encoding WRS were confirmed by peptide sequencing. Using high resolution column chromatography and sucrose gradient centrifugation combined with assays for WRS and eRF activities, we show that functionally distinct WRS and eRF proteins can be completely separated from each other. Moreover, a putative anti-eRF monoclonal antibody appears incapable of immunoprecipitating the eRF activity or binding to protein(s) possessing eRF activity. This antibody binds to protein fractions which coincide in various separation procedures with rabbit WRS activity, and to pure bovine WRS. The protein expressed in *Escherichia coli* from the original cDNA clone initially reported to encode eRF, has WRS activity but not eRF activity. Resequencing of the fragment of the original rabbit cDNA demonstrates the presence of the previously overlooked HXGH motif typical of class I aminoacyl-tRNA synthetases. Consequently, mammalian WRS and eRF are different proteins, and the cDNA clone formerly assigned as encoding eRF encodes rabbit WRS.**

*Key words:* mammalian peptide chain release factor/ mammalian tryptophanyl-tRNA synthetase/protein biosynthesis

## Introduction

The prokaryotic protein synthesizing apparatus is well characterized structurally; amino acid sequences are known for all the aminoacyl-tRNA synthetases, ribosomal proteins, polypeptide chain initiation, elongation and release factors, as well as the nucleotide sequences for all the ribosomal RNAs and tRNAs (for reviews see Spedding, 1990; Schimmel, 1991; Moras, 1992). Far less information is available concerning the structure of these components in higher eukaryotes; for example the amino acid sequence of only seven out of at least 20 aminoacyl-tRNA synthetases is known, and the primary structures of the ribosomal proteins are far from complete (for a review see Trachsel, 1991). The elucidation of the amino acid sequence of the components of the translation apparatus is a significant limiting step in understanding protein synthesis in higher eukaryotes. Moreover it is not always relevant to extrapolate the data available for prokaryotes to multicellular organisms, because of the much more complex organization and regulation found in higher eukaryotic cells.

Recently, cDNA clones encoding the bovine tryptophanyl-tRNA synthetase, bWRS (Garret *et al.*, 1991) and human tryptophanyl-tRNA synthetase, hWRS (Frolova *et al.*, 1991) were sequenced; the deduced amino acid sequences are very similar and exhibit structural features typical of class I aminoacyl-tRNA synthetases (Eriani *et al.*, 1990; reviewed in Moras, 1992), and limited although significant homology with bacterial WRS (Garret *et al.*, 1991). Independently, it was shown that a major protein whose synthesis is stimulated in human cell cultures by interferon $\gamma$ (Fleckner *et al.*, 1991; Bange *et al.*, 1992; Buwitt *et al.*, 1992) and interferons $\gamma$ or $\alpha$ (Rubin *et al.*, 1991) exhibits WRS activity, and that its amino acid sequence is identical to that of hWRS. Surprisingly this amino acid sequence is very similar to that deduced from a sequenced cDNA reported by Lee *et al.* (1990) to encode the rabbit polypeptide chain release factor (eRF). The close similarity between mammalian WRS and eRF is unexpected given the distinct functional properties of these proteins (Frolova *et al.*, 1993). Consequently, the question arises as to whether the mammalian WRS and eRF activities reside on identical or very similar polypeptides as has already been suggested (Garret *et al.*, 1991; Bange *et al.*, 1992; Buwitt *et al.*, 1992), or whether the rabbit cDNA encodes a protein other than eRF (Frolova *et al.*, 1991, 1993).

Several immunochemical, biochemical and physicochemical properties of mammalian WRS and eRF have been compared (Frolova *et al.*, 1993). Rabbit (r) WRS and eRF have distinct thermostabilities, and dissimilar chromatographic profiles on phosphocellulose. Both anti-bWRS polyclonal antibodies and the anti-bWRS monoAb $Am_2$ strongly inhibit the bWRS and hWRS aminoacylation activities, but not the rabbit eRF activity. Moreover, neither pure bWRS nor purified hWRS exhibits eRF activity. It was therefore concluded (Frolova *et al.*, 1993) that (i) the functional centres responsible for the WRS and eRF activities are structurally and functionally distinct and could hardly belong to the same protein domain, (ii) eRF and WRS probably reside on different polypeptide chains encoded by different genes, although it could not be excluded that the eRF- and WRS-specific domains might be situated on the

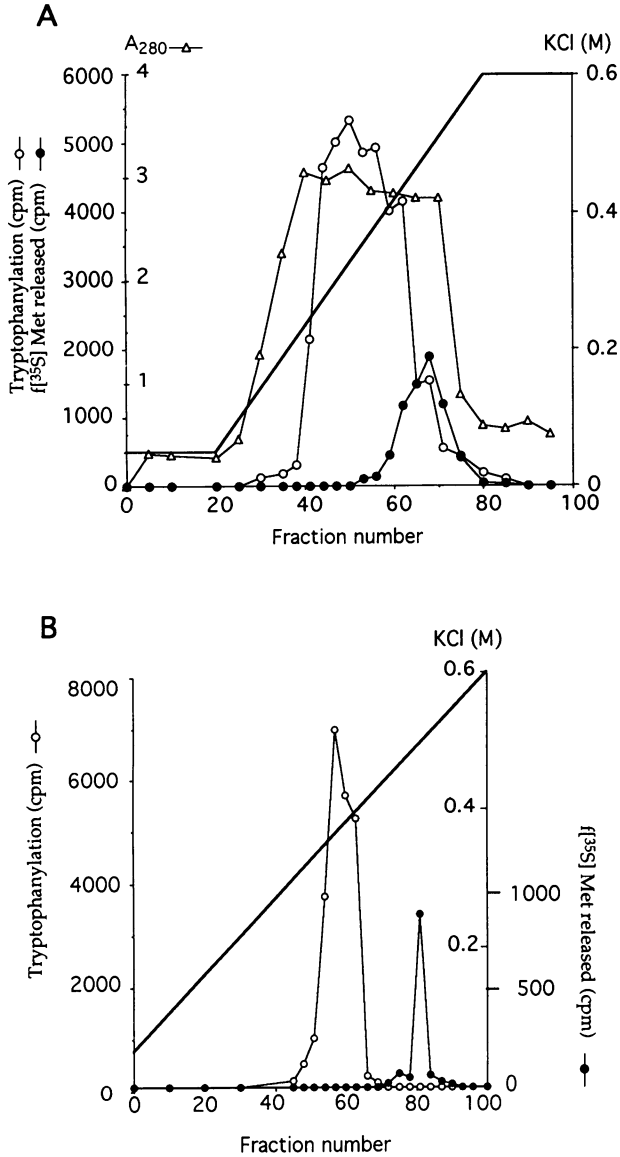


**Fig. 1.** Column chromatography of the eRF and WRS activities. (**A**) Q-Sepharose (Fast Flow) chromatography. The S100 supernatant from rabbit reticulocyte lysates was treated with ammonium sulfate: the fraction precipitating between 30 and 70% (3000 $A_{280}$) was dialysed against buffer B (20 mM Tris−HCl, pH 7.8, 1 mM DTT and 0.1 mM EDTA) and applied onto a Q-Sepharose column (2 × 11 cm) washed with buffer B containing 50 mM KCl followed by elution with 60 ml of a linear 50−600 mM KCl gradient. Fractions of 1 ml were collected. The WRS and eRF activities were measured in 10 μl samples. Fractions 60−75 containing the eRF activity were pooled and dialysed against buffer B containing 50 mM KCl. (**B**) Mono Q chromatography. Five $A_{280}$ units of the pooled fractions after the Q-Sepharose column and containing eRF activity were applied onto a Mono Q column HR 5/5 using FLPC; the column was washed with 10 ml of buffer B and proteins were eluted with 20 ml of a linear 50−600 mM KCl gradient in buffer B. Fractions of 0.2 ml were collected. Samples (10 μl) from the gradient fractions were used to measure the WRS and eRF activities. Fractions 50−62 (WRS activity) and 72−82 (eRF activity) were pooled and stored at −70°C.

same polypeptide, and (iii) the rabbit cDNA reported to encode eRF, more likely encodes rWRS; this would explain why its deduced amino acid sequence has no similarity with bacterial RFs but is very similar to mammalian WRS.

To gain further insight into the relationship between WRS and eRF in higher eukaryotes, new purification techniques involving chromatographic carriers with high resolution have been used to separate the WRS and eRF activities. These fractionation procedures were combined with immunochemical methods to detect antigen−antibody complex formation. Furthermore, a detailed examination of the product expressed by the cDNA clone described by Lee *et al.* (1990) was undertaken based on its close similarity with the mammalian WRS sequences.

It has been possible for the first time to resolve completely the WRS and eRF activities present in reticulocyte lysates. In addition, the 'anti-eRF' monoAb used previously to isolate the 'eRF' cDNA clone bound only to the protein in the fractions containing the WRS activity and not to the protein(s) in the fractions containing the eRF activity. The same monoAb did not immunoprecipitate native rabbit eRF. The protein programmed with the cDNA assigned earlier as encoding rabbit eRF and expressed in *Escherichia coli*, possessed strong WRS activity but no eRF activity. Consequently, the rabbit cDNA encodes WRS but not eRF, and these two mammalian proteins are different.

## Results

### *Complete separation of RF and WRS activities*

Using a combination of DEAE−cellulose and phosphocellulose column chromatography it was previously not possible to separate completely the eRF activity from the WRS activity (Frolova *et al.*, 1993). These two activities have now been separated by sequential Q-Sepharose and Mono Q column chromatography. Figure 1A shows that the peaks of eRF and WRS activities partially overlap during the Q-Sepharose step of purification. However, complete separation of these two activities from the enriched eRF fractions eluted from Q-Sepharose was achieved on a Mono Q column (Figure 1B). The WRS and eRF activities reproducibly eluted from the Mono Q column as two sharp and symmetrical peaks at 370 mM KCl and 450 mM KCl respectively, reflecting the effectiveness of the fractionation procedure. After these purification steps, the WRS activity remained much more stable than the eRF activity (not shown).

An alternative strategy made use of a partially purified sample containing both WRS and eRF activities from a DEAE−Sephadex column which was further purified on heparin−Sepharose before being fractionated by sedimentation through a 5−20% (w/v) sucrose gradient. Partial separation of the two activities was achieved with a KCl concentration of 50 mM in the gradient (Figure 2A); however, the WRS partially resolved into two peaks, both containing the same single polypeptide of 54 kDa when fractions were examined (not shown) by SDS−PAGE. The eRF activity was not coincident with the 54 kDa band, but rather with a slightly smaller and much less prominent polypeptide of ~50 kDa (not shown).

When the salt concentration in the gradient was raised (Figure 2B) from 50 to 300 mM KCl (following an unpublished observation of W.P.Tate that the eRF had different apparent $M_r$s when sedimented under different salt concentrations), the eRF was seen to shift to a position further down the gradient (from fraction 16/17 to fraction 9/10) and thereby have an apparent higher $M_r$; the WRS was unchanged in its sedimentation peak (fractions 19−21). In this case the separation of the two activities was complete,

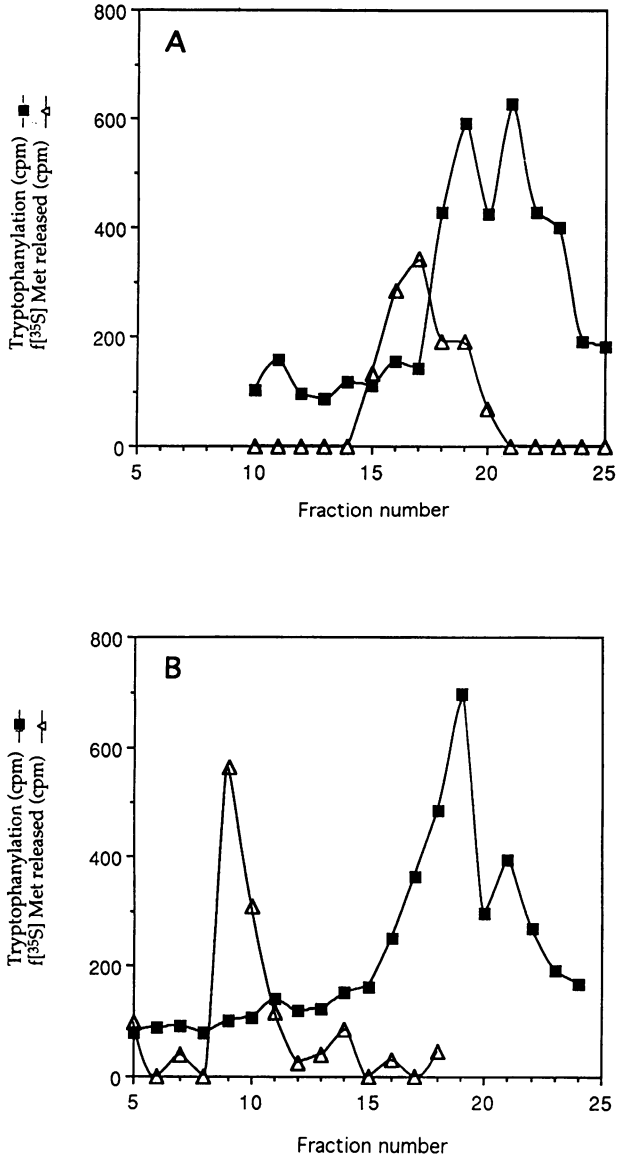


**Fig. 2.** Sedimentation of WRS and eRF activities in a sucrose gradient. (**A**) A purified eRF fraction (~100 μg of protein) containing significant WRS activity was sedimented through 5–20% (w/v) sucrose in a solution of 20 mM Tris–HCl, pH 7.8, 50 mM KCl, 0.1 mM EDTA and 1 mM DTT for 48 h at 38 000 r.p.m. at 4°C in a Beckman SW 41Ti rotor. Approximately 40 fractions, each of ~300 μl, were collected and assayed for the activities of the two proteins; 5 μl for WRS (up to 100 ng/50 μl assay; tryptophanylation c.p.m. must be multiplied by 10) and 10 μl for eRF (up to 25 ng/50 μl assay). The figure shows the part of the gradient containing the activities. (**B**) The same purified eRF fraction as in (A) was sedimented under the same conditions except that the KCl concentration in the gradient was 300 mM instead of 50 mM. Sedimentation was from right to left.

and as above, the 54 kDa band coincided with the WRS activity but not with the eRF activity. Obviously, the different sedimentation properties of eRF and WRS are not due to their molecular masses since these proteins are similar, both in their dimeric and monomeric states, and therefore other properties must account for this difference in behaviour.

### *The monoAb raised against purified rabbit eRF recognizes WRS but does not recognize eRF*

It was previously inferred (Frolova *et al.*, 1991, 1993) that the monoAb used to screen the rabbit cDNA library (Lee

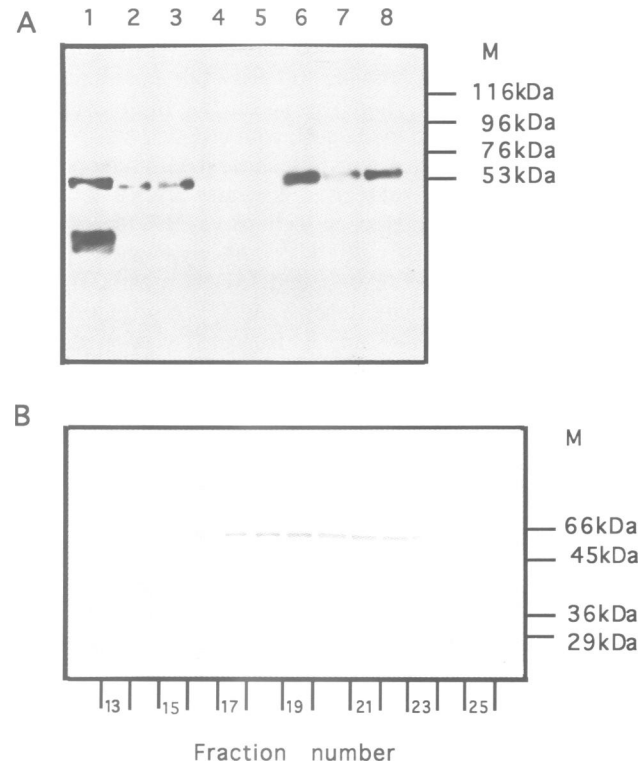


**Fig. 3.** Immunoblot analysis of the anti-eRF monoAb with the proteins contained in the eRF and WRS fractions. (**A**) Fractions from Q-Sepharose and Mono Q column chromatography. The protein fractions containing eRF and WRS activities (Figure 1A and B) were separated by SDS–PAGE and blotted on to an Immobilon P membrane (Procedure 1). Lane 1: 5 μg of pure bWRS; lanes 2 and 3: 3 μg of protein containing WRS activity from the combined fractions 50–62 after two separate Mono Q column chromatographies; lanes 4 and 5: 10 μg of protein containing eRF activity from combined fractions 78–82 after two separate Mono Q column chromatographies; lanes 6–8: 10 μg of protein containing both WRS and eRF activities from the combined fractions 60–75 after three separate Q-Sepharose column fractionations. The lower band corresponding to ~40 kDa observed after immunoblotting of bWRS (lane 1) is due to limited proteolysis of bWRS. All samples are from the same gel. (**B**) Fractions from sedimentation through a sucrose gradient. Samples (16 μl) of the fractions shown in Figure 2A containing WRS or eRF activity were separated by SDS–PAGE and blotted on to a nitrocellulose membrane (Procedure 2). In (A) and (B), protein markers (M) were analysed in a parallel well of the same gels and detected by amido black.

*et al.*, 1990) might have been raised against a protein other than against rabbit eRF. To test this possibility, a highly purified rabbit eRF preparation was immunoprecipitated with the monoAb mentioned above, and the supernatant assayed for its eRF activity. Under these conditions, half of the protein was immunoprecipitated, but almost all the eRF activity remained in the supernatant (not shown). This means that this monoAb, although capable of forming an immunoprecipitate, does not recognize native active eRF. There are two possible explanations for this result. (i) Part of the eRF protein has been denatured during the purification steps and it is this fraction which is now recognized by the monoAb raised against eRF. (ii) The monoAb binds to a protein different from the native or denatured eRF. If the second possibility is correct, what is the identity of the protein that binds to the monoAb?

It has been proposed (Frolova *et al.*, 1991, 1993) that the monoAb might have been raised against rWRS because it

binds efficiently to pure bWRS. Since complete separation of the eRF and WRS activities was achieved (Figures 1B and 2B), it became possible to verify this possibility. As shown in Figure 3A, there was no binding of this monoAb to proteins contained in the peak with eRF activity from the Mono Q column (lanes 4 and 5). Since the immunoblotting procedure denatures proteins, this result proves that besides being incapable of interacting with native eRF, the monoAb does not recognize partially or completely denatured eRF. In contrast, the monoAb bound to the protein fraction containing WRS activity (lanes 2 and 3). This monoAb also recognized pure bWRS and its truncated version (lane 1) generated by limited proteolysis of intact bWRS (Lemaire *et al.*, 1975; Prassolov *et al.*, 1975). When the rabbit eRF and WRS activities were present in the same fraction, as after Q-Sepharose chromatography (lanes 6–8), the monoAb still bound, demonstrating that eRF did not interfere with binding of the monoAb to WRS. The lack of competition between these two proteins for the same antibody precludes structural identity between rabbit WRS and eRF.

This conclusion was further strengthened by the results of Western blot analysis (Figure 3B) of the fractions from the sucrose gradient. In the gradient containing 50 mM KCl (Figure 2A), a single polypeptide of 54 kDa reacting with the monoAb was detected in fractions 17–23. The band with slightly faster mobility which was stained very faintly by Coomassie Blue (not shown) and coincided with the eRF activity (fractions 15–17) did not react with the monoAb. No immunoreacting species was detected in the region of the eRF in the higher salt gradient (Figure 2B) where this activity was better separated from the WRS activity (not shown). In this experiment, a characteristic degradation product of the WRS (~40 kDa) was also just visible although this was not seen on gels after they had been stained with Coomassie Blue.

### *The original rabbit cDNA encodes a protein with an ATP binding motif typical of class I aminoacyl-tRNA synthetases*

Since the monoAb recognizes WRS rather than eRF, the clone immuno-selected with this monoAb most likely encodes rabbit WRS. To date all class I aminoacyl-tRNA synthetases including WRS from bovine (Garret *et al.*, 1991), human (Fleckner *et al.*, 1991; Frolova *et al.*, 1991; Rubin *et al.*, 1991) and mouse (B.Pajot, J.Bonnet and M.Garret, in preparation; accession number X69657) contain the ATP binding motif, HXGH (Eriani *et al.*, 1990; reviewed in Moras, 1992 and in Cavarelli and Moras, 1993). Lee *et al.* (1990) however, did not find this motif in the deduced protein sequence encoded by this rabbit cDNA clone. To examine this point a segment of the cDNA was resequenced. Frolova *et al.* (1991, 1993) had previously suggested that the absence of this motif might have been due to sequence misinterpretation caused by band compression in the GC-rich region of this part of the cDNA. Indeed if one C residue is added at the level of nucleotides 644–646, and one A residue is omitted downstream (at position 663 or 664) the open reading frame is maintained and the same amino acid sequence is preserved as in the other mammalian WRS. Resequencing of the region encompassing nucleotides 644–664 in the forward and the reverse directions was performed automatically (not shown) and manually (Figure 4A). This demonstrated that a C had indeed been missed

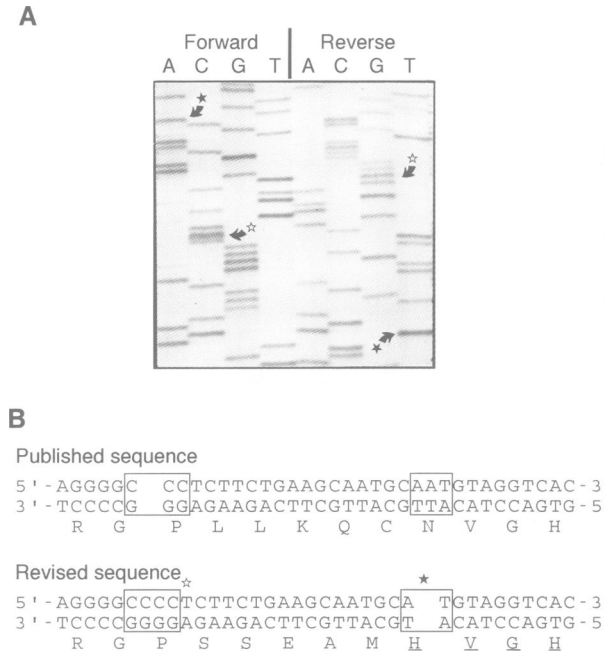


**Fig. 4.** Sequencing of the region of the putative rabbit eRF cDNA clone. (**A**) The sequence is shown for both strands in the region of the putative eRF clone where the ATP binding motif characteristic of class I aminoacyl-tRNA synthetases is located. The open star shows the site where a C compression from four to three Cs was suspected (G in the opposite strand) and the closed star shows the site scored as two As in the original sequence (T in the opposite strand). (**B**) Sequence showing the changes made to the original sequence determined by Lee *et al.* (1990). The revised amino acid sequence now includes the HXGH motif of class I aminoacyl-tRNA synthetases (underlined).

and an A had been inadvertently added. Although the C compression was hard to score as three or four Cs (open star, Figure 4A), the sequence from the other strand was unequivocally four Gs (open star). In the case of the additional A residue both strands gave a sequence which was quite unequivocal; there was only one A, and the other strand contained a single T at the position in question (closed star, Figure 4A). The corrected sequence of this region is shown in Figure 4B.

Taking these corrections into account, all the mammalian WRS sequences available can be aligned (Figure 5). It is noteworthy that the nucleotide sequence divergence between any two of the mammalian species belonging to four different mammalian orders is ~10% at the amino acid level. The alignment of the four mammalian WRS sequences, including the rabbit sequence formerly believed to encode eRF, strongly favours the proposition (Frolova *et al.*, 1991, 1993) that the cloned rabbit cDNA encodes WRS.

### *The rabbit cDNA encodes a protein that exhibits WRS activity*

The rabbit cDNA was expressed by Lee *et al.* (1990) in *E.coli*, and although the WRS activity was not determined, the authors did note some homology with the bacterial WRS sequences available at that time. We have re-examined the properties of this protein induced by isopropyl β-D-thio-galactopyranoside (IPTG) and have shown that the protein has $tRNA^{Trp}$ aminoacylation activity (Figure 6), but no eRF activity. Consequently, the properties of the expressed cDNA

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                                    18

MPNSE      ASLLELFNSIATQGELVRSLKAGNASKDEIDSAVKMLVS   45 hWRS
MADMSNGEQGCG*P****H***A******D***R**A**********L*   50 bWRS
MADVTNGER C**PQ***S*********K****RK*P*E*********L*   49 rWRS
MADMPSGE SCT*P**********************P**********L*   49 mWRS

LKMSYKAAAGEDYKADCPPGNPAPTSNHGPDATEAEEDFVDPWTVQTSSA   95 hWRS
**T*****T*****V*****D***E*GE*L*****D************** 100 bWRS
**T***E*M***********ST*D*HGD*E*VDDK*********R****   99 rWRS
********M**E***G******TAGR*CDS***K*S*********R****   99 mWRS

KGIDYDKLIVRFGSSKIDKELINRIERATGQRPHHFLRRGIFFSHRDMNQ 145 hWRS
********************V***********R*************H*  150 bWRS
**********Q*********V***********R*************** 149 rWRS
*********QP*********************R*************** 149 mWRS

VLDAYENKKPFYLYTGRGPSSEAMHVGHLIPFIFTKWLQDVFNVPLVIQM 195 hWRS
I************************************************** 200 bWRS
**************************************D****V**  199 rWRS
I*********************L**V********I***********  199 mWRS

                SY
TDDEKYLWKDLTLDQAYGDAVENAKDIIACGFDINKTFIFSDLDYMGMSS 245 hWRS
****************GY********  T********************P 249 bWRS
S************E*V*GYTL******MP****V***************P 249 rWRS
S************E***SYT*********************E***Q*P 249 mWRS

GFYKNVVKIQKHVTFNQVKGIFGFTDSDCIGKISFPAIQAAPSFSNSFPQ 295 hWRS
************************************************** 299 bWRS
************************************************** 299 rWRS
***R*****************************S****V**********K 299 mWRS

IFRDRTDIQCLIPCAIDQDPYFRMTRDVAPRIGYPKPALLHSTFFPALQG 345 hWRS
*******V****************************************** 349 bWRS
**HGQA**********************************************D 349 rWRS
*********************************H**************** 349 mWRS

AQTKMSASDPNSSIFLTDTAKQIKTKVNKHAFSGGRDTIEEHRQFGGNCD 395 hWRS
*********************************************V********** 399 bWRS
********************************************************* 399 rWRS
*********************S************V**********E 399 mWRS

VDVSFMYLTFFLEDDDKLEQIRKDYTSGAMLTGELKKALIEVLQPLIAEH 445 hWRS
**********************R************E*********** 449 bWRS
************************S**********E**D*****V*** 449 rWRS
****************R*******************T**D********* 449 rWRS

QARRKEVTDEIVKEFMTPRKLSFDFQ                       471 hWRS
************************Y***                      475 bWRS
**********M********Q*C*HY*                       475 rWRS
*****A**E*T********Q***H**CFCFDT                  481 mWRS
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**Fig. 5.** Comparison of the amino acid sequences of mammalian WRS. bWRS (Garret *et al.*, 1991), hWRS (Frolova *et al.*, 1991), mouse (m) WRS (B.Pajot, J.Bonnet and M.Garret, in preparation; accession number X69657) and rWRS (Lee *et al.*, 1990 and Figure 4). Asterisks denote residues identical with hWRS. Two adjacent amino acids in hWRS, Ser (position 213) and Tyr (position 214), indicated above the hWRS sequence, reflect differences in nucleotide sequence between two hWRS cDNA clones (Frolova *et al.*, 1991). The ATP (HXGH) and tRNA (KMSAS) binding motifs in the WRS are boxed.

product prove that the clone encodes biologically active rWRS that in our hands lacks eRF activity.

In the bacterially expressed cDNA clone, the eRF activity of the synthesized product was originally measured in the presence of 10% ethanol (Lee *et al.*, 1990). Therefore, although this cDNA encodes rWRS as shown above, the possibility remained that in the presence of ethanol the expressed protein might undergo a conformational change leading to the acquisition of a new catalytic property, hydrolysis of peptidyl-tRNA resembling that of eRF. To address this issue, the activity of WRS was measured in the presence of 10% ethanol, but with no appearance of eRF activity (not shown). Consequently, it seems most unlikely that 10% ethanol can confer RF activity to WRS.

## Discussion

### *Mammalian WRS and RF are different proteins*

In our earlier studies (Frolova *et al.*, 1993) we carried out initial experiments to test whether WRS and eRF might in fact be distinct proteins, despite the prevailing view that both

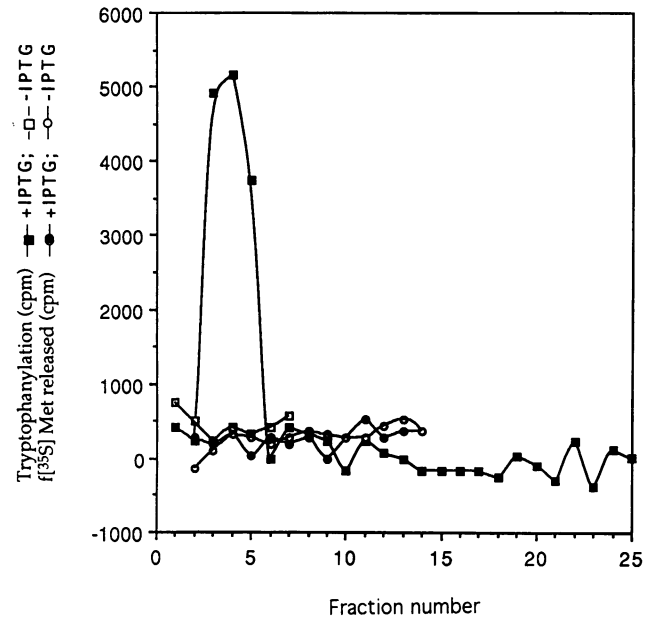


**Fig. 6.** WRS and eRF activities of partially purified extracts of *E.coli* carrying the plasmid encoding the putative rabbit eRF after DEAE-5PW fractionation using FPLC. *E.coli* cells containing the putative eRF cDNA in pBluescript were induced with IPTG. As control, extracts were also prepared from cells grown for the same period but not induced with IPTG. Fractions were assayed for WRS activity using total yeast tRNA, and for eRF activity. The eRF assay was only performed on fractions containing material which reacted with monoAb.

activities were exhibited by the same or similar proteins (Garret *et al.*, 1991; Bange *et al.*, 1992; Buwitt *et al.*, 1992). In the current experiments conclusive evidence has been obtained that the two proteins are distinct.

First, the peaks of WRS and eRF activities are completely separated on a Mono Q column (Figure 1B) and also by sucrose gradient sedimentation (Figure 2B). In other words, two independent strategies relying on different parameters distinguish these activities. Second, as demonstrated in Figure 3, the monoAb used to isolate the 'eRF' cDNA binds to the protein fractions containing WRS but not to those with eRF activity, highlighting the structural differences between these two proteins. We have also shown previously that the WRS activity is remarkably more thermostable than the eRF activity.

Given the entirely different roles of eRF and WRS in protein synthesis, the conclusion that these two activities reside on different polypeptide chains is perhaps not surprising. It is noteworthy that in prokaryotes there have been no reports of similarity between any of the 20 aminoacyl-tRNA synthetases and the two RFs.

In spite of these differences, RF and aminoacyl-tRNA synthetases could have a limited degree of 3-dimensional structural similarity since both proteins specifically recognize nucleotide triplets (the anticodons in the case of the majority of aminoacyl-tRNA synthetases and the termination codons in the case of RF). Therefore a certain undetermined domain of the RF structure could resemble the anticodon binding sites of aminoacyl-tRNA synthetases as revealed by X-ray crystallography of the tRNA–aminoacyl-tRNA synthetase complexes (reviewed in Cavarelli and Moras, 1993).

Mammalian WRS is composed of 475 amino acids with a deduced $M_r$ of 53 728 for the bovine species (Garret

*et al.*, 1991) and 471 amino acids ($M_r$ of 53 027) for the human species (Fleckner *et al.*, 1991; Frolova *et al.*, 1991; Rubin *et al.*, 1991). The native enzyme is a metalloprotein containing one Zn atom per dimer, essential for its structural integrity and catalytic activity (Kisselev *et al.*, 1981). WRS catalyses the aminoacylation of $tRNA^{Trp}$ coupled to ATP hydrolysis yielding AMP and $PP_i$. It recognizes the anticodon in $tRNA^{Trp}$. Native bWRS possesses no GTPase activity; however, after removal of $Zn^{2+}$, it acquires the ability to split purine nucleoside triphosphates into NDP and $P_i$. Peptidyl-tRNAs are usually not substrates for aminoacyl-tRNA synthetases (reviewed in Kisselev *et al.*, 1984).

The eRF purified from rabbit reticulocyte lysates is also composed of two subunits of ~56 kDa (Konecki *et al.*, 1977). It possesses ribosome-dependent GTPase activity and participates in ribosome-dependent cleavage of peptidyl-tRNA into the peptide moiety and free tRNA in the presence of a termination codon and GTP. It specifically recognizes termination signals containing UAA, UAG or UGA (reviewed in Caskey, 1980; Tate and Brown, 1992). It is unknown whether rabbit eRF is able to form tryptophanyl-adenylate or to bind to tryptophan and/or ATP; such activities have been described for bWRS (reviewed in Kisselev *et al.*, 1979).

One essential functional difference between these two proteins resides in their dissimilar recognition of tRNAs: eRF, if it participates directly in the hydrolysis reaction, should recognize any peptidyl-tRNA whereas WRS specifically interacts with $tRNA^{Trp}$. This indicates that at least a part of their tRNA binding sites must be different. In the case of WRS an ester bond is formed, whereas in the RF-dependent reaction hydrolytic cleavage occurs between the peptidyl moiety and the tRNA. The hydrolytic activity of aminoacyl-tRNA synthetases towards aminoacyl-tRNAs is low and does not require NTP hydrolysis (reviewed in Kisselev *et al.*, 1984). It is unknown whether WRS can hydrolyse peptidyl-tRNA, and we have not been able to detect such an activity.

From a comparison between the properties of bovine WRS and rabbit eRF (Frolova *et al.*, 1993) it seems highly improbable that both reactions, the ATP-dependent tryptophanylation of $tRNA^{Trp}$, and the ribosome-, termination codon- and GTP-dependent hydrolysis of peptidyl-tRNAs, are catalysed by the same functional centre or protein domain.

Multidomain proteins do exist however; for example, a multidomain protein encoding prolyl- and glutamyl-tRNA synthetase activities in *Drosophila* has been described (Cerini *et al.*, 1991). Nevertheless the occurrence of the WRS and eRF activities on the same polypeptide chain would seem functionally unfavourable. Furthermore, both activities would interfere with each other were they to reside on the same polypeptide chain, and this seems to be a property hardly likely to have been fixed during evolution.

### *The original rabbit cDNA clone encodes WRS*

It was suggested earlier (Frolova *et al.*, 1991, 1993) that the close resemblance between the deduced amino acid sequences of WRS and eRF might be due to incorrect assignment of the rabbit cDNA clone, and it was proposed that this rabbit cDNA encodes WRS rather than eRF. The following observations strongly support this assumption: (i) the nucleotide sequence of the cloned cDNA encodes a protein that possesses all the features of mammalian WRS (Figure 5) including a high degree of similarity with these proteins and the ATP binding motif previously overlooked (Figure 4), (ii) the bacterially expressed cDNA exhibits WRS activity but no eRF activity in our hands (Figure 6), and (iii) no similarity is detected between bacterial RFs and the putative eukaryotic RF, whereas bacterial and mammalian WRS exhibit a certain degree of structural homology (Lee *et al.*, 1990; Frolova *et al.*, 1991; Garret *et al.*, 1991).

The rabbit cDNA clone of Lee *et al.* (1990) should be redefined as encoding rWRS (with corrections of the nucleotide sequence) in the appropriate databanks. In the light of these results, the studies of Buwitt *et al.* (1992) on hWRS induced by interferon should be re-interpreted taking into account that the apparent anti-suppression activity of the induced protein is unlikely to be caused by eRF activity. We have shown previously that hWRS has no eRF activity (Frolova *et al.*, 1993).

To screen the rabbit cDNA library, a monoAb raised against eRF purified from a reticulocyte lysate was used (Lee *et al.*, 1990). This monoAb bound to a single polypeptide of ~56 kDa (Konecki *et al.*, 1977) thought to be eRF, and on this basis was believed to have been generated against eRF. This monoAb does not inhibit eRF activity (Frolova *et al.*, 1993), and now we have shown that it neither binds to eRF (Figure 3), nor immunoprecipitates active eRF. In conclusion, the epitope recognized by the antibody must reside on a polypeptide other than eRF. It is most likely (Figure 3) that it was generated against WRS present as a contaminant, due to the similar size of these two proteins. Several observations are in favour of this possibility: (i) the monoAb binds to pure bWRS and to fractions containing rWRS activity (Figure 3A, lanes 1–3; and Figure 3B), (ii) the monoAb used to screen the cDNA library selected a cDNA encoding rWRS, (iii) the standard purification scheme (Caskey *et al.*, 1974) does not yield eRF completely devoid of WRS (Frolova *et al.*, 1993), and (iv) the eRF preparation used as antigen to generate the monoAb was not tested for WRS activity. This monoAb could be very useful as a tool for studying rWRS.

## Materials and methods

#### *Materials*

Poly(A,G,U), AUG, *E.coli* $tRNA^{fMet}$, CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], heparin–Sepharose, peroxidase-linked goat anti-mouse IgG, and the substrates for alkaline phosphatase were from Sigma. Chemicals for PAGE were from Bio-Rad. Q-Sepharose and Mono Q HR 5/5 were from Pharmacia. The DEAE-5PW column was from Waters. Peroxidase-linked anti-mouse IgG for the detection of membrane-bound mouse primary antibodies, protein markers, ECL Western blotting reagents, L-[$^{35}$S]methionine (>30 TBq/mmol), L-[$^{3}$H]methionine (2.6 Tbq/mmol), L-[$^{3}$H]tryptophan (1.1 TBq/mmol) and [$^{35}$S]dATP (>37 TBq/mmol) were from Amersham. The tetranucleotide UAAA was synthesized by polynucleotide phosphorylase from UA and ADP (Tate and Caskey, 1990).

#### *Formyl[$^{35}$S or $^{3}$H]methionyl-tRNA$^{fMet}$ (f[$^{35}$S or $^{3}$H]Met-tRNA$^{fMet}$)*

This compound was synthesized using *E.coli* $tRNA^{fMet}$ and L-[$^{35}$S]-methionine or [$^{3}$H]methionine as described by Goldstein *et al.* (1970).

#### *Rabbit reticulocyte ribosomes*

The ribosomes were isolated according to Caskey *et al.* (1974). They were further purified by centrifugation through a 10% sucrose cushion containing 20 mM Tris–HCl, pH 7.3, 3 mM $MgCl_2$, 350 mM KCl and 1 mM dithiothreitol (DTT). The f[$^{35}$S or $^{3}$H]Met-$tRNA^{fMet}$–ribosome intermediates were prepared as described by Caskey *et al.* (1974) in the presence of 0.05 $A_{260}$ of AUG per 2 $A_{260}$ of ribosomes in 50 μl of incubation mixture and were used immediately for the release assay.

Rabbit eRF was purified (at 4°C) from reticulocyte lysates. The first purification steps included isolation of the S100 supernatant, and ammonium

sulfate precipitation. This was followed either by chromatography on Q-Sepharose (Fast Flow) and then Mono Q columns using FPLC (for details, see legend of Figure 1), or by DEAE–Sephadex, heparin–Sepharose (firstly batch elution, and then gradient elution) and sucrose gradient sedimentation.

### *Release assay*

The incubation mixture (50 μl) for the release assay contained 20 mM Tris–HCl, pH 7.5, 11 mM $MgCl_2$, 40–60 mM KCl, 1 mM GTP, 3 pmol of $f$[$^{35}$S or $^{3}$H]Met-tRNA$^{fMet}$–ribosome intermediate, 0.2 $A_{260}$ unit of poly(A,G,U) or 0.1 $A_{260}$ unit (4 nmol) of UAAA, and appropriate protein fractions as indicated. Quantitation of $f$[$^{35}$S or $^{3}$H]methionine ($f$[$^{35}$S or $^{3}$H]Met) released was performed as described by Tate and Caskey (1990). The radioactivity of the samples was measured in scintillation liquid with 25% Triton X-100.

### *WRS purification and assay*

Bovine WRS was purified according to Kisselev *et al.* (1979) as modified by Kovaleva *et al.* (1992), and was kindly provided by G.Kovaleva. The WRS activity was measured by tryptophanylation of yeast tRNA enriched in tRNA$^{Trp}$ (kindly provided by G.Keith) according to Kisselev *et al.* (1979).

### *Antibodies*

A crude 'anti-eRF' monoAb raised against purified rabbit eRF was kindly provided by C.T.Caskey.

### *Protein immunoblotting*

Two procedures were used.

*Procedure 1.* Proteins were separated on a 0.1% SDS–12.5% polyacrylamide gel (Laemmli, 1970) and transferred to an Immobilon-P membrane (Millipore) by electrophoresis for 10–12 h at 0.5 mA/cm$^2$ of membrane at 4°C in a trans blot apparatus (Bio-Rad), in a solution containing 50 mM CAPS buffer, pH 11. The filter was washed for 2–5 min with buffer A (20 mM Tris–HCl, pH 8.0 and 150 mM NaCl), containing 0.05% Tween-20 (buffer AT). After blocking the membrane with 5% bovine serum albumin (BSA) in buffer A containing 0.5% Tween-20 for 2 h at room temperature, the filter was washed three times for 10 min with buffer AT. It was then incubated for 2 h at 37°C in buffer AT containing 1% BSA and 'anti-eRF' monoAb at a 1:1000 dilution. Unbound monoAb was removed by washing the filter three times for 10 min with buffer AT. The filter was incubated for 1 h at room temperature with anti-mouse IgG conjugated with peroxidase, and washed four times for 10 min with buffer AT and once with water. To detect the blotted antigen, the filter was transferred to the ECL solution containing the substrate for peroxidase. The filter was exposed for 10–20 s to a Fuji RX X-ray film and developed.

*Procedure 2.* Alternatively the membrane was first treated with 1% milk $T_3N$ [1% (w/v) of non-fat milk powder in 40 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20 and 0.01% (w/v) thiomersal]. The primary 'anti-eRF' monoAb, diluted 1:1000 in $T_3N$ but containing 0.1% milk powder, was applied to the filter for 1 h, and the filter rinsed extensively in $T_3N$. The secondary antibody, goat anti-mouse IgG conjugated to alkaline phosphatase diluted in $T_3N$ containing 0.1% milk powder, was incubated with the filter for 1 h and the filter washed again extensively with $T_3N$ buffer. The filter was rinsed in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$ (AP buffer). The substrates, nitro blue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.167 mg/ml) were added to the blot in AP buffer and incubated for ~5 min until the bands appeared.

### *Sequencing of a segment of the rabbit cDNA*

Double-stranded template was purified from the cloned cDNA as a recombinant in pBluescript, a kind gift from C.C.Lee. Primers were synthesized spanning the region of the putative eRF clone (Lee *et al.*, 1990) whose sequence was in question. The forward primer starting at position 521 was 5′-AGCCACCGGCCAGAGACCG-3′ whereas the complementary reverse primer starting at position 757 was 5′-GCGTGAGGTCCTT-CCACAGG-3′. Sequencing was performed using a Pharmacia T7 sequencing kit and [$^{35}$S]dATP, and repeated with an ABI Automated Sequencer with identical results.

### *Expression of the putative rabbit eRF gene in E.coli*

*E.coli* cells containing the putative eRF gene in the expression vector pBluescript were grown in LB to an $A_{600}$ of 0.5. The cells were then induced with 10 mM IPTG and grown for 3 h more at 37°C, washed and harvested. They were then lysed by grinding with alumina, suspended in eRF buffer (20 mM Tris–HCl, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, pH 7.8 at 4°C) and centrifuged at 100 000 *g* for 3 h at 4°C. The S100 supernatant was precipitated by ammonium sulfate to 80% saturation, the protein pellet resuspended in eRF buffer and dialysed overnight against the same buffer. The protein solution (~10 mg) was chromatographed on a DEAE-5PW column using a 0–1 M stepwise KCl gradient in eRF buffer.

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