TRANSLATION OF NATURAL mRNA IN CELL-FREE SYSTEMS FROM A POLYAMINE-REQUIRING MUTANT OF ESCHERICHIA COLI

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SUMMARY - Cell-free extracts of polyamine-deficient and supplemented bacteria were used to study the translation of MS 2 bacteriophage RNA. The decreased synthetic activity observed with preparations of starved cells depends on a deficient initiation of polypeptide synthesis, as indicated by a lower capacity for the binding of formyl-methionyl-tRNA to ribosomes, induced by the trinucleotide AUG. The analysis of polypeptides formed showed that extracts from polyamine-depleted and supplemented cells are both able to synthesize complete protein chains.

Multiple functions have been proposed for polyamines, such as the regulation of nucleic acids and protein biosynthesis, the stabilization of membranes and organelles, and the participation in a variety of biochemical processes involved in cell growth and differentiation (1-4).

Many studies carried out with cell-free systems obtained from prokaryotic and eukaryotic cells have shown that polyamines can partially replace the Mg^{++} requirement and stimulate different steps of polypeptide synthesis (5-9). Furthermore recent reports have suggested that putrescine, spermidine and spermine are also able to enhance the fidelity of translation (10).

The isolation of bacterial mutants blocked in the biosynthesis of putrescin (11, 12) has provided a new and powerful tool for studying the mechanisms which are altered under conditions of polyamine deficiency. Using this approach several *in vivo* studies have indicated that polyamines may play a role in protein synthesis (13-15).

We have investigated the translation process and its machinery in cell-free systems derived from polyamine auxotrophs of *E. coli* cultivated in the absence and presence of putrescine. Our results have demonstrated that polypeptide synthesis directed by poly U or natural mRNA was markedly decreased in extract from polyamine-deficient cells (16, 17). This low activity is due to the pre-

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sence of defective 30S ribosomal subunits in the polyamine-starved bacteria (17). These experiments gave unambiguous support to the conclusion that polyamines play a direct role in translation, independently from their effect on RNA synthesis. Furthermore our results strongly suggested a new physiological function for polyamines, namely, the participation in the biogenesis or the assembly of ribosomal particles.

In the present paper we have examined in detail the translation of RNA from MS 2 bacteriophage using cell-free systems obtained from polyamine-starved or supplemented bacteria. Our aim was to find out whether the decreased activity for protein synthesis observed in extracts of polyamine-deficient cells is due to one or more of the following possibilities: a) a reduced capacity for the initiation of protein synthesis, b) a lower rate of polypeptide chain elongation and c) the synthesis of unfinished proteins.

MATERIALS AND METHODS

Putrescine dihydrochloride was purchased from Sigma. MS 2 phage RNA and the trinucleotide AUG were obtained from Miles; E. coli W stripped tRNA from General Biochemicals and [^{14}C]valine (280 Ci/mole) from New England Nuclear Corporation. [^{14}C]Formyl-methionyl-tRNA (55 cpm/pmole) was a generous gift of Dr. M. Krauskopf.

The polyamine auxotroph E. coli MA 261, kindly supplied by Dr. W. K. Maas has been used in all the experiments described in the present work. The growth media and culture conditions have been previously described (18).

For polyamine starvation of the cells, an exponentially growing culture containing putrescine was appropriately diluted in fresh medium lacking putrescine and allowed to grow overnight at 37°C. The resulting polyamine-depleted bacteria were then cultivated in the absence or presence of putrescine (MMO and MMOP media, respectively) and collected after slow cooling at the exponential phase of growth.

The S_{30} supernatant fractions and crude ribosomal preparations were obtained as reported previously (16).

The incubation mixture used to measure the polypeptide synthesis induced by MS 2 phage RNA was essentially as already described (17), omitting the preincubation of S_{30} extracts and the addition of S_{150} supernatant fractions.

In order to determine the val-tRNA formation the standard reaction mixture (without mRNA) was used in the presence or absence of E. coli W tRNA. The radioactivity of cold 5% trichloroacetic acid insoluble material was measured.

Analysis of the polypeptides formed during incubation with MS 2 phage RNA. After incubation for the periods indicated in each experiment, the standard reaction mixtures were treated with 0.3 ml of 0.3 N KOH for 15 min at 37°C in order to discharge aa-tRNAs and release the nascent peptide chains. One ml of cold 15% trichloroacetic acid was then added, the precipitate was collected by centrifugation and resuspended in 20 µl of a cracking solution containing 0.05 M Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 2 mM EDTA and 10% glycerol (19). The samples were heated for 2 min at 100°C and submitted to SDS-polyacrylamide gel electrophoresis according to Studier (19), using slabs 1.8 mm thick and 105 mm long. The gels were loaded with the different samples or appropriate standards (10 to 40 µg of protein per sample well) and the electrophoresis was carried out for 5 h at 20 mA. After staining with Coomassie brilliant blue R to locate the standard proteins and destaining in a solution of 7.5% acetic acid and 5% methanol, the gels were

sliced into 3 mm pieces and crushed in 1.2 ml H_2O. The resulting suspensions corresponding to each slice were counted with 20 ml Bray solution in a Packard scintillation counter. About 50-60% of the total radioactivity applied with each sample could be recovered from the gels.

AUG induced binding of fMet-tRNA to ribosomes. The reaction mixture contained in a total volume of 0.1 ml: 50 mM Tris-HCl, pH 7.2, 62 mM NH_4Cl, 0.16 mM GTP, 0.05 A_{260} units of AUG, 42 pmoles (2,300 cpm) of [^{14}C]formylmethionyl-tRNA, magnesium acetate and crude ribosomes as indicated in each case. The complete mixture was incubated for 20 min at 24°C. The reaction was stopped by adding 1 ml of cold 0.1 M Tris-HCl buffer, pH 7.4, containing 20 mM magnesium acetate and 50 mM KCl. After filtering immediately through Millipore filters (HAWP, 0.45 μm pore size) with gentle suction, the retained material was washed three times with 3 ml of the same buffer, dried and counted in a Packard scintillation counter.

RESULTS AND DISCUSSION

Previous experiments with S_{30} extracts obtained from E. coli MA 261 have indicated that polypeptide synthesis directed either by poly U or MS 2 phage RNA was 2-4 fold higher in cell-free systems derived from bacteria grown in the presence of putrescine than the corresponding activities of extracts prepared from polyamine-depleted cells (16, 17). We have also demonstrated that although the Mg^{++} concentrations required for maximal incorporation depend on the kind of mRNA used, the optimal cation levels did not change for cell-free systems derived either from polyamine starved or unstarved bacteria (16, 17).

In order to investigate further the translation of a natural mRNA we have followed the time course of [^{14}C]valine incorporation using S_{30} extracts programmed with MS 2 phage RNA. Fig. 1 shows that incorporation of [^{14}C]valine into total protein with cell-free systems from polyamine-supplemented bacteria was linear for about 20 min after a short lag of 2-3 min. On the other hand, with S_{30} extracts prepared from cells grown in the absence of putrescine, the lag period was much longer and the rate of protein synthesis was lower. This dissimilar behaviour is not due to differences in the formation of [^{14}C]val-tRNA, since the amino acid activation step had virtually the same kinetics in both systems (Fig. 2), with or without the addition of exogenous tRNA. Protein synthesis was also measured after a 5 min preincubation of the reaction mixtures in the absence of mRNA, in order to allow the charging of tRNA with radioactive amino acid. Under these conditions the kinetics of [^{14}C]valine incorporation was similar to that shown in Fig. 1, except for the disappearance of the lag period observed with S_{30} extracts from polyamine-supplemented bacteria. On the contrary, with systems from polyamine-starved cells a lag period of about 5 min was still clearly evident.

The results described above strongly suggest that the initiation of polypeptide synthesis in extracts from polyamine-depleted cells may be deficient.

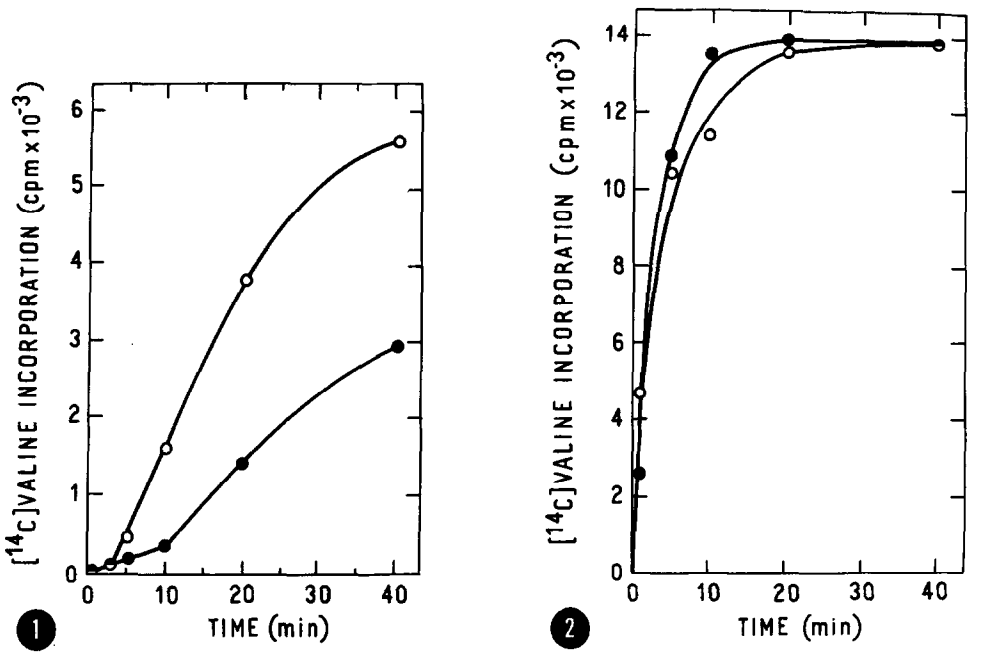

Fig. 1. Time course of valine incorporation induced by MS 2 phage RNA using S30 extracts from starved or unstarved cells.
Reaction mixtures as described in Materials and Methods containing 8.5 mM magnesium acetate. Symbols: ● and ○ correspond to extracts from bacteria grown in the absence and presence of putrescine, respectively.

Fig. 2. Kinetics of valine activation in S30 extracts from starved and unstarved bacteria.
Reaction mixtures as described in Materials and Methods using 8.5 mM magnesium acetate and 40 μg of stripped E. coli tRNA. Symbols as in Fig. 1.

In order to test this possibility we have measured the binding of fMet-tRNA to ribosomes of bacteria grown in the absence and presence of polyamine, induced by the trinucleotide AUG.

Table I shows that the formation of the complex fMet-tRNA-ribosomes-AUG was 3-5 fold higher when ribosomal particles from bacteria grown in the presence of putrescine were used. This result is not due to a different Mg^{++} concentration requirement, since the level of this cation at which maximal binding occurred was 6 mM with ribosomes from either polyamine-starved or unstarved bacteria. It should be pointed out that the reaction of fMet-tRNA binding did not require supernatant fraction because the crude ribosomal preparations contained sufficient amounts of initiation factors.

In order to compare the elongation process and the length of polypeptides

TABLE I

AUG-Dependent Binding of [^{14}C]Formyl-Methionyl-tRNA to Ribosomes from Starved or Unstarved Bacteria

The reaction mixtures described in Materials and Methods contained 2 A_{260} units of ribosomal suspension and the Mg^{++} concentration indicated in each experiment. The blank values obtained in the absence of AUG were subtracted in each case.

Ribosomes source	Mg^{++} concentration (mM)	[^{14}C]fMet-tRNA bound (pmoles/mg ribosomes)
Polyamine-depleted bacteria	6	7.6
	10	5.7
Polyamine-supplemented bacteria	6	38.6
	10	13.1

synthesized by cell-free systems from polyamine-depleted and supplemented cells we have analyzed the reaction products obtained after different periods of incubation by means of SDS-polyacrylamide gel electrophoresis. Fig. 3 gives the distribution of polypeptides formed when the reactions were carried out in the following way: The complete mixtures were incubated for 30 min in the presence of radioactive valine, and after the addition of a ten-fold excess of unlabeled amino acids, they were incubated again for 20 min. It is assumed that under these conditions the unfinished labeled polypeptides should be completed. The radioactive peaks obtained using cell-free extracts derived from polyamine-depleted bacteria had the same mobilities as those synthesized by extracts obtained from cells cultivated in the presence of putrescine. This fact is a good indication that in both cases polypeptides of identical or very similar similar sizes were formed. A large excess of a 14,000-16,000 daltons polypeptide (presumably MS 2 phage coat protein) could be detected, in accordance with many studies carried out on *in vivo* and *in vitro* translation of single-stranded RNA from bacteriophages (10, 20-22). Similar results were obtained after short incubations of reaction mixtures containing cell-free systems from starved or unstarved bacteria. The two minor peaks (about 65,000 and 35,000 daltons, respectively) which also appeared in the gels may correspond to the RNA synthetase or related polypeptides resulting from translation of partially degraded messenger molecules (22).

Although we cannot yet rule out that polypeptide chain growth rates may be different in both extracts, the electrophoretic patterns seem to indicate that the cell-free system prepared from polyamine-depleted cells had not a

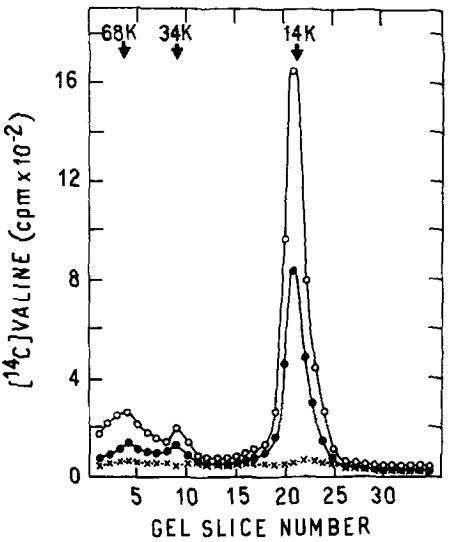

Fig. 3. SDS-polyacrylamide gel electrophoretic analysis of the translation products obtained with S30 extracts of polyamine-depleted or supplemented-bacteria, using MS 2 phage RNA as messenger. Reaction mixtures and all other details of the assays and analyses were described in Materials and Methods and in the text. Direction of migration was from left to right. Bromophenol-blue tracking dye migrated to slice 35. The arrows indicate the positions of proteins used as molecular weight standards: 68,000, 34,000 and 14,000 daltons, respectively. Symbols as in Fig. 1. A control reaction mixture without mRNA was also analyzed (×).

decreased fidelity of translation and was able to produce finished polypeptides.

The results described in the present paper led to the conclusion that the reduced protein synthesis induced by a natural mRNA in cell-extracts from polyamine-starved bacteria is mainly produced by some deficiency occurring at the level of polypeptide initiation. We have previously demonstrated that extracts from bacteria grown in the absence of polyamine contain a mixture of normal and defective 30S ribosomal subunits (17). The defective 30S particles could be unable to form active initiation complexes; therefore only the normal small subunits would bear nascent peptide chains. Since limiting amounts of ribosomes were used in the assays of protein synthetic activity, the above mentioned possibilities would mean a decreased number of growing polypeptide chains in cell-free systems from polyamine-depleted bacteria. This conclusion is in agreement with the results presented in Fig. 1 and Table I. Once the peptide chains are initiated, they probably grow until completion in a normal way.

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