RIBOSOMAL SYNTHESIS OF POLYLEUCINE ON POLYURIDYLIC ACID AS A TEMPLATE

Contribution of the elongation factors

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1. Introduction

The ability of the ribosomal machinery to miscode was discovered more than 15 years ago. Incorporation of phenylalanine into peptide synthesized in a cell-free translation system with poly(U) was shown to be accompanied by incorporation of small amounts of leucine and isoleucine, amino acids close to phenylalanine in their code specificity [1–4]. It was also observed that in the absence of phenylalanine poly(U)-programmed *Escherichia coli* ribosomes were capable of incorporating leucine [5,6] or isoleucine [7] into acid-insoluble product, presumably polyleucine or polyisoleucine, respectively. We have demonstrated ribosomal poly(U)-directed polyleucine incorporation in the *E. coli* cell-free system containing pure elongation factors (EF-Tu and EF-G) and leucyl-tRNA as the only substrate [8]. The product was identified as polyleucine using pepsin digestion and subsequent analysis of the oligopeptides produced [8].

Earlier it was shown that poly(U)-directed synthesis of polyphenylalanine can be performed by *E. coli* ribosomes in the absence of one or both elongation factors (EF-Tu or/and EF-G) [9]. Here, in order to check possible contributions of the elongation factors to substrate selectivity, we have used one-factor-promoted translation systems, as well as factor-free translation systems for the poly(U)-directed synthesis of polyleucine from leucyl-tRNA. We have demonstrated that poly(U)-programmed ribosomes synthesize polyphenylalanine ~10–30-times faster than polyleucine and have found that the presence of elongation factors does not contribute drastically to the selectivity of the ribosomal machinery at high $[Mg^{2+}]$ ($\geq$10 mM); polyleucine synthesis is accelerated by EF-Tu and/or EF-G to roughly the same extent as is polyphenylalanine synthesis. However, at low $[Mg^{2+}]$ (<10 mM Mg^{2+}) leucine polymerization, as compared with phenylalanine polymerization, becomes sharply reduced in EF-Tu-containing systems.

2. Materials and methods

2.1. *Ribosomal particles, elongation factors and aminoacylated tRNA*

Escherichia coli MRE 600 cells were cultivated, the ribosomes were isolated and the 30 S and 50 S ribosomal subparticles separated by sucrose gradient zonal centrifugation as in [9,10].

The elongation factors were obtained and checked for the purity ($\geq$90%) and for the absence of the mutual contaminations as in [9].

Aminoacylation of total *E. coli* tRNA (Serva, FRG) was done with [^{14}C]leucine, spec. act. 342 Ci/mol, or with [^{3}H]phenylalanine, spec. act. 880 Ci/mol or 17 000 Ci/mol, or with [^{14}C]phenylalanine, spec. act. 486 Ci/mol (Amersham, England). The enzyme fraction for aminoacylation was prepared as in [11] and purified from possible free amino acid contaminations by Sephadex G-25 gel-filtration. The activity of the obtained preparations was 1200–2600 pmol [^{14}C]-leucine, 700–1000 pmol [^{14}C]phenylalanine or 650 pmol [^{3}H]phenylalanine/mg total *E. coli* tRNA.

2.2. *Factor-free, one-factor-promoted and complete factor-promoted translation systems* [9]

All the reaction mixtures were prepared in buffers containing 10 mM Tris–HCl (pH 7.3 at 37°C), 100 mM KCl and 1 mM dithiothreitol; the $[MgCl_2]$ varied from 5–25 mM.

For polyleucine synthesis each 60 μl or 100 μl

sample contained 13 μg 30 S and 26 μg 50 S subparticles, 20 μg poly(U) and 200 μg total *E. coli* tRNA acylated with [^{14}C]leucine. In the spontaneous factor-free system no other components were introduced into the mixture. In the factor-promoted translation systems 0.4 mM GTP, 10 μg EF-Tu and/or 1 μg EF-G were also present.

For polyphenylalanine synthesis each 60 μl or 100 μl sample contained the same amounts of the same components as for polyleucine synthesis, except that [^{14}C]phenylalanine-tRNA was present instead of [^{14}C]leucyl-tRNA.

Incubation was at 37°C. The reaction was stopped by adding 3 ml 5% trichloroacetic acid. The samples were hydrolyzed for 20 min at 90°C and cooled; the precipitates were placed onto nitrocellulose filters and washed with 5% trichloroacetic acid; the filters were dried and the radioactivity was counted in the standard toluene–PPO–POPOP mixture using a Beckman LS100 scintillation spectrometer.

2.3. *Preparation of ribosomes carrying pre-synthesized oligophenylalanines*

Pre-synthesis of [^{3}H]oligophenylalanine on ribosomes with poly(U) was done in the factor-free or the EF-G-promoted translation systems. Reaction mixture contained (per ml): 0.42 mg 30 S and 0.84 mg 50 S ribosomal subparticles; 0.57 mg poly(U); and 5.0 mg total tRNA acylated with [^{3}H]phenylalanine. In the case of the EF-G-promoted system 62.5 μg EF-G, 0.4 mM GTP, 1 mM phosphoenolpyruvate and 62 μg phosphoenolpyruvate kinase were also present in the mixture. Incubation was at 37°C, either with 12 mM $MgCl_2$ for 40 min for the factor-free system, or with 16 mM $MgCl_2$ for 15 min for the EF-G-promoted system. After incubation the excess [^{3}H]phenylalanyl-tRNA was removed from the ribosome · poly(U) · [^{3}H]oligophenylalanyl-tRNA complex on a Bio-Gel A-5 m or A-0.5 m column (1.5 × 20 cm).

2.4. *Factor-free and EF-G-promoted systems with ribosomes carrying pre-synthesized oligophenylalanine*

The ribosome · poly(U) · [^{3}H]oligophenylalanyl-tRNA complex was used in experiments on the elongation of pre-synthesized oligophenylalanine with [^{14}C]leucine or [^{14}C]phenylalanine in factor-free or in EF-G-promoted systems. The composition of the mixtures and conditions for these experiments are given in the legend to fig.3. In the stimulated factor-free system 0.2 mM *p*CMB was added to the mixture instead of 1 mM dithiothreitol. The reaction was stopped by adding 5% trichloroacetic acid and the samples were hydrolyzed for 20 min at 90°C. The precipitates after hydrolysis were washed twice with 5% trichloroacetic acid, twice with 96% ethanol, dissolved in 0.5 ml Hyamine 10-X, put in the standard scintillation mixture and the [^{3}H]phenylalanine and [^{14}C]leucine radioactivities counted.

3. Results

3.1. *Poly(U)-directed polyleucine synthesis in the complete factor-promoted translation system*

Fig.1 (upper curves) shows that the formation of a hot trichloroacetic acid-insoluble product containing [^{14}C]leucine takes place in the complete factor-promoted system consisting of ribosomes, poly(U), EF-Tu, EF-G, GTP and [^{14}C]leucyl-tRNA (at 10 mM

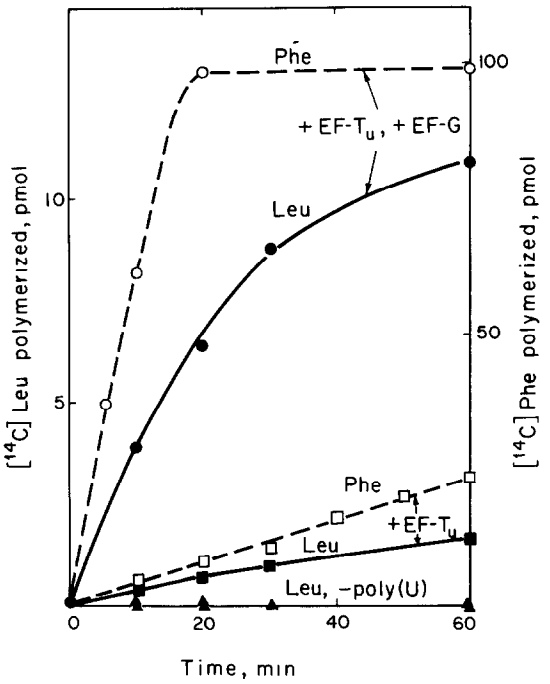

Fig.1. Kinetics of poly(U)-directed polyleucine synthesis in the complete factor-promoted (●——●) and in the EF-Tu-promoted (■——■) translation systems, as compared with the kinetics of poly(U)-directed polyphenylalanine syntheses in the same systems (○——○ and □——□, respectively). Experiments were also done in the presence of [^{14}C]leucyl-tRNA, but without poly(U) (▲——▲). Incubation was at 10 mM $MgCl_2$, 37°C.

Mg^{2+}, 37°C). We have identified this product as polyleucine [8]. The formation of polyleucine is shown to depend completely on the presence of the template polynucleotide; without poly(U) incorporation of [^{14}C]leucine into the acid-insoluble product is not observed (fig.1, lowest symbols). It is seen that synthesis of the leucine-containing product on a poly(U) template proceeds ~20-times slower than polyphenylalanine synthesis under the same conditions (fig.1, table 1).

Fig.2A shows the dependence of poly(U)-directed polyleucine synthesis on [Mg^{2+}] in the complete factor-promoted translation system, as compared with that of polyphenylalanine synthesis. It is seen that the optimum [Mg^{2+}] for polyleucine synthesis is slightly shifted towards higher levels. Separate experiments have shown that the ratio of the rates of polyphenylalanine and polyleucine syntheses becomes ~800 at 6 mM Mg^{2+}.

3.2. *Poly(U)-directed polyleucine synthesis in the EF-Tu-promoted translation system*

Fig.1 (lower curves) shows that if only EF-Tu with GTP (without EF-G) is added to ribosomes with poly(U) and [^{14}C]leucyl-tRNA, polyleucine synthesis also takes place, just as with the complete factor-promoted translation system. Comparison of the rates of poly(U)-directed polyphenylalanine and polyleucine syntheses in the parallel EF-Tu-promoted systems under the same conditions (at 10 mM Mg^{2+}, 37°C) shows that polyleucine synthesis is ~10–20 times slower than polyphenylalanine synthesis (fig.1, table 1).

Fig.2B shows the comparison of the dependence of poly(U)-directed polyleucine and polyphenylalanine syntheses in the EF-Tu-promoted translation systems on [Mg^{2+}]. It is seen that the optimal [Mg^{2+}] in the EF-Tu-promoted systems is somewhat higher for polyleucine synthesis than for polyphenylalanine synthesis, as in the case of the corresponding complete factor-promoted systems. The rate of the polyleucine synthesis at [Mg^{2+}] <10 mM is greatly reduced.

3.3. *Poly(U)-directed polyleucine synthesis in the EF-G-promoted translation system*

It has been found that usually no visible synthesis of polyleucine can be detected during 1 h incubation at 37°C, if only EF-G with GTP (without EF-Tu) is added to ribosomes, poly(U) and [^{14}C]leucyl-tRNA. Control experiments have demonstrated that under the same conditions the substitution of [^{14}C]phenylalanyl-tRNA for [^{14}C]leucyl-tRNA results in stimulation of polyphenylalanine synthesis by EF-G with GTP (without EF-Tu).

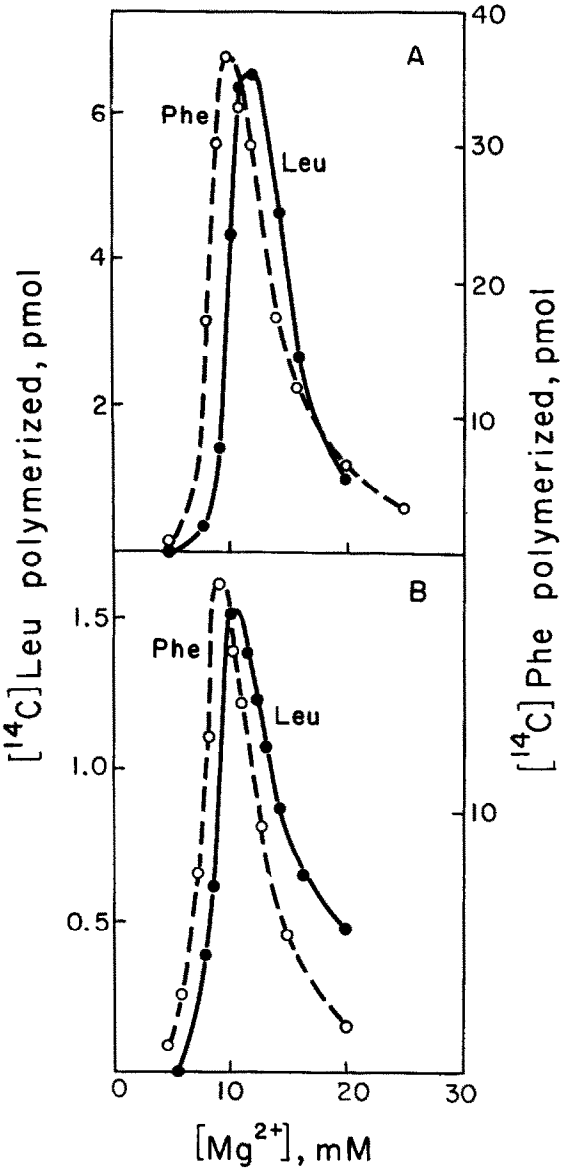

Fig.2. Dependence of poly(U)-directed polyleucine synthesis on [Mg^{2+}] (•——•) as compared with the Mg^{2+}-dependence of polyphenylalanine synthesis (o– – –o) in the same system. (A) complete factor-promoted translation system; incubation was at 37°C for 15 min in the case of polyleucine synthesis and 5 min in the case of polyphenylalanine synthesis. (B) EF-Tu-promoted translation system; incubation was at 37°C for 50 min in the case of polyleucine synthesis and 25 min in the case of polyphenylalanine synthesis.

Table 1
Rates of polyphenylalanine or polyleucine synthesis on poly(U)-programmed ribosomes

Presence of EF	[$MgCl_2$] (mM)	No. expt.	Phe (pmol/min)	Leu (pmol/min)	Rates (Phe/Leu)
+EF-Tu, +EF-G[a]	10	7	10 ± 4	0.5 ± 0.2	20 ± 4
+EF-Tu[a]		5	0.6 ± 0.2	0.04 ± 0.01	16 ± 6
+EF-Tu, +EF-G[b]	15	3	4.0 ± 0.5	0.15 ± 0.04	24 ± 6
+EF-G[b]		3	1.1 ± 0.3	0.04 ± 0.01	27 ± 4

[a] Figures are calculated per 40 μg ribosomes
[b] Figures are calculated per 1 A_{260} unit of the ribosome · poly(U) · oligophenylalanyl-tRNA complex

Mean rates of Phe and Leu incorporation, mean ratios of the rates and the corresponding standard deviations are given

It was logical to assume that the absence of polyleucine synthesis in the EF-G-promoted system could be the result of difficulties in passing the initiation stage when the binding factor EF-Tu is absent. To check this assumption the experiments were done with the ribosomes which had already passed the initiation stage and had synthesized oligophenylalanines. [^{14}C]-leucyl-tRNA was added to the ribosomes carrying the presynthesized peptide and the poly(U). The question was whether EF-G with GTP would stimulate a further growth (elongation) of the pre-synthesized peptide by adding leucine residues. The results are given in fig.3 (upper curves). It is seen that in the presence of EF-G with GTP reasonably good polyleucine synthesis proceeds on poly(U)-programmed ribosomes which have passed the initiation stage and are carrying oligophenylalanines.

Table 1 gives the comparison of the rates of the

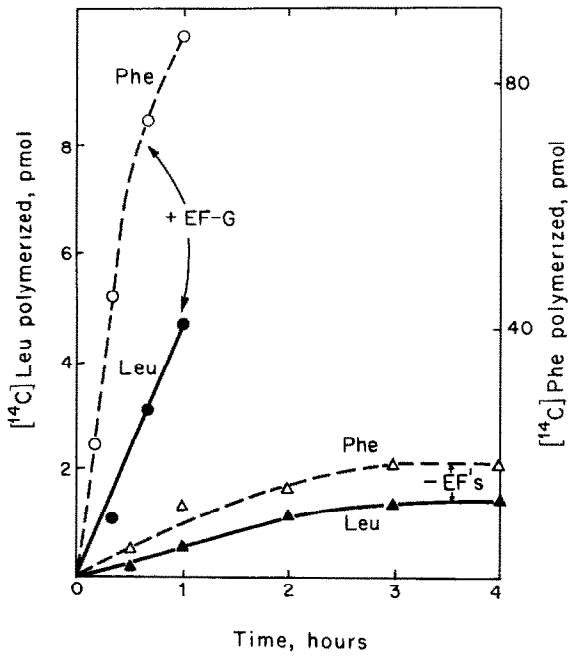

Fig.3. Poly(U)-directed polyleucine synthesis in the EF-G-promoted (●——●) and in the spontaneous factor-free (▲——▲) translation systems proceeding on ribosomes carrying pre-synthesized oligophenylalanine, as compared with the kinetics of poly(U)-directed polyphenylalanine syntheses in the same systems (○——○ and △——△, respectively). Pre-synthesis of [^{3}H]oligophenylalanine on ribosomes with poly(U) was done in the EF-G-promoted or in the factor-free system, respectively. In the experiments on the elongation of the pre-synthesized peptide by leucines or by phenylalanines in the EF-G-promoted system each 125 μl sample contained 0.38 A_{260} units of the ribosome · poly(U) · [^{3}H]-oligophenylalanyl-tRNA complex, 190 μg total *E. coli* tRNA acylated with [^{14}C]leucine, or [^{14}C]phenylalanine, 0.4 mM GTP, 1 mM phosphoenolpyruvate, 0.5 μg pyruvate kinase and 2 μg EF-G. Incubation in buffer with 10 mM Tris–HCl, 100 mM KCl, 1 mM dithiothreitol and 15 mM $MgCl_2$ (pH 7.3 at 37°C). In the experiments on the elongation of the pre-synthesized peptide by leucine in the factor-free system each 340 μl sample contained 1.8 A_{260} units of the ribosome · poly(U) · [^{3}H]oligophenylalanyl-tRNA complex and 500 μg *E. coli* total tRNA acylated with [^{14}C]leucine. In the experiments with phenylalanine each 113 μl sample contained 0.6 A_{260} units of the ribosome · poly(U) · [^{3}H]oligophenylalanyl-tRNA complex and 210 μg *E. coli* total tRNA acylated with [^{14}C]phenylalanine. Incubation at 37°C in the buffer with 10 mM Tris–HCl, 100 mM KCl, 1 mM-dithiothreitol and 12 mM $MgCl_2$ (pH 7.3).

poly(U)-directed polyphenylalanine and polyleucine elongation in parallel assays using ribosomes carrying pre-synthesized oligophenylalanines, both for complete and EF-G-promoted systems at 15 mM $MgCl_2$.

3.4. *Poly(U)-directed polyleucine synthesis in the factor-free translation system*

It has been established earlier that *E. coli* ribosomes are able to read out poly(U) and synthesize polyphenylalanine without the participation of the elongation factors (EF-Tu and EF-G) and GTP [11–13]. At present two kinds of factor-free translation systems are known for *E. coli* ribosomes [9]: the spontaneous factor-free translation systems [11,12,14,15] and the *p*CMB-activated factor-free translation system [13,16].

Our attempts to synthesize polyleucine using poly(U) as a template in the factor-free translation systems have given the following results. In the absence of the elongation factors and GTP, poly(U)-directed polyleucine synthesis did not take place in the spontaneous factor-free translation system, though under the same conditions noticeable polyphenylalanine synthesis proceeded. The reason for the absence of polyleucine synthesis in the factor-free translation system has proved to be the same as in the case of the EF-G-promoted poly(U)-directed polyleucine synthesis; difficulties in passing the initiation stage of translation with leucyl-tRNA in the absence of the binding factor EF-Tu.

It is seen in fig.3 (lower curves) that in the absence of the elongation factors and GTP, the poly(U)-programmed ribosomes, having passed the initiation stage and carrying oligophenylalanines, are capable of further reading-out poly(U) and adding leucine residues to the pre-synthesized peptides. Comparison of the rates of phenylalanine and leucine incorporation in factor-free translation experiments is given in table 2.

On the contrary, *p*CMB-activated ribosomes effectively synthesizing polyphenylalanine on poly(U) have proved to be very slightly active in synthesizing polyleucine on poly(U), whether the ribosomes had or had not passed the initiation stage (table 2).

4. Discussion

Comparisons of the rates of polyphenylalanine and polyleucine syntheses by poly(U)-programmed *E. coli* ribosomes in analogous systems show that in most cases their ratio (Phe/Leu) is within the limits from 10–30. Only at <10 mM [Mg^{2+}] does the ability for polyleucine synthesis on poly(U) become abruptly reduced, at least in the EF-Tu-containing systems (fig.2A,B), so the ratio of polyphenylalanine and polyleucine syntheses can reach ~10^3 (at 6 mM Mg^{2+}). At ⩾10 mM [Mg^{2+}], polyphenylalanine is synthesized roughly 20-times faster than is polyleucine.

Comparison of the complete (EF-Tu + EF-G) and the EF-Tu-promoted systems demonstrates that the presence of EF-G results in ~1 order of magnitude stimulation of polypeptide synthesis rate under the given ionic conditions (table 1). At the same time, EF-G seems to manifest neither a negative nor a positive gross contribution to the selectivity of the ribosomal machinery in this case; the polymerization of leucine and that of phenylalanine are equally accelerated by addition of EF-G. The same conclusion can be drawn for other [Mg^{2+}] from analysis of the curves of fig.2. The above observations contradict the conclusion made by us in [9] that EF-G increases the level of miscoding. The basis for that conclusion was the same level of miscoding in the complete (EF-Tu + EF-G) and the EF-G-promoted systems at high [Mg^{2+}] (20 mM or 13 mM, 25°C), when misincorporation in

Table 2
Rates of polyphenylalanine and polyleucine syntheses on poly(U)-programmed ribosomes in the factor-free (–EF-Tu, –EF-G) systems

Type of system	[$MgCl_2$] (mM)	Phe (pmol/min)	Leu (pmol/min)	Rates (Phe/Leu)
Spontaneous (–*p*CMB)	12	0.10	0.005	20
*p*CMB-activated (+*p*CMB)	15	0.40	⩽0.0035	>100

Rates of incorporation are given per 1 A_{260} unit of the ribosome · poly(U) · oligophenylalanyl-tRNA complex

the factor-free and the EF-Tu-promoted systems was undetectable. However, now we believe that the activity of the factor-free and the EF-Tu-promoted systems in those experiments was too low to determine the real level of miscoding there.

As to the contribution of the other elongation factor, EF-Tu, the information available, unfortunately, is less conclusive. Comparison of the elongation rates in parallel experiments with the complete (EF-Tu + EF-G) and the EF-G-promoted systems at 15 mM $MgCl_2$ (table 1) shows no clearly visible contribution of the presence of EF-Tu to the selectivity of the ribosomal machinery; in both systems the ratio of the rates of phenylalanine and leucine incorporation is 25 ± 6. At the same time, EF-Tu manifests a strong stimulatory effect on the polypeptide synthesis under the given conditions. A stimulatory effect and the absence of discriminatory effect of EF-Tu can be also seen from comparison of the EF-Tu-promoted (table 1) and the spontaneous factor-free (table 2) systems. This suggests that the evident involvement of EF-Tu in the codon-dependent binding of aminoacyl-tRNA does not increase significantly the discrimination between correct and incorrect substrates, at least at >10 mM $[Mg^{2+}]$.

On the other hand, as mentioned already, the discrimination becomes much higher in EF-Tu-containing systems at <10 mM $[Mg^{2+}]$ (fig.2). However, in this case we cannot decide now whether this is due to the presence of EF-Tu or to the low $[Mg^{2+}]$ as such.

It is interesting that pre-treatment of ribosomes with *p*CMB, which is known to stimulate factor-free translation of poly(U) [9,13], significantly increases the selectivity of the ribosomal machinery even at high $[Mg^{2+}]$ (table 2). This is consistent with our observations that miscoding is very low in the *p*CMB-activated factor-free translation system [9,17], i.e., the *p*CMB-modified ribosomes incorporate very little leucine into the polyphenylalanine synthesized on poly(U). Thus the idea that the ability for miscoding is governed by structural features of the ribosome itself is further supported.

Acknowledgements

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