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Coupling of *lac* mRNA transcription to translation in *Escherichia coli* cell extracts

(*in vitro* protein synthesis/N gene mRNA/calcium ions/*lac* operon/coupling)

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ABSTRACT **In an extract containing all the components for *lac* gene expression except washed ribosomes, *lac* mRNA formation was increased 4- to 6-fold by the addition of washed ribosomes. The formation of β-galactosidase mRNA and enzyme showed very different dependency on added ribosomes. Enzyme was formed in proportion to the number of ribosomes added, whereas 10% of the standard level of ribosomes promoted full levels of transcription. Consistent with their action *in vivo*, chloramphenicol and erythromycin blocked the ribosome-dependent *lac* transcription. The same inhibition was seen with RNA pulse-labeled for 1 or 5 min, so that the effect was truly a blockage of formation rather than an increased hyperlability of nascent mRNA. The effect was specific for some RNA species, as it is *in vivo*: phage λ N gene transcription was increased rather than inhibited in the presence of chloramphenicol. Chloramphenicol did not stop *lac* transcription as a result of its blockage of formation of the regulatory nucleotide tetraphosphate (ppGpp), because addition of the nucleotide did not restore mRNA formation in chloramphenicol-treated extracts. Rather, the data are consistent with the ideas that one or a few ribosomes moving closely behind RNA polymerase can prevent its arrest and that, when ribosome movement is blocked by chloramphenicol, the RNA polymerase is exposed to factors that provoke premature RNA chain termination.**

When the translation of certain mRNA species in *Escherichia coli* is arrested prematurely, the portion of the mRNA distal to the blocked ribosomes becomes undetectable (1–11). The effect is comparable when ribosomes are stopped by a nonsense codon in a gene ("polarity") or by an antibiotic such as chloramphenicol ("coupling"). Studies with cell cultures suggested that the missing mRNA sequences are not formed or are formed and rapidly degraded.

Recently it has been found that the formation of distal sequences is restored, at least for the case of polarity, in cells in which "rho," a protein that stops transcription, has been modified by mutation. The strong inference is made that polarity results primarily from the arrest of RNA polymerase (12). However, the way in which ribosome arrest leads to polymerase arrest, and any similar polymerase arrest in the case of coupling, has not been analyzed in detail.

To analyze the mechanisms of polarity and coupling, an *in vitro* system seems indispensible. Use of the DNA-directed system of Zubay (13) has helped in the analysis of many mechanisms of gene regulation, but little or no polarity or coupling has been observed in many studies (14, 15). Here we report conditions for the consistent observation of coupling of *lac* mRNA transcription to translation in a DNA-directed system (16). A partial analysis of the phenomenon, with the elimination of some models for coupling, has been achieved.

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METHODS

Preparation of Extracts and DNA. S-30 extracts were made from *E. coli* strains 514 (13) and 514K2 (16). Template DNA was prepared from lysogenic *E. coli* strain RV (λh80d *lac*p^s-c1857t68, λh80c1857St68) (17) and *E. coli* strain CSH44 (λh80d*lac* c1857St68, λh80c1857St68) (18). The DNA used as a hybridization probe was prepared from phage produced in strains W3110 (λc1857S7) (19) and M7133 (λp*lac*5c1857) (20).

Two different probes were used to detect *lac*-specific mRNA: intact λp*lac*5 DNA, and a restriction fragment isolated from that DNA. (See ref. 21 for the appropriate restriction map.) The *lac*-containing restriction fragment was prepared by digesting λp*lac*5 with restriction enzyme *Hin*dIII (22). The corresponding restriction fragment of λ, which includes the region in which *lac*DNA is inserted in λp*lac*5, was prepared by digesting λ DNA with *Eco*RI. The fragments were heated, separated by electrophoresis (23), eluted by electrophoresis into a dialysis bag, denatured, and then deposited on filters.

β-Galactosidase and mRNA Formation and Assay. Fractionation of the crude S-30 extract [into soluble proteins (S-100), ribosomal wash, and ribosomes] and enzyme formation have been described in detail (16). Reaction mixtures contained either 6.5 mg of S-30 protein or 5.2 mg of S-100, 0.26 mg of ribosomal wash, and 2.2 mg of ribosomes per ml. To label RNA, 200-μl reaction mixtures were first incubated at 37° for 3–5 min. Freshly prepared chloramphenicol in 10 mM Tris·acetate, pH 8.2/10 mM magnesium acetate (final concentration as in legends) was then added to some samples. After 16–18 min, 100 μCi of [5,6-^{3}H]UTP (lyophilized and resuspended at 10 mCi/ml in 10 mM magnesium acetate) was added for 4 min. DNase was added (final concentration, 5 μg/ml), and RNA was phenol-extracted in buffer [0.2 M Tris·acetate, pH 5.7/10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid/0.5% sodium dodecyl sulfate]. RNA was then extracted once with chloroform, precipitated twice with ethanol, and stored at −20° in 0.1 × standard saline-citrate (SSC)/0.2% sodium dodecyl sulfate until assayed for its hybridization to DNA.

Assay of Specific mRNA. [^{3}H]*lac* mRNA was detected by hybridization to denatured DNA immobilized on a 0.45 μm pore size nitrocellulose filter. DNA was denatured in 0.1 M NaOH for 20 min at room temperature, neutralized with 0.1 M HCl, and brought to 6 × SSC with 20 × SSC. A solution containing either 25 μg of intact DNA or 7 μg of a restriction fragment was filtered through a 27-mm membrane filter at low suction, washed with 50–100 ml of 6 × SSC, air-dried for at least 3 hr, and incubated at 85° for 1 hr 45 min. Ten small filters

Abbreviations: S-100, soluble proteins of S-30 extract; SSC, standard saline-citrate (0.15 M sodium chloride/0.015 M sodium citrate); cAMP, cyclic AMP.

Table 1. Chloramphenicol inhibits *lac* mRNA synthesis *in vitro*

Extract and conditions	cpm hybridized to different probes					
	λ*plac*5	λ	*lac*	III-*plac*5	RI·λ	*lac*
(A) 514 S-30						
−CHL	1819	834	985	1226	23	1203
+CHL	675	609	66	168	37	131
(B) 514K2 S-230						
+cAMP, −CHL	1643	520	1123			
+cAMP, + CHL	406	271	135			
−cAMP, −CHL	805	445	360			
−cAMP, +CHL	301	371	−70			

The equivalent of RNA extracted from a reaction mixture of 35 μl or 25 μl was used to hybridize to intact DNA or restriction fragments, respectively. The *lac* mRNA is the difference in counts hybridized to the "*lac*" probes and the homologous λ probes. (A) λh80d*lac* template DNA; (B) λh80d*lac*p^s template. Initial input cpm for hybridization from an *in vitro* reaction varied from 10,000–20,000. Prehybridization to λ DNA removed 70–80% of the total λ-specific transcripts. *lac* mRNA represents 4–5% of the total RNA made in the absence of chloramphenicol. CHL, chloramphenicol (final concentration 100 μg/ml); cAMP, cyclic AMP. III-*plac*5, *Hin*dIII restriction fragment of λ*plac*5 DNA. RI·λ, *Eco*RI restriction fragment of λ DNA, corresponding to III-*plac*5.

were punched out of each 27-mm filter. Hybridization assays were incubated at 66° for 20 h in 6 × SSC/0.2% sodium dodecyl sulfate. Filters were then removed from the mixture, washed with 2 × SSC, treated with pancreatic RNase (20 μg/ml for 20 min at 37°), washed again with 2 × SSC, dried, and assayed for radioactivity in a toluene-based scintillator. RNA homologous to the λ genome was removed from each RNA sample by prehybridization to excess λ DNA (four small filters each bearing 10 μg of λ DNA). The hybridization fluid was then divided into two portions. To one was added a filter bearing λ*plac*5 DNA (or a restriction fragment) and to the second, a filter containing λ DNA (or a restriction fragment) for the specific assay of *lac* mRNA.

N gene-specific mRNA was assayed as the difference in the hybridization to 1 μg of l-strands of transducing phages λ*bio*10 and λ*bio*3h-1 (24). The appropriate hybridization protocol was as described (25). The separated strands were a generous gift of H. Lozeron.

Reagents. The chelator and chloramphenicol were obtained from Sigma Chemical Co. Marine Colloids agarose was used. [^{3}H]UTP (1 mCi/14 μg) was from New England Nuclear. DNase was electrophoretically pure from Worthington; restriction enzymes *Eco*RI and *Hin*dIII were obtained from Bethesda Research Laboartories; ppGpp was purchased from P-L Biochemicals, Inc.

RESULTS

Coupling Can Be Observed in Subcellular Systems. *lac* mRNA, assessed by DNA·RNA hybridization, decreases to 10% or less in induced cultures treated with chloramphenicol (7). In extracts of strains 514 and 514K2 programmed with λh80d*lac* DNA, an equivalent decrease in the synthesis of *lac* mRNA was seen in the presence of chloramphenicol (Tables 1 and 2). In trials with four independent extracts, the inhibition varied from 77–93%. Comparable inhibition was seen in systems reconstituted from ribosomes and soluble proteins, with pulse-labeling durations of 1–5 min (Table 2 and Fig. 1) and also with erythromycin instead of chloramphenicol.

When the template DNA contained a p^s mutation in the *lac* promoter (17), *lac* mRNA and enzyme synthesis became partially independent of cyclic AMP (cAMP); but in that case, both cAMP-independent and cAMP-dependent synthesis were sensitive to chloramphenicol (Table 1). Thus, the control by coupling is separate from the positive control by cAMP.

Table 2. Transcription of the λ N gene is resistant to inhibition by chloramphenicol

Extracts and conditions	cpm hybridized to different probes			
	*bio*10	*bio*3h-1	N	*lac*
(A) 514K2 S-30				
−CHL	151	134	17	1123
+CHL	174	52	122	135
(B) 514K2 "S-100"				
−Ribs, −CHL	173	83	90	186
−Ribs, +CHL	204	103	101	185
+Ribs, −CHL	512	349	163	1074
+Ribs, + CHL	519	74	445	249

The above experiments were done in similar but separate reactions. For details see Table 1. The counts in (A) are from the trial in Table 1, experiment (B). The N mRNA is the difference in radioactivity hybridized to λ*bio*10 and λ*bio*3h-1 (see *Materials and Methods*). 2.3 μl of [^{3}H]RNA was used to assay for N mRNA and 35 μl of RNA was used for *lac* mRNA. "S-100": reconstituted fraction containing S-100 and ribosomal wash. Ribs, ribosomes; other abbreviations as in Table 1.

In these initial trials, *lac* mRNA was determined as the difference in hybridization between λ*plac*5 and λ DNAs. To decrease the background of λ hybridization (as in Table 1, lines 2, 4, and 6), hybridization trials were also run with appropriate restriction fragments. Nearly all the remaining assays (Figs. 1 and 2, and tables as indicated) were carried out with restriction fragment probes.

Ca^{2+}, which is not required for translation (16), was specifically included in some reactions and consistently decreased the effect of chloramphenicol on transcription from 10-fold down to 0.2- to 2-fold. For example, in a trial in which *lac*-specific cpm were decreased from 781 to 53 by chloramphenicol in the absence of Ca^{2+}, the decrease was only from 733 to 600 cpm in parallel reactions in the presence of Ca^{2+}.

Coupling *In Vitro* Is Promoter-Specific. The template λh80d*lac* formed much more λ-specific RNA than *lac* mRNA. Much of this RNA consisted of sequences of "immediate early" mRNA that are formed at an undiminished or even accelerated rate in cells treated with chloramphenicol (9, 10). The chloramphenicol resistance can be seen *in vitro* by assaying for λ N gene mRNA as the difference between hybridization to two appropriate DNA strands. Table 2 includes sample results from an experiment with an S-30 extract and another trial with fractionated ribosomes and S-100 soluble proteins. As in whole cells, *lac* mRNA production was reduced to 1/5 to 1/11 in the presence of chloramphenicol, whereas N gene mRNA was increased up to 3-fold.

Transcription Is Dependent on Functioning Ribosomes. Table 2 shows that washed ribosomes apparently increase the rate of *lac* mRNA formation. Fig. 1 shows additional evidence that (*i*) in a mixture of soluble proteins and ribosome wash, RNA polymerase transcribes *lac* DNA at the same low rate in the presence or absence of chloramphenicol; (*ii*) washed ribosomes increase the level of transcription (4- to 6-fold in various trials); and (*iii*) the positive effect of functioning ribosomes is blocked by chloramphenicol.

Differential Dose-Response of β-Galactosidase mRNA and Protein Formation to Added Ribosomes. Transcription and translation of *lac* mRNA showed different responses to increasing amounts of ribosomes (Fig. 2). The yield of enzyme

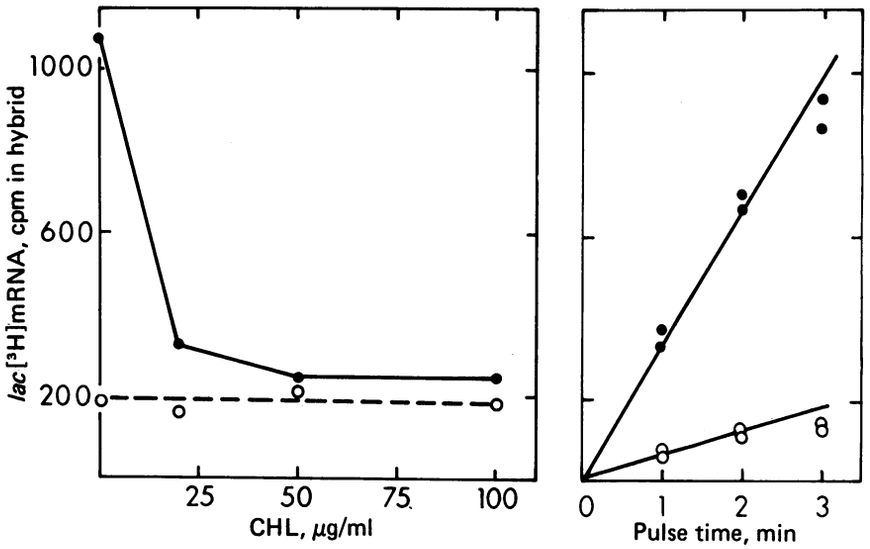

FIG. 1. Pulse-labeling of *lac* mRNA is blocked by chloramphenicol (CHL) by a reaction mediated through ribosomes. The reconstituted system consisting of S-100 and ribosomal wash, with or without ribosomes, was used for this experiment. *lac* mRNA was assayed by hybridization to restriction fragments after prehybridization to λDNA. The data are corrected for the background radioactivity (10–60 cpm) hybridized to the RI restriction fragment of λDNA. (*Left*) *lac* mRNA was pulse-labeled for 5 min in presence of different levels of chloramphenicol, with (●) or without (○) ribosomes. (*Right*) Pulse-labeling was for shorter times in the presence of ribosomes, with (○) or without (●) chloramphenicol.

was proportional to the amount of ribosomes present (16), but *lac* mRNA production was already maximal with the addition of less than 10% of the amount of ribosomes required to give maximum enzyme production. In controls, ribosome-dependent mRNA and enzyme production were again both completely sensitive to chloramphenicol.

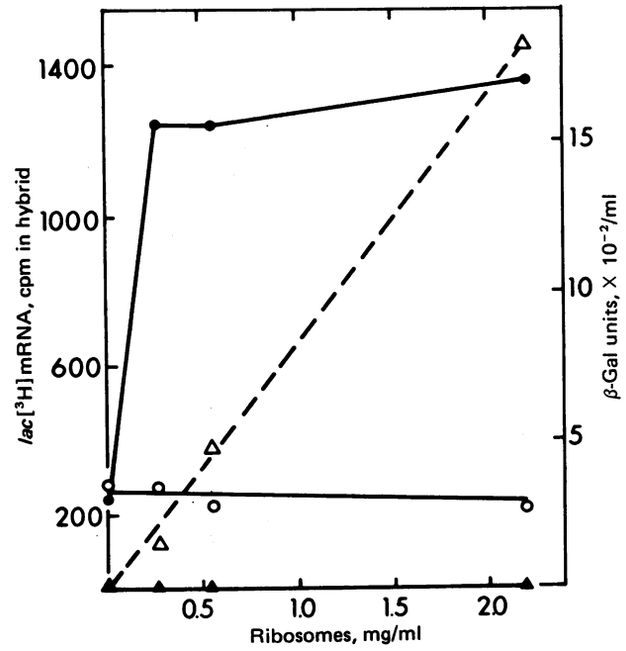

FIG. 2. Dose-response of β-galactosidase synthesis (triangles) and *lac* mRNA synthesis (circles) to ribosomes in the presence (○, ▲) and absence (●, △) of chloramphenicol (100 μg/ml). The reconstituted system consisted of S-100, ribosomal wash, and different concentrations of ribosomes. β-Galactosidase was measured in separate reactions after 60-min incubations at 37°. *lac* mRNA was assayed by hybridization to restriction fragments prepared from λp*lac*5 and λ phage DNA. The data are corrected for the background radioactivity (17–43 cpm) which hybridized to the RI restriction fragment of λDNA.

Ribosome-Dependent *lac* mRNA Synthesis Is Not Mediated through ppGpp. One possible mechanism for the promotion of *lac* formation by ribosomes involves the tetraphosphorylated derivative of guanine, ppGpp (26), which is formed on ribosomes, stimulates *lac* (27) and *his* specific mRNA (28) formation *in vitro*, and decreases sharply in chloramphenicol-treated cells (29). If ppGpp were a positive effector that was progressively destroyed in presence of chloramphenicol, then addition of ppGpp to chloramphenicol-treated extracts should alleviate coupling.

The optimal level of ppGpp (0.2 mM) stimulated *lac* gene expression 1.7- to 2-fold in these extracts, in agreement with previous reports (27). There was, however, a similar decrease in *lac*-specific mRNA (10-to 20-fold) when chloramphenicol was added in the presence or absence of ppGpp (from 1031 to 45 cpm hybridized *lac* mRNA in the absence of added ppGpp; from 1714 to 169 in its presence). Therefore, ppGpp alone does not seem to counteract the effect of chloramphenicol.

DISCUSSION

In extracts containing all the factors for gene expression, *lac* mRNA production is dependent on functioning ribosomes (Table 2 and Figs. 1 and 2). In the presence of chloramphenicol or erythromycin, which block ribosome movement, the decrease in *lac* mRNA is equivalent to that observed with whole cells (7) and, as *in vivo*, species like λ N mRNA are still made (Table 2).

Because the levels of *lac* mRNA pulse-labeled for 1 or 5 min were decreased to a comparable extent (Fig. 1), chloramphenicol is inferred to inhibit transcription (3) rather than to increase mRNA decay. This effect on transcription is independent of the positive control by cAMP (Table 1); and the number of ribosomes that support only 10% of the maximal rate of enzyme synthesis are sufficient to promote full levels of transcription (Fig. 2).

Gros and Crepin (30) have shown evidence that certain ribosomal proteins, 30S ribosomes, and 70S initiation complexes exert a considerable general stimulation of the action of RNA polymerase on λ DNA. However, such an action must be dis-

tinct from the process studied here because the λ RNA formation was stimulated in the absence of protein synthesis and still occurred in the presence of chloramphenicol (Table 2).

Ribosomes might also make some product that promotes transcription; its formation would be blocked in presence of chloramphenicol. An example of such a product is the tetraphosphorylated guanine nucleotide, ppGpp. However, at least that particular product of ribosomes does not counteract chloramphenicol action.

More in keeping with recent results is the notion that ribosomes antagonize polymerase arrest. In the case of both polarity (12, 31) and of coupling (32), a ribosome moving close behind RNA polymerase might physically prevent the binding to polymerase of the rho factor (12, 31) or other proteins that would otherwise provoke RNA chain termination. In that case, one or a few ribosomes could give maximal transcription rates, as observed (Fig. 2), even without high levels of protein formation.

Earlier attempts to demonstrate coupling or polarity *in vitro* have shown less pronounced effects (33). One reason for this may have been the traditional inclusion in reaction mixtures of Ca^{2+}, which alleviate coupling. With the availability of a system that tests for coupling *in vitro*, fractionation of soluble proteins should permit determination of the site of action of Ca^{2+} in relation to their other effects *in vitro* (16) and further definition of the mechanism of coupling.

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