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Ribonucleic Acid Regulation in Permeabilized Cells of *Escherichia coli* Capable of Ribonucleic Acid and Protein Synthesis[1]

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A cell permeabilization procedure is described that reduces viability less than 10% and does not significantly reduce the rates of ribonucleic acid and protein synthesis when appropriately supplemented. Permeabilization abolishes the normal stringent coupling of protein and ribonucleic acid synthesis.

To test proposed regulator compounds for their involvement in transcriptional regulation it is convenient to use permeabilized cells fully capable of normal rates of ribosomal ribonucleic acid (rRNA) and protein synthesis. Several techniques have been used to permeabilize bacterial cells (2, 3, 5, 6, 8; H. A. Raué and M. Cashel, J. Biol. Chem., in press). Smeaton and Elliott found that *Bacillus subtilis* became permeable to small molecules when exposed to cold-shock treatment (10). Lazzarini and Johnson (5) adapted cold-shock treatment to *Escherichia coli* but found that, when the cells became permeabilized to [^{14}C]adenosine 5'-triphosphate, RNA synthesis was reduced by about 70% of the control culture rate, and the ability for protein synthesis was essentially eliminated. Raué and Cashel (J. Biol. Chem., in press) also modified the cold-shock procedure and were successful in establishing protein synthesis in the permeabilized cells but simultaneously reduced permeability in the treated cells. This communication described a technique that produces highly permeable cells capable of [^{3}H]uridine 5'-triphosphate (UTP) uptake into RNA and [^{14}C]phenylalanine uptake into protein at nearly normal rates with only a 10% reduction in viability. This system is used to test the effect of added ppGpp on the rate of rRNA synthesis in *E. coli rel*$^{+}$ and *rel*$^{-}$ strains.

Data presented in Fig. 1A show the rate of [^{3}H]UTP incorporation into cells cold-shocked for 5 min in the presence of 1.0 M tris(hydroxymethyl)aminomethane Tris) (pH 7.6 at 25 C) and 10 mM magnesium. Magnesium (10 mM) is necessary to maintain protein synthetic capacity. For comparison, the rate of uptake of [^{3}H]UTP into nonshocked cells is shown; no [^{3}H]UTP incorporation is observed in nonshocked cells. The rate of [^{3}H]UTP uptake in cold-shocked cells is rapid initially but slowly decreases thereafter. Incorporation of [^{3}H]UTP continues for at least 3 h (data not shown). Also shown in Fig. 1A is the rate of [^{3}H]uridine uptake by shocked and nonshocked cells. The amount and rate of [^{3}H]uridine uptake by cold-shocked cells is greatly reduced from that by nonshocked cells, suggesting that intermediates in formation of UTP are leaking from the cells.

Viability after cold-shock of strain NF-58 was reduced less than 10%. The initial and final viable counts of the experiment, shown in Fig. 1, are 2.0×10^{8} cells/ml for the cold-shocked cells, but with nonshocked cells the viable count increased from 2×10^{8} cells/ml to 5×10^{8} cells/ml during the course of the incubation.

The data shown in Fig. 1B present a comparison of the rates of [^{14}C]phenylalanine incorporation by cold-shocked cells and nonshocked cells. Initially (0 to 20 min), the rate of protein synthesis in shocked and nonshocked cells is very similar; after 60 min, however, the rate of protein synthesis in cold-shocked cells has decreased to 50% of the rate of normal cells. This can be partly ascribed to the differential in viable count of shocked and nonshocked cells at the two time periods. From these data (Fig. 1) it is concluded that the rates of RNA and protein synthesis in cells of strain NF-58 cold-shocked in 1.0 M Tris, 10 mM $MgCl_2$ for 5 min compares favorably with rates found in nonshocked cells. However, in contrast to normal cells, these permeabilized cells continue RNA synthesis in the absence of required amino acids when protein synthesis is inhibited (data not shown).

De novo RNA and protein synthesis in cold-

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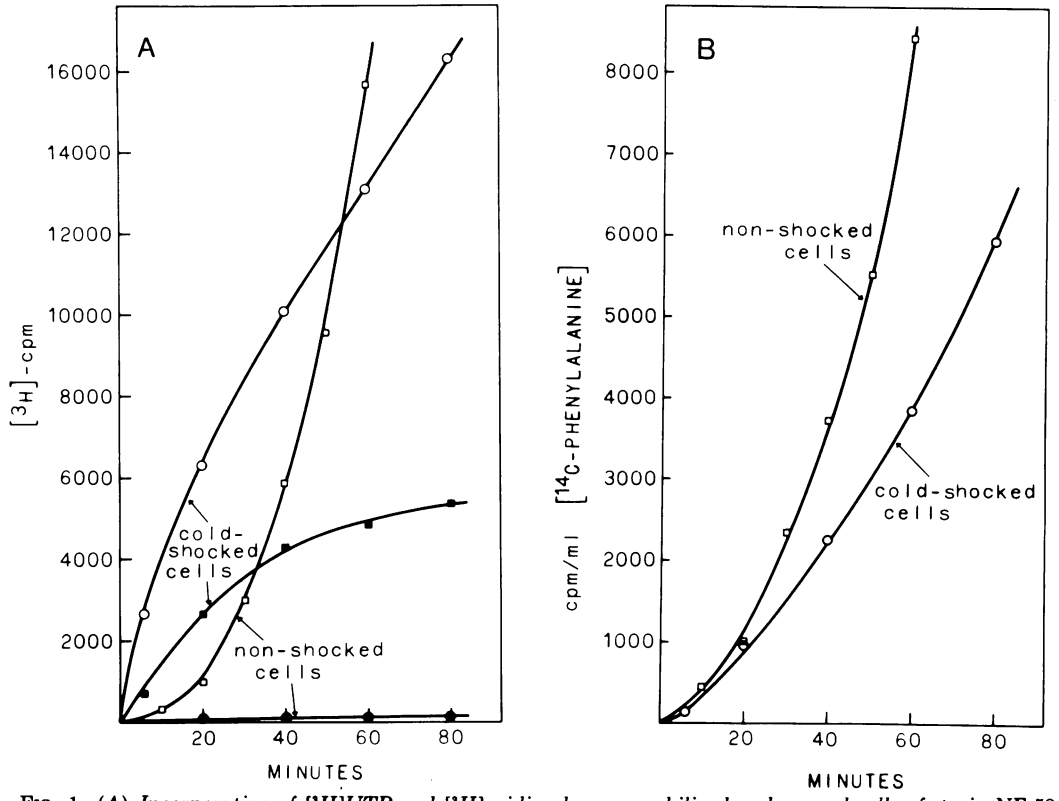

FIG. 1. *(A) Incorporation of [^3H]UTP and [^3H]uridine by permeabilized and normal cells of strain NF-58. Cells were permeabilized by slowly injecting a 20 times concentrated culture of cells (at 23 C in 0.05 M Tris, pH 7.4, 10 mM $MgCl_2$) into a rapidly stirring solution of 1 M Tris, 10 mM $MgCl_2$ at 5 C. The solution was stirred 5 min, and cells were collected by centrifugation. For measurement of RNA and protein synthesis cells were incubated with the appropriate radioactive compounds at a cell density of 4 × 10^8 per ml. The complete reaction mixture contained, per milliter: Tris-hydrochloride, pH 7.7, 50 μmol; $MgCl_2$, 10 μmol; $MnCl_2$, 0.2 μmol; KCl, 10 μmol; ATP, GTP, and CTP, 0.5 μmol; UTP, 0.10 μmol; phosphoenolpyruvate, 6 μmol; pyruvate kinase, 40 μg; β-mercaptoethanol, 0.3 μmol; 20 amino acids, 0.10 μmol, and 0.10 ml of permeabilized cells at 2 × 10^9 per ml. Reaction mixture were incubated at 30 C unless otherwise stated. Samples were removed (90 μliters) at intervals and combined with 3 ml of 5% trichloroacetic acid. For determination of [^{14}C]phenylalanine incorporation, samples were heated for 30 min at 90 C. The precipitates were collected on membrane filters, washed and dried, and radioactivity was counted in a model 3320 Packard scintillation spectrometer as previously described (1). Symbols: ○, [^3H]UTP into shocked cells; ■, [^3H]uridine into shocked cells; ●, [^3H]-UTP into normal cells; □, [^3H]uridine into normal cells. (B) Incorporation of [^{14}C]phenylalanine into normal and permeabilized cells. Conditions were identical to (A) except the presence of [^{14}C]phenylalanine (0.20 μCi/μmol) incorporated into hot trichloroacetic acid-precipitable material. Symbols: □, normal cells; ○, cold-shocked cells of strain NF-58.*

shocked cells is demonstrated by their ability to synthesize β-galactosidase. Table 1 presents data describing the rates of β-galactosidase synthesis in cold-shocked and nonshocked cells. The rate of β-galactosidase synthesis in cold-shocked cells is reduced to 15% of the expected rate observed in nonshocked cells and also the rate of β-galactosidase synthesis is not stimulated by 1 mM cyclic adenosine 5'-monophosphate. This finding is in contrast to Raué and Cashel who observed that cyclic adenosine 5'-monophosphate stimulated β-galactosidase synthesis eightfold in their permeabilized cells (J. Biol. Chem., in press). Rifampin (2 μg/ml) completely inhibits β-galactosidase synthesis in cold-shocked cells, but has no observable effect on normal cells. This would suggest that the cells have also become highly permeable to rifampin by cold-shocking.

The ability of normal and cold-shocked cells to synthesize ppGpp and guanine 5'-triphosphate (GTP) is shown in Table 2. Strain 10B6 *rel*$^+$, a severe temperature-sensitive mutant for valyl-transfer RNA synthetase, was used to

TABLE 1. *Rate of β-galactosidase synthesis*

Additions[a]	Units per min per ml[b]
Nonshocked cells (strain NF-58)	0.230
Nonshocked + rifampin (2 μg/ml)	0.230
Cold-shocked cells (strain NF-58)	0.035
Cold-shocked + rifampin (2 μg/ml)	0.000
+ cAMP (1 mM)	0.036

[a] Shocked cells of strain NF-58 (*met, arg, rel*$^+$, B_1) were combined with the complete reaction mixture at 2×10^8 per ml and induced with 1 mM isopropyl β-D-thiogalactopyranoside. Samples (100 μliters) were removed at timed intervals and put into 0.01 ml of toluene and 0.02 ml of 5% sarcosyl, shaken vigorously, and the level of enzyme was measured as previously described (9). Nonshocked cells were grown to 2×10^8 per ml in enriched media (1) with glycerol as a carbon source and induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Incubations were done at 30 C. Rates of enzyme activity were estimated from the initial slopes of the induction curves.

[b] A unit is defined as change in optical density at 420 nm × 100 by using cells at 10^8/ml.

completely stop protein synthesis. From the data presented in Table 3 it is seen that ppGpp synthesis is greatly stimulated in normal cells in the absence of protein synthesis (40 C). Also, in cold-shocked cells ppGpp synthesis is stimulated in the absence of protein synthesis (40 C) but to only 15% of the amount found in normal cells. The levels of GTP are quite similar in normal and cold-shocked cells. However, the true level of ppGpp synthesis in cold-shocked cells cannot be accurately determined since the $^{32}PO_4$ label being acquired in ppGpp is from newly synthesized [^{32}P] adenosine 5′-triphosphate and [^{32}P]GTP which are leaching from the cells. This conclusion is substantiated from experiment 2, given in Table 2. The level of labeled GTP and ppGpp present in the extracellular fluid was determined. As can be seen from the data presented, approximately 6% of the label ppGpp and 50% of GTP have leached from the cold-shocked cells during the 10-min incubation.

The relative amount of rRNA synthesized by permeabilized cells was estimated by RNA-deoxyribonucleic acid (DNA) hybridization (Table 3). Unlabeled competitor rRNA and labeled RNA synthesized by cold-shocked cells were hybridized to filter-bound denatured *E. coli* (strain AB 1206) DNA in a sequential manner (4). RNA synthesized by cold-shocked cells formed 30% ± 5% ribonuclease-resistant hybrids, in both *rel*$^+$ and *rel*$^-$ strains and was independent of the presence of charged valyl-transfer RNA. These results demonstrate that the differential rate of rRNA synthesis in cold-shocked cells is four to five times higher than that observed in vitro with purified components. Permeabilized cells retain amino acid-dependent protein synthesis but permeabilization abolishes the normal stringent coupling of protein and RNA synthesis. Thus, if ppGpp does control rRNA biosynthesis its effect must be mediated by some component which is lost or inactivated in permeabilized cells.

Addition of exogenous ppGpp to cold-shocked cells results in a reduction of total RNA synthesis. A 30% reduction in the amount of incorporation of [^{3}H]uridine 5′-triphosphate was seen as both 2 mM and 0.05 mM ppGpp (data not shown). The reduction in [^{3}H] uridine 5′-triphosphate uptake into RNA suggests that cold-shocked cells are permeable to ppGpp. However, permeability is limited as evidenced by

TABLE 2. *Synthesis of ppGpp in permeabilized cells of strain 10B6 rel*$^+$

Expt[a]	Cells tested	Growth temp (C)	Counts per min per 3×10^7 cells per 10 min	
			ppGpp	GTP
1	Nonshocked	30	780	16,300
	Nonshocked	40	16,000	8,200
	Cold-shocked	30	200	6,000
	Cold-shocked	40	2,070	8,200
2[b]	Cold-shocked	40	20,000	27,300
	Cold-shocked	40	1,300	13,000
	Supernatant liquid			

[a] The reaction mixture to measure accumulation of radioactive ppGpp contained, per ml: $MgCl_2$, 10 μmol; KCl, 10 μmol; phosphoenolpyruvate, 6 μmol; Tris-hydrochloride, pH 7.6, 10 μmol; β-mercaptoethanol, 0.3 μmol; $MnCl_2$, 0.20 μmol; 20 amino acids, 0.10 μmol; K_2HPO_4, pH 7.0, 0.10 μmol; $^{32}PO_4^{+3}$, 125 μCi, and cold-shocked cells, 4×10^8. Reaction mixtures were incubated at 30 C and samples were removed (50 μliters) and put into 50 μliters of 2N formic acid at timed intervals. The cells were centrifuged to remove cellular material, and a portion of the supernatant liquid was spotted on a thin-layer chromatography sheet of polyethylene-imine cellulose, plastic-backed, obtained from Brinkmann Inst. Co. Ascending elution was carried out in 1.5 M KH_2PO_4 (pH 3.4).

[b] In experiment 2, after 10 min of incubation of cold-shocked cells in the presence of 1 mCi of $^{32}PO_4$ per ml the cells were rapidly centrifuged (42 C) and the supernatant liquid was removed. The amount of ppGpp and GTP was then measured in the supernatant liquid. Zero-time counts per minute were subtracted from the values given.

TABLE 3. *Hybridization of RNA synthesized in cold-shocked cells to DNA*

Expt[a]	Strain	Synthesis temp (C)	Additions	Composition (% rRNA)
1	10B6 *rel*$^+$	30		31
		40		34
	10B6 *rel*$^-$	30		34
		40		34
2	10B6 *rel*$^+$	30		31
		30	0.05 mM ppGpp	28
3	NF-58 *rel*$^+$	30		33
		30	2 mM ppGpp	25

[a] ^{3}H-labeled RNA was prepared from the appropriate permeabilized cells by incubating 10 min at 30 or 40 C as indicated. The complete reaction mixture was used (Fig. 1) deleting UTP and including 50 μliters of 500 μCi of [^{3}H]UTP per ml at 26.9 Ci/mM specific activity. [^{3}H]RNA was then obtained by phenol extraction and contained 2,000 to 3,000 counts per min per μg of RNA. Filters (25 mm) from Schleicher and Schuell, Inc. (B-6) were loaded with 200 μg of denatured DNA isolated from *E. coli* strain AB-1206, diploid, for the region of 70 to 80 min on the *E. coli* genetic map. DNA was prepared by the procedure described by Miura (7). Radioactive RNA (0.5 μg) was added to 0.50 ml of 6XSSC in the presence of a DNA-loaded filter with and without 200 μg of ribosomal RNA prepared as previously described (4). The mixture was incubated 20 h at 66 C in a sealed vial, washed on both sides with 50 ml of 2XSSC, and incubated 1 h at 25 C in 2.0 ml of 2XSSC containing 40 μg of DNAase-free RNAase per ml. Both sides of the filters were again washed with 50 ml of 2XSSC, the filters were dried, and radioactivity was determined. The percentage of rRNA was then estimated from amount of counts per minute completed out by the presence of added cold rRNA. Seventy-five percent of the input counts per minute were hybridized in uncompeted controls.

the fact that only 6% of synthesized ppGpp leached from the permeabilized cells during a 10-min incubation (Table 2).

The effect of exogenous ppGpp on stable RNA synthesis in permeabilized cells was tested. From the data presented in Table 3, it can be seen that neither added 0.05 mM nor added 2 mM ppGpp to either strain 10B6, *rel*$^+$ or strain NF58, *rel*$^+$ has any appreciable effect on rRNA synthesis in cold-shocked cells. Repeated attempts to specifically inhibit rRNA synthesis in cold-shocked cells by added ppGpp were unsuccessful.

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