Ribosomal tRNA binding studies and functional tests were performed at 6 mM Mg²⁺ using the mRNA analogue C₃₅:AUGA₃₁₇ which contains three unique codons in its central region. The following results were obtained. 1) The relative binding affinities of 20 different deacylated tRNAs to nonprogrammed 70 S ribosomes were assessed and were found to vary substantially. 2) When added as the first tRNA, fMet-tRNA and deacylated tRNAs (but not N-acetylated aminoacyl-tRNAs) can bind to internal codons of the mRNA and are therefore suitable for setting the reading frame via codon-anticodon interaction in the peptidyl-tRNA site (P site). 3) After prefilling the P site with deacylated tRNA, the exit site for deacylated tRNA (E site) can be quantitatively occupied only if the cognate codon is present at that site. 4) The translocation of peptidyl-tRNA from the aminoacyl-tRNA site (A site) to the P site is not accompanied by a release of deacylated tRNA. The codon sequence excludes a release and rebinding of deacylated tRNA to the newly exposed A site. Rather, the deacylated tRNA is cotranslocated from the P site to the E site where it remains stably bound. 5) After one round of elongation, addition of an A site ligand triggers the dissociation of deacylated tRNA from the E site. Conversely, E site occupation reduces the affinity of the A site for N-acetylated aminoacyl-tRNA. Thus, A and E sites are allosterically linked via negative cooperativity. The results support the allosteric three-site model as an appropriate description of the ribosomal elongation cycle.

The allosteric three-site model for the ribosomal elongation cycle is characterized by the following features (11-14) see also Fig. 4). 1) The ribosome contains three tRNA-binding sites, A, P, and E, the latter being specific for deacylated tRNA. 2) During translocation the deacylated tRNA does not fall off the ribosome but moves from the P to E site. 3) Both tRNAs present on the ribosome before and after translocation simultaneously undergo codon-anticodon interaction. 4) The ribosome can adopt two conformational states; the first is the pretranslational state, which is characterized by high affinities for tRNA in the A and P sites and a low affinity for tRNA in the E site. The second is the post-translational state, in which the P and E sites display high affinities for tRNA, whereas that of the A site is weak. The respective transitions from one state to the other are induced in each case by occupation of the previously low affinity binding site. Thus, the A and E sites are allosterically linked via negative cooperativity, and, as a consequence, deacylated tRNA is released from the E site concomitantly with aminoacyl-tRNA binding to the A site.

Various groups have reported observations supporting the existence of a third tRNA binding site (5-8). Nonetheless, the allosteric three-site model has provoked criticisms in the literature. The reported release of deacylated tRNA from the E site triggered by A site occupation (3) was reinterpreted (9) as a chase of E site-bound tRNA by deacylated tRNA present in the A site substrate Phe-tRNA. A more rigorous criticism was recently raised by Baranov and Ryabova (10) who confirmed our observation of translocation without tRNA dissociation (2) but explained this finding in the frame of the two-site model as a release and rebinding of deacylated tRNA to the A site. 

These differences in the interpretation of functional experiments can only be partially ascribed to differences in preparation and experimental performance but rather reflect an intrinsic difficulty in the experimental strategy applied by both us and our colleagues, namely the use of the homopolymeric poly(U) as mRNA. The advantage of a homopolymeric system is that differences observed at the various ribosomal binding sites reflect different ribosomal properties at these sites, since the tRNAs and codons are identical at each position. The disadvantage lies in the fact that an assessment of the site location of a deacylated tRNA is often difficult and can only be made in a circumstantial manner. A heteropolymeric mRNA exposing three different codons at A, P, and E sites would solve this problem; a tRNA which had left the P site could a priori not rebind to the A site, and a deacylated tRNA contaminating an A site substrate could not chase the tRNA from the E site, since only a deacylated tRNA cognate to the codon at the E site would be an effective chasing substrate.

In this paper we analyze the ribosomal reactions during elongation using the synthetic oligoribonucleotide C₃₅:AUGA₃₁₇ as a mRNA which carries three defined codons in its central region. The results fully confirm the allosteric three-site model.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tightly coupled ribosomes from *Escherichia coli* were isolated as described (11). 1 A₂₆₀ unit of 70 S ribosomes was taken to be equivalent to 24 pmol. tRNAₘ₆₇ (E. coli), tRNAₘ₆₇ (E. coli), poly(A), poly(C), and poly(U) were obtained from Boehringer Mannheim. tRNAₘ₆₇ (E. coli), poly(I), and L-amino acids were from Sigma.

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°° The abbreviations used are: A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; E site, exit site for deacylated tRNA; Ac-acyl-tRNA, N-acetyl-aminoacyl-tRNA; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EF, elongation factor.
Characteristics of the Binding System

Relative Binding Affinities of Decacyl-tRNAs to Nonprogrammed 70 S Ribosomes—Since we are working here with various tRNA species, we first explore the intrinsic binding affinities of the tRNAs to nonprogrammed ribosomes. [14C]tRNA\textsubscript{Glu} is prebound to nonprogrammed 70 S ribosomes, then various tRNAs are added, and the chasing efficiency is determined. The chasing effects vary remarkably as shown in Fig. 1. [14C]tRNA\textsubscript{Glu} was chased most effectively by tRNAs specific for Pro, Aap, Phe, Leu-2 (i.e. tRNA\textsubscript{Leu-2}), Gln, His, Glu-2, Leu-5, Ala-2, and fMet. A group of tRNAs which chase with intermediate efficiency consists of tRNAs specific for Thr-1, Ile, Leu-4, Leu-1, Thr-3, Ser-1, Tyr, and Val-1, and the tRNAs with the lowest chasing efficiency are those specific for Lys and Gly-1.

Setting the Reading Frame by Binding the First tRNA—First we compare the binding of decacyl-, acyl-, and N-blocked acyl-tRNAs to 70 S in the absence and presence of the synthetic oligoribonucleotide C\textsubscript{73}AUGA\textsubscript{C\textsubscript{73}} (Table I). Decacyl-tRNAs bind well to nonprogrammed ribosomes, and the extent of binding agrees with their chasing efficiencies (Fig. 1). Low binding (\(\nu \leq 0.65\)) is observed with tRNAs specific for Gly-1, Lys, and Thr-3, which belong to the group of lowest or intermediate chasing efficiency, whereas tRNA\textsubscript{Met}, tRNA\textsubscript{Asp}, and tRNA\textsubscript{Trp} (stronger binding, \(\nu \geq 0.75\)) are members of the group with highest chasing efficiency.

All decacyl-tRNAs which are coded for by C\textsubscript{73}AUGA\textsubscript{C\textsubscript{73}} show an increased binding of up to one molecule/ribosome in the presence of this mRNA, whereas the noncoded tRNAs (tRNA\textsubscript{Asp} and tRNA\textsubscript{Trp}) fail to bind in an mRNA-dependent manner and even show a slight decrease in the presence of the noncognate mRNA.

In contrast to decacyl-tRNA, acyl-tRNAs (with or without EF-Tu) hardly bind to nonprogrammed ribosomes, in agreement with previous observations (16, 17). In the presence of mRNA significant but relatively low binding is found with Lys-tRNA and Glu-tRNA, both of which are complementary to codons within the internal CAUGAAAACC stretch of the mRNA.

A different picture is seen with N-acetylated acyl-tRNAs. Addition of mRNA hardly increases the low binding if the corresponding codon is internal (compare 0.01 with 0.05 for AcGlutRNA, and 0.13 with 0.18 for AcLys-tRNA in Table I). However, a strong stimulatory effect is found if the mRNA carries cognate codons near to the 5'-end, as seen in the case of poly(A)-dependent binding of AcLys-tRNA (0.89) and poly(C)- or C\textsubscript{73}AUGA\textsubscript{C\textsubscript{73}}-dependent binding of AcPro-tRNA (0.64 and 0.50, respectively). Clearly, given that the first tRNA, N-acetylated acyl-tRNA does not bind efficiently to internal codons at the P site. The initiator fMet-tRNA is an exception. It is the only one of the N-blocked acyl-tRNAs.
### Table I

**Binding of the first added tRNA to programmed 70 S ribosomes**

tRNA and mRNA were simultaneously added to 70 S ribosomes. A 20-μl aliquot contained 8 pmol of 70 S, 80 pmol of C17AUGA4Cl7, and 12 pmol of deacyl-tRNA or 8 pmol of acyl-tRNA.

<table>
<thead>
<tr>
<th>State of tRNA</th>
<th>tRNA species</th>
<th>Codons on C17AUGA4Cl7</th>
<th>tRNA bound per 70 S ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>−mRNA</td>
</tr>
<tr>
<td>Deacyl-tRNA</td>
<td>tRNA^tia</td>
<td>CAU</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>tRNA^tib</td>
<td>AUG</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>tRNA^tib</td>
<td>GAA</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>tRNA^tis</td>
<td>AAA</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>tRNA^tis</td>
<td>ACC</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>tRNA^tis</td>
<td>CCC</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>tRNA^tis</td>
<td>GAA</td>
<td>0.93</td>
</tr>
<tr>
<td>Acyl-tRNA</td>
<td>Glu-tRNA</td>
<td>GAA</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>−EF-Tu-GTP</td>
<td>GAA</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Lys-tRNA</td>
<td>AAA</td>
<td>0.04</td>
</tr>
<tr>
<td>N-Blocked acyl-tRNA</td>
<td>fMet-tRNA</td>
<td>AUG</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Glu-tRNA</td>
<td>GAA</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Lys-tRNA</td>
<td>AAA</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Pro-tRNA</td>
<td>CCC</td>
<td>0.15</td>
</tr>
</tbody>
</table>

### Table II

**Binding of Ac-acyl-tRNA as the second added tRNA to programmed 70 S ribosomes**

One aliquot contained 8 pmol of 70 S and, where indicated, 80 pmol of C17AUGA4Cl7 (mRNA), 12 pmol of deacyl-tRNA, and 8 pmol of AcLys- and/or AcPro-tRNA. Specific activities: [32P]tRNA? (300 dpm/pmol), Ac[14C]Lys-tRNA (1800 dpm/pmol), Ac[14C]Pro-tRNA (500 dpm/pmol).

<table>
<thead>
<tr>
<th>tRNA species</th>
<th>mRNA state</th>
<th>Expected tRNA bound per 70 S (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[32P]tRNA^tia</td>
<td>Ac[14C]Lys-tRNA</td>
<td>0.69 0.08</td>
</tr>
<tr>
<td>[32P]tRNA^tia</td>
<td>Ac[14C]Pro-tRNA</td>
<td>0.41 0.07</td>
</tr>
<tr>
<td>tRNA^tia</td>
<td>Ac[14C]Lys-tRNA</td>
<td>0.97 0.57</td>
</tr>
<tr>
<td>tRNA^tia</td>
<td>Ac[14C]Pro-tRNA</td>
<td>0.05 0.69</td>
</tr>
</tbody>
</table>
tested which binds efficiently to an internal AUG (in the absence of initiation factors and a Shine-Dalgarno interaction).

The results suggest that a deacylated tRNA or fMet-tRNA has to be used as the first tRNA for the fixation of the mRNA in the P site at an internal codon.

In the next experiment we analyzed whether this fixation defines the reading frame sufficiently for the binding of the second tRNA. When the mRNA is fixed by a deacylated tRNA in the P site, an AAA codon is exposed at the A site. Now AcLys-tRNA can bind as expected (Table II, experiment 1; \( \text{v} = 0.57 \)), whereas it cannot when added as first tRNA, i.e., when the heteropolymeric mRNA has not been prefixed by deacylated tRNA (compare 0.57 with 0.18 in Table I).

If the mRNA is fixed via a P site bound deacyl-tRNA, and a mixture of N-acetyl-acyl-tRNAs is added, only that acyl-tRNA can bind which corresponds to the codon exposed at Acyl-tRNA and AcLYs-tRNA remained unchanged during the translocation. The complex was pelleted, re-translation. to whether an elongator deacyl-tRNA (experiment 1, Table IV). The binding of both tRNAs amounts to 1.82 in the presence of mRNA, as compared to 0.90 in the absence of mRNA.

In contrast, only very low binding is observed for tRNA (\( \text{v} = 0.12 \)) which is noncognate to the AAA codon exposed at the E site, although this tRNA species binds normally in the presence of cognate mRNA (Table I). It follows that the E site, similar to the A site, can be filled as a second site in a codon-dependent fashion.

Translocation Is Not Accompanied by a Release of Deacyl-
tRNA—For the translocation experiment the mRNA is fixed by a deacyl-tRNA at the P site, then Ac-acyl-tRNA is bound to the A site. After EF-G-dependent translocation the extent of translocation is determined by a puromycin reaction and the binding of deacylated tRNA by nitrocellulose filtration.

Table IV demonstrates that the translocation is not accompanied by a significant release of deacyl-tRNA, regardless as to whether an elongator deacyl-tRNA (experiment 1, mRNA) or the initiator deacyl-tRNA (experiment 2) is used for the initial occupation of the P site. In experiment 1, a translocation of 0.24 is accompanied by a release of 0.06 eq of deacyl-tRNA, the corresponding numbers in experiment 2 being 0.48 and 0.03. The use of the heteropolymeric mRNA excludes the interpretation that the deacyl-tRNA is released from the P site in the course of translocation and rebinds to the vacated A site. It follows that the deacyl-tRNA is cotranslocated from the P to the E site.

Experiment 2 illustrates in addition the stability of the post-translocational complex. The complex was pelleted, re-suspended, and the tRNAs bound to ribosomes again determined by nitrocellulose filtration. The amounts of both deacyl-tRNA and AcLys-tRNA remained unchanged during the centrifugation treatment. It is clear that the deacyl-tRNA is not transiently and unstably bound at the E site in a manner which would allow it to fall off easily, but rather it is firmly located in this site and can only be released by an active mechanism.

Addition of an A Site Ligand Triggers the Release of Deacyl-
tRNA—In this experiment the dipeptidyl-tRNA fMet-Lys-
tRNA is formed at the A site. After translocation the fMet-
Lys-tRNA is located at the P site and correspondingly the tRNA is at the E site. Then Thr-tRNA is bound to the A site and the effect on the E site ligand studied (Table V).

\[ \text{f}[^{[H]}]_{\text{Met}}[^{[P]}]_{\text{tRNA}^{\text{Al}}_{\text{Al}}^{\text{Al}}_{\text{Al}}^{\text{Al}}} \] is first bound to the P site. The arrow in \([^{[H]}]_{\text{Lys}}\) indicates a low specific activity of 100 dpm/pmol. The puromycin reaction with this substrate was not quantitative (2 h/0 °C), so that the puromycin values in Table V can be used only as a qualitative measure. The addition of \([^{[C]}]_{\text{Lys-tRNA}}\) (without EF-Tu) severely reduces the puromycin sensitivity of the fMet label (0.06 versus 0.27), indicating the formation of fMet-Lys-tRNA at the A site. An EF-G-dependent translocation restores the puromycin reactivity of the fMet label (0.21 versus 0.06), and the Lys label also becomes reactive (0.23 versus 0.03). As in the previous experiments the translocation is not accompanied by a release of tRNA (0.01). Addition of \([^{[H]}]_{\text{Thr-tRNA}}\) (\([^{[H]}]_{\text{Thr}}\) denotes a high specific activity of 2300 dpm/pmol) again reduces the puromycin radioactivity of the Lys label (0.08 versus 0.23), indicating the formation of the tripeptidyl-tRNA fMet-Lys-
Thr-tRNA at the A site. EF-Tu is omitted throughout the assay in order to prevent spontaneous translocation (for fur-
TABLE III

Binding of deacyl-tRNA as the second added tRNA to programmed 70 S ribosomes

70 S ribosomes (8 pmol/aliquot) were preincubated for 15 min at 37°C with 11 pmol of \([^{32}P]tRNA^{Thr}\) (330 dpm/pmol) and, if indicated, 80 pmol of C\(_5\)AUGA\(_5\). Then 22 pmol of \([^{3}H]tRNA^{Lys}\) (340 dpm/pmol) or 22 pmol of \([^{35}S]tRNA^{Gly}\) (580 dpm/pmol) were added per aliquot followed by a second incubation for 15 min at 37°C.

<table>
<thead>
<tr>
<th>1. Incubation</th>
<th>2. Incubation</th>
<th>Expected binding state</th>
<th>tRNA bound per 70 S (u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>tRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_5)AUGA(_5)</td>
<td>([^{32}P]tRNA^{Thr})</td>
<td>E</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>([^{32}P]tRNA^{Thr})</td>
<td>([^{3}H]tRNA^{Lys})</td>
<td>0.51</td>
</tr>
<tr>
<td>([^{35}S]tRNA^{Gly})</td>
<td>([^{32}P]tRNA^{Thr})</td>
<td>AAAACCCCC</td>
<td>0.58</td>
</tr>
<tr>
<td>+ ([^{35}P]tRNA^{Thr})</td>
<td>AAAACCCCC</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>+ ([^{32}P]tRNA^{Thr})</td>
<td>AAAACCCCC</td>
<td>0.92</td>
<td>0.90</td>
</tr>
<tr>
<td>+ ([^{35}S]tRNA^{Gly})</td>
<td>AAAACCCCC</td>
<td>0.93</td>
<td>0.12</td>
</tr>
</tbody>
</table>

It is very unlikely that deacyl-tRNA\(^{Thr}\) present in the Thr-tRNA preparation could chase the tRNA\(^{Ase}\) from the E site, since deacyl-tRNA\(^{Thr}\) is noncognate to the AUG-coded E site. The experiment also excludes the possibility that the translocation of the tripeptidyl-tRNA (fMet-Lys- Thr-tRNA) triggers the release of tRNA\(^{Ase}\) from the E site, because the majority of the Thr label is still present at the A site (see the low puromycin reactivity of the Thr label (0.04) in Table V).

Allosteric Interactions between A and E Sites—In the next experiment we want to analyze whether the occupation of the E site influences the A site affinity for Ac-acyl-tRNA and vice versa.

When the P site is blocked with deacyl-tRNA\(^{Thr}\) the A site can be readily filled with AcPro-tRNA (\(r = 0.69\), Table VI, experiment 1; see also Table II, experiment 2). However, if
Allosteric 3-Site Model for Ribosomal Elongation Cycle

**TABLE IV**

**Translocation and the release of deacyl-tRNA**

One aliquot contains 8 pmol of 70 S, 80 pmol of C17AUG & C17, 12 pmol of deacyl-tRNA, and 8 pmol of N-acetyl-aminooacyl-tRNA (Ac-acyl-tRNA); PM, puromycin; specific activities: [32P]tRNAHS (370 dpm/pmol), Ac[3H]Glu-tRNA (2300 dpm/pmol), [32P]tRNAHS (1200 dpm/pmol), Ac[3H]Lys-tRNA (620 dpm/pmol). For step 4 in experiment 2, 200 pmol of 70 S in 715 µl were subjected to centrifugation, and the pellet was resuspended (15 min/4 °C) in 500 µl of a buffer with the ionic milieu of the assay (20 mM Hepes/KOH, pH 7.8 (0 °C), 6 mM Mg**, 150 mM NH₃, 0.6 mM spermine, and 0.4 mM spermidine). The suspension contained 160 pmol of 70 S according to the absorption at 260 nm. The tRNAs bound per ribosome were determined by a nitrocellulose filtration. The puromycin reaction was performed for 2.5 h at 0 °C in experiment 1 (the reaction not being quantitative) and for 10 min at 37 °C in experiment 2.

<table>
<thead>
<tr>
<th>Incubation steps and additions</th>
<th>Expected state</th>
<th>tRNA bound per 70 S</th>
<th>Ac-acyl-PM per 70 S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. [32P]tRNAHS (**\H)</td>
<td>CCCAUGAAAAC</td>
<td>1.02</td>
<td>0.39</td>
</tr>
<tr>
<td>2. Ac[3H]Glu-tRNA (**\H)</td>
<td>CCCAUGAAAAC</td>
<td>0.96</td>
<td>0.41</td>
</tr>
<tr>
<td>3. EF-G + GTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Release of deacyl-tRNA 0.06</td>
<td>Translocation of AcGlu-tRNA 0.24</td>
<td></td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. [32P]tRNAHS (**\H)</td>
<td>CCCAUGAAAAC</td>
<td>0.94</td>
<td>0.61</td>
</tr>
<tr>
<td>2. Ac[3H]Lys-tRNA (**\H)</td>
<td>CCCAUGAAAAC</td>
<td>0.91</td>
<td>0.57</td>
</tr>
<tr>
<td>3. EF-G + GTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Release of deacyl-tRNA 0.03</td>
<td>Translocation of AcLys-tRNA 0.48</td>
<td></td>
</tr>
<tr>
<td>4. 2.5 h/70,000 × g</td>
<td>CCCAUGAAAAC</td>
<td>0.92</td>
<td>0.56</td>
</tr>
</tbody>
</table>

the E site is blocked in addition to the P site, the binding to the A site is severely reduced (compare 0.23 with 0.69 in Table VI).

On the other hand, if the P and A sites are filled first with deacyl-tRNA** and AcPro-tRNA, respectively, the binding of tRNA to the E site is also impaired (Table VI, experiment 2; compare 0.61 with 0.91). The effect is somewhat lower than the reversed one seen in experiment 1, since some of the deacyl-tRNA** binds to the E site and triggers a release of the prebound AcPro-tRNA (0.21 = 0.77 - 0.56). Evidently, the E site can bind a deacyl-tRNA so tightly under the conditions applied that it even induces the release of an Ac-acyl-tRNA from the A site.

It is clear that the reduced A site binding seen upon addition of the E site ligand is not caused by a competition of the tRNA** with AcPro-tRNA, since the A site does not contain a codon corresponding to tRNA**. Therefore, the experiments shown in Table VI demonstrate that an occupation of
**Table V**

*Allosteric 3-Site Model for Ribosomal Elongation Cycle*

One aliquot contained 8 pmol of 70S, 80 pmol of C17AUGAC17, and 8 pmol of [3H]Met-[32P]tRNA

The sample was incubated for 10 min at 37°C, and 8 pmol of [3H]Lys-tRNA was then added at 0°C (no further incubation). For the EF-G-dependent translocation the mixture was incubated for 10 min at 37°C, and, after addition of [3H]Thr-tRNA

For the puromycin reaction two samples were withdrawn, incubated in the presence of puromycin for 2 h at 0°C (no quantitative puromycin reaction), and subjected to a nitrocellulose filtration. The difference between the bound material found in the absence and the presence of puromycin indicates the puromycin-reactive material. Specific activities: [3H]Met (100 dpm/pmol), [32P]tRNA

Incubation steps and additions | Expected state | [3P]tRNA<sup>10</sup> | [3H]Met | [3C]Lys | [3H]Thr |
--- | --- | --- | --- | --- | --- |
1. fMT-[3P]tRNA<sup>10</sup> | CCCAUGAAA ACC | 0.64 | 0.57 | 0.27 |
2. [3C]Lys-tRNA | CCCAUGAAA ACC | 0.66 | 0.60 | 0.96 | 0.64 | 0.03 |
3. EF-G + GTP | CCC AUGAAAACC | 0.65 | 0.53 | 0.21 | 0.61 | 0.23 |
4. [3H]Thr-tRNA<sup>10</sup> | CCC AUGAAAACC | 0.43 | 0.49 | 0.08 | 0.34 | 0.04 |

Control

1. fMT-[3P]tRNA<sup>10</sup> + 2. [3C]Lys-tRNA
2. [3C]Lys-tRNA<sup>10</sup>

<table>
<thead>
<tr>
<th>Binding (bdg) and puromycin reaction (PM) per 70S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation steps and additions</td>
</tr>
<tr>
<td>1. fMT-[3P]tRNA&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>2. [3C]Lys-tRNA</td>
</tr>
<tr>
<td>3. EF-G + GTP</td>
</tr>
<tr>
<td>4. [3H]Thr-tRNA&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Discussion

The Heteropolymeric mRNA System—The simple heteropolymeric mRNA C17AUGAC17 used in this study contains in two reading frames a sequence of three defined codons in
Allosteric 3-Site Model for Ribosomal Elongation Cycle

### Table VI

<table>
<thead>
<tr>
<th>Binding state</th>
<th>Starting complex</th>
<th>Final complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tRNA&lt;sup&gt;β&lt;/sup&gt;</td>
<td>tRNA&lt;sup&gt;θ&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>E P A</td>
<td>AAAACCC CCC</td>
</tr>
<tr>
<td></td>
<td>AAAACCCCC</td>
<td></td>
</tr>
<tr>
<td>Exp. 2</td>
<td>AAAACCCCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAA ACCCCC</td>
<td></td>
</tr>
</tbody>
</table>

### Cotranslocation of Peptidyl-tRNA and Decacyl-tRNA, Stable Binding of Decacyl-tRNA at the E Site

A corollary of the allosteric three-site model is the uncoupling of translocation and decacyl-tRNA release (18), whereas the two-site model requires a strict coupling of these two phenomena. In the frame of the latter model EF-G was thought to function as a decacyl-tRNA release factor (19), and, indeed, various authors (20–26) reported an EF-G-induced release of decacyl-tRNA. However, there is not one case where the necessity of a coupling between translocation and release of decacyl-tRNA could be demonstrated in an unequivocal fashion (for discussion see Refs. 18, 27, and 28). One essential drawback of the early experiments (19–23) was the following. It was not known at that time that significant amounts of ribosomes carrying two decacylated tRNAs in the P and E sites have to be avoided in translocation experiments. EF-G induces a release of a decacylated tRNA from such ribosomes, and this release is not related to translocation (17).

Translocation without a significant reduction of the binding value for decacylated tRNA was recently reported by Baranov...
and Ryabova (10). However, they concluded that the deacyl-tRNA is released from the P sites upon translocation but rebinds quantitatively to the vacated A sites. This conclusion is based on four arguments; after translocation the interaction between deacyl-tRNA and ribosome was found to be reversible, Mg\(^{2+}\)-dependent, codon-specific, and could be inhibited by tetracycline. However, these arguments do not withstand a closer inspection. 1) It is not clear why the E site binding should not be reversible. 2) A, P, and E site binding depend on Mg\(^{2+}\) to the same extent (29). 3) Codon specificity is not an exclusive property of the A site; and 4) the authors did not study the inhibition effects of tetracycline alone but rather those induced by a mixture of tetracycline and edeine. Edeine inhibits the binding of mRNA to ribosomes (30) as well as the binding of tRNA to P and A sites \(^{1}\) and thus does not specifically block the P sites as assumed by the authors (10). Therefore, the observed inhibition may not be related solely to the A site. Furthermore, tetracycline inhibits the binding of deacyl-tRNA to the E site, although the inhibition is significantly weaker than that of the A site binding (31). The uncoupling of translocation and deacyl-tRNA release postulated by the three-site model could be demonstrated in the poly(U) system at 15 mM Mg\(^{2+}\) (2) and in the presence of polyamines at 6 and 3 mM Mg\(^{2+}\) (29) as well as in the poly(A) system at 15 mM Mg\(^{2+}\) (4). The experiments in Tables IV and V confirm these observations. The particular advantage of the heteropolymeric mRNA system used here is seen in the fact that the A site does not harbor a codon complementary to the deacyl-tRNA. Therefore, an EF-G-induced release of deacyl-tRNA followed by a re-binding to vacated A sites can definitely be excluded.

Wintermeyer and co-workers also observed an uncoupling of translocation and deacyl-tRNA release (9, 32). However, they described the post-translocation complex with an occupied E site as a labile transition state, which immediately proceeds to a post-translocational complex with a free E site via diffusion of the deacyl-tRNA into solution. According to these authors a stable enrichment of the E site complex is only possible at unphysiologically high Mg\(^{2+}\) concentrations, and EF-G-dependent cleavage of GTP is required for an efficient release of deacyl-tRNA from the E site.

A lability of the E site complexes could not be confirmed in the experiments presented here. Deacylated tRNAs present at the E site of post-translocational ribosomes are able to withstand not only nitrocellulose filtration but even centrifugation (Table IV). Further, it has already been shown that the Mg\(^{2+}\) argument \(^{2}\) is not valid, since a decrease in the Mg\(^{2+}\) concentration destabilizes A, P, and E sites in a coordinate fashion and not only the E site (29). A "strong" E site binding is found again under optimized conditions (low Mg\(^{2+}\) (6 mM) and polyamines; see Tables IV-VI). The uncoupling of translocation and deacyl-tRNA release has been observed at various conditions (see above) in the presence of EF-G and GTP.

EF-G-dependent hydrolysis of GTP is therefore not involved in the release of deacyl-tRNA from the E site. One reason for the labile E site binding observed by Robertson and Wintermeyer (9) is the use of a heterologous system with E. coli ribosomes and yeast tRNA\(^{\text{TM}}\). This tRNA\(^{\text{TM}}\) has an E site affinity which is 20-fold lower than that of the homologous tRNA\(^{\text{TM}}\). This second reason is the employment of NH\(_4\)Cl-washed ribosomes. The washing procedure partially removes some proteins (S1, S5, S6, S16, L1, L3, L6, L10, L11, L7/L12, L24, L29, L30, L32/L33) and thus generates a heterogeneous ribosome population. NH\(_4\)Cl-washed ribosomes apparently have a "weaker" E site for unwashed ribosomes (9). Finally, the reported dissociation rate of the E site complex (0.3/s (32)) is 10 times slower than the elongation rate measured under comparable conditions (3/s (33)). Therefore, a mere passive diffusion of the E site-bound tRNA seems also to be improbable from kinetic considerations.

Codon-Anticodon Interaction at the Ribosomal E Site—The mRNA C\(_1\)AUGA\(_{C7}\); the third frame contains an UGA codon and is not shown. B, defined fixation of the heteropolymeric mRNA by deacyl-tRNA\(^{\text{TM}}\). C, defined occupation of the E site.

\(^{1}\) M. Krause, H.-J. Reinheimer, and K. H. Nierhaus, unpublished observation.

\(^{2}\) U. Fehner and K. H. Nierhaus, unpublished observation.
ever, deacyl-tRNAs from the high affinity group, such as deacyl-tRNA\textsuperscript{Phe}, do bind up to 1.5 molecules/nonprogrammed ribosome in the 6 mM Mg\textsuperscript{2+}/polyamine system indicating a significant E site binding (data not shown; note that at 15 mM Mg\textsuperscript{2+} we could not find any E site interaction (1)). It follows that the P site and, in the case of deacyl-tRNAs belonging to the high affinity group, the E site can be occupied in the absence of mRNA. However, in the presence of mRNA, codon-anticodon interaction takes place at both sites, thereby significantly increasing the stability of the tRNA binding.

Allosteric Interactions between A and E Sites—The allosteric three-site model distinguishes two ribosomal conformers in the course of an elongation cycle: the pretransloca
tional conformation with occupied A and P sites (low affinity at E site) and the post-transloca
tional conformation with occupied P and E sites (low affinity at A site). The situation where only the P site is filled does not naturally occur during elongation, but such a complex formed in vitro can be easily transformed to one or the other elongation conformers.

If for example the mRNA C\textsubscript{17}AUG\textsubscript{C17} is fixed with a deacyl-tRNA\textsuperscript{Th} at the P site, a tRNA\textsuperscript{Lys} complementary to the E site codon is bound with high efficiency (Table VI). The same is true for AcPro-tRNA, which is correspondingly specific for the A site codon. If, however, tRNA\textsuperscript{Lys} has been bound to the E site prior to the addition of the A site ligand AcPro-tRNA, then the binding of AcPro-tRNA to the A site is severely impaired. This inhibition can be best explained by allostery; deacyl-tRNA\textsuperscript{Lys} occupies the E site, induces the post-transloca
tional conformation with a low affinity A site, and thus operates as an allosteric effector. A competition of both tRNA species for the A site can be excluded by virtue of the heteropolymeric mRNA system used.

The influence of an A site prefilled with AcPro-tRNA on the subsequent binding of tRNA\textsuperscript{Lys} to the E site is clear, although somewhat less pronounced (Table VI). In this case the binding of deacyl-tRNA\textsuperscript{Lys} causes the transition from the pre- to the post-transloca
tional state in a fraction (about 20\%) of the ribosomal population, as indicated by a release of the prebound AcPro-tRNA. Thus, the post-transloca
tional state is energetically favored over the pretransloca
tional state under the experimental conditions used, again underlining the strength and stability of the E site binding.

In order to demonstrate the transition from the post- to the pretransloca
tional state, a more physiological model reaction is evidently required. Indeed, the addition of an acyl-tRNA after one elongation cycle was able to trigger a significant release of the E site ligand (Table VI). Here we cannot dis
tinguish between A site occupation and peptide-bond formation, but in the poly(U) system the A site binding has been identified as the triggering reaction (3). The essential outcome of the experiments shown in Table VI is the demonstration of a mutual influence of the A and E sites in a negatively cooperative manner, whereas the binding at the intervening P site remains unchanged.

The Physiological Importance of an Allosteric Three-site Ribosome—According to the experimental data reported here the allosteric three-site model, which is summarized in Fig. 4, represents an appropriate description of the ribosomal elongation cycle. What is the significance of the three tRNA
binding sites and the observed allosteric interactions?

In vivo the ribosome runs through the elongation cycle about 15 times/s, and during this process the mRNA must be precisely positioned and exactly moved relative to the ribo
some in order to preserve the reading frame. The fixation of the mRNA is essentially mediated by mRNA-tRNA interactions on the one hand and tRNA-ribosome contacts on the other, and there is some evidence that the tRNAs are actively moved during translocation, whereas the mRNA follows pas
tively (34). Because of the stability and precision of the movement, it makes sense to maintain two codon-anticodon interactions continuously and to anchor both tRNAs tightly in their respective binding sites before and after translocation.

On the other hand, a low stability of the tRNA-ribosome complex is advantageous for both the quick dissociation of a tRNA from the E site and an efficient selection of the correct aminoa
cyl-tRNA at the A site. In general, weak tRNA-ribosome contacts at the A site should result in short "sticking times" and thus contribute to a quick selection of the correct substrate. We assume that the successful formation of codon-anticodon interaction at the A site induces the transition to the pretransloca
tional state. If so, only aminoa
cyl-tRNAs with complementary or near complementary anticodons can trigger this transition from low affinity to high affinity A site. In contrast, the noncomplementary aminoa
cyl-tRNAs, which represent by far the majority, cannot trigger this transition and thus do not interfere with the rate and accuracy of tRNA selection.

The ribosome has to fulfill two opposing requirements. On the one hand, two continuous codon-anticodon interactions and two high affinity binding sites are necessary for the maintenance of the mRNA frame; on the other hand, a quick selection and release of tRNA presupposes weak tRNA-ribo
some contacts. The ribosome with allosterically linked affini
ties at A and E sites represents a translational apparatus which fulfills these requirements for a translation of the genetic information which is both rapid and precise.

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Allosteric 3-Site Model for Ribosomal Elongation Cycle

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