REPRESSION OF TRYPTOPHAN OPERON RNA SYNTHESIS BY *trp* REPRESSOR IN AN IN VITRO COUPLED TRANSCRIPTION–TRANSLATION SYSTEM

Nobuyoshi SHIMIZU, Yoshiko SHIMIZU,
Frank K. FUJIMURA and Masaki HAYASHI

Department of Biology, University of California, San Diego, La Jolla, California 92037, USA

Received 4 January 1974

1. Introduction

The expression of the tryptophan (*trp*) operon in *E. coli* is negatively controlled by the interactions of at least three factors: the product of the regulatory gene (*trp* R), the intact *trp* operator and excess L-tryptophan [1–5]. Recently we have purified the protein product of the *trp* R gene and have characterized it in a purified in vitro transcription system directed by DNAs from *trp* transducing phages [6]. These in vitro studies were in agreement with the classical model of repression of the *trp* operon [1, 7]. In this model, the product of the regulatory gene is an inactive aporepressor, which by interaction with L-tryptophan is converted to the active repressor. The activated *trp* repressor binds to the DNA at the *trp* operator to block transcription initiating at the *trp* promoter. Similar conclusions from in vitro studies have been reported by Rose et al. [8]. However, the possible contribution of coupling between transcription and translation in the regulation of the *trp* operon [9–12] remains to be elucidated.

A DNA-directed RNA-dependent in vitro protein synthesizing system has been developed in which the faithful expression of the genetic information coded on ΦX174 replicative form DNA and T7 DNA has been observed [13, 14]. In this paper, we have applied this cell-free system to study the bacterial *trp* operon. *Trp* specific RNA was transcribed from the correct strand of template DNA from a *trp* transducing phage and was furnished to the translational machinery. Addition of the isolated *trp* repressor into this coupled transcription–translation system specifically inhibited the expression of the *trp* operon at the transcriptional level. The *trp* repression depended upon the concentration of L-tryptophan.

2. Materials and methods

2.1. *Coupled system extracts* were prepared from *E. coli* D24 (RNaseI$^-$, λ$^-$, F$^-$, met$^-$) cells according to the method of Bryan et al. [13].

2.2. *Synthesis of RNA and protein* in the coupled system were measured by the method of Bryan et al. [13]. The standard reaction mixture (0.13 ml) contained 1.65 μmoles Tris–HCl (pH 7.8), 0.1 μmole NaCl, 1.5 μmoles $Mg(COOCH_3)_2$, 0.9 μmole 2-mercaptoethanol, 0.6 μmole phosphoenol pyruvate, 0.25 μmole ATP, 0.05 μmole each of α-^{32}P-GTP (42 Ci/mole), CTP and UTP, about 4 nmoles each of 20 amino acids (^{14}C-leucine, 263 Ci/mole), 5 μg pyruvate kinase, 5 μg λpt60-3 DNA, 5 μg RNA polymerase, 5 A_{260} units ribosome fraction, and 0.75 A_{260} unit soluble fraction. When appropriate, 20 μl of partially purified *trp* repressor were added to the reaction mixture, which was then kept for 5 min at 0°C prior to the addition of the RNA polymerase. After incubation at 33°C for the desired time intervals, radioactivities in the cold 5% CCl_3COOH (TCA)-precipitable materials and the hot 7% $HClO_4$ (PCA)-precipitable materials were measured as the amounts of RNA and protein synthesized, respectively.

2.3. *trp Repressor* was partially purified from *E. coli* W3110 Ilv$^-$, leu$^-$, pro$^-$, *trp* A_{9865} *trp* R$^+$ rec A$^-$ J_{273}/KLFH (thr$^+$, leu$^+$, pro$^+$, *trp* R$^+$) [15] cells as

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described previously [6]. The DNA–cellulose fraction (1 mg protein/ml) was used for the present experiments.

2.4. *Other experimental methods* including the preparation of phage stocks, the extraction of phage DNAs, the separation of DNA strands, and the purification of DNA-dependent RNA polymerase have been described elsewhere [16].

3. Results and discussion

Fig. 1 shows the kinetics of RNA and protein synthesis in the in vitro coupled system using λpt60-3 DNA, which carries an intact *trp* operon [12], as the template. RNA synthesis proceeded linearly for 20 min then reached a plateau level. After a lag of about 4 min protein synthesis continued for at least 45 min, indicating that translation closely follows the transcriptional process. Very little RNA and protein was synthesized in the absence of the template DNA. The standard concentration of L-tryptophan in the coupled system is 0.0248 mM which is adequate for de novo protein synthesis in vitro [13, 14] but is insufficient to repress the *trp* operon in vivo [17, 18]. Since all the experiments described here were carried out with bacterial extracts prepared from a strain of *E. coli* carrying the intact *trp* R gene it is expected that the RNA and protein synthesis would be repressed by the action of endogenous *trp* repressor when the L-tryptophan concentration was increased to 0.509 mM, which is a sufficient level to cause repression in vivo and in vitro [3, 6, 12, 16]. However, no obvious inhibition of RNA and protein synthesis was observed when the concentration of L-tryptophan was increased (upper columns in table 1). We believe that some *trp* repressor molecules were removed from the soluble fraction of the cell extracts and/or were inactivated during preparation of the coupled system. Thus, most of the template DNA molecules, unhindered by *trp* repressor molecules, would be accessible for the synthesis of *trp* RNA and

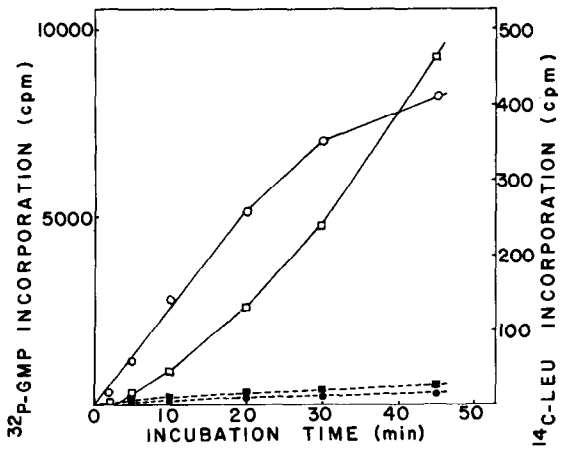

Fig. 1. Kinetics of RNA and protein synthesis in the coupled system under standard conditions. At the designated intervals, 20 μl samples were withdrawn from the standard reaction mixture (see Materials and methods) and pipetted into 2 ml of ice-cold 1% casamino acid solution to terminate the reaction. Radioactivities in the cold 5% TCA precipitable materials and the hot 7% PCA precipitable materials were measured, respectively. [^{32}P] GMP incorporation per 10 μl with (o——o——o) or without (●——●——●) λpt60-3 DNA. [^{14}C]Leu incorporation with (□——□——□) or without (■——■——■) λpt60-3 DNA.

Table 1
RNA and protein synthesis in the coupled system under different conditions

Addition of *trp* repressor	Synthesis	Time of incubation	[^{32}P]GMP or [^{14}C]leu incorporated (cpm/10 μl) L-Trp conc. 0.0248 mM	0.509 mM	b/a
None	RNA	5′	1088 (a)	1055 (b)	0.97
		10′	2226	2309	1.04
		30′	6109	6303	1.03
None	protein	5′	32	31	0.97
		10′	74	71	0.96
		30′	196	206	1.05
Added	RNA	5′	885	726	0.82
		10′	1715	1489	0.87
		30′	6211	5442	0.88
Added	protein	5′	27	22	0.79
		10′	66	56	0.85
		30′	180	167	0.93

Reaction mixtures (0.13 ml) were set up as described in Materials and methods with L-tryptophan added to the final concentrations indicated. At the designated intervals, 20 μl samples were withdrawn from each reaction mixture to measure the net RNA and protein synthesis.

Table 2
Repression of *trp* RNA synthesis

Addition of *trp* repressor	Time of incubation	*trp* ED RNA (% of input) L–Trp conc. 0.0248 mM	*trp* ED RNA (% of input) L–Trp conc. 0.509 mM	*trp* CBA RNA (% of input) L–Trp conc. 0.0248 mM	*trp* CBA RNA (% of input) L–Trp conc. 0.0509 mM
None	5′	8.4	7.8	2.6	2.1
	10′	8.9	8.0	6.3	5.9
Added	5′	5.3	1.2	2.1	0.87
	10′	6.5	1.5	2.4	1.1

[^{32}P] GMP labeled RNAs were synthesized under various conditions as described in table 1 and the RNAs were purified from the reaction mixtures by phenol extraction [16]. 10 μl aliquots of the purified RNA samples dissolved in 110 μl of 0.2% SDS were separately hybridized to excess amounts (1.0 μg) of separated *l*-strands of Φ80ptED, Φ80ptCBA and Φ80 wild DNAs. Hybridizations were carried out in 0.15 ml of 0.30 M NaCl–0.03 M sodium citrate, pH 7.4 (2 × SSC) at 65° C for 4 hr. After RNase treatment (6 μg/ml of RNase A, Worthington Biochem. Co., and 3 units/ml of RNase T_1, Sankyo Co.) at 25° C for 30 min, the RNase-resistant RNA–DNA complex was collected on a presoaked membrane filter (Schleicher and Schuell Co., Bac-T-Flex, Type B6, 27 mm diam.) and washed with 50 ml of cold 2 × SSC and the radioactivity on a filter was counted. Hybridization efficiency was 95%. The difference in hybridization values between Φ80pt and Φ80 wild type was taken as a measure of the *trp* operon specific RNA, and is represented by percentage of input counts.

protein. When purified *trp* repressor together with a high concentration of L-tryptophan were added to the coupled system, RNA and protein synthesis was significantly inhibited (lower columns in table 1).

In order to know whether or not the reduction of RNA synthesis is due to the specific repression of transcription of the *trp* operon, we measured the amounts of *trp* RNA in the total RNA synthesized under various conditions. The results of DNA–RNA hybridization experiments are summarized in table 2. Without addition of *trp* repressor about 8 to 9% of the total RNA is *trp* RNA which is complementary to operator proximal *trp* E–D genes, regardless of the concentration of L-tryptophan. When *trp* repressor was added to the coupled system *trp* ED RNA synthesis was remarkably repressed by the higher concentration of L-tryptophan. Significant repression of *trp* ED RNA synthesis was also observed at the lower concentration of L-tryptophan (about 40% repression at 5 min). The concentration of L-tryptophan that causes 50% repression in our coupled system is calculated to be about 0.03 mM, which seems to be several fold higher than that in other in vitro systems not involving translation [8, 19], our unpublished results [0.014 mM]).

During the initial 5 min of synthesis, there is much less RNA which is complementary to *trp* C–B–A genes than that complementary to *trp* E–D genes, indicating that transcription of the *trp* operon occurs sequentially from E gene to A gene as observed in vivo [20]. It is noteworthy that synthesis of significant amounts of *trp* RNA was observed even under fully repressed conditions (in the presence of both *trp* repressor and a high concentration of L-tryptophan). This could be explained by the following possibilities. (a) Some transcription initiated at the λ *N* gene promoter proceeds into the *trp* operon even in the presence of active *trp* repressor on the *trp* operator [6, 12, 16]. (b) Transcription on the *trp* operon initiates mainly at the principal *trp* promoter but some transcription occurs at second minor promoter located between the *trp* D and C genes [21, 22], and the latter is not subject to the action of *trp* repressor [23].

In summary, we have demonstrated the repression of *trp* specific RNA synthesis in a coupled transcription–translation system. Repression was dependent upon the action of the *trp* repressor in the presence of excess L-tryptophan. This system would be useful for the further study of regulation of the *trp* operon.

Acknowledgements

The authors are very grateful to Drs. C. Yanofsky, A. Matsushiro, F. Imamoto for providing us with phage and bacterial strains used in these experiments. This investigation was supported in part by grants from the U.S. Public Health Service, GM-12934, and the National Science Foundation, GB-29170X. M.H. is the recipient of a Career Development Award from the U.S. Public Health Service (GM-42360). N.S. is grateful to the Naito Science Foundation of Japan for a travel grant.

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