FURTHER EVIDENCE FOR THE FOUR-SITE MODEL OF RIBOSOMAL FUNCTION: BACTERIOPHAGE fr mRNA CODED BINDING OF AA–tRNA

D. SWAN and H. MATTHAEI
Arbeitsgruppe Biochemie, Max-Planck-Institut für experimentelle Medizin, Göttingen, Germany

Received 29 July 1971

1. Introduction

The generally accepted ribosome model [1, 2], recently supported by sophisticated experimental evidence [3, 4], assumes two tRNA binding sites on the 70 S particle. This model furthermore proposes that the peptidyl group is transferred from peptidyl–tRNA in the donor site to the amino group of the AA–tRNA in the neighbouring acceptor site by virtue of a peptidyl transferase [5]. Various experimental observations [6–9], however, have suggested additional binding sites for AA–tRNAs. In order to ascertain the number of tRNA binding sites and their function, initiation complexes coded by the mRNA of bacteriophage fr were studied [10]. According to results with the RNA from a closely related bacteriophage [11] it could be expected that ribosomes would bind primarily at the beginning of the coat protein cistron of this RNA, and therefore, that each tRNA binding site would contain a codon for a different amino acid. Fractionated 30 S and 50 S subunits from *E. coli* ribosomes, initiation factors F_1, F_2, F_3, and f[^{3}H]Met–$tRNA^{fMet}_{E.\ coli}$ were prepared and combined with fr RNA and GTP to form 70 S initiation complexes [12]. These were used for binding studies with either total tRNA, charged with single ^{14}C-labelled amino acids, or in subsequent experiments with fractionated elongation factors T_u and T_s and purified tRNA fractions capable of accepting only one of the following amino acids: Ala, Asn, Glu, Phe, or Ser. Bound AA– or peptidyl–tRNAs were assayed by adsorption of ribosomal complexes to cellulose nitrate filters [13]. The products were analysed by thin-layer electrophoresis after hydrolysis of the complexes in alkaline solution. Besides Ala, Asn, fMet, and Ser only the one dipeptide, fMet–Ala was found. Several further observations made with this system are briefly reported which support a four-site-model of ribosomal function.

2. Materials and methods

Ribosomes and initiation factors F_1, F_2, and F_3 were prepared from *E. coli* MRE 600 according to Revel et al. [14]. Factor F_1 was purified further by chromatography on phosphocellulose [15], factor F_2 by chromatography on hydroxylapatite [16]. Transfer factors T_u, T_s, and G, were separated on DEAE-Sephadex A-50 [17]. Bacteriophage fr was grown under lysis-inhibiting conditions [18], and the RNA extracted by phenol-sodium dodecyl sulphate treatment [19]. 30 S and 50 S ribosomal subunits were separated in an MSE Type B XV zonal rotor [20]. tRNA fractions accepting either Ala, Asn, fMet, Glu, Phe or Ser were prepared from *E. coli* tRNA (Schwarz BioResearch) by reversed phase chromatography [21] and/or benzoylated DEAE-cellulose [22]. Product analysis was performed by electrophoresis on MN 300 cellulose thin-layer plates (Macherey and Nagel, Düren) by a modified procedure of Capecchi [23].

* Dedicated to Professor K. Mothes at the occasion of his 70th birthday.

Table 1
Specificity of AA–tRNA binding coded by natural mRNAs.

AA–tRNA	+fr RNA*	–fr RNA blanks
	(pmole/ml)	
Ala–	*24.8*	5.2
Arg–	1.0	9.7
Asn–	*24.1*	4.5
Asp–	–1.1	4.3
Cys–	0.7	5.8
Gln–	0.8	9.6
Glu–	0.0	5.4
Gly–	–1.8	3.1
His–	–0.3	3.8
Ile	1.4	3.9
Leu–	0.3	3.2
Lys–	0.2	1.5
fMet–	*24.6*	7.8
Met	0.7	4.2
Phe–	*3.1*	14.3
Pro–	0.2	4.2
Ser–	*19.2*	11.7
Thr–	0.1	1.0
Trp–	1.1	2.2
Tyr–	0.3	5.6
Val–	0.9	7.8

Duplicate 50 μl reaction mixtures contained 50 mM Tris-HCl pH 7.5, 80 mM NH_4Cl, 10 mM $MgCl_2$, 0.4 mM GTP, 7 mM β-mercaptoethanol, 30 pmoles f[^{3}H]Met–tRNA (2.6% charged with [^{3}H]Met, 5400 Ci/mole) and 30 pmoles tRNA charged with one [^{14}C]amino acid, 16 pmoles of both 30 S and 50 S ribosomal subunits, 5 μg factor F_1 (DEAE-cell), 10 μg factor F_2 (DEAE-cell), and 6 μg factor F_3 (DEAE-cell). Alternate tubes contained 20 pmoles phage mRNA. After a 15 min incubation at 37°, the binding was assayed by filtration through Schleicher & Schüll Selectron filters (0.45 μm pore size) and by counting in a Tricarb liquid scintillation spectrometer (efficiencies for ^{3}H = 14% and ^{14}C = 35%).

* minus mRNA blanks subtracted.

Table 2
Characteristics of the fr RNA-coded binding of fMet– and Asn–tRNA.

System	fMet–tRNA bound (pmole/ml)	Asn–tRNA bound (pmole/ml)
Complete	18.0	18.1
–30 S	1.4	1.5
–50 S	11.0	1.8
–Init. factor f_1 (A)	5.0	8.1
–Init. factor f_3 (B)	5.7	1.4
–Init. factor f_2 (C)	5.1	0.2
–fr RNA	4.5	3.0
–fMet–tRNA	–	4.3
–Asn–tRNA	20.8	–
–GTP	4.3	1.0
+GMP–PCP	17.0	1.8
–GTP, + GMP–PCP	7.6	1.0
–GTP, –50 S	4.5	0.8
–GTP, +GMP–PCP, –50 S	4.8	0.4
+0.04 mM NEM	4.2	1.0
+0.5 mM fusidic acid	16.2	9.1
+1.5 mM fusidic acid	7.7	0.6
+1.0 mM amicetin	18.1	18.6

50 μl reaction mixtures contained 30 pmole f[^{3}H]Met–tRNA (2.6% charged with [^{3}H]Met, 5400 Ci/mole), 30 pmole [^{14}C]Asn–tRNA (2.0% charged with [^{14}C]Asn, 218 Ci/mole), 25 pmole fr RNA, 7.5 μg factor F_1 (DEAE-cell), 15 μg factor F_2 (DEAE-cell), 9 μg factor F_3 (DEAE-cell) and where indicated 0.4 mM GMD. Further components and method of assay were as in the legend to table 1.

3. Results and discussion

In the cell-free system described under table 1, fr RNA stimulated the binding of roughly equimolar amounts of Ala–, Asn–, fMet–, and Ser–tRNAs (see table 1). No other AA–tRNAs were coded except a trace amount of Phe–tRNA which, however, completely disappeared in later experiments with more highly purified initiation factor preparations. The AA–tRNAs bound under these conditions correspond to the first four amino acids

Table 3
The dependence of the binding of the first three elongator-tRNAs on the transfer factors T_u and T_s.

AA–tRNA	$-(T_u + T_s)$		Δ	$+(T_u + T_s)$		Δ
	–fr RNA	+fr RNA		–fr RNA	+fr RNA	
	(pmole/ml)			(pmole/ml)		
fMet–	8.2	26.3	18.1	6.9	25.2	18.3
Ala–	5.0	4.6	–0.4	5.7	21.4	15.7
Ser–	7.6	8.5	0.9	10.3	28.0	17.7
Asn–	3.3	3.9	0.6	4.8	21.9	17.1

50 μl reaction mixtures contained 5 mM $MgCl_2$, 120 pmole fractionated f[^{3}H]Met–tRNA (41% charged with [^{3}H]Met, 100 Ci/mole), and either 120 pmole fractionated [^{14}C]Ala–tRNA (4.5% charged with [^{14}C]Ala, 156 Ci/mole), [^{14}C]Ser–tRNA (4.7% charged with [^{14}C]Ser, 153 Ci/mole) or [^{14}C]Asn–tRNA (3.5% charged with [^{14}C] Asn, 218 Ci/mole), 1 μg factor F_1 (P-cell), 0.7 μg factor F_2 (hydroxylapatite), 13 μg factor F_3 (DEAE-cell). Where indicated, reaction mixtures also contained 20 μg T_u, 1 μg T_s and 25 pmole fr RNA. Further components were as in the legend to table 1.

Table 4
Test of the model by studies of binding of fractionated elongator–AA–tRNAs to ribosomes translocated into different positions on the mRNA.

Composition of preincubation reaction mixtures	f[^{3}H]Met–tRNA (pmole/ml)		[^{14}C]Asn–tRNA* (pmole/ml)		[^{14}C]Phe–tRNA* (pmole/ml)		[^{14}C]Glu–tRNA* (pmole/ml)	
	–fr RNA	+fr RNA	–fr RNA	+fr RNA	–fr RNA	+fr RNA	–fr RNA	+fr RNA
f[^{3}H]Met–tRNA	6.2	22.0	4.9	18.0	16.3	15.9	3.8	4.3
f[^{3}H]Met–tRNA Ala–tRNA	5.8	20.7	6.9	22.8	16.9	17.8	–	–
f[^{3}H]Met–tRNA Ala–tRNA Factor G	6.3	26.9	–	–	15.8	31.6	4.2	5.6
f[^{3}H]Met–tRNA Ala–tRNA Ser–tRNA Factor G	5.9	24.7	–	–	–	–	2.5	15.3
f[^{3}H]Met–tRNA Ala–tRNA Ser–tRNA Factor G, amicetin	7.2	24.6	6.2	20.3	18.4	16.3	6.3	5.8

500 μl preincubation reaction mixtures contained 5 mM $MgCl_2$, 1.2 nmole f[^{3}H]Met–tRNA (41% charged with [^{3}H]Met, 100 Ci/mole), 160 pmole of both 30 S and 50 S subunits, 10 μg factor F_1 (P-cell), 7 μg factor F_2 (hydroxylapatite), 130 μg factor F_3 (DEAE-cell), 200 μg factor T_u, 10 μg factor T_s and, when indicated 1 nmole Ala–tRNA (4.5% charged with Ala, as concluded from a parallel charging with labelled Ala), 1 nmole Ser–tRNA (4.7% charged with Ser, as concluded from a parallel charging with labelled Ser), 400 μg factor G, 1 mM amicetin. Two tubes for each of the complexes were preincubated for 15 min at 37°, one containing 250 pmole fr RNA and the other no fr RNA. 100 pmole of the [^{14}C]AA–tRNAs given in the table were then added individually to 50 μl aliquots of the preincubation mixtures; the concomitant change in ionic conditions was corrected, and the binding assayed after a further 15 min incubation at 37°. Other conditions as in the legend to table 1.
* All reaction mixtures finally contained f[^{3}H]Met–tRNA and *one* [^{14}C]AA–tRNA. If the binding of more than one [^{14}C]AA–tRNA was measured, this was performed in parallel mixtures.

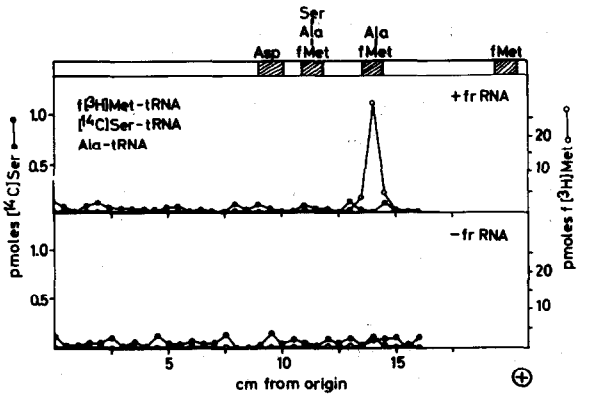

Fig. 1. Electrophoretic separation of amino acids and peptides released from the initiation complex by hydrolysis. 250 μl reaction mixtures contained 300 pmole fractionated f[^{3}H]Met–tRNA (41% charged with [^{3}H]Met, 100 Ci/mole), 300 pmole Ala–tRNA (4.5% charged with Ala, as concluded from a parallel charging with labelled Ala), 300 pmole fractionated [^{14}C]Ser–tRNA (9.5% charged with [^{14}C]Ser, 153 Ci/mole), 100 pmole of both 30 S and 50 S subunits, 5 μg factor F_1 (P-cell), 3.5 μg factor F_2 (hydroxylapatite), 65 μg factor F_3 (DEAE-cell), 100 μg factor T_u, and 5 μg factor T_s. Ionic conditions were as in table 3. The first tube contained in addition 100 pmole fr RNA. After incubation for 15 min at 37°, the tubes were cooled in ice and the ribosomal complexes pelletted by centrifugation for 2.5 hr at 150,000 g. The supernatant was removed and the pellet treated with 0.5 ml 1 N NaOH for 5 hr at room temperature. The hydrolysate was then lyophilised and dissolved in 10 μg of an aqueous solution containing 50 nmole fMet, fMet–Ala, fMet–Ala–Ser, Ser and Asn. 5 μl were applied in 1μl aliquots to a line drawn along the middle of a 20 cm × 20 cm cellulose thin-layer plate. Electrophoresis was for 1 hr at 800 V in a Varsol tank cooled to 5°. The buffer system was according to Capecchi [23]. The thin-layer plate was then dried and cut into strips, 1 cm wide, parallel to the direction of migration. These were then cut every 0.5 cm perpendicular to the direction of migration and counted in a Packard scintillation counter. The strips containing the NH_2-blocked markers were sprayed with an iodine azide-starch solution [25], and the others with ninhydrin.

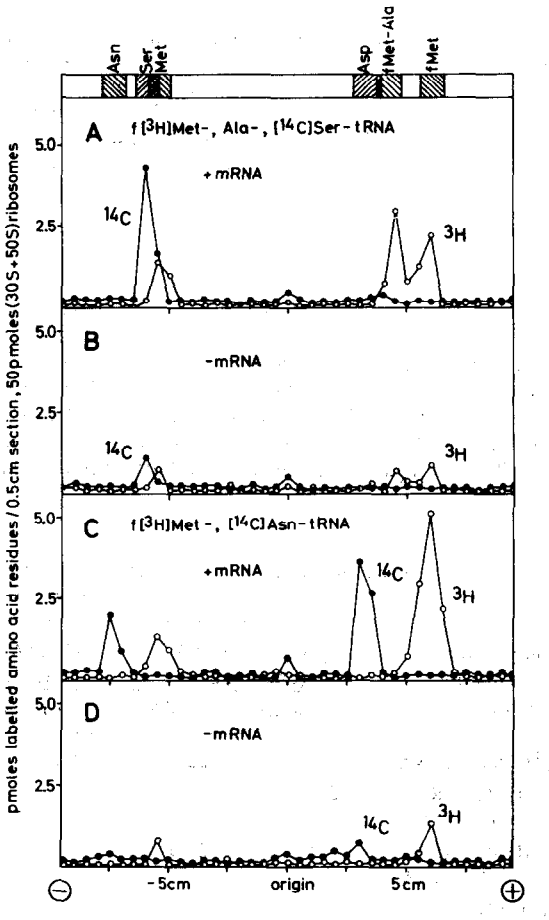

Fig. 2. Electrophoretic analysis of hydrolysates of initiation complexes. 250 μl reaction mixtures contained the following AA–tRNAs: A and B, 600 pmole fractionated f[^{3}H]Met–tRNA (41% charged with [^{3}H]Met, 100 Ci/mole), 500 pmole fractionated Ala–tRNA (see fig. 1), 500 pmole fractionated [^{14}C]Ser–tRNA (9.5% charged with [^{14}C]Ser, 153 Ci/mole) C and D, 600 pmole f[^{3}H]Met–tRNA (as above) and 500 pmole [^{14}C]Asn–tRNA (6.9% charged with [^{14}C]Asn, 218 Ci/mole). A and C also contained 100 pmole fr RNA. Ionic conditions as given under table 3. Electrophoresis was carried out for 45 min at 800 V, otherwise as indicated under fig. 1.

of the aminoterminal sequence of the fr coat protein: fMet–Ala–Ser–Asn–Phe–Glu... [24]. This specificity and the approximate equimolarity suggest that binding of the 3 elongator tRNAs occurs next to the initiator–tRNA on the same ribosome. This conclusion was supported by the demonstration that stoichiometric amounts of both fMet–tRNA and labelled fr RNA are bound to a 70 S complex [12].

Characteristics of fr RNA-coded fMet– and Asn–tRNA binding are shown in table 2. Asn–tRNA binding was dependent on the presence of both ribosomal subunits, fMet–tRNA, the three initiation factors, GTP, and fr RNA. It was inhibited by guanylyl–methenyl–diphosphonate (GMD), *N*-ethyl maleimide (NEM), or fusidic acid, all of which can act as translocation inhibitors. It was not affected by the transfer

inhibitor amicetin. The transfer factors T_u and T_s were presumably contained in the F_2 factor preparation used in the experiments documented by tables 1 and 2, since later results (table 3) showed that the binding of Ala–, Asn– and Ser–tRNAs was strictly dependent on T_u and T_s, whereas fMet–tRNA binding was unaffected by these factors.

The possibility still remained that the Asn–tRNA binding might be due to a residual amount of G factor on the ribosome allowing formation of the tripeptidyl–tRNA into the donor site, and the subsequent binding of Asn–tRNA in the acceptor site. Peptide analysis, however, showed that no tripeptide was synthesized (fig. 1) and that all the [^{14}C] Ser was electrophoresed as free Ser towards the cathode (fig. 2A). Likewise, Ala [12] and Asn (fig. 2C) were found as free amono acids; fMet and Ala were, however, partly converted into fMet–Ala (fig. 2A), the only peptide formed in this system [12]. Part of the Asn was found to be converted into Asp in the reaction mixtures (fig. 2C).

Further support for the four-site-model was obtained by translocation experiments with fractionated AA–tRNAs free of relevant mutually cross-contaminating acceptor activities. Transfer and translocation events could thus be stopped at certain points by omission of one or two AA–tRNA preparations required for a continuous mRNA translation. Binding of Phe–tRNA could be demonstrated in the presence of G factor, fMet– and Ala–tRNAs (table 4), when both Ser– and Asn–tRNAs had been omitted. Glu–tRNA binding occurred only when Ser-tRNA was included in addition to fMet– and Ala–tRNAs. In this experiment, Asn– and Phe–tRNAs were absent; fMet–Ala–Ser–tRNA had presumably been formed and translocated into the donor site. Both Phe– and Glu–tRNA binding could be prevented by the transfer inhibitor amicetin; binding of Asn–tRNA, however, was not affected. The evidence reported here suggests that *E. coli* ribosomes have three decoding sites in addition to the donor site. These function in coded AA–tRNA binding only in the presence of T factor. The negative evidence obtained by others [3, 4, 26] in respect to two of the three decoding sites could possibly be explained by: (1) different purity and properties of the ribosome preparations, (2) differences in the AA–tRNA preparations, (3) inhibitory side effects of the anti-G-immunoglobulin fraction.

Acknowledgements

The expert technical assistance of Miss Roswitha Zöllner is gratefully acknowledged. Qβ RNA was a kind gift from Dr. Thor Eikhom (Bergen), fr RNA from Mr. Jürgen Vogt (Tübingen). The Deutsche Forschungsgemeinschaft has supported this work.

References

[1] J.D. Watson, Science 140 (1963) 17.
[2] H. Matthaei, F. Amelunxen, K. Eckert and G. Heller, Ber. Bunsenges. physikal. Chem. 68 (1964) 735.
[3] D.J. Roufa, B.P. Doctor and P. Leder, Biochem. Biophys. Res. Commun. 39 (1970) 231.
[4] D.J. Roufa, L.E. Skogerson and P. Leder, Nature 227 (1970) 567.
[5] R.E. Monro, J. Mol. Biol. 26 (1967) 147.
[6] H. Matthaei and M. Milberg, Biochem. Biophys. Res. Commun. 29 (1967) 593.
[7] W.J. Culp, W.L. McKeehan and B. Hardesty, Proc. Natl. Acad. Sci. U.S. 63 (1969) 1431.
[8] D. Swan, G. Sander, E. Bermek, W. Krämer, T. Kreuzer, C. Arglebe, R. Zöllner, K. Eckert and H. Matthaei, Cold Spring Harbor Symp. Quant. Biol. 34 (1969) 179.
[9] P. Leder, A. Bernardi, D. Livingston, B. Loyd, D. Roufa and L. Skogerson, Cold Spring Harbor Symp. Quant. Biol. 34 (1969) 411.
[10] D. Swan, M. Milberg, D. Schwarz, R. Zöllner, A. Fehse, T. Focke and H. Matthaei, Federal European Biochem. Soc. Meeting, Madrid, Abstr. 120 (1969).
[11] H. Lodish, J. Mol. Biol. 32 (1968) 681.
[12] D. Swan, C. Arglebe and H. Matthaei, manuscript in preparation.
[13] M.W. Nirenberg and P. Leder, Science 145 (1964) 1399.
[14] M. Revel, M. Herzberg, A. Becarevic and F. Gros, J. Mol. Biol. 33 (1968) 231.
[15] R.E. Thach, J.W.B. Hershey, D. Kolakofsky, K.F. Dewey and E. Remold-O'Donnell, Cold Spring Harbor Symp. Quant. Biol. 34 (1969) 277.
[16] M. Herzberg, J.C. Lelong and M. Revel, J. Mol. Biol. 44 (1969) 297.
[17] J.M. Ravel, R.L. Shorey, S. Froehner and W. Shive, Arch. Biochem. Biophys. 125 (1968) 514.
[18] H.H. Gschwender and P.H. Hofschneider, Biochim. Biophys. Acta 190 (1969) 454.
[19] C.G. Kurland, J. Mol. Biol. 2 (1960) 83.
[20] W. Leifer and T. Kreuzer, Anal. Biochem., in press.
[21] J.F. Weiss, R.L. Pearson and A.D. Kelmers, Biochemistry 7 (1968) 3479.
[22] I. Gillam, S. Millward, D. Blew, M. von Tigerstrom, E. Wimmer and G.M. Tener, Biochemistry 6 (1967) 3043.
[23] M.R. Capecchi, Biochem. Biophys. Res. Commun. 28 (1967) 773.

[24] B. Wittmann-Liebold and H.G. Wittmann, Molec. Gen. Genet. 100 (1967) 358.
[25] J.L. Bailey, Techniques in Protein Chemistry (Elsevier, Amsterdam, 1962) p. 24.
[26] D.L. Roufa and P. Leder, J. Biol. Chem. 246 (1971) 3160.