Three tRNA Binding Sites in Rabbit Liver Ribosomes and Role of the Intrinsic ATPase in 80S Ribosomes from Higher Eukaryotes†

Anna V. El'skaya,‡ Galina V. Ovcharenko,‡ Sergey S. Palchevskii,‡ Zoja M. Petrushenko,‡ Francisco J. Triana-Alonso,§,|| and Knud H. Nierhaus*,§

Institute of Molecular Biology & Genetics of the National Academy of Sciences of Ukraine, Kiev, Ukraine, and Max-Planck-Institut für Molekulare Genetik, AG Ribosomen, Ihnestrasse 73, D-14195 Berlin, Germany

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ABSTRACT: Three tRNA binding sites have been found in organisms of all domains (former kingdoms) with only one exception: Four binding sites have been reported for cytoplasmic 80S ribosomes from rabbit liver. Therefore, the issue was reconsidered, and the data revealed that rabbit liver ribosomes contain three tRNA binding sites, underlining the universal character of this ribosomal feature. Furthermore, a first analysis of the role of the ribosome intrinsic ATPase was performed. This ATPase is found in ribosomes of higher eukarya but not in lower eukarya such as yeast or ribosomes of the domains archea and bacteria. The results suggest that the intrinsic ATPase fulfills the same function as the essential third elongation factor EF-3, an ATPase in higher fungi (yeast etc.), that facilitates the release of the deacylated tRNA from the E site.

The mechanism of protein synthesis is explained in the frame of models which comprise three tRNA binding sites, the A,[1] P, and E sites, since three tRNA binding sites have been found on ribosomes of eubacterial organisms (*1*, *2*, *3*) as well as archaebacterial (*4*) and lower eukaryotic organisms (yeast, *5*).

However, in higher eukaryotes (rabbit liver), four tRNA binding sites were reported (*6*). This observation rejects the view that three tRNA binding sites are a universal feature of ribosomes. However, a reconsideration of this issue seems to be justified, since only three sites for ribosomes from the same organism were previously reported (*7*), and again three tRNA binding sites were observed in 80S ribosomes derived from another mammalian organism (human placenta, *8*).

Here we reexamine the issue and, furthermore, analyze the importance of the ATPase activity which is an intrinsic feature of 80S ribosomes from higher organisms. Arguments have been raised against (*9*) and in favor of the view (*10*) that this activity might fulfill a role similar to the third elongation factor (EF-3) in lower eukaryotes such as yeast: EF-3 is a ribosome-dependent ATPase that facilitates the release of the deacylated tRNA from the E site (*5*). The data reported here demonstrate that rabbit liver ribosomes have only three tRNA binding sites, suggesting that three sites are a universal characteristic of ribosomes. Furthermore, ATP hydrolysis by the 80S ribosomes facilitates the release of deacylated tRNA from the E site.

EXPERIMENTAL PROCEDURES

Poly(U), ATP, GTP, AM*PP*[NH]*P*, GM*PP*[NH]*P*, puromycin, DTT, alkaline phosphatase, and polynucleotide kinase were purchased from Boehringer; HEPES, creatine kinase (CPK), and creatine phosphate (CP) from Calbiochem; spermine and spermidine from Fluka. Venom phosphodiesterase was from Worthington, and T7 RNA polymerase and *Ssp*I were obtained from Biolabs. Nitrocellulose filters were from Sartorius; GF/C glass microfiber filters were from Whatman, and radioactively labeled compounds were from Amersham. Chemicals were of analytical grade and purchased from Merck.

In order to obtain a consistent and reliable set of data, the biological components used should be of the same origin. Therefore, 80S ribosomes and their subunits, elongation factors, crude tRNA preparation, total aminoacyl-tRNA synthetase fraction, highly purified phenylalanyl-tRNA synthetase (PheRS), and several individual tRNAs were isolated from rabbit liver.

Highly active ribosomal subunits were obtained according to (*10*). In short, polysomal fractions were incubated in a complete translation system for 45 min at 37 °C and then for 10 min at 37 °C in the presence of 0.2 mM puromycin. KCl was added to reach a final concentration of 0.66 M monovalent cations (KCl plus NH_4Cl), and the subunits were separated by zonal centrifugation in a 7.5−37% (w/v) sucrose gradient containing 20 mM Tris-HCl, pH 7.5 (0 °C), 3.5 mM $MgCl_2$, 0.35 M KCl, and 6 mM 2-mercaptoethanol. 80S ribosomes were obtained by reassociation of 40S and 60S subunits with a 1.2-fold molar excess of 60S subunit. Reassociation was confirmed by analytical centrifugation in an SW60 rotor. The ribosome concentrations were calculated assuming the following ratios: 56 pmol/A_{260} for 40S subunits, 27 pmol/A_{260} for 60S subunits, and 18 pmol/A_{260} for 80S ribosomes.

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* Correspondence should be addressed to this author at the MPI für Molekulare Genetik, AG Ribosomen, Ihnestrasse 73, D-14195 Berlin, Germany. Telephone: *49-30-8413-1217. Fax: *49-30-8413-1380. E-mail: Nierhaus_KH@mpimg-berlin-dahlem.mpg.de.

‡ Institute of Molecular Biology & Genetics of the National Academy of Sciences of Ukraine.

§ Max-Planck-Institut für Molekulare Genetik.

|| Current address: Centro de Investigaciones Biomedicas, Universidad de Carabobo, Maracay, Venezuela.

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[1] Abbreviations: A site, aminoacyl-tRNA binding site (decoding center of the ribosome); P site, ribosomal binding site of the peptidyl-tRNA before peptide bond formation; E site, exit site specific for deacylated tRNA.

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Elongation Factors Were Isolated as Follows: EF-1α was isolated using a combination of gel-filtration and ion-exchange chromatographies according to (*11*). EF-2 was obtained by a procedure described in (*12*) with slight modifications. The purity of both factors was about 95% as judged from the SDS−polyacrylamide gel electrophoresis. The factors were highly active in stimulation of poly(Phe) synthesis. EF-1α was also assayed by stimulation of Phe-tRNA^{Phe} binding to the ribosomal A site and EF-2 by stimulation of AcPhe-tRNA^{Phe} translocation from the A to the P site determined by puromycin reaction. The factors were free of cross-contamination according to these assays.

The total aminoacyl-tRNA synthetase preparation, free of tRNA, was obtained by chromatography of the S-100 fraction on DEAE-cellulose as detailed in (*13*). PheRS was highly purified from the polyribosomal fraction by ion exchange chromatography as described in (*14*).

Crude tRNA from rabbit liver was isolated according to (*13*). Individual tRNA$_i^{Met}$, tRNA^{Val}, and tRNA^{Phe} from rabbit liver and their aminoacyl and acetylaminoacyl forms were obtained by reversed phase HPLC purification on a Nucleosil C4 column in a methanol gradient as outlined in (*5*). tRNA$_i^{Met}$ was purified in the form of *N*-formylmethionyl-tRNA^{Met}, obtained by using formylase in the S-100 fraction from *E. coli* and formyl donor (N^{10}-formyltetrahydrofolic acid); deacylated tRNA$_i^{Met}$ was obtained using a peptidyl-tRNA hydrolase isolated from *E. coli* as described in (*15*). The 5′-ends of deacylated tRNA^{Phe} and tRNA$_i^{Met}$ were labeled with [^{32}P]phosphate according to (*16*), after which the 3′-ends were labeled (*17*). After separation in a sequencing gel, the pure radioactive tRNA^{Phe} and tRNA$_i^{Met}$ were diluted with corresponding cold tRNA to a specific activity of 3000−5000 cpm/pmol as described in (*16*).

Heteropolymeric MF-mRNA is 46 nucleotides long with the sequence GGG-$(A_4G)_3$-AAA-AUG-UUC-$(A_4G)_3$--AAAU and was obtained in large-scale transcription assays where the UTP concentration was 0.1 of that of the ATP concentration in order to increase the yield of correct transcripts (*5*, *18*). The MVF-mRNA is 42 nucleotides long and contains the sequence C_{17}-AUG-GUC-UUC-C_{16} and was chemically synthesised as described (*5*).

Poly(Phe) synthesis was performed in an incubation mixture of 25 μL containing 20 mM HEPES·KOH, pH 7.6 (0 °C), 100 mM NH_4Cl, 0.6 mM spermidine, 0.8 mM spermine, 5 mM $MgCl_2$, 1 mM ATP, 0.4 mM GTP, 2 mM 2-mercaptoethanol, 10 mM CP, 1 μg/μL CPK, 50 g poly-(U), 2.3 pmol of 80S ribosomes, 10 pmol of EF-1, 5 pmol of EF-2, and 47 pmol of [^{14}C]Phe-tRNA^{Phe}. In some experiments, no charged [^{14}C]Phe-tRNA^{Phe} was added, but instead tRNA^{Phe} was preincubated with PheRS, ATP, and [^{14}C]Phe for 10 min at 37 °C in conditions optimal for aminoacylation. Poly(Phe) synthesis was performed at 37 °C for the time periods indicated. The reaction was terminated by the addition of 1 mL of 10% trichloroacetic acid, and unreacted aminoacyl-tRNA was destroyed by incubation at 90 °C for 10 min.

[^{32}P]tRNA^{Phe} binding to 80S ribosome was carried out in a mixture of 30 μL containing 20 mM HEPES·KOH, pH 7.6 (0 °C), 100 mM NH_4Cl, 4 mM $MgCl_2$, 0.6 mM spermidine, 0.8 mM spermine (buffer A), 3.2 pmol of 80S ribosomes, 50 μg of poly(U), and amounts of [^{32}P]tRNA^{Phe} (1650 pmol/A_{260}) as indicated. The incubation time was 15 min at 37 °C. Binding of labeled tRNA to ribosomes was measured by nitrocellulose filtration.

Translocation Assay. To form a pretranslocated complex, the ribosomal P site was occupied by incubating 2.0 pmol of 80S ribosomes with 14 pmol of MF-mRNA (or MVF-mRNA) and 3 pmol of [^{32}P]tRNA$_i^{Met}$ in 25 μL of buffer A for 10 min at 37 °C. Then the A site was filled by adding 4 pmol of Ac[^{14}C]Phe-tRNA^{Phe} (or Ac[^{3}H]Val-tRNA^{Val}) and incubating for 10 min at 37 °C. Translocation was performed by adding 4 pmol of EF-2 and 0.2 mM GTP and incubating the mixture for 20 min at 37 °C. The level of translocation was determined by a puromycin reaction for 10 min at 30 °C (under these conditions, the amount of puromycin-reactive acetylaminoacyl-tRNA before translocation did not exceed 10% of that after translocation).

When MVF-mRNA was used, the A site of the posttranslocational complexes was occupied by incubating the mixture with [^{14}C]Phe-tRNA^{Phe} in a ternary complex (4 pmol of Phe-tRNA^{Phe}, 8 pmol of EF-1α, and 0.4 mM GTP were preincubated for 3 min at 37 °C) for 15 min at 37 °C.

Chasing Experiments. [^{32}P]tRNA$_i^{Met}$ was chased from the E site of the posttranslocational complexes by adding a 10-fold molar excess of nonlabeled deacylated tRNA$_i^{Met}$ and incubating the mixture for 5 min at 37 °C.

ATPase Assay. The standard incubation mixture for the ATPase assay contained 5 pmol of 80S ribosomes and 0.6−3 mM [γ-^{32}P]ATP in 50 μL of buffer A. Incubation time was 15 min at 37 °C. The extent of ATP hydrolysis was determined by measuring the amount of ^{32}P released according to (*19*). The ribosomes used hydrolyzed 10 pmol of ATP/min per ribosome, similar to the ribosomal activity described in (*10*).

RESULTS

The ionic conditions were optimized for poly(Phe) synthesis and for tRNA binding to programmed ribosomes from rabbit liver. Figure 1A,B shows examples of the dependence of poly(Phe) synthesis on the concentrations of Mg^{2+} and spermine, respectively; further conditions are as described under Experimental Procedures. The optima found in both systems [poly(Phe) synthesis and tRNA binding] were very similar: 100−150 mM NH_4Cl, 4−5 mM $MgCl_2$, 0.6 mM spermine, and 0.8 mM spermidine in the presence of 20 mM HEPES·KOH, pH 7.6 (0 °C). All further experiments were performed under these conditions.

Maximum Number of tRNA Binding Sites on Rabbit Liver Ribosomes. The determination of the number of tRNA binding sites requires knowledge of the fraction of ribosomes active in tRNA binding. We assessed the active fraction of ribosomes *via* saturating the ribosomes with AcPhe-tRNA. In the absence of poly(U), up to 0.9 molecule could be bound per 80S ribosome, whereas the saturation curve leveled off at 1.66 AcPhe-tRNAs per 80S ribosome (Figure 2A) in the presence of poly(U). Since a maximum of 2 AcPhe-tRNAs can be bound per 80S ribosome from rabbit liver (*6*, *7*), 83% of the input ribosomes participate in tRNA binding.

AcPhe-tRNA can occupy A and P sites, whereas deacylated tRNA can bind to all available sites (*6*). Saturation of poly(U)-programmed ribosomes with deacylated [^{32}P]-tRNA^{Phe} should therefore give the maximal number of tRNA

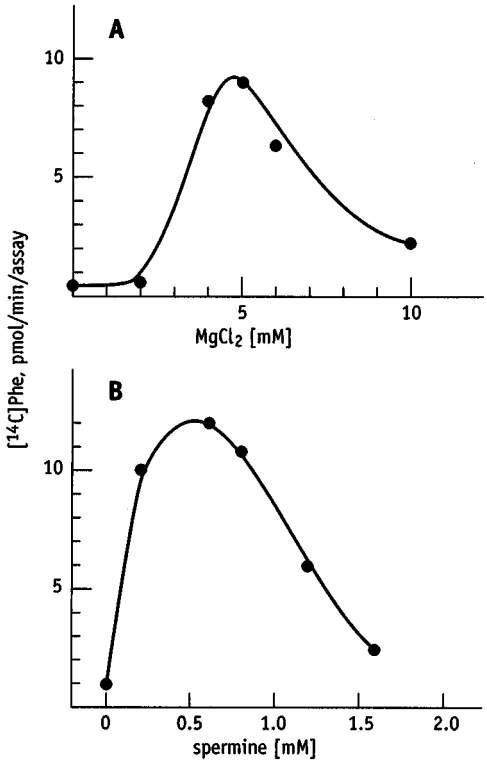

FIGURE 1: Optimization of poly(Phe) synthesis of 80S ribosomes from rabbit liver. (A) Dependence on $MgCl_2$ concentrations. (B) Dependence on spermine concentrations.

binding sites. In the presence of poly(U), about 2.9 $tRNA^{Phe}$ can be bound per *active* 80S ribosomes, in the absence about 1.25 $tRNA^{Phe}$ (Figure 2B). Saturation is achieved at a 25-molar excess of tRNAs over ribosomes. Kinetics at the corresponding concentration of tRNAs over 80S ribosomes demonstrate that the same maximal binding numbers are found (Figure 2C), indicating that the observed maximal binding numbers are indeed representing the extent of reaction.

ATPase Activity Strongly Associated with the 80S Ribosome Facilitates tRNA Dissociation from the E Site. A point of controversy is the functional importance of the ATPase strongly associated with 80S ribosomes of higher eukaryotes. We tested the possibility that the intrinsic ATPase of ribosomes of higher eukarya fulfills a function equivalent to that of EF-3, an essential elongation factor and ATPase in higher fungi such as yeast, and analyzed the diagnostic feature of the ATP-dependent EF-3 function, *i.e.*, the improved accessibility of the E site in the presence of EF-3·TP. The increased accessibility is revealed by an increase of the chasing efficiency of E site bound tRNA (5).

To this end, a posttranslocational complex was enzymatically constructed in the presence of the heteropolymeric MVF-mRNA which contains three unique codons, AUG-GUC-UUC, in the middle. The codons code for methionine, valine, and phenylalanine. The posttranslocational complex carried $[^{32}P]tRNA_i^{Met}$ at the E site and $Ac[^3H]$Val-tRNA at the P site (Figure 3A). No more than 20% of the tRNA could be chased from the E site upon addition of a 10-molar excess of nonlabeled $tRNA_i^{Met}$ over ribosomes. However, in the presence of 3 mM ATP, the chasing efficiency increased to about 50% (Figure 3B; AcVal-tRNA at the P site could not be chased at all; not shown) The dependence on the ATP concentration is shown with an equivalent

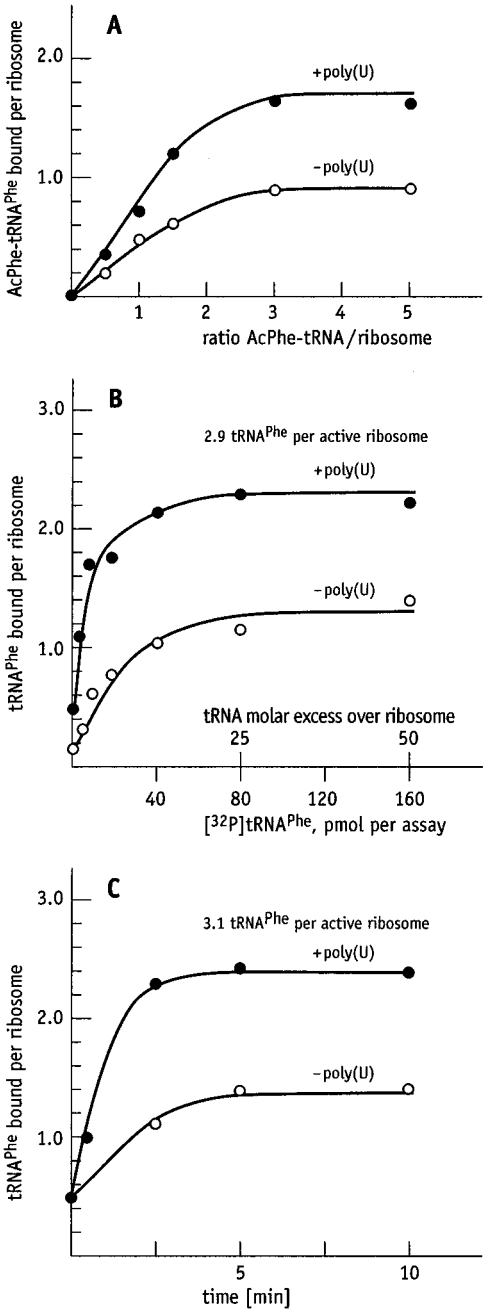

FIGURE 2: tRNA binding to 80S ribosomes. (A) Saturation curve with $Ac[^{14}C]$Phe-tRNA (940 cpm/pmol; AcPhe-tRNA per *input* 80S) in the presence and absence of poly(U). (B) Saturation curves with $[^{32}P]tRNA^{Phe}$ (4800 cpm/pmol; tRNA per *active* 80S). (C) Kinetics of $[^{32}P]tRNA^{Phe}$ (4300 cpm/pmol) binding in the presence of a 25-molar excess of tRNA over ribosomes.

posttranslocational complex where the MF-mRNA carrying the AUG-UUC codon in the middle was used (Figure 4A,B). GTP had also a significant but lower effect, whereas the noncleavable analogues AMPPNP and GMPPNP even reduced the chasing efficiency from 15% (no nucleotide triphosphates) to 5% (Figure 4C). No effect of ATP hydrolysis was found on $tRNA_i^{Met}$ dissociation from the P site before translocation. In control experiments, 3′-labeled $[^{32}P]tRNA_i^{Met}$ was also used to be sure that the terminal A of the acceptor end is preserved. Equivalent results were obtained.

A second translocation round was performed with the posttranslocational complex shown in Figure 3A by adding a ternary complex containing $[^{14}C]$Phe-tRNA. After the

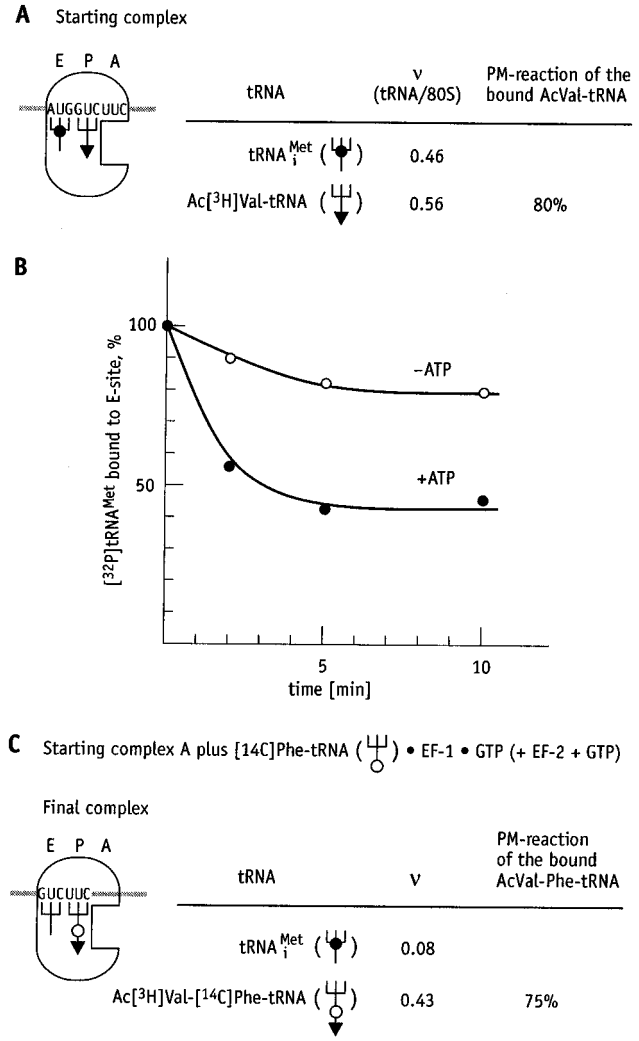

FIGURE 3: Chasing experiment. (A) Start complex: the posttranslocational complex was enzymatically prepared in the presence of MVF-mRNA and carried [^{32}P]tRNA$_i^{Met}$ at the E site and Ac[^{3}H]-Val-tRNA^{Val} at the P site. The specific activities were 1750 and 1960 dpm/pmol, respectively. (B) Kinetics of chasing the E site bound tRNA caused by a 10-molar excess of nonlabeled tRNA$_i^{Met}$ in the presence and absence of 3 mM ATP. The AcVal-tRNA was not chased at all. (C) Final complex after a second translocation: a ternary complex containing [^{14}C]Phe-tRNA^{Phe}•EF-1•GTP was added to the start complex (A), and a translocation was performed with EF-2 and GTP. The [^{14}C]Phe-tRNA^{Phe} had a specific activity of 1200 dpm/pmol. For more details, see Experimental Procedures.

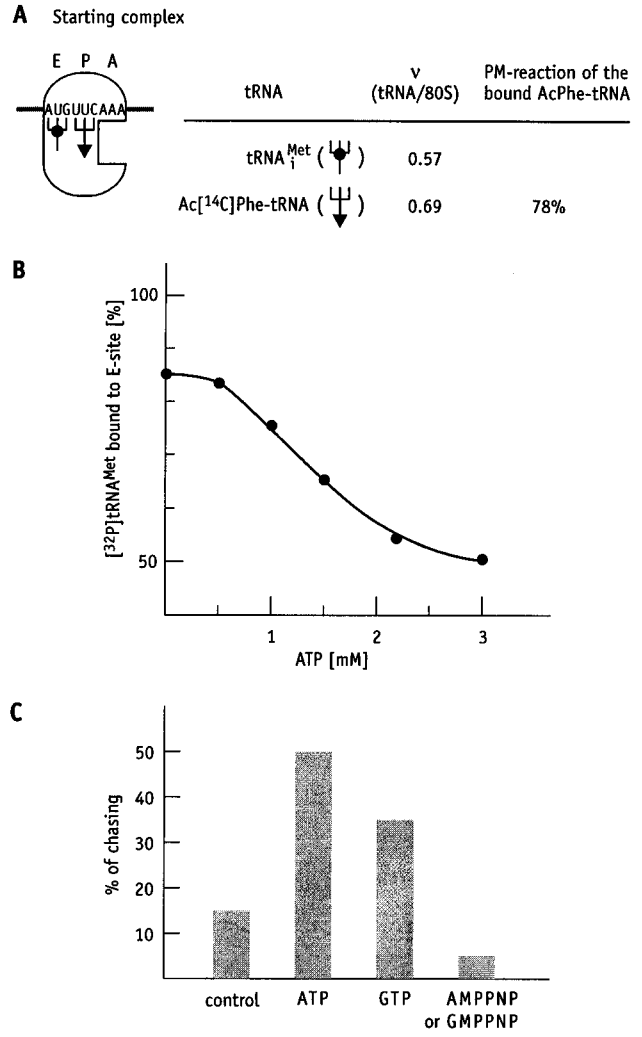

FIGURE 4: Dependence of the chasing efficiency of the tRNA present at the E site on various nucleotides. (A) Start complex: the posttranslocational complex was enzymatically constructed in the presence of MF-mRNA and carried [^{32}P]tRNA$_i^{Met}$ at the E site and Ac[^{14}C]Phe-tRNA^{Phe} at the P site. (B) Chasing efficiency (25-molar excess of nonlabeled tRNA$_i^{Met}$) at various ATP concentrations. (C) Chasing efficiency as in (B) but in the presence of 3 mM ATP, GTP, or their noncleavable analogues.

second translocation, 75% of the ^{14}C-labeled material reacted with puromycin, indicating that at least this amount of the bound Phe-tRNA was present at the P site presumably in the form of AcVal-Phe-tRNA. The important point is that this posttranslocational complex (Figure 3C) has lost almost all the [^{32}P]RNA$_i^{Met}$ ($\nu = 0.08$) which was so stably bound in the starting posttranslocational complex ($\nu = 0.46$; Figure 3A,B).

DISCUSSION

Three tRNA Binding Sites on Ribosomes of Higher Eukaryotes (Rabbit Liver). Ribosomes from bacteria (*1*, *2*, *3*), archaea (*4*), lower eukarya (yeast, *5*), and mammals (*8*) were shown to contain three ribosomal binding sites. Only in rabbit liver was a fourth site, the "S" site, postulated in addition to the canonical A, P, and E sites (*6*). Both E and S sites were considered to be specific for deacylated tRNA. Since poly(U) was used as the mRNA, the bound deacylated tRNAs could not be unequivocally assigned to the various sites. The assignment followed qualitative criteria: The E site binding was described as labile and being independent of codon−anticodon interaction, which were thought to be features of the E site of prokaryotic ribosomes. In contrast, the S site binding was described as stable and also being independent of codon−anticodon interaction; it was thought that this site did not participate in the tRNA turnover during the elongation phase of protein synthesis.

Here we show that 80S ribosomes from rabbit liver contain only three tRNA binding sites. Saturation experiments do not reveal any evidence for a fourth site (Figure 2A), and kinetics demonstrate that we achieved the maximal extent of the tRNA binding reaction (Figure 2B). [^{32}P]tRNA$_i^{Met}$ at the P site of pretranslocational ribosomes was translocated to the E site where it was stably bound (Figure 3A,B), but this tRNA was almost completely in the next round of elongation (Figure 3C); *i.e.*, the E site behaves as the corresponding site in prokaryotic ribosomes (*20*), and the

existence of an "S" site keeping its tRNA during several rounds of elongation could not be confirmed.

Several points involved in this misassignment can be identified: (1) The authors (*6*) did not perform saturation experiments which, together with an assessment of the active fraction of the ribosome preparation, should have given a clean-cut answer. (2) The criteria for the E site assignment did not take into consideration published data from other groups. For example, stable E site binding was observed when more physiological conditions were applied (Mg^{2+} at 6 mM or lower concentrations in the presence of polyamines); under these conditions, quantitative occupation of the E site with tRNA requires codon−anticodon interaction. (3) tRNAs and ribosomes from heterologous sources were frequently used which can produce artifacts (for discussion and references, see refs *20*, *21*).

The maximal binding of two peptidyl-tRNA analogs (AcPhe-tRNA) per ribosome represents a new quality of ribosomal tRNA binding, since ribosomes from bacteria (*22*), archaea (*4*), and lower eukaryotes (*5*) bind up to one AcPhe-tRNA, which can be present either at the A site or at the P site. The question of whether bacterial ribosomes from *E. coli* can bind one or two AcPhe-tRNA is still controversial (an example of a report of two AcPhe-tRNAs bound per ribosome is found in ref *3*). These discrepancies have been analyzed in a series of papers, and several points to be taken into consideration concerning tRNA saturation experiments have been identified (see Discussion in ref *22* and Experimental Procedures in ref *5*).

Ribosomes from bacteria, archaea, and lower and higher eukarya contain three tRNA binding sites, supporting the conclusion that three tRNA binding sites, A, P, and E, are a universal feature of the translational machinery.

Role of the Intrinsic ATPase Activity of 80S Ribosomes from Higher Eukaryotes. It is well documented that 80S ribosomes from higher eukaryotes possess a firmly associated ATPase activity (*23*, *24*), whereas purified prokaryotic 70S ribosomes or totally reconstituted *E. coli* ribosomes do not contain any NTPase activity (R. Adlung and K. H. Nierhaus, unpublished results). The analysis presented here sheds some light on the functional role of the intrinsic ATPase of 80S ribosomes from higher eukaryotes.

Deacylated tRNA is remarkably stable when bound at the E site of 80S ribosomes from rabbit liver: Only about 15−20% of the tRNA present at this site can be chased by cognate nonlabeled tRNA (Figures 3 and 4). Three observations characterize the effects of nucleotides on the stability of the tRNA present at the E site: (1) The presence of ATP improves the chasing efficiency 2−3-fold, raising the chasing efficiency up to 50%; *i.e.*, ATP weakens the tight binding of the deacylated tRNA at the E site (Figure 3B). (2) ATP hydrolysis is required for this effect; noncleavable ATP analogues have no effect. (3) The dependence on cleavable nucleotide triphosphates is not restricted to ATP; GTP shows a similar although somewhat lower effect (Figure 4C).

All three points correspond to the EF-3-dependent functions observed with yeast ribosomes (*5*): The E site of yeast ribosomes binds deacylated tRNA so tightly that the A site cannot be occupied due to the reciprocal coupling between both sites. When the E site is occupied, an aminoacyl-tRNA cannot bind stably to the A site and *vice versa*; the E site loses its binding capacity upon occupation of the A site. EF-3 facilitates the release of the E site bound tRNA in an ATP-dependent manner and thus enables the occupation of the A site. EF-3 hydrolyzes GTP almost as well as ATP (*5*). These observations are fully compatible with the features of the allosteric three-site model first derived from functional studies with *E. coli* ribosomes (for review, see *20*). Recently published data were interpreted as a refutation of the features of the allosteric three-site model in *E. coli* (*25*). However, the interpretation did not withstand a closer inspection; a detailed analysis will be published elsewhere (K. H. Nierhaus, Ralf Jünemann, C. M. T. Spahn, and F. J. Triana-Alonso, manuscript submitted for publication).

A number of observations were taken as evidence arguing against the view that the ATPase EF-3 of yeast ribosomes and the intrinsic ATPase of 80S ribosomes from higher eukaryotes (pig liver) fulfill the same functions: The kinetic parameters of ATPases from these two sources were found to be different, tRNA binding to programmed 80S ribosomes stimulated only the EF-3-dependent ATPase, and AMPPNP did not reduce the poly(Phe) synthesis from pig liver 80S ribosomes (*9*). The results of another study did not agree with these findings: The binding of cognate tRNA to programmed 80S ribosomes from higher eukaryotes (rabbit liver) was reported to stimulate the intrinsic ATPase, and, furthermore, AMPPNP impaired the enzymatic occupation of the A site. The extent of occupied E sites in the latter experiments could not be assessed since poly(U) was used as mRNA displaying the same codons in P and E sites (*10*). Possible explanations of these controversial results are the following: (i) Pig liver ribosomes were not washed in high-salt buffers and thus might contain still other ATPases which mask the effects of the ribosome-intrinsic ATPase. (ii) The poly(Phe) synthesis of pig liver ribosomes in the presence of AMPPNP may just reflect the "promiscuous" character of the ribosome−intrinsic ATPase to accept and cleave GTP nearly as well (Figure 4C).

Here we demonstrate that the diagnostic effect of the EF-3-dependent ATPase is also observed with the ATPase activity intrinsic to higher eukaryotic 80S ribosomes, namely, to stimulate the chasing efficiency of the tRNA present at the E site. This finding makes it likely that both ATPases fulfill the same function during protein synthesis, *i.e.*, enabling tRNA release from the E site, thus facilitating A site occupation. The obvious equivalence between the ATP requirements of lower (yeast) and higher eukaryotes (rabbit liver) suggests that EF-3 has become a ribosomal protein in the course of evolution or, alternatively, at least a ribosomal component has taken over the function of EF-3.

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