Factor-free and one-factor-promoted poly(U,C)-dependent synthesis of polypeptides in cell-free systems from *Escherichia coli*

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1. INTRODUCTION

Ribosomes from *Escherichia coli* can read out a template and synthesize a polypeptide from aminoacyl-tRNA corresponding to the code specificity of messenger polynucleotide, without the participation of protein elongation factors and GTP [1–3]. Homopolynucleotides, such as poly(U) [1–6] and poly(A) [7] were used as templates in those experiments.

Here, we present a study of the ribosomal synthesis of polypeptides in factor-free and one-factor-promoted systems from *E. coli* using a heteropolynucleotide as a template. The random copolymer of uridylic and cytidylic acids with a U/C ratio of 1:1 was taken as a template heteropolynucleotide. It is shown that the poly(U,C)-dependent synthesis of trichloroacetic acid-insoluble polypeptides takes place with the participation of *E. coli* ribosomes without elongation factors and GTP. The factor-free poly(U,C)-dependent polypeptide synthesis is stimulated either by EF-Tu with GTP or EF-G with GTP, just as observed in analogous experiments with poly(U) as a template [6]. The amino acid composition of peptides synthesized on poly(U,C)-programmed ribosomes without the elongation factors or with only one of them corresponds to the code specificity of the template polynucleotide.

2. MATERIALS AND METHODS

Ribosomes, ribosomal 30 S and 50 S subparticles, and the enzyme fraction for aminoacylation of tRNA were isolated from *Escherichia coli* MRE-600 cells as in [6].

The elongation factors EF-Tu (in the form of the EF-Tu·GDP complex) and EF-G free from mutual contaminations were purified as in [8,9] with some modifications.

Aminoacylation of total *E. coli* tRNA (Serva) was done with amino acid mixtures whose compositions are shown in the figure legends. The following labeled amino acids were used: [^{14}C]phenylalanine, spec. act. 513 Ci/mol (Amersham); [^{3}H]phenylalanine, spec. act. 2520 Ci/mol (Amersham); [^{14}C]leucine, spec. act. 342 Ci/mol (Amersham) or 211 Ci/mol (Institute of Investigation, Production and Utilization of Radioisotopes, Czechoslovakia). The specific activity of the aminoacyl-tRNA was 800–1200 pmol [^{14}C]phenylalanine, 1700 pmol [^{3}H]phenylalanine or 2600–3200 pmol [^{14}C]leucine/mg total *E. coli* tRNA.

All the reaction mixtures were prepared in a buffer containing 10 mM Tris–HCl (pH 7.3 at 37°C), 100 mM KCl and 1 mM dithiothreitol, with the exception of *p*-hydroxymercuribenzoate (*p*HMB)-stimulated factor-free system where dithiothreitol was absent [6]. The [$MgCl_2$] was optimal for each system: 12 mM for the spontaneous factor-free; 15 mM for the *p*HMB-stimulated factor-free; 10 mM for the EF-Tu-promoted; 16–17 mM for the EF-G-promoted; and 10–12 mM for the complete factor-promoted systems [6]. Each 160–200 μl sample contained 27 μg 30 S and 54 μg 50 S subparticles, 16 μg poly(U,C) with U:C = 1:1 (random copolymer, Serva), and 320–800 μg amino-

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acylated tRNA. 30 S subparticles pretreated with 0.2 mM *p*HMB for 1 h at 25°C and freed from excess *p*HMB by Sephadex G-50 gel-filtration were used in the *p*HMB-activated factor-free system. In the factor-free systems no other components were introduced into the mixture. In the factor-promoted translation systems 80 nmol GTP, 200 nmol phosphoenolpyruvate, 2 μg pyruvate kinase, 20 μg EF-Tu and/or 4 μg EF-G were also present.

Incubation was at 37°C. The reaction was stopped by adding KOH to 0.15 M. The samples were hydrolyzed in KOH for 30 min at 37°C, acidified by addition of 5 ml 5% trichloroacetic acid, incubated for 20 min at 90°C and cooled. If only ^{14}C-labeled aminoacyl-tRNA was used, the radioactivity of the trichloroacetic acid-insoluble polypeptide was determined by a nitrocellulose filter assay with a counting efficiency of 80%. If the sample contained mixture of ^{3}H- and ^{14}C-labeled aminoacyl-tRNAs, the precipitate was collected on glass-fiber filters (GF/B, Whatman), washed successively with 10 ml 5% trichloroacetic acid and 10 ml 96% ethanol, dissolved in 1 ml Hyamine 10-X (Packard or Koch-Light) for 1–2 min at 70°C; then 10 ml standard toluene–PPO–POPOP mixture was added, and the radioactivity of the [^{14}C]/[^{3}H]polypeptide was measured in a scintillation spectrometer. Counting efficiencies in the two channels were 17% and 0.1% for ^{3}H and 5.6% and 57% for ^{14}C, respectively.

3. RESULTS

Fig.1 shows the kinetics of poly(U,C) (1:1)-dependent synthesis of trichloroacetic acid-insoluble peptides in factor-free systems (without EF-Tu, EF-G and GTP): the spontaneous one [2,3,6] and the system activated with SH-reagent, *p*HMB [4,5,10,11]. Total *E. coli* tRNA acylated with amino acids coded by poly(U,C), i.e., with phenylalanine, leucine, serine and proline, was used as a substrate in these experiments. Only phenylalanine or leucine were ^{14}C-labeled. *p*HMB was used as an SH-reagent. It has been shown to activate factor-free translation of poly(U), similar to *p*-chloromercuribenzoate [4]. The presence of the SH-reagent in the reaction mixture, however, inhibited the poly-(U,C)-dependent peptide synthesis, in contrast to the reading out of poly(U) [4], poly(A) [7] and poly(U,I) (unpublished); inactivation of tRNA^{Ser}

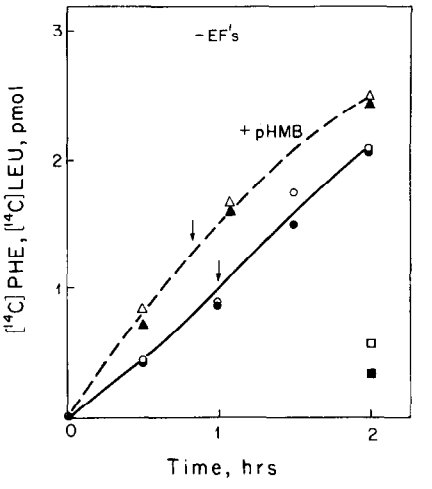

Fig.1. Kinetics of poly(U,C)-dependent peptide synthesis in the factor-free translation systems at 12 mM $MgCl_2$ for the spontaneous system (—, •, ○) and at 15 mM $MgCl_2$ for the *p*HMB-activated system (- - -, ▲, △, ■, □). Incorporation of [^{14}C]Phe (•, ▲, ■) or [^{14}C]Leu (○, △, □) into trichloroacetic acid-insoluble peptides. Without poly-(U,C) (■, □); with poly(U,C) (—, •, ○, - - -, ▲, △). Each 160–200 μl sample contained 160 μg equimolar 30 S and 50 S subparticle mixture and 32 μg poly(U,C) (1:1). 30 S subparticles were pretreated with *p*HMB in the case of the *p*HMB-activated system (see section 2). Each sample also contained 800 μg tRNA-acylated with either [^{14}C]phenylalanine, unlabeled leucine, serine and proline (•, ▲, ■) or [^{14}C]leucine, unlabeled phenylalanine, serine and proline (○, △, □) at the beginning of the reaction. The second portion of aminoacyl-tRNA (800 μg sample) was added after 1 h to the spontaneous system and after 50 min to the *p*HMB-activated system (→, moment of addition).

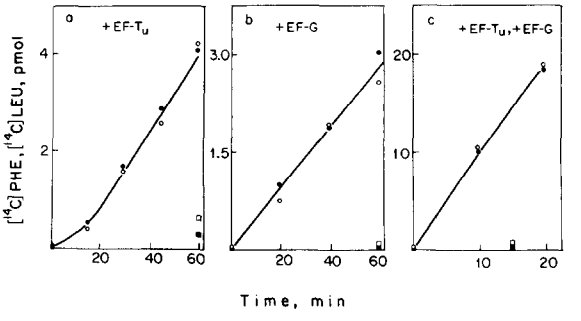

Fig.2. (legend opposite)

Table 1

Rates of phenylalanine and/or leucine incorporation into peptides, synthesized on poly(U,C) (1:1)-programmed ribosomes in factor-free and factor-promoted systems (per 80 μg ribosomes)

Presence of elongation factors	Preparation of ribosomes (no.)	$[MgCl_2]$ (mM)	Phe (pmol/h)	Leu (pmol/h)	Ratio of the rates (Phe/Leu)	Mean ratio of the rates (Phe/Leu)
– EF's	1	12	0.63	0.50	1.26	1.13
	1	12	0.52	0.52	1.00	
– EF's + *p*HMB	1[a]	15	1.08	1.00	1.08	1.01
	1[a]	15	0.71	0.73	0.97	
	2	15	0.55	0.57	0.96	
	2[a]	15	0.77	0.74	1.04	
+EF-Tu	1[a]	10	8.13	8.35	0.97	1.04
	3	10	5.53	4.62	1.20	
	4[b]	10	4.46	4.47	1.00	
	4[b]	10	3.82	3.90	0.97	
+EF-G	3	16	5.61	5.46	1.02	1.06
	3	16	3.27	2.69	1.22	
	4[b]	17	8.68	9.20	0.94	
	4[b]	17	5.13	4.83	1.06	
+EF-Tu, +EF-G	2	12	245	247	0.99	1.03
	4[b]	10	129	125	1.03	
	4[b]	12	106	107	0.99	
	4[b]	12	99.8	90.6	1.10	

[a] Additional portions of aminoacyl-tRNA were added to the reaction mixture during incubation (cf. fig.1)

[b] tRNA, acylated with [^{14}C]leucine, [^{3}H]phenylalanine, unlabeled serine and proline was used. In all other experiments tRNA acylated with [^{14}C]phenylalanine, unlabeled leucine, serine and proline or tRNA acylated with [^{14}C]leucine, unlabeled phenylalanine, serine and proline was used

Rates of phenylalanine and leucine incorporations are calculated from the linear part of the slope of the corresponding kinetic curves (cf. fig.1,2)

←

Fig.2. Kinetics of poly(U,C)-dependent peptide synthesis in the factor-promoted translation system in the presence of (a) EF-Tu and GTP at 10 mM $MgCl_2$; (b) EF-G and GTP at 16 mM $MgCl_2$; (c) EF-Tu, EF-G and GTP at 12 mM $MgCl_2$. Incorporation of [^{14}C]Phe (●, ■) or [^{14}C]Leu (○, □) into trichloroacetic acid-insoluble peptides: without poly(U,C) (■, □); with poly(U,C) (○, ●). In (a) and (b) each 80–100 μl sample contained 40 μg equimolar 30 S and 50 S subparticle mixture, 8 μg poly(U,C), 10 μg EF-Tu (a) or 2 μg EF-G (b). In (a) each sample also contained 400 μg aminoacyl-tRNA; in (b) 160 μg aminoacyl-tRNA. In (c) each 50 μl sample contained 20 μg of the equimolar 30 S and 50 S subparticle mixture, 4 μg poly(U,C), 100 μg aminoacyl-tRNA, 5 μg EF-Tu and 1 μg EF-G. The tRNA was acylated with either [^{14}C]phenylalanine and unlabeled leucine, serine and proline or [^{14}C]leucine and unlabeled phenylalanine, serine and proline.

and/or $tRNA^{Pro}$ by *p*HMB could be responsible for this. To avoid the effect of inhibition by the SH-reagent we used ribosomes whose 30 S subparticles were pretreated with *p*HMB and freed of its excess.

The kinetics of poly(U,C)-dependent syntheses of trichloroacetic acid-insoluble peptides in one-factor-promoted systems, containing either EF-Tu with GTP (without EF-G) or EF-G with GTP (without EF-Tu) [6], are shown in fig.2a and 2b, respectively. The kinetic curve of poly(U,C) translation in the complete factor-promoted system (containing both elongation factors and GTP) is given for comparison (fig.2c).

It is seen in fig.1 and 2 that the poly(U,C)-dependent increase of [^{14}C]phenylalanine or

[^{14}C]leucine incorporation into trichloroacetic acid-insoluble peptides takes place in all the systems tested: the factor-free ones and the one-factor-promoted as well as in the normal complete factor-promoted cell-free system.

Table 1 summarizes the results of experiments on on poly(U,C)-dependent polypeptide syntheses. Rates of phenylalanine and/or leucine incorporation into peptides synthesized, calculated from the linear parts of the kinetic curves (similar to those presented in fig.1,2), are given. There were two types of experiments.

(1) Kinetics of phenylalanine or leucine incorporation into peptides were measured simultaneously in different tubes using the same ribosome preparation but different tRNA preparations: either tRNA acylated with [^{14}C]phenylalanine and unlabeled leucine, serine and proline, or tRNA acylated with [^{14}C]leucine and unlabeled phenylalanine, serine and proline (table 1, experiments without an asterisk).

(2) Rates of phenylalanine and leucine incorporation into peptides were determined in one tube; tRNA acylated with [^{3}H]phenylalanine, [^{14}C]-leucine and unlabeled serine and proline was used as a substrate in this type of experiment.

The lowest rate of poly(U,C)-dependent peptide synthesis is characteristic for the spontaneous factor-free system (table 1). The SH-reagent, *p*HMB, only slightly stimulates the factor-free poly-(U,C)-dependent peptide synthesis as compared with the case when poly(U) was used as a template [6]. Addition of only EF-Tu with GTP or only EF-G with GTP to the factor-free system noticeably stimulates the peptide synthesis, although it does not reach the rate of the synthesis in the complete system, where the two elongation factors and GTP are present. Neither of the elongation factors has an essential predominance in stimulation of the poly-(U,C)-dependent peptide synthesis.

Poly(U,C) with U/C ratio of 1:1 was used as a template. This U/C ratio provides the equal probability for the triplet pairs corresponding to each of the 4 poly(U,C)-coded amino acids. Consequently, polypeptides synthesized on the poly(U,C) (1:1) template have to contain all the 4 amino acids in equimolar amounts. The data of fig.1,2 and table 1 show that this is actually the case. Phenylalanine and leucine are incorporated at practically equal rates into polypeptides synthesized on poly(U,C) (1:1)-programmed ribosomes in all the systems used. This means that polypeptides synthesized on poly(U,C)-programmed ribosomes correspond in their amino acid composition to the code specificity of the template in factor-free and one-factor-promoted systems as well as in the normal complete factor-promoted system.

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