STIMULATION OF LAC mRNA SYNTHESIS BY CYCLIC AMP IN CELL FREE EXTRACTS OF ESCHERICHIA COLI

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Cyclic 3',5'-AMP increases the synthesis of lac mRNA in a cell-free extract of E. coli using λh80dlac DNA as template. Lac mRNA was measured by DNA-RNA hybridization to F'lac DNA and shown to be specific by competition of the ^{3}H-lac mRNA with unlabeled lac mRNA prepared from whole cells.

Although many of the steps in the synthesis of RNA and protein by bacteria are fairly well elucidated, the exact mechanisms controlling the rate and specificity of gene expression are obscure. With respect to the lac operon, genetic and biochemical studies in whole cells indicate that two small molecules, an inducer such as isopropyl-β-D-thiogalactoside (IPTG) and cyclic 3',5'-adenosine monophosphate (cAMP) are required for its transcription and therefore for synthesis of the gene product β-galactosidase. Zubay and coworkers have developed a cell free system which will make β-galactosidase upon the addition of DNA containing the genes of the lac operon (1). This system also requires cAMP and responds to the lac repressor and IPTG (1,2). However, it has not thus far been possible to demonstrate transcriptional effects of these substances by direct measurement of lac mRNA in a cell-free system.

Recently we have developed DNA-RNA hybridization methods for measuring lac mRNA (4). Here we report that in cell free extracts lac mRNA accumulation is stimulated by cAMP.

TABLE 2

Reduction of Cytochrome c by GSH and by GSH plus GSSG under Anaerobic as Compared to Aerobic Conditions

Buffer medium: 0.025 M Tris + 0.175 M KCl, pH 7.45; cytochrome c 10 μM; Temp., 25°.

Additions	Rate of Reduction (mμmoles/ml/min.)	
	Aerobic	Anaerobic
1 mM GSH	0.52	0.42
10 mM GSH	4.06	3.42
1 mM GSH + 5 mM GSSG	17.6	40
500 μM Cysteine	3.37	2.33

Rates are initial rates. The anaerobic rate with GSH + GSSG may be considerably greater than 40, which is based on tracings obtained following the slightly greater delay after mixing in the anaerobic cuvette.

tion for reduction of cytochrome c by GSH is much faster under anaerobic conditions than under aerobic conditions. Since earlier experiments by Schneider et al. (3) have indicated that oxidation of GSH in a GSH + GSSG mixture by dissolved O_2 could be only partially inhibited by 10 mM EDTA, it is very likely that GSSG also catalyzes a reaction with O_2. We propose that the slower rate of reduction of cytochrome c under aerobic conditions is due to competition between O_2 and cytochrome c for an activated form of GSH which exists as the result of interaction with GSSG. The data in Table II do not necessarily answer the question about the possible role of O_2 in the metal catalyzed reaction. The experiment was conducted under conditions (high ionic strength) under which the metal ion catalyzed reaction would be greatly inhibited.

The catalysis by GSSG is not specific for GSH, nor is acceleration of thiol oxidation specific for GSSG. Several thiols and disulfide compounds exhibit similar properties. There is ample evidence to indicate that GSSG catalysis is due to the disulfide

TABLE I

Exp't	DNA	cAMP (10^{-3}M)	β Gal. u x 10^{-2}	Cpm Input	F'lac cpm	% Stim.
I	None	-	-	4,500	0 ± 2	-
	λh80	+	5.2	12,500	46 ± 2	-
	λh80dlac	-	0.3	12,500	129 ± 11	-
	λh80dlac	+	27.7	12,500	209 ± 7	96
II	None	-	-	10,600	39 ± 6	-
	λh80dlac	-	0.3	30,500	476 ± 35	-
	λh80dlac	+	10.4	30,200	787 ± 20	88
III	λh80dlac	-	3.2	20,300	383 ± 36	-
	λh80dlac	+	45.3	22,400	587 ± 36	72
	λh80dlac	5'AMP	4.7	17,400	354 ± 25	0
IV	None	-	-	16,500	56 ± 3	-
	λh80	-	1.4	51,000	257 ± 10	-
				169,000	323 ± 3	
	λh80	+	3.7	52,500	282 ± 55	7
				175,000	334 ± 30	3

Results of transcription and translation in a cell-free β-galactosidase synthesizing system. The template used in each reaction mixture is indicated in the DNA column. Enzyme synthesis was measured by hydrolysis of o-nitrophenyl-β-D-galactoside for 20 hrs. at 28°C. Cpm used for hybridization were determined by 5% trichloroacetic acid precipitation and are also a measure of overall RNA synthesis in each reaction. Cpm specifically bound to F'lac, shown with S.E. of mean, are used to calculate the percent stimulation of lac mRNA accumulation by cAMP, after subtraction of values for F'lac transcribed from a λh80 DNA template. For other details, see text.

tution of 5'-AMP for cAMP results in very little enzyme synthesis. Similar data has previously been published by Chambers and Zubay (2). The small amount of enzyme produced in the presence of λh80 DNA is due to minor contamination with λh80dlac DNA.

Parallel incubations were performed with ^{3}H-CTP and following incubation for 15-30 minutes the RNA was extracted. In general, overall ^{3}H-RNA synthesis, as measured by acid-precipitable radioactivity, varies by less than 10% among the experimental samples. Samples incubated without added DNA incorporate only 30-40% as much label into RNA, demonstrating a moderate dependence upon added phage DNA (Table 1). CAMP has no detectable effect on overall RNA synthesis. This was to be expected if cAMP only stimulates lac mRNA synthesis, since lac genes comprise only

about 5% of the λh80dlac genome. In order to detect RNA transcribed specifically from the E. coli lac genes on the λh80dlac DNA template, it is necessary to hybridize the ^{3}H-RNA with a DNA preparation containing lac genes but free of λh80 sequences. For these experiments we employed lac DNA previously F-ducted from E. coli to PM-1. Equal amounts of ^{3}H-RNA synthesized in cell-free extracts were incubated with filters containing equal amounts of PM-1 or PM-1 F'lac DNA. The amount of RNA homologous to F'lac was determined by subtracting the counts/minute bound to PM-1 DNA from those bound to PM-1 F'lac DNA. These results are recorded in Table 1. The principal finding is the increased accumulation of ^{3}H-RNA homologous to F'lac DNA observed when cAMP is added to reaction mixtures containing λh80dlac DNA template; 5'AMP is inactive. In general, the range of stimulation by cAMP is 50-100% after subtracting the largely non-lac material produced from λh80 DNA template. Virtually no detectable ^{3}H-RNA homologous to F'lac DNA is synthesized in the absence of phage DNA. However, a small amount is found in preparations extracted from reaction mixtures containing λh80 DNA. This is probably due to the presence of unidentified sequences homologous to F DNA in the λh80 molecule and minor contamination of the λh80 DNA with λh80dlac DNA. CAMP does not significantly affect the synthesis of this species of RNA, suggesting that it differs from the RNA species whose production is stimulated when cAMP is added in the presence of λh80dlac template. Moreover, as shown in Table 1, when large amounts of ^{3}H-RNA transcribed from a λh80 DNA template are hybridized to PM-1 F'lac DNA, less than 350 cpm of F'lac homologous material is observed. Since these DNA filters have the capacity to hybridize 1400 cpm of F'lac homologous RNA, made from a λh80dlac template, it is apparent that contamination of λh80 DNA with λh80dlac is small.

Competition Hybridization Experiments. Although it appeared highly probable that we were detecting an increase in the amount of lac mRNA, it was also possible that we were measuring message transcribed from

non-lac DNA also present in the F′lac episome. To exclude this possibility, we performed competition hybridization experiments designed to determine whether unlabeled RNA, prepared from a wild-type strain of E. coli induced for β-galactosidase synthesis, would compete with ^{3}H-RNA for lac DNA sites. For controls, unlabeled RNA was prepared from an uninduced culture of the wild type strain and a lac deletion strain.

First, increasing amounts of ^{3}H-RNA produced in the presence of cAMP from a λh80dlac template were hybridized with filters containing 4.0 μg of PM-1 Flac ^{14}C DNA to determine the saturation point. This proved to be approximately 1400 cpm. Using equivalent amounts of ^{3}H-RNA obtained from a λh80 DNA template, about 350 cpm could be hybridized. Therefore, it was assumed that a maximum of 1050 cpm could be competed by unlabeled lac mRNA, if those counts all represented ^{3}H-RNA transcribed from the correct strant of the transduced lac operon. As can be seen in Figure 1, 144 μg of unlabeled RNA from an induced strain competes successfully with over 600 cpm (about 55%) of the potentially competable material for available DNA sites. The lac deletion strain RNA demonstrates only trivial competition, of the magnitude observed when ^{3}H-RNA from a λh80 DNA template is competed by this quantity of unlabeled RNA. There is slightly more competition by RNA from the uninduced culture, perhaps indicating low but measurable levels of lac mRNA in such cells.

A similar experiment, performed with ^{3}H-RNA transcribed from a λh80dlac template in the absence of cAMP, is depicted in Figure 1B. Again, unlabeled RNA from an induced culture of the wild type E. coli competes much more efficiently with the labeled material than does unlabeled RNA from an uninduced culture of the same strain. However, in this case, the extent of competition is less than 350 cpm (about 30%), despite the fact that the experiment was performed with less labeled material homologous to F′lac in the hybridization mixtures.

Since the unlabeled lac mRNA synthesized *in vivo* represents read-

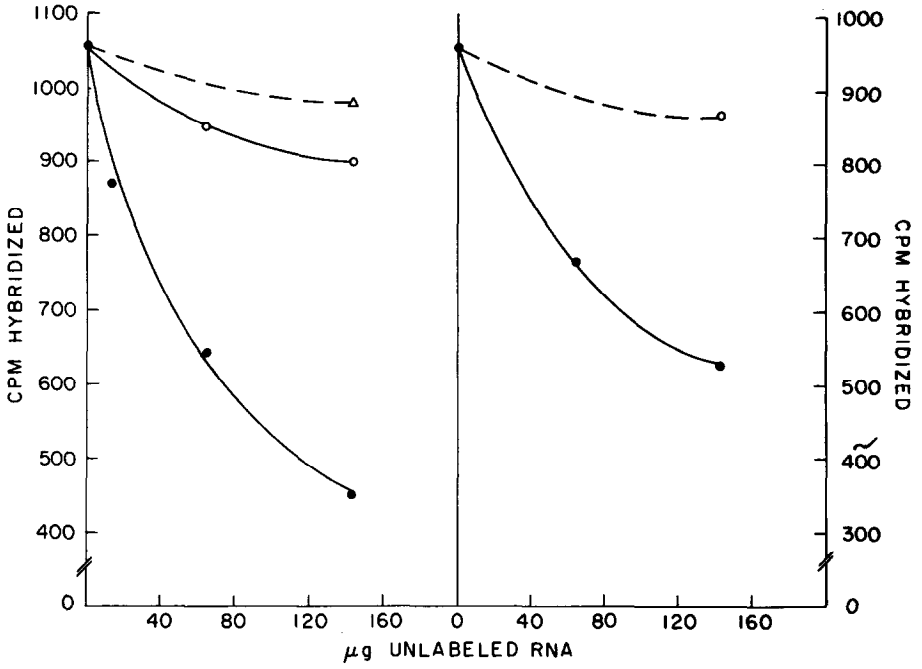

Figure 1A and 1B. Competition hybridization. Saturating amounts of ^{3}H-RNA made in the presence (1A) or absence (1B) of cAMP in a cell-free β-galactosidase synthesizing system were hybridized with PM-1 F′lac DNA with increasing amounts of unlabeled RNA extracted from growing cells: wild strain induced for β-galactosidase production, ●——●; uninduced wild strain, O——O; lac deletion strain, Δ- - Δ. For additional details, see text.

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ing of only the correct strand of DNA, only those labeled messages made in vitro from the correct strand can be competed. The increase in the capacity of the ^{3}H-RNA made in the presence of cAMP to compete with lac mRNA prepared in vivo suggests that cAMP may confer strand specificity upon the transcription process.

DISCUSSION. Attempts to study regulation of bacterial gene transcription in a cell-free system have been frustrated by the apparently rigid requirements for control; by the high frequency of random, symmetrical initiation of strand reading in the absence of cellular organization; and by the need to isolate the gene under study sufficiently to detect its RNA product with high accuracy. The development of a cell-free system

for the synthesis of β-galactosidase from lac DNA by Zubay and co-workers (1,2) seemed to offer a unique opportunity for investigating in vitro transcription. Correct reading of DNA into RNA occurs in this system, as evidenced by the production of apparently normal enzyme; moreover, the rate of enzyme production can be greatly altered by compounds---lac repressor, IPTG, and cAMP---believed to act at the transcriptional level in vivo. Using hybridization to F'lac DNA to detect lac mRNA transcribed from a λh80dlac DNA template, we have shown that cAMP increase the accumulation of lac mRNA by 50-100%. This effect is not observed with 5'-AMP, the principal degradation product of cAMP; and cAMP does not affect either total RNA synthesis or production of RNA homologous to F'lac from a λh80 DNA template. Moreover, most of the ^{3}H-RNA measured by our assay appears to be identical to unlabeled lac mRNA synthesized in whole cells as indicated by competition experiments.

Stimulation of β-galactosidase synthesis by cAMP is 10-20 fold, whereas accumulation of lac mRNA is increased only 50-100%. Because the hybridization assay detects all lac DNA reading---including random starts, incomplete chains, and transcription of the incorrect strand---the degree of stimulation by cAMP may appear much lower than can be detected by enzyme synthesis, which responds only to correctly transcribed messages. Nevertheless, it is not possible at present to rule out an additional translational effect of cyclic AMP in vitro.

The mechanism by which cAMP affects transcriptional events has not as yet been clarified by this approach. We have recently performed experiments demonstrating that λh80 RNA or lac mRNA synthesized in vitro in the absence of cAMP is degraded with a half-life of approximately six minutes; the degradation of lac mRNA made in the presence of cAMP is at least as rapid. This suggests that the effect of cAMP is to stimulate synthesis, not to retard degradation, of lac mRNA. Similar results have been obtained in vivo where lac mRNA synthesis is directly proportional

to the rate of β-galactosidase synthesis when cAMP levels are lowered by glucose or restored by exogenous cAMP (3). Clear definition of the mechanism of cAMP action, however, will require the development of a fully reconstituted system.

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