DNA-DEPENDENT IN VITRO SYNTHESIS OF *ESCHERICHIA COLI* RIBOSOMAL PROTEIN S20 AND ISOLEUCYL-tRNA SYNTHETASE

Effect of guanosine-5′-diphosphate-3′-diphosphate

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1. Introduction

Recent analysis by means of a direct chemical assay has shown that the numbers of aminoacyl-tRNA synthetase molecules per bacterial cell vary with the growth rate [1,2]. This type of control ('metabolic regulation') is responsible for the fact that under different rates of unrestricted growth the ratio of aminoacyl-tRNA synthetases relative to other components of the protein synthesis apparatus, especially ribosomes, remains more or less balanced [2]. Upon growth restriction, e.g., by amino acid starvation, however, aminoacyl-tRNA synthetases and ribosomes display a somewhat different response. In contrast to the well known 'stringent response' of ribosome synthesis under this condition, the amino acid activating enzymes seem to be only weakly affected by this control mechanism [3,4].

A suitable system for the in vitro investigation of the coordinate or non-coordinate regulation of synthesis of aminoacyl-tRNA synthetases and ribosomal components was provided by the isolation of a specialized transducing phage λ carrying the structural genes for ribosomal protein S20 and for isoleucyl-tRNA synthetase [5]. DNA from this phage was used in this work to study how guanosine-5′-diphosphate-3′-diphosphate (ppGpp) affects synthesis of isoleucyl-tRNA synthetase and of ribosomal protein S20 in a coupled transcription and translation system [6] since the different growth-rate-dependent basal levels of ppGpp have been implicated as a possible effector in the metabolic regulation of ribosome synthesis [7,8] during balanced growth. In addition, this nucleotide was demonstrated to drastically affect the in vitro synthesis of ribosomal RNA, ribosomal proteins and elongation factors [9–11].

2. Material and methods

2.1. *Chemicals*

L-[^{3}H]Lysine and L-[^{14}C]lysine were obtained from the Radiochemical Centre, Amersham. L-[^{35}S]-Methionine was from New England Nuclear. Goat antibodies directed against rabbit γ-globulins were purchased from the Behringwerke AG, Marburg. Purified preparations of isoleucyl-tRNA synthetase and ribosomal protein S20 were kindly provided by E. Holler, Regensburg and H. G. Wittmann, Berlin, and guanosine-5′-diphosphate-3′-diphosphate was a generous gift from D. Richter, Hamburg, FRG.

2.2. *In vitro protein synthesis system*

The system described by Zubay [6] was used for cell-free λ-DNA directed protein synthesis. Two modifications were employed: i) λ-DNA which was prepared from λcI857S7, λddapB119 and λddapB254 [5] was isolated by precipitation with 0.5 M KCl, and ii) the final assay volume was 50 μl and each assay received 13 nmol of 19 unlabelled amino acids and 10 μCi of L-[^{3}H]lysine (8 Ci/mM) together with 1.3 nmol non-radioactive L-lysine. In the experiment illustrated by fig.1 L-[^{35}S]methionine was used for labelling. In this case 20 μCi of the labelled amino

acid (16 Ci/mM) were added together with 1.3 nmol. non-radioactive methionine. S30 extracts were prepared from *E. coli* strain A19 (met$^-$ RNAaseI$^-$ λ^+).

2.3. *Preparation of ribosomes labelled with L-[^{14}C]-lysine*

E. coli strain A19 was grown in minimal medium supplemented with 40 μg/ml L-methionine and 0.4% glucose. At A_{436} 0.4, L-[^{14}C]lysine (0.2 μCi/ml) was added to the culture and the aeration continued until A_{436} 1.2 was reached. The ribosomes were prepared from these cells according to Hardy et al. [12].

2.4. *Determination of the amount of isoleucyl-tRNA synthetase and ribosomal protein S20 synthesized*

For the determination of the total amount of [^{3}H]lysine incorporated into protein 5 μl samples of the in vitro assay were mixed with 1 ml of a solution of bovine serum albumine (1 mg/ml) and 2 vol. cold 10% trichloroacetic acid; the samples were heated for 20 min at 90°C, the precipitate was collected on Whatman GF/C glass fibre filters, washed two times with cold 10% trichloroacetic acid and finally with 70% ethanol (–20°C). The filters were dried under an infrared lamp and the radioactivity determined in a liquid scintillation spectrometer employing 0.5% diphenyloxazol in toluene as scintillation liquid.

For the determination of the amount of ribosomal protein S20 synthesized suitable samples from the in vitro system were mixed with 4 mg [^{14}C]lysine-labelled ribosomes (about 10^6 cpm) prepared as described above. After extraction of ribosomal proteins with 66% acetic acid [12] samples were subjected to two-dimensional polyacrylamide gel electrophoresis according to Kaltschmidt and Wittmann [13]. After staining, the spots for ribosomal protein S20 were cut out from the gels, the slices were burnt in a Packard sample oxidizer Tricarb 306 and the separated ^{3}H and ^{14}C radioactivity determined in a liquid scintillation spectrometer. ^{14}C counts detected in ribosomal protein S20 were in the range from 800–1200 cpm. To correct for yield, ^{3}H counts were normalized to 1000 ^{14}C counts.

For the measurement of the amount of isoleucyl-tRNA synthetase made in the in vitro system 10 μl samples were taken and added to 20 μl sodium dodecylsulfate (SDS) containing sample buffer described by Laemmli and Favre [14]. After the addition of 0.5 μg purified isoleucyl-tRNA synthetase the samples were boiled for 3 min and then applied to a 10–20% SDS polyacrylamide-gradient slab gel. Electrophoresis (at 4°C) was first for 45 min at 20 mA and then for 150 min at 30 mA. The gels were soaked in 200 ml of 50% trichloroacetic acid for 30 min followed by two washes in deionized water for 30 min, then stained with 0.2% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid. After destaining by diffusion the electrophoretic bands indicated by the purified isoleucyl-tRNA synthetase were cut out, oxidized and their radioactivity determined as described above. Fluorographs of the dried slab gels were made as described [15,16].

2.4. *Immunological identification of isoleucyl-tRNA synthetase*

The procedures for the preparation of anti isoleucyl-tRNA-synthetase serum and for the determination of the equivalence concentration between the rabbit γ-globulins and the goat anti rabbit-γ-globulin serum were as described [17,18]. Immunoprecipitation of isoleucyl-tRNA synthetase was done by incubation of 20 μl of the in vitro system for 1 h at 0°C with that amount of the rabbit anti isoleucyl-tRNA-synthetase serum which inhibits the activity of 1 μg pure enzyme to 50%. An equivalent amount of anti rabbit-γ-globulin serum from the goat was then added, the mixture was incubated for 15 h at 0°C and the immunoprecipitate washed three times with 10 mM potassium phosphate buffer, pH 7.5, containing 150 mM NaCl. After solubilisation in sample buffer, the proteins were separated electrophoretically and the radioactivity present in the isoleucyl-tRNA synthetase band was determined as described above.

3. Results and discussion

Figure 1 shows the time dependence of the synthesis of the total hot trichloroacetic acid precipitable material, of ribosomal protein S20 and of isoleucyl-tRNA synthetase. As synthesis is optimal after 60 min incubation, this time was chosen for further experiments. As a control, the incorporation of radioactivity in response to λ-DNA not carrying the isoleucyl-tRNA synthetase and S20 structural genes (λcI857S7) was measured. These background values were less than

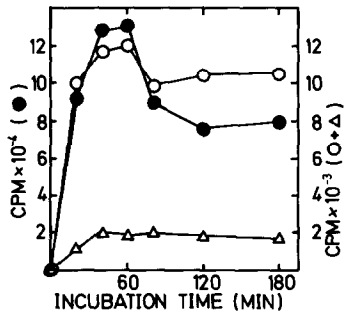

Fig.1. Time dependence of in vitro incorporation of L-[^{35}S]-methionine into hot trichloroacetic acid-precipitable material (●), ribosomal protein S20 (○) and isoleucyl-tRNA synthetase (△). In this experiment, separation of labelled products was by one-dimensional slab gel electrophoresis. Bands co-migrating with purified protein S20 and isoleucyl-tRNA synthetase were cut out from the gel, the slices were solubilized by incubation with 0.7 ml 90% NCS (Amersham/Searle) and the radioactivity determined by means of a toluene based 0.5% diphenyloxazole scintillation fluid.

10% in the case of ribosomal protein S20, about 25% for isoleucyl-tRNA synthetase when the products of the in vitro system were applied to the slab gel without any prior purification by immunoprecipitation and less than 10% when immunoprecipitation was employed.

The results of fig.2 show that the synthesis of protein S20 can be almost totally inhibited by ppGpp in concentrations reported to accumulate in vivo under amino acid starvation conditions [19]. The amount of S20 made from λddapB119 (3800 cpm/10 μl assay) and λddapB254 (3600 cpm/10 μl assay) was

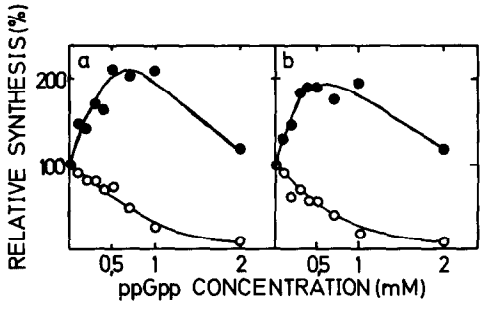

Fig.2. Effect of ppGpp on synthesis of ribosomal protein S20 (○) (average of two experiments) from: (a) λddapB119 DNA; (b) λddapB254 DNA. (●) Hot trichloroacetic precipitable material.

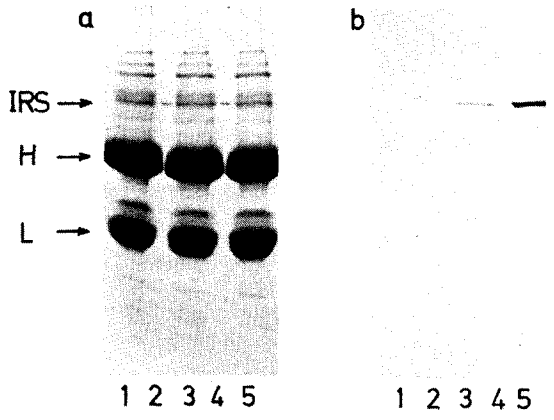

Fig.3. Immunological identification of the isoleucyl-tRNA synthetase synthesized. (a) Stained gel; (b) fluorograph of the gel. Lanes 2 and 4 contain 0.4 μg purified isoleucyl-tRNA synthetase, each. Immunoprecipitates of material synthesized from λcI857S7 DNA in the absence of ppGpp, from λddapB254 DNA in the absence and in the presence of 0.5 mM ppGpp were separated in lanes 1, 3 and 5, respectively. IRS, H and L denote the position of the bands for isoleucyl-tRNA synthetase and for the heavy and light chains of immunoglobulins.

nearly identical and ppGpp seems to affect synthesis in an equal manner and also in a concentration range previously shown to effectively inhibit the synthesis of other ribosomal components [9–11].

Figure 3 illustrates that the radioactively-labelled material migrating in the position of purified isoleucyl-tRNA synthetase actually consists of this enzyme. Whereas antiserum directed against isoleucyl-tRNA synthetase cannot precipitate any radioactive material from the λcI857S7 DNA-primed in vitro system, labelled material comigrating with isoleucyl-tRNA synthetase can be detected in immunoprecipitates from the λddapB254 DNA (and also λddapB119 DNA, not shown) encoded assay.

In contrast to that of ribosomal protein S20 (see fig.2) in vitro synthesis of isoleucyl-tRNA synthetase is not inhibited but rather stimulated in the presence of ppGpp (fig.4). An up to 4-fold stimulation could be observed at a ppGpp concentration of 0.5 mM. Values obtained by immunoprecipitation were identical to those obtained by direct separation of the material synthesized. DNA from both transducing phages showed a similar response to ppGpp although the absolute amount of isoleucyl-tRNA

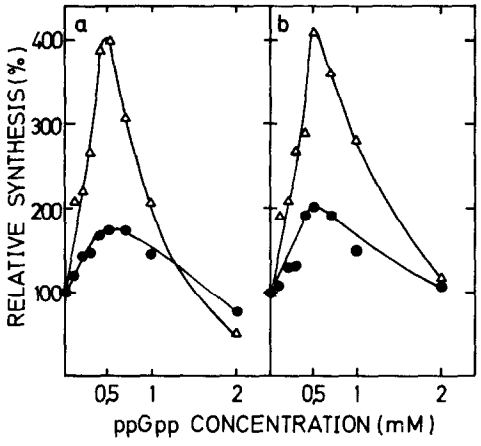

Fig.4. Effect of ppGpp on synthesis of isoleucyl-tRNA synthetase (△) (a) directed by λddapB119 DNA and (b) λddapB254 DNA (average of three experiments). (•) Hot trichloroacetic acid-precipitable material.

synthetase made was two times higher with DNA from λddapB254 than with DNA from λddapB119.

The identical results obtained with two transducing phages which contain different amounts of *E. coli* DNA (P. Buckel, unpublished results) are in favour of the assumption, though do not prove it, that the structural genes investigated are still under the control of bacterial promoters.

4. Conclusion

The results described show that in vitro synthesis of ribosomal protein S20 and isoleucyl-tRNA synthetase is differentially affected by ppGpp. Synthesis of protein S20 responds in a similar way to ppGpp as that of other ribosomal components [9,10], which demonstrates that this effector also affects the transcription of structural genes for ribosomal proteins located singly and not in any common ribosomal proteins located singly and not in any common ribosomal protein transcriptional unit. In vitro synthesis of isoleucyl-tRNA synthetase, on the other hand, is distinctly stimulated by ppGpp. This response is identical to that found for the arabinose, lactose, tryptophan and histidine operons [20–22] and indicates that control of synthesis of aminoacyl-tRNA synthetases is quite different from that of the other components of the bacterial translational apparatus.

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