hydr. Res. 29, 397-403.
Ogawa, K., Watanabe, T., Tsurugi, J., and Ono, S. (1972), *Carbohydr. Res. 23,* 399-405.
Rees, D. A. (1968), *Adv. Carbohydr. Chem. Biochem. 24,* 267-332.
Rees, D. A. (1972), *Biochem. J. 126,* 257-273.
Rees, D. A., and Scott, W. E. (1969), *Chem. Commun.,* 1037-1038.
Saito, H., Misaki, A., and Harada, T. (1968), *Agric Biol. Chem. 32,* 1261-1269.
Saitô, H., Ohki, T., Yoshioka, Y., and Fukuoka, F. (1976), *FEBS Lett. 68,* 15-18.
Saitô, H., Sasaki, T., and Yoshioka, Y. (1977), Abstracts of the VIIIth International Symposium on Carbohydrate Chemistry, Kyoto, Japan, 1976, p 121; manuscript in preparation.
Saitô, H., and Smith, I. C. P. (1973), *Arch. Biochem. Biophys. 158,* 154-163.
Sathyanarayana, B. K., and Rao, V. S. R. (1971), *Biopolymers 10,* 1605-1615.
Schaefer, J. (1973), *Macromolecules 6,* 882-889.
Smith, I. C. P., Jennings, H. J., and Deslaurier, R. (1975), *Acc. Chem. Res. 8,* 306-313.
Sundaralingam, M. (1968), *Biopolymers 6,* 189-213.
Takeda, H., Yasuoka, N., and Kasai, N. (1977), *Carbohydr. Res.* (in press).
Yokota, K., Abe, A., Hosaka, S., Sakai, I., and Saitô, H. (1977), Abstract of the 15th Nuclear Magnetic Resonance Symposium, Fukuoka, 1975, pp 228-231; manuscript in preparation.

Escherichia coli DNA-Directed β-Galactosidase Synthesis in Presence and Absence of Ca^{2+}†

Kenneth A. Jacobs and David Schlessinger*

ABSTRACT: DNA-dependent synthesis of β-galactosidase was optimized in extracts made from cells lysed by a standard French pressure cell. Extracts made at 3200 psi synthesized up to 25-fold more β-galactosidase than extracts made at 7500 psi. β-Galactosidase synthesis was cyclic 3′,5′ AMP dependent, as expected, and in optimal conditions transcription and translation proceeded at 8.6 nucleotides and 2.7 amino acids per s, respectively. The high pressure extracts were stimulated 3- to 5-fold by Ca^{2+}, especially at low Mg^{2+} concentrations. In contrast, extracts prepared at low pressure were inhibited as much as 50-fold by Ca^{2+} ions. The inhibition by Ca^{2+} was analyzed further. Addition of kasugamycin, an antibiotic that acts on ribosomes, to reactions containing Ca^{2+} stimulated β-galactosidase synthesis to nearly control levels. Extracts from a kasugamycin resistant mutant were neither inhibited by Ca^{2+} nor stimulated by the addition of kasugamycin to in vitro reactions containing Ca^{2+}. The change in the mutant was ascribed to the ribosomes by testing combinations of soluble proteins, ribosome wash, and ribosomes from parental and mutant strains. These results suggest that Ca^{2+} ions inhibit translation by ribosomes, very likely at an initiation step; and that they enhance enzyme synthesis only in conditions where translation is inefficient (high-pressure extracts at low concentrations of Mg^{2+}, for example). This latter effect is probably a consequence of increased RNA stability in the presence of Ca^{2+} (Cremer, K., and Schlessinger, D. (1974), *J. Biol. Chem. 249,* 4730).

The DNA-coupled system of Zubay has provided a powerful way to reproduce in vitro many features of gene expression (Zubay, 1973). Major components required for DNA-directed synthesis of enzymes have been fractionated to a high state of purity (Kung et al., 1973, 1975a), and the systems have been used to analyze the regulation of transcription and translation of the *lac* operon (de Crombrugghe et al., 1971a; Kung et al., 1975a,b: Zubay et al., 1970), *gal* operon (Parks et al., 1971; Wetekam et al., 1972; Schumacher and Ehring, 1975), *ara* operon (Wilcox et al., 1974), *trp* operon (Pouwels and Van Rotterdam, 1972, 1975; Zalkin et al., 1974), the genes coding for the β and β′ subunits of *E. coli* RNA polymerase (Austin, 1974), the ribosomal proteins of *E. coli* (Kaltschmidt et al., 1974), and the N gene of bacteriophage λ (Dottin and Pearson, 1973; Greenblatt, 1973). Of singular importance to all these studies is an understanding of the components of the in vitro system which affect protein synthesis. For example, three features of the results have varied widely in different reports: the absolute levels of enzyme formed (Austin, 1974; Kung et al., 1973; Parks et al., 1971; Wetekam et al., 1971; Wilcox et al., 1974; Zubay et al., 1970); the dependence or lack of dependence on Ca^{2+} for enzyme synthesis (de Crombrugghe et al., 1970; Kung et al., 1973; Parks et al., 1971; Wetekam et al., 1972; Zubay et al., 1970); and the levels of Mg^{2+} required for efficient protein synthesis (de Crombrugghe et al., 1970; Parks et al., 1971; Pouwels and Van Rotterdam, 1972, 1975; Wetekam et al., 1972; Zalkin et al., 1974; Zubay et al., 1970).

We report here that these three parameters are interrelated and give conditions for their optimization for the case of β-galactosidase synthesis directed by DNA. This has required a further analysis of the effects of Ca^{2+}. The results indicate that, in addition to its inhibitory effect on nucleases (Cremer and Schlessinger, 1974), Ca^{2+} is an effective inhibitor of translation, probably at the initiation step. This inhibition is overcome by modifications of the ribosome which are induced either by certain antibiotics or by a specific ribosomal mutation.

† From the Department of Microbiology and Immunology, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110. *Received September 10, 1976.* Supported by National Science Foundation Grant BMS74-11779.

Experimental Procedure

Bacterial Strains. E. coli K12 strain 514 (Zubay et al., 1970) and two kasugamycin resistant derivatives, 514K1 and 514K2, were used in these studies. Strains 514K1 and 514K2 were obtained after two independent mutagenic treatments with nitrosoguanidine to 50% survival as described by Miller (1972); they were selected as colonies resistant to 300 μg/mL of kasugamycin on Luria broth plates (Miller, 1972). Growth of the parental strain was totally inhibited by 100 μg/mL of the antibiotic.

Bacteriophage Strains and DNA Preparation. Bacteriophage were prepared by heat induction of both *E. coli* RV (λh80dlacp^{s}c1857St68,λh80c1857St68) (de Crombrugghe et al., 1971b; Nakanishi et al., 1975) (a gift of Dr. N. Brot) and *E. coli* strain CSH444 (λh80dlac$^+$c1857St68, λh80c1857St68) (Miller, 1972; a gift of Dr. D. Zipser), growing in 2YT medium (Miller, 1972). The phage were purified by banding to equilibrium in a CsCl density gradient and selectively removing the denser band containing the defective transducing phage. The purified phage were then diluted into a solution 0.1 M in NaCl and 0.1 M in sodium phosphate buffer at pH 7.1. Template DNA was extracted as described in Zubay et al. (1970). The final solution was extracted once with an equal volume of chloroform and then dialyzed for 24 h against 50 to 100 volumes of 20 mM Tris-acetate, 5 mM EDTA[1] (pH 7.8), with two changes of buffer, and for 48 h more against 50 to 100 volumes of 10 mM Tris-acetate (pH 8.2), with three changes of buffer. The final solution was stored at 4 °C over chloroform.

Growth of Cells and preparation of S-30 Extract. Extracts were prepared as described previously (Cremer and Schlessinger, 1974) except for the modifications given below and in Results. Cells were grown in the medium described by Zubay et al. (1970), except for the omission of thiamine and the addition of $MgSO_4$ to a final concentration of 1 mM. The cells were poured onto ice, collected by continuous flow centrifugation at 4 °C, and stored unwashed at −70 °C until they were extracted. The lysate (S-30) was prepared as described by Zubay et al. (1970), from thawed, washed cells, with the modifications suggested by Wetekam et al. (1971) and the additional modifications given in the text. Preincubation of the S-30 extract was according to Nirenberg (1963) as modified by Wetekam et al. (1971). A buffer (buffer A), containing 10 mM Tris-acetate, pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, and 0.2 mM dithiothreitol, was used throughout the preparation. After dialysis against buffer A (three changes for a total of 6 h), aliquots of the S-30 extract were frozen and stored in liquid nitrogen. Extracts stored in this way were stable for at least a year.

Preparation of S-100, Ribosomal Wash, and Washed Ribosomes. After preincubation the S-30 was centrifuged for 90 min at 175 000*g* in the Beckman Spinco rotor 65. The top 80% of the supernatant layer (S-100) was removed, dialyzed against buffer A for 1 h, concentrated by dialysis against 20% poly(ethylene glycol) in buffer A, and dialyzed again against two changes of buffer A. The ribosomal wash was prepared by resuspending the remaining supernatant and ribosomes in their original volume in buffer A containing 1 M ammonium chloride and shaking gently for 1 h at 4 °C. After a low-speed centrifugation (10 000*g* for 10 min in a Sorval centrifuge) to remove aggregates, the ribosomes were pelleted again. The top 80% of the supernatant layer (ribosomal wash; RW in Table I) was removed and dialyzed as described for the S-100. Cloudy material which had formed a loose layer above the ribosomes was removed by gentle rinses with buffer A. The ribosomes were then resuspended in one-half their original volume and dialyzed against buffer A. The dialyzed S-100, ribosomal wash, and washed ribosomes were stored in liquid nitrogen.

TABLE I: Kasugamycin Resistance of Strain 514K2 Is Associated with the Ribosome.[a]

S-100 + RW	RIBS	Experiment 1[b] −Ksg	Experiment 1[b] +Ksg	Experiment 1[b] % Inhibition	Experiment 2[b] −Ksg	Experiment 2[b] +Ksg	Experiment 2[b] % Inhibition
S	S	1790	1030	36	2930	2090	39
		1680	1200				
S	R	2530	2360				
		2440	2170	9	4000	3660	8
R	R	2270[c]	1960[c]	14	5260	4650	12
R	S	2920	1000				
		2530	1580	53	4860	2110	57

[a] The in vitro system was reconstituted from supernatant (S-100), ribosomal wash (RW), and washed ribosomes (RIBS) from strains sensitive (S) and resistant (R) to kasugamycin, strains 514 and 514K2, respectively. After 8 min at 37 °C kasugamycin was added to some reactions (final concentration 0.5 mM); an equal volume of water was added to control reactions. Incubation was continued for 52 min at which time the amount of β-galactosidase synthesized was assayed. [b] Units/mL of β-galactosidase synthesized in reactions with and without kasugamycin (Ksg). The samples in experiment 1 were incubated at 13 mM Mg^{2+}, in experiment 2 at 14 mM. The percent of enzyme synthesis inhibited by kasugamycin is given. [c] Duplicate samples were lost.

Components of in Vitro Reactions and Order of Addition. The incubation conditions for the Zubay coupled system are the same as described earlier (Cremer and Schlessinger, 1974; Zubay et al., 1970), except for the omission of various coenzymes. Comparable changes have been made by other investigators (Kung et al., 1973; Pouwels and Van Rotterdam, 1972). The complete system (100 μL) contained the following ingredients added in this order: 2.1 μmol of phosphoenolpyruvate; 4.4 μmol of Tris-acetate (pH 8.2); 0.14 μmol of dithiothreitol; 5.5 μmol of potassium acetate; 2.7 μmol of ammonium acetate; 0.02 μmol each of 20 amino acids; 0.22 μmol of ATP; 0.05 μmol of GTP, CTP, and UTP; 10 μg of *E. coli* tRNA stripped of amino acids; 3.0 μg of folinic acid; 0.025 μmol of isopropyl β-D-thiogalactopyranoside; 0.057 μmol of cAMP; 2.0 mg of poly(ethylene glycol) 6000; 5.0 μg of template DNA; 0.55 μmol of calcium acetate as indicated (except for the experiments of Figure 1, in which 0.60 μmol was used in the reaction); 1.6–1.8 μmol of magnesium acetate; and 0.65 mg of S-30 protein or 0.36 mg of S-100, 0.12 mg of ribosomal wash, and 0.22 mg of ribosomes. (Each of these last three components was necessary for β-galactosidase synthesis in a reconstituted system as in Table I; data not given.) The necessary amount of phosphoenolpyruvate was weighed before each reaction. The amounts of Tris-acetate and potassium acetate are accurate to within 20% since the S-30, S-100, ribosomal wash, and ribosomes were dialyzed against a buffer containing these compounds (buffer A). The amount of magnesium acetate indicates a range of concentrations because S-30 preparations vary in the optimum for enzyme synthesis (see Results). The amount of enzyme synthesized varied as much as threefold, even for trials with portions of the same

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; ksg, kasugamycin; psi, pounds per square inch; rif, rifampicin.

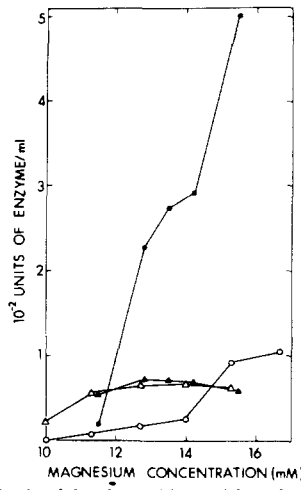

FIGURE 1: Synthesis of β-galactosidase with and without added Ca^{2+} at different concentrations of Mg^{2+}; comparison of extracts made at low and high pressure. Extracts were made from cells disrupted by passage through a French pressure apparatus at either low pressure (3200 psi) or high pressure (7500 psi). Other details of the extract preparation are given in Experimental Procedure. Portions of each extract were then used for in vitro synthesis reactions, as described in Experimental procedure, with or without Ca^{2+} at different concentrations of Mg^{2+}. Incubations were terminated after 60 min at 37 °C, and β-galactosidase was then assayed as described in Experimental Procedure. (●—●) Low-pressure extracts, no Ca^{2+}; (▲—▲) low-pressure extract, 6.0 mM Ca^{2+}; (○—○) high-pressure extract, no Ca^{2+}; (△-△) high-pressure extract, 6.0 mM Ca^{2+}.

extract tested over a period of time. Standardized positive controls were run in each experiment.

β-Galactosidase Assay. The assay used has been described elsewhere (Zubay et al., 1970). To stop protein synthesis portions of the in vitro reaction were pipetted into separate tubes containing chloramphenicol (100 μg per mL final concentration). After the completion of the reaction, β-galactosidase assay buffer containing 100 μg/mL of chloramphenicol was added to each tube, which was then incubated at 30 °C until sufficient yellow color developed. Each assay was terminated by addition of 1 drop of glacial acetic acid, chilled, and spun at low speed to pellet the protein–DNA precipitate. An equal volume of 1 M sodium carbonate was added to the supernatant and the absorption at 420 nm was read against a distilled water blank. One unit of enzyme produced 0.001 A_{420} unit/min at 30 °C. Enzyme units were normalized to a standard of 0.1 mL of in vitro reaction plus 0.7 mL of assay buffer, followed by 0.8 mL of 1 M sodium carbonate after the acid precipitation.

Protein Determination. Protein concentration of the preparations was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as a standard.

Chemicals. Rifampicin, phosphoenolpyruvate, dithiothreitol, amino acids, nucleotide triphosphates, isopropyl β-D-thiogalactopyranoside, and *O*-nitrophenyl β-D-galactopyranoside were purchased from Sigma Chemical Co. *E. coli* tRNA stripped of amino acids was purchased from General Biochemicals, leucovorin from Lederle, cyclic adenosine 3′,5′-monophosphate from Plenum Scientific Research, and poly(ethylene glycol) 6000 from Union Carbide Corp. Kasugamycin·HCl·H_2O was purchased from Calbiochem. RNase-free DNase was the Worthington Corp. product, and streptolidigin was from the Upjohn Co.

Results

Starting with standard extracts [30 000*g* supernatant (S-30)] of *E. coli* strain 514, we first optimized the in vitro system for β-galactosidase synthesis in the absence of Ca^{2+}. The effects of Ca^{2+} on ribosome function were then analyzed, using antibiotics and bacterial mutants.

Optimization and Characterization of the in Vitro System in the Absence of Ca^{2+}. The DNA-coupled system developed by Zubay to study regulation of *lac* operon expression has been used to study the regulation of a number of operons. In several cases the protocol for preparation of the S-30 has been modified to optimize expression of the operon being studied (Austin, 1974; Parks et al., 1971; Wetekam et al., 1972; Wilcox et al., 1974). For β-galactosidase synthesis a most critical feature in the S-30 preparation was the pressure employed in the French pressure apparatus used to disrupt the cells.

Figure 1 shows a comparison of the amounts of enzyme formed in extracts made from cells broken at low and high pressures, 3200 and 7500 psi, respectively. A batch of cells was divided in two and each half was run through the French press with a rapid flow rate, but high pressures were used for one half and low pressure for the other. Portions of each preparation were then tested for the efficiency of β-galactosidase synthesis over a range of Mg^{2+} concentrations.

Initially, tests were run in absence of Ca^{2+}. Results for a particular extract are plotted in Figure 1 and show that Mg^{2+} was required for substantial enzyme formation, as expected, and that at optimum concentrations of Mg^{2+} the low pressure extract synthesized 50-fold more enzyme than the high pressure extract. More generally we have found that S-30 preparations made at low pressure synthesized 1000–5000 units of β-galactosidase per mL whereas preparations made at high pressure made only 100–200 units/mL. Therefore extracts made at low pressure were used in further experiments.

Extracts made in this way mimicked a number of features of in vivo *lac* operon expression. In particular, expression of β-galactosidase was dependent upon cAMP, and the rates of transcription and translation were similar.

The *lac* operon requires cAMP for expression in vivo and in vitro (de Crombrugghe et al., 1970; Zubay et al., 1970). In the optimized system this was verified with template DNAs bearing either a wild-type *lac* promoter or a *lac* promoter mutation resulting in partial CAP independence (de Crombrugghe et al., 1971a,b). Reactions which contained the wild-type *lac* operon synthesized 10 units/mL of β-galactosidase in the absence of cAMP and 570 units/mL when cAMP was added to the reaction. Reactions which contained the mutant *lac* promoter synthesized 284 units/mL in absence and 1440 units/mL in presence of cAMP.

With the assurance that transcription was initiated at the correct promoter, the rates of transcription and translation were next measured. Because it was not possible to induce *lac* expression instantaneously *in vitro,* the experimental design was a modification of the experiment of Jacquet and Kepes (1971), who used isopropyl β-D-thiogalactopyranoside and actinomycin D to make such a determination for whole cells. After the in vitro reaction had reached the steady state rifampicin was added to block further initiation of RNA synthesis, and streptolidigin to block elongation of nascent RNA. The rate of transcription was then determined as follows.

At time zero in Figure 2, rifampicin (final concentration 100 μg/mL) was added to an in vitro reaction. At the subsequent times indicated on the abscissa portions of this reaction were carefully withdrawn, pipetted into prewarmed tubes containing streptolidigin (final concentration 0.15 mM), and incubated at 37 °C for an additional 30 min. At this concentration streptolidigin stopped transcription within 30 s (data not shown). The amount of enzyme synthesized at the end of the

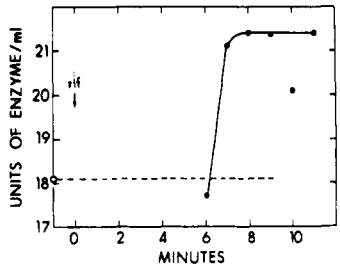

FIGURE 2: Measurement of the rate of transcription of the β-galactosidase gene in the absence of Ca^{2+}, using rifampicin and streptolidigin. Time zero is 13 min after the reaction was placed at 37 °C. At −1 min a 50-μL aliquot was withdrawn from the reaction, transferred to a second tube containing 2 μL of rifampicin (final concentration 100 μg/mL), and incubated at 37 °C for an additional 30 min to allow completion of enzyme synthesis (O- - -). At time zero (↓ rif), rifampicin was added to a final concentration of 100 μg per mL to the remainder of the reaction. At subsequent times, 50-μL aliquots were withdrawn, transferred to another tube containing 2 μL of streptolidigin (final concentration 0.15 mM), and incubated at 37 °C for an additional 30 min to allow completion of enzyme synthesis (●—●). After this time, enzyme activity was assayed as in Experimental Procedure.

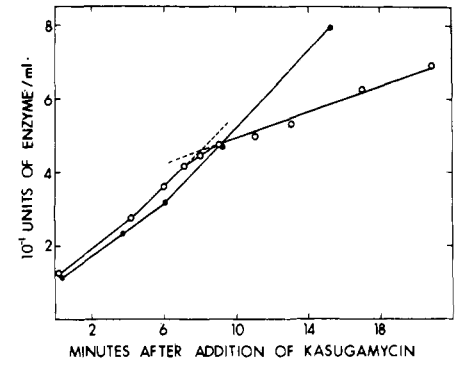

FIGURE 3: Measurement of the rate of translation of β-galactosidase mRNA in the absence of Ca^{2+} using kasugamycin. Equivalent reactions were prepared and, after 16 min at 37 °C (time zero in the figure), kasugamycin (final concentration 0.5 mM) was added to one reaction and an equivalent volume of water to the other. Samples were removed at various times, and enzyme synthesis was terminated by 100 μg per mL of chloramphenicol. Enzyme activity was determined as described in Experimental Procedure. (●—●) Control; (O—O) kasugamycin; (- - -) extrapolations.

30-min incubation was determined for each sample, and the values are plotted in Figure 2. Thus enzyme production was measured after all ribosomes had finished translation. As a result, in each sample, mRNA chains that had been initiated before rifampicin addition, but still had an incomplete β-galactosidase gene transcript when streptolidigin was added, could not code for any enzyme. In other words, for each time at which streptolidigin was added, the amount of enzyme formed gave an estimate of the relative number of nascent mRNA chains that had reached a point distal to the β-galactosidase gene.

The earliest time after rifampicin addition at which streptolidigin added to the reactions no longer affected the final level of enzyme was 7 min. Because this method measures all transcripts which are completed, it is biased toward the slowest polymerases. Seven minutes was, therefore, the maximum length of time necessary for an RNA polymerase to initiate and completely transcribe the β-galactosidase gene.

In Figure 2 data are also presented to indicate that in vitro all RNA polymerases transcribe at that same rate. At −1 min in the figure a portion of the main reaction was transferred to a separate tube containing rifampicin (final concentration 100 μg/mL) and incubated at 37 °C for an additional 30 min as described above. The β-galactosidase activity at the end of the incubation, 18 units per mL, is indicated by the horizontal dashed line. This intersects the solid line (the streptolidigin curve) at 6 min, indicating that all polymerases that started by −1 min also completed transcription within 7 min (see Discussion).

With the same S-30 and under identical conditions the rate of translation was estimated using kasugamycin (Figure 3), an antibiotic which has been shown to act on ribosomes, blocking the initiation of translation without affecting elongation (Tai et al., 1973). Because it specifically inhibits initiation, there should be no effect on the rate of enzyme synthesis until the last ribosome to initiate before addition of kasugamycin completes translation. After this time, which thus equals the translation time of the mRNA, the rate of enzyme synthesis will decrease because of the effect of the antibiotic.

The inhibitory action of kasugamycin is itself antagonized by concentrations of Mg^{2+} higher than 8 mM (Tai et al., 1973), and the DNA coupled system requires at least twice this concentration, so that only a partial block in enzyme synthesis was expected. As determined by the ratio of the final slopes in Figure 3, the inhibitor was 70% effective in this experiment at 15.9 mM Mg^{2+} (cf. Table 1 and Helser et al. (1972) for levels of inhibition with systems reconstituted from ribosomes and soluble factors).

As shown by the intersection of the dashed lines in Figure 3, there was a lag of 8 min between the time of addition and the time of measurable effect of kasugamycin. For the inference that all ribosomes translate β-galactosidase mRNA at this rate, see Discussion. (The comparable rates of enzyme synthesis in the control and kasugamycin-treated reactions during the 8-min lag are further evidence that as expected, the antibiotic neither affects elongation nor induces massive miscoding by the ribosomes.)

Effect of Ca^{2+} on Efficiency of β-Galactosidase Synthesis in Vitro. Concerning the effects of Ca^{2+} on *lac* gene expression, reports have differed concerning the requirement for Ca^{2+} for in vitro protein synthesis (see Discussion). Here it was found that some types of extracts required Ca^{2+} but that in others enzyme synthesis was inhibited.

As described in Figure 1, S-30 preparations made at low and high pressure were tested for their efficiency in β-galactosidase synthesis in reactions which contained Ca^{2+}. Over a wide range of Mg^{2+} concentrations the extracts synthesized comparable amounts of enzyme but differed in their response to Ca^{2+}. At lower concentrations of Mg^{2+} (less than 15 mM) the high pressure extracts were dependent upon Ca^{2+} for enzyme synthesis. In contrast, the low-pressure extracts were inhibited by Ca^{2+}.

Mechanism of Ca^{2+} Inhibition in Low-Pressure Extracts. Even under optimum conditions in presence of Ca^{2+}, both high- and low-pressure extracts were inefficient when compared with low-pressure extracts run without Ca^{2+} (see Figure 1). For the particular low-pressure extract and conditions shown enzyme synthesis was depressed more than 7-fold by Ca^{2+}. More generally, Ca^{2+} depressed enzyme synthesis 50–100-fold. (But even at these low levels enzyme production was cAMP dependent. For example, a set of samples which used a template DNA containing a wild type *lac* operon synthesized 15.1 units/mL of enzyme in the presence of cAMP and 1.6 units/mL in the absence of cAMP. Reactions which used as a template DNA containing the *lac* promoter mutant

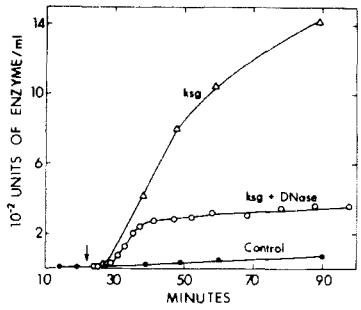

FIGURE 4: Stimulation of β-galactosidase synthesis by kasugamycin in the presence of Ca^{2+}. A complete reaction was prepared and incubated at 37 °C. After 22 min (↓) portions of the reaction were carefully withdrawn and transferred to tubes containing either kasugamycin (final concentration 0.5 mM), kasugamycin plus DNase (final concentrations of 0.5 mM and 5 μg per mL respectively), or water (curve marked control), and incubation at 37 °C was continued. At subsequent times aliquots were removed and assayed for enzyme as in Experimental Procedure.

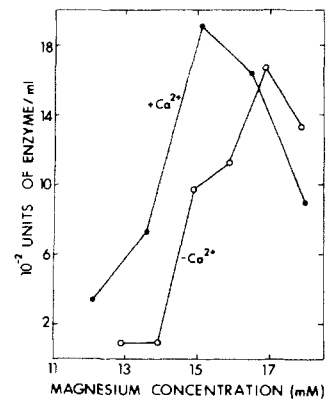

FIGURE 5: Synthesis of β-galactosidase with and without added Ca^{2+} ions at different concentraions of Mg^{2+} in extracts of strain 514K2. Strain 514K2 was isolated, and low-pressure extracts of this strain were prepared as described in Experimental Procedure. Reaction mixtures were prepared at different concentrations of Mg^{2+} either in the presence or absence of Ca^{2+}. At the termination of the incubations, 60 min at 37 °C, β-galactosidase was assayed as described in Experimental Procedure.

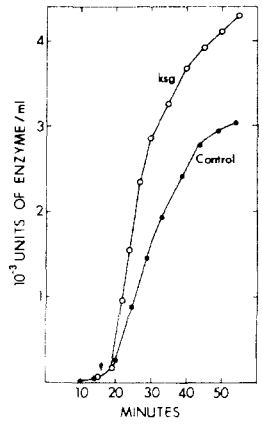

FIGURE 6: Effect of kasugamycin on β-galactosidase synthesis in extracts of strain 514K2 in the presence of Ca^{2+}. Two equivalent reactions were prepared. After 16 min (↓) at 37 °C kasugamycin (final concentration 0.5 mM) was added to one reaction and water to the other (curve marked control). At subsequent times aliquots were withdrawn and enzyme assayed as in Experimental Procedure.

described earlier synthesized 234 and 7.9 units/mL of enzyme in the presence and absence of cAMP.)

For several reasons it seemed likely that Ca^{2+} was exerting its inhibitory effect on ribosomes (see Discussion). If so, it seemed possible that changes in the ribosome induced by antibiotics or mutations might affect the interaction with Ca^{2+}. Kasugamycin and kasugamycin resistant ribosomes provided such effects.

Kasugamycin added to reactions which contained no Ca^{2+} had inhibited enzyme synthesis (Figure 3). In contrast, when it was added to reactions which contained Ca^{2+} kasugamycin alleviated the inhibitory effect of these ions on β-galactosidase synthesis. Portions of a reaction containing Ca^{2+} were withdrawn and transferred to tubes which contained either kasugamycin or water (curve labeled "control" in Figure 4). Subsequent enzyme synthesis was followed by withdrawing aliquots from each reaction. The reaction treated with kasugamycin synthesized 20-fold more enzyme than did the untreated control reaction.

To determine if the stimulation observed was an effect on translation, DNase was used to uncouple translation and transcription, taking advantage of the stabilizing effect of Ca^{2+} on mRNA (Cremer and Schlessinger, 1974). Since DNase effectively blocked transcription in less than 1 min under these conditions (data not shown), any subsequent increase in the amount of β-galactosidase had to come from translation of preexisting mRNA. As shown in Figure 4, enzyme synthesis was stimulated in reactions to which kasugamycin and DNase were added simultaneously. Similar results were seen using streptolidigin instead of DNase (data not shown).

These results suggested that Ca^{2+} and kasugamycin somehow affect one another's action at the ribosome. Evidence to support this suggestion was obtained by studying extracts of kasugamycin resistant strains. Mutants resistant to 300 μg/mL of kasugamycin, strains 514K1 and 514K2, were selected from strain 514 after separate mutagenic treatments (see Experimental Procedure). In initial trials extracts of strain 514K1 showed responses to Ca^{2+} and kasugamycin similar to the parental extracts (data not shown) and were not further studied. In contrast, extracts of 514K2 showed altered responses to Ca^{2+} and kasugamycin.

Figure 5 illustrates the effects of Mg^{2+} and Ca^{2+} on a S-30 extract of this strain. β-Galactosidase synthesis was no longer inhibited by Ca^{2+} in these extracts. A repetition of the experiment of Figure 4 is shown in Figure 6 for an extract of 514K2. The extract was stimulated no more than 30% when kasugamycin was added to reactions containing Ca^{2+}.

To correlate the observed resistance to the inhibitory effects of Ca^{2+} with a change in the ribosome, trials of enzyme synthesis were repeated with fractionated components. S-100, ribosomal wash, and ribosome fractions were prepared (see Experimental Procedure) from S-30 extracts of 514K2 and its kasugamycin sensitive parent 514. The S-100 and ribosomal wash from each strain were then mixed with ribosomes from each strain (see Table I) to reconsitute an S-30 system. Each of the four mixtures was tested for resistance to kasugamycin. The two experiments in Table I show that both sensitivity and resistance to kasugamycin are, as expected (Helser et al., 1972; Sparling et al., 1973), a property of the ribosomes and not the soluble components.

Discussion

The amount of enzyme synthesized in standard DNA coupled systems depends on interactions that prove to include three parameters: the pressure used to lyse the cells, and the relative concentrations of Mg^{2+} and Ca^{2+} in the reactions. Depending

on the type of extract (low or high pressure) and the concentration of Mg^{2+} in the reactions, Ca^{2+} may either stimulate or inhibit β-galactosidase synthesis. These results provide at least a partial explanation of why reports from different labs have varied concerning the level of enzyme synthesis and the need for Ca^{2+} for in vitro protein synthesis.

β-Galactosidase Synthesis in Absence of Ca^{2+}. To find optimal conditions for β-galactosidase synthesis in the absence of Ca^{2+}, extracts made from cells broken at low and high pressures, 3200 and 7500 psi, respectively, were compared.

Low-pressure extracts were 5- to 25-fold more efficient than high-pressure extracts (Figure 1 and text). Other authors have also noticed effects of pressure on the synthesis of other enyzmes (Austin, 1974; Wilcox et al., 1974). We have no explanation at present for the clear inactivation of extracts by high-pressure extraction. One testable speculation implicates ribosomes, which are known to undergo pressure-dependent changes in vitro (Schwarz and Landau, 1972; Smith et al., 1975), and might adopt an inactive conformation in such extracts. For the moment, the results suggest a practical prescription for preparing efficient cell-free extracts.

The efficiency of the optimized system was such that, during the phase of linear synthesis of enzyme by either S-30 (Figure 6) or S-100, ribosomes, and ribosomal wash (Table I), 50 to 100 units of enzyme each minute was produced by extracts containing 2 mg per mL of ribosomes. These levels are 3- to 30-fold more than in other published reports, and enzyme production could potentially be boosted even further by the addition of guanosine tetraphosphate (Kung et al., 1973).

Both low- and high-pressure extracts retained the cellular regulation of *lac* expression by cAMP (see text). In the low-pressure extracts, the rates of transcription and translation were calculated to be 8.3 and 7.3 nucleotides per s, respectively (from data in Figures 2 and 3), assuming the β-galactosidase gene to be 3500 nucleotides long (Zabin and Fowler, 1970). This transcription rate is 2–3-fold slower than the rate measured for the *trp* operon in vitro by Zalkin et al. (1974), and 5-fold slower than the rate measured for *lac* operon in vivo (Jacquet and Kepes, 1971); but the comparable rates of transcription and translation suggest that, as in whole cells, the ribosomes are close behind the RNA polymerase.

That all RNA polymerases transcribe at comparable rates can be inferred from the data of Figure 2 as follows. The RNA polymerases which initiated transcription by −1 min had completed transcription by 6 min. The RNA polymerases which initiated transcription by 0 min completed transcription by 7 min. In other words, the cohort of RNA polymerases which initiated transcription within the 1-min interval between −1 and 0 min also completed transcription within a 1-min interval 6–7 min later.

The comparable rate of movement of all the RNA polymerases can be used to establish that all ribosomes translate β-galactosidase mRNA at similar rates. The slowest ribosomes complete translation within 8 min (Figure 3), and the fastest ribosomes, because they are limited by the movement of the RNA polymerase, cannot be more than 1 min faster.

Thus, in the optimized in vitro system, transcription and translation proceed at comparable rates on all DNA templates.

Effects of Ca^{2+} and Kasugamycin on β-Galactosidase Synthesis. Further studies with extracts made at high and low pressure indicated that Ca^{2+} and kasugamycin could be either stimulatory or inhibitory, depending on the conditions of the reaction. In low-pressure extracts of the sensitive strain 514, kasugamycin was partially inhibitory (Figure 3) and Ca^{2+} highly inhibitory (Figure 1) for enzyme synthesis; but when they were added together, nearly all inhibition was relieved (Figure 4). The mechanism of the antagonism is not known, but, from these studies and those of others (Gordon and Lipmann, 1967; Likover and Kurland, 1967; Zitomer and Flaks, 1972), it is likely that Ca^{2+} itself, like kasugamycin (Tai et al., 1973), inhibits some step at the initiation of protein synthesis.

The mutual antagonism by Ca^{2+} and kasugamycin appeared to be at the level of translation (Figure 4). In support of an overlapping site of action for Ca^{2+} and kasugamycin, a changed ribosomal component (Table I) conferred resistance to kasugamycin in vivo, prevented Ca^{2+} from inhibiting enzyme synthesis in vitro (Figure 5), and blocked stimulation of enzyme synthesis when kasugamycin was added to reactions containing Ca^{2+} (cf. Figures 4 and 6). Candidates for the possible modification include changes in specific rRNA methyl groups (Helser et al., 1972; Sparling et al., 1973) or in ribosomal protein S2 (Yoshikawa et al., 1975).

Zalkin et al. (1974) have added kasugamycin to a DNA-directed system for *trp* gene expression, but in that case no stimulation was observed. This could be due to differences in the reaction conditions used (low- vs. high-pressure extract, for example); or alternatively, different ribosome initiation sites may respond differentially to kasugamycin in presence of Ca^{2+}.

Ca^{2+} Effects in Different Systems, and Use of Strain 514K2. In an earlier report it was demonstrated that Ca^{2+} conferred stability on mRNA transcribed in vitro (Cremer and Schlessinger, 1974). Our present data demonstrate that they also inhibit translation. These two effects of Ca^{2+} could have differential results in different types of assays. Where maximum enzyme production is sought starting from DNA, the inhibitory effects of Ca^{2+} would clearly be disadvantageous (Figure 1). On the other hand, there are cases in which the inhibition of nucleases might be more critical; for example, Ca^{2+} promoted protein synthesis in trials in which RNA was first transcribed and only later translated (Figure 4) or extracted and added back to extracts for translation (Gordon and Lipmann, 1967; Likover and Kurland, 1967; Schumacher and Ehring, 1973, 1975). The dependence of high-pressure extracts on Ca^{2+} at lower Mg^{2+} concentrations (Figure 1) may also result from mRNA stabilization that more than compensates for the inhibitory effect of Ca^{2+} on translation.

It should be emphasized that one cannot necessarily ascribe all the variability in the system—or even all the effects of Ca^{2+}—to the parameters studied here. For example, in recent experiments in which the template was DNA from λplac5 (Dickson et al., 1975; Shapiro et al., 1969), synthesis of β-galactosidase was independent of cAMP (Eron et al., 1971; confirmed in these trials). In that case enzyme synthesis was Ca^{2+} dependent, and high rates were observed only in strain 514K2. Whether such phenomena can also be attributed to Ca^{2+} effects on nucleases or translation remains to be seen.

With respect to these different effects of Ca^{2+}, extracts of strain 514K2, and analogous kasugamycin resistant strains, may have special experimental value, for they permit studies in which, in presence of Ca^{2+}, maximum translation yield (Figure 5) is accompanied by stabilization of mRNA (Cremer and Schlessinger, 1974, and work in progress).

Acknowledgments

We thank Dr. Rhadey Shyam Gupta, Dr. Takashi Kasai, and Dr. B. Vijaya Kumar for many helpful discussions.

References

Austin, S. (1974), *Nature (London) 252,* 596.

Cremer, K., and Schlessinger, D. (1974), *J. Biol. Chem. 249,* 4730.

de Crombrugghe, B., Chen, B., Anderson, W. B., Gottesman, M. E., Perlman, R. L., and Pastan, I. (1971a), *J. Biol. Chem. 246,* 7343.

de Crombrugghe, B., Chem, B., Gottesman, M., Pastan, I., Varmus, H. E., Emmer, M., and Perlman, R. L. (1971b), *Nature (London), New Biol. 230,* 37.

de Crombrugghe, B., Varmus, H. E., Perlman, R. L., and Pastan, I. H. (1970), *Biochem. Biophys. Res. Commun. 38,* 894.

Dickson, R. C., Abelson, J., Barnes, W. M., and Reznikoff, W. A. (1975), *Science 187,* 27.

Dottin, R. P., and Pearson, M. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1078.

Eron, L., Arditti, R., Zubay, G., Connaway, S., and Beckwith, J. R. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 215.

Gordon, J., and Lipmann, F. (1967), *J. Mol. Biol. 23,* 23.

Greenblatt, J. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 421.

Helser, T. L., Davies, J. E., and Dahlberg, J. E. (1972), *Nature (London), New Biol. 235,* 6.

Jacquet, M., and Kepes, A. (1971), *J. Mol. Biol. 60,* 453.

Kaltschmidt, E., Kahan, L., and Nomura, M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 446.

Kung, H., Fox, E., Spears, C., Brot, N., and Weissbach, H. (1973), *J. Biol. Chem. 248,* 5012.

Kung, H., Morrissey, J., Revel, M., Spears, C., and Weissbach, H. (1975a), *J. Biol. Chem. 250,* 8780.

Kung, H., Spears, C., and Weissbach, H. (1975b), *J. Biol. Chem. 250,* 1556.

Likover, T. E., and Kurland, C. G. (1967), *J. Mol. Biol. 25,* 497.

Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,*265.

Miller, J. H. (1972), in Experiments in Molecular Genetics, Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 125, 433.

Nakanishi, A., Adhya, S., Gottesman, M., and Pastan, I. (1975), *J. Biol. Chem. 250,* 8202.

Nirenberg, M. W. (1963), *Methods Enzymol. 6,* 17.

Parks, J. S., Gottesman, M., Perlman, R. L., and Pastan, I. (1971), *J. Biol. Chem. 246,* 2419.

Pouwels, P. H., and Van Rotterdam, J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1786.

Pouwels, P. H., and Van Rotterdam, J. (1975), *Mol. Gen. Genet. 136,* 185.

Schumacher, G., and Ehring, R. (1973), *Mol. Gen. Genet. 124,* 329.

Schumacher, G., and Ehring, R. (1975), *Mol. Gen. Genet. 136,* 41.

Schwarz, J. R., and Landau, J. V. (1972), *J. Bacteriol. 112,* 1222.

Shapiro, J., Machattie, L., Eron, L., Ihler, G., Ippen, K., and Beckwith, J. (1969), *Nature (London) 224,* 768.

Smith, W., Pope, D., and Landau, J. V. (1975), *J. Bacteriol. 124,* 582.

Sparling, P. F., Ikeya, Y., and Elliot, D. (1973), *J. Bacteriol. 113,* 704.

Tai, P.-C., Wallace, B. J., and Davis, B. D. (1973), *Biochemistry 12,* 616.

Wetekam, W., Staack, K., and Ehring, R. (1971), *Mol. Gen. Genet. 112,* 14.

Wetekam, W., Staack, K., and Ehring, R. (1972), *Mol. Gen. Genet. 116,* 258.

Wilcox, G., Meuris, P., Bass, R., and Englesberg, E. (1974), *J. Biol. Chem. 249,* 2946.

Yoshikawa, M., Okuyama, A., and Tanaka, N. (1975), *J. Bacteriol. 122,* 796.

Zabin, I., and Fowler, A. V. (1970), in The Lactose Operon, Beckwith, J. R., and Zipser, D., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Press, p 27.

Zalkin, H., Yanofsky, C., and Squires, C. L. (1974), *J. Biol. Chem. 249,* 465.

Zitomer, R. A., and Flaks, J. G. (1972), *J. Mol. Biol. 71,* 263.

Zubay, G. (1973), *Annu. Rev. Genet. 7,* 267.

Zubay, G., Chambers, D. A., and Cheong, L. C. (1970), in The Lactose Operon, Beckwith, J. R., and Zipser, D., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Press, p 375.