Regulation of Synthesis of Ribosomal Proteins during Pyrimidine Starvation in *Escherichia coli*

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The synthesis of ribosomal proteins during pyrimidine starvation was investigated. A progressive turn-off of protein synthesis, with a decay half-time of about 5 min, was observed when *Escherichia coli* cells were starved of uridine. By means of double-labelling, the syntheses of different ribosomal proteins were shown to be turned off unequally during the starvation. Comparison of the turn-off patterns for some proteins and the known polycistronic organization of the structural genes for these proteins suggests that a major cause of the unequal turn-off was the degradation of mRNA molecules for the ribosomal proteins from the 5'-end toward the 3'-end.

Although the structure and function of bacterial ribosomal proteins are being rapidly elucidated, relatively little is known about the regulation of their biosynthesis. Previously, we observed that pyrimidine starvation of *Escherichia coli* cells brought about a rapid but unequal turn-off of the synthesis of the different ribosomal proteins (Beatty & Wong, 1970, 1971). The mechanism for this unequal turn-off could be examined only on the basis of a precise identification of the proteins and information on the genetic organization of their coding cistrons. The identification of ribosomal proteins is now possible with two-dimensional gel electrophoresis (Kalt-schmidt & Wittmann, 1970), and polycistronic models for ribosomal proteins are also beginning to be defined (Nomura & Engbaek, 1972; Molin *et al.*, 1974; Carpenter & Sells, 1974). In the present study the relative turn-off of synthesis of various ribosomal proteins during uridine starvation has been analysed, in order to gain insight into the mode of metabolism of their mRNA molecules.

Experimental

Isolation of proteins

A pyrimidine auxotroph of *E. coli* (A.T.C.C. 12632) was grown in mineral medium '63' with 0.5% glycerol as carbon source, as previously described (Beatty & Wong, 1970). Uridine starvation was brought about by growing cells in medium containing only 7 µg of uridine/ml. Depletion of uridine from the medium was marked by a sharp break in the growth curve monitored by E_{450} measurement, and the growth of the starved cells could be reinitiated by the readdition of 23 µg of uridine/ml. The cells were radioactively labelled with leucine during exponential growth, during uridine starvation, or after re-addition of uridine. On harvesting, the cells were chilled by pouring them

over crushed frozen mineral medium containing a final concentration of 20 mM-NaN₃. The cells were then pelleted by centrifugation at 6000g for 15 min, re-suspended in 10 mM-Tris-acetate-10 mM-magnesium acetate, pH 7.4 (TM buffer) and lysed in a French pressure cell at 82 700 kPa (12 000 lb/in²). Ribosomal particles were sedimented at 220 000g for 1 h. After another wash in TM buffer and two more washes in TM buffer containing 0.5 M-NH₄Cl and 10 mM-2-mercaptoethanol (cf. Hindennach *et al.*, 1971), the ribosomal proteins were extracted with 66% (v/v) acetic acid-33 mM-magnesium acetate (Hardy *et al.*, 1969). After freeze-drying, they were separated by two-dimensional polyacrylamide-gel electrophoresis as described by Kaltschmidt & Wittmann (1970), and identified on the basis of the pattern established by these workers for *E. coli* B.

Radioactive labelling scheme

The rates of synthesis of the different ribosomal proteins relative to total synthesis of cellular proteins were determined by the method of double-labelling, by using such short pulses of [¹⁴C]- and [³H]-leucine that under all experimental conditions they were completely incorporated into cellular proteins within 0.5 min. Altogether three types of ³H/¹⁴C ratios were obtained, namely under conditions of exponential growth, uridine starvation and recovery from uridine starvation.

(1) *Control ³H/¹⁴C ratio.* One 200 ml batch of cells, growing in the presence of excess of uridine (30 µg/ml), was pulse-labelled with 0.2 mCi of [¹⁴C]leucine (254 mCi/mmol) in early exponential phase, and harvested in mid-exponential phase. Another 200 ml batch was pulse-labelled with 2 mCi of [³H]leucine (29.8 Ci/mmol) under similar conditions. The two batches were combined, and the different ribosomal

proteins from the combined sample were extracted from the two-dimensional polyacrylamide gel by the method of Zaitlin & Hariharasubramanian (1970). The ^3H and ^{14}C contents of each protein were determined by the channels-ratio method with external standard by using a scintillant containing 4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene and a Nuclear-Chicago mark II liquid-scintillation spectrometer. The $^3\text{H}/^{14}\text{C}$ ratio so obtained constituted the control $^3\text{H}/^{14}\text{C}$ ratio.

(2) 'Starvation' $^3\text{H}/^{14}\text{C}$ ratio. One 200ml batch of cells was pulse-labelled with 0.2mCi of [^{14}C]leucine in the presence of excess of uridine, similarly to the control samples. Another 200ml batch was pulse-labelled with 2mCi of [^3H]leucine at 5min after the onset of uridine starvation and harvested 20min later. The two batches were combined, and the ^3H and ^{14}C contents of each ribosomal protein from the combined sample were determined, to yield the 'starvation' $^3\text{H}/^{14}\text{C}$ ratio.

(3) 'Recovery' $^3\text{H}/^{14}\text{C}$ ratio. One 200ml batch of cells was pulse-labelled with 0.2mCi of [^{14}C]leucine in the presence of uridine, similarly to the control samples. Another 200ml batch was allowed to undergo uridine starvation. After 20min of starvation, recovery from starvation was initiated by adding 23 μg of uridine/ml to the starved cells. At some pre-selected time after recovery, the cells were pulse-labelled with 2mCi of [^3H]leucine, and harvested 20min later. The two batches were combined, and the ^3H and ^{14}C contents of each ribosomal protein from the combined sample were determined, to yield the 'recovery' $^3\text{H}/^{14}\text{C}$ ratio.

(4) Determination of F values. Changes in the incorporation of leucine into any ribosomal protein could be monitored in terms of an F value defined as follows:

$$F = \frac{\text{Experimental } ^3\text{H}/^{14}\text{C} \text{ ratio}}{\text{Control } ^3\text{H}/^{14}\text{C} \text{ ratio}}$$

The F value is a measure of the fraction of [^3H]leucine that enters into any ribosomal protein on the ribosome during uridine starvation or recovery, relative to the fraction that normally enters during exponential growth. An F value greater than 1 indicates an increased incorporation relative to exponential incorporation, and an F value smaller than 1 indicates a decreased incorporation relative to exponential incorporation.

Results

When cells of *E. coli* A.T.C.C. 12632 strain were starved of uridine, RNA synthesis stopped (Fig. 1), and protein synthesis decreased progressively with time (Fig. 2). The synthesis of ribosomal proteins

decreased more than the synthesis of soluble proteins, such that a smaller fraction of the same [^3H]leucine pulse entered into the ribosomal proteins during uridine starvation (Fig. 3). This decrease was different for different ribosomal proteins and, since it could not be reversed by re-addition of uridine subsequent to [^3H]leucine labelling, indicated a differential turn-off of the synthesis of the different ribosomal proteins (Beatty & Wong, 1970, 1971). The extent of turn-off for various ribosomal proteins at 5min after uridine starvation, measured on the basis of their F values, are shown in Tables 1 and 2.

Both the small- and large-subunit proteins can be separated into three groups. The group I proteins, especially S4, S5, L2, L17 and L3, showed an extensive fall in F value after uridine starvation. The group II proteins showed a smaller fall in F value; and the group III proteins, which include S2, S12, S9, S20/L26, S21, L25, L28 and L21, showed no significant fall and in some cases a significant rise in F value.

Fig. 1. Effects of uridine starvation on cell growth, RNA synthesis and protein synthesis

Cell growth (○) was followed by change in E_{450} . Protein (●) and RNA (▲) synthesis were followed by incorporation of [^{14}C]leucine (15 $\mu\text{g}/\text{ml}$, 0.01 $\mu\text{Ci}/\text{ml}$) and [^{14}C]adenine (8 $\mu\text{g}/\text{ml}$, 0.015 $\mu\text{Ci}/\text{ml}$) into material precipitable by cold 5% (w/v) trichloroacetic acid. Cells were grown in 7 μg of uridine/ml. Uridine starvation set in at about 170 min, and was relieved at 200 min by the addition of 23 μg of uridine/ml.

Fig. 2. Initial rate of [^{14}C]leucine incorporation into total cellular protein before and after pyrimidine starvation

Cells were grown in $7\text{ }\mu\text{g}$ of uridine/ml. Samples (40 ml) were removed during exponential growth before starvation ($\circ$), and at 5 ($\bullet$), 10 (Δ) and 15 ($\square$) min after the onset of starvation. Each sample was immediately pulsed with $0.025\text{ }\mu\text{Ci}$ of [^{14}C]leucine/ml (240 mCi/mmol), and the radioactivity in the acid-precipitable material was determined at the indicated times. Insert shows the initial rates of incorporation estimated from the time-curves.

However, the number of proteins with F values smaller than 1 exceeds the number of proteins with F values greater than 1.

When uridine was re-added to the starved cells, RNA synthesis was quickly restored (Fig. 1). As determinations at 10 s and 1 min after re-addition show, a rapid recovery also occurred in the F values of the group I and II proteins, indicating that the decreases in F values occasioned by uridine starvation were not due to any irreversible damage to the cells. Indeed, the recovery actually led to F values greater than 1 in many instances, suggesting that the synthesis of many of the ribosomal proteins was actually more favoured after the relief of uridine starvation compared with synthesis under exponential growth conditions.

Discussion

The observations recorded in Fig. 2 revealed that a progressive turn-off of protein synthesis, with a decay half-time of about 5 min, occurred when *E. coli* cells

Fig. 3. Effect of uridine starvation on the distribution of an [^3H]leucine pulse into different cell fractions

The cells, grown in limited uridine ($7\text{ }\mu\text{g/ml}$), were pulsed with $0.01\text{ }\mu\text{Ci}$ of [^{14}C]leucine/ml (240 mCi/mmol) in early exponential phase ($E_{450} = 0.2$) to allow uniform labelling of the cells before uridine depletion. Samples (100 ml) were removed at the times indicated and pulsed with $0.1\text{ }\mu\text{Ci}$ of [^3H]leucine (29.8 Ci/mmol)/ml. The cells were incubated for 15 min and chased with leucine ($20\text{ }\mu\text{g/ml}$) for an additional 15 min. The cells were harvested and broken in a French pressure cell. The debris fraction (Δ) was obtained by spinning the broken-cell suspension twice at 15000 rev./min for 15 min in a Sorvall RC-2B centrifuge, and the two pellets were combined and resuspended. The soluble protein fraction ($\bullet$) was the supernatant obtained by spinning the crude extract at 65000 rev./min for 2 h in a Beckman Spinco L2-65 centrifuge. The unwashed ribosomal-protein fraction (Δ) was contained in the pellet from this centrifugation. The ribosomal pellets were washed, as described in the text, to produce the 'washed' ribosomal-protein fraction ($\circ$). Duplicate samples of $100\text{ }\mu\text{l}$ from each fraction were precipitated in cold 5% trichloroacetic acid. The precipitate was collected on Millipore filters and the radioactivity was determined in a Nuclear-Chicago Mark I liquid-scintillation counter.

were starved of uridine. The progressive decay of functional mRNA molecules offers the simplest, if not the only, explanation for this turn-off in the rate of protein synthesis. The turn-off was not the same for all cellular proteins, and Fig. 3 suggests that the synthesis of ribosomal proteins decayed more rapidly than that of soluble proteins. Moreover, the turn-off was not the same for different ribosomal proteins, and Tables 1 and 2 show a spread of over tenfold in the F values for these proteins. Since these F values could not be enhanced by the re-addition of uridine after [^3H]leucine labelling (Beatty & Wong, 1970, 1971), the spread was indicative of a differential turn-off of synthesis, rather than of assembly, of the ribosomal proteins. Several model mechanisms capable of bringing

Table 1. *F* values of proteins from small ribosomal subunit

The *F* values were determined by adding the [^3H]leucine pulse to the experimental cells at 5 min after uridine starvation, or at 10s and 1 min after re-addition of uridine to cells which had been starved of uridine for 20 min. In each case the cells were harvested 20 min after [^3H]leucine addition.

Group	Protein	F value		
		5 min after uridine starvation	10s after uridine re-addition	1 min after uridine re-addition
I	S4	0.16	0.94	1.05
	S5	0.17	0.75	0.95
	S8	0.25	1.38	1.48
	S7	0.32	1.66	1.71
	S3	0.34	0.87	1.06
II	S16	0.41	1.53	1.38
	S17	0.48	0.71	1.11
	S13	0.52	1.35	1.29
	S6	0.56	1.79	1.60
	S14	0.70	1.40	1.72
	S11	0.79	1.69	1.49
	S10	0.84	1.23	1.55
	S19	0.88	1.40	1.72
	S1	0.91	1.30	1.61
	S15	0.93	1.12	1.48
	S18	0.96	1.56	1.35
III	S2	1.09	1.27	1.44
	S12	1.36	1.87	1.93
	S9	1.38	2.20	1.95
	S20/L26	1.44	3.02	3.14
	S21	1.55	2.37	2.40

about this differential turn-off of synthesis can be recognized.

Model I: incomplete transcription

Kaplan (1973) and Molin *et al.* (1974) suggested that most *E. coli* mRNA molecules are polycistronic in nature. An mRNA coding for two ribosomal proteins, A and B, and other proteins as well, can be represented as:

Transcription proceeds from the 5' end. Under uridine starvation, if the transcription process were interrupted, the interruption might lead to a premature termination giving rise to incomplete mRNA transcripts. The message for protein B, which is located closer to the 3' end, would be subject to a greater loss of transcription compared with the message for protein A, which is located closer to the 5' end. Accordingly, this model would predict a more severe turn-off in the synthesis of protein B compared with protein A during uridine starvation.

Model II: 3' to 5' degradation

If the production of mRNA was inhibited by uridine starvation, the relative amounts of functional messages in the cells for proteins A and B would depend on the direction of degradation of the pre-formed polycistronic mRNA. Degradation proceeding from the 3' toward the 5' end would result in a lower amount of message for protein B than for A, resulting again in a more severe turn-off of the syn-

thesis of protein B compared with protein A during uridine starvation.

Model III: 5' to 3' degradation

If the production of mRNA was inhibited by uridine starvation, and degradation of the pre-formed polycistronic mRNA proceeded from the 5' toward the 3' end, there would be a lower level of message for A than for B. Accordingly the turn-off in the synthesis of protein A would be more severe compared with protein B.

Table 2. *F* values of proteins from large ribosomal subunit

For details see Table 1.

Group	Protein	F value		
		5 min after uridine starvation	10s after uridine readdition	1 min after uridine readdition
I	L2	0.12	0.46	0.55
	L17	0.16	0.65	0.76
	L3	0.16	0.99	1.28
	L18	0.23	0.96	1.20
	L16	0.24	0.95	0.70
	L11	0.26	1.19	1.38
	L15	0.26	0.87	0.91
	L1	0.27	1.30	1.39
	L6	0.30	0.97	1.19
	L5	0.32	1.52	1.39
	L14	0.36	1.35	1.12
	L12	0.37	0.92	0.93
	L22	0.39	0.86	1.03
II	L13	0.44	1.70	1.65
	L4	0.45	1.08	1.17
	L27	0.50	1.14	1.20
	L29	0.52	0.95	1.20
	L23	0.53	1.25	1.19
	L10	0.56	1.29	1.24
	L7	0.58	1.37	1.40
	L9	0.58	1.28	1.54
	L19	0.65	1.18	1.27
	L8	0.71	1.37	1.36
	L24	0.81	2.20	1.71
	L20	0.88	2.59	1.90
III	L25	1.02	1.82	1.47
	L26/S20	1.44	3.02	3.14
	L28	1.86	1.82	1.97
	L21	2.04	1.98	2.03

Model IV: different mRNA molecules

Finally, if proteins A and B were coded by different mRNA molecules altogether, their relative turn-offs during uridine starvation would be governed by the rates of metabolism of their respective mRNA molecules.

These different mechanisms need not be mutually exclusive, and more than one of them might have operated to bring about the unequal turn-offs recorded in Tables 1 and 2. Model IV is inadequate by itself, since at least some of the ribosomal proteins are coded by polycistronic mRNA molecules (Nomura & Engbaek, 1972; Carpenter & Sells, 1974; Molin *et al.*, 1974). A meaningful decision between the other models may be attempted only if the order of the genes within an operon is known. One such favourable instance concerns the genes for streptomycin, spectinomycin and erythromycin resistances. From genetic experiments, Nomura & Engbaek (1972) suggested that these three genes belonged to the same operon, and the order was *ery*

spc str. On further analysis, Wittmann *et al.* (1973) found that the order was *spc ery str* instead in some strains of *E. coli*, which corresponded to an order of S5–L22, L4–S12 in terms of the ribosomal proteins responsible for the drug resistances. In this order, protein S12 would stand closest to the 3' end, and S5 closest to the 5' end of the mRNA. At 5 min after uridine starvation, the F values for proteins S5, L22, L4 and S12 were 0.17, 0.39, 0.45 and 1.36 respectively. These values suggest that the syntheses of L22 and L4 were turned off to an extent intermediate between those of S5 and S12, being closer to that of S5 than that of S12. This behaviour conformed to the gene order suggested by Wittmann *et al.* (1973). Further, since protein S12 was closest to the 3' end on the mRNA and underwent the least turn-off in synthesis compared with the others, these findings would favour Model III over Models I and II, and suggest that in this instance the relative turn-offs of the four proteins were largely determined by the 5' to 3' degradation of the polycistronic mRNA.

A directional decay of polycistronic *E. coli* mRNA from the 5' to the 3' end has been proposed for the *trp* operon (Morikawa & Imamoto, 1969; Morse *et al.*, 1969) and the *lac* operon (Leive & Kollin, 1967; Jacquet & Kepes, 1971). Therefore the suggestion that the mRNA for proteins S5-L22, L4-S12 decayed from the end coding protein S5 toward that for S12 is in agreement with these other experimental systems, with the necessary recognition that the precise mode of mRNA inactivation in these other systems also might be open to some complex modulation (Blundell *et al.*, 1972; Kennell & Bicknell, 1973). In general, if the 5' to 3' directional decay of mRNA can be regarded as the major factor responsible for the unequal F values for different ribosomal proteins, the F values recorded in Tables 1 and 2 would suggest that the genes for group I proteins were located closer to the 5' end of an mRNA, and those for group III proteins closer to the 3' end. Although the exact number and nature of the mRNA molecules coding for the various ribosomal proteins remain to be determined, the F values obtained during uridine starvation should provide a useful basis, to be combined with information from other experimental approaches, for understanding the regulation and metabolism of these informational molecules.

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