Anti-mRNA: Specific inhibition of translation of single mRNA molecules

(β-galactosidase mRNA/complementary RNA/lactose operon)

SIDNEY PESTKA, BRUCE L. DAUGHERTY, VINCENT JUNG, KUNIMOTO HOTTA, AND ROBERT K. PESTKA

Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

ABSTRACT   A plasmid was constructed to generate RNA complementary to the β-galactosidase mRNA under control of the phage λ P_L promoter. When this anti-mRNA was produced, synthesis of β-galactosidase was dramatically inhibited (98%). Syntheses of galactoside permease and transacetylase, whose coding sequences are downstream of the β-galactosidase coding region, are inhibited to a lesser degree, 80% and 55%, respectively. The generation of anti-mRNA that can be targeted to inhibit a single species of mRNA molecule within cells provides a potent mechanism by which specific transcripts can be translationally inactivated. This can be used to determine the function of proteins as well as to select cloned genes in a single rapid and convenient step.

Inhibition of translation has been efficiently carried out by antibiotics and other inhibitors of protein synthesis (1). In specific instances, physiological mechanisms have been manipulated to turn on or off genes such as the *lac* operon of *Escherichia coli* grown in the presence or absence, respectively, of a β-galactoside (see ref. 2). However, no general method has been described to block the translation of a specific mRNA molecule.

It has been known that RNA with substantial structure, such as double-stranded RNA, serves as a poor template for protein synthesis (1, 3). Knowledge of this has been used effectively to block translation in cell-free extracts. *In vitro*, hybrid-arrested translation has been reported to prevent the synthesis of specific proteins (4, 5) and has been used to detect recombinant DNA molecules containing sequences complementary to a given mRNA. The procedures of hybrid-arrested translation involve DNA·RNA hybridization, wherein the double-stranded DNA is denatured by heating and annealed with a population of mRNA molecules. Hybridization of DNA to the mRNA effectively blocked translation of these mRNA molecules that formed hybrids.

To develop a method to block the translation of specific mRNA molecules in intact cells, we postulated that RNA complementary to mRNA would also effectively block translation if RNA·RNA hybridization would occur readily under physiological conditions within the cell. Because single-stranded complementary RNA molecules can be readily generated within cells by appropriate vectors, the hypothesis could be tested conveniently. The objective was to generate anti-mRNA intracellularly to block translation of specific proteins. The present communication describes the construction and use of complementary RNA generated *in vivo* to block translation of only a single species of mRNA molecules to which it can specifically hybridize.

PRINCIPLE OF THE PROCEDURE

Because the *lac* operon of *E. coli* is well understood, it was chosen as the focus of these initial studies. In addition, since

the mRNA transcribed upon induction of this operon is polycistronic, it would be possible to determine the extent of polarity of the inhibition of translation if RNA complementary only to the *lacZ* (β-galactosidase) coding sequence was used as the anti-mRNA.

An 831-base-pair DNA fragment of the lactose operon, cloned in phage M13mp7 (6), was the source of a DNA fragment containing the sequence coding for the NH$_2$ terminus of β-galactosidase. This fragment was isolated and placed in reverse orientation under control of the phage λ P_L promoter. The gene coding for a temperature-sensitive repressor of P_L, *c*Its, is carried on a compatible plasmid pRK248*c*Its (7–9). Thus, at 30°C, no anti-mRNA would be made, but at 42°C anti-mRNA would be synthesized extensively. On hybridizing with the mRNA, the anti-mRNA would block translation of the message for β-galactosidase highly specifically, acting as a precisely directed anti-mRNA "missile."

EXPERIMENTAL PROCEDURES

Media, Enzymes, and Electrophoresis. M9 medium consisted of 50 mM Na$_2$HPO$_4$, 28 mM KH$_2$PO$_4$, 8.5 mM NaCl, 19 mM NH$_4$Cl, 0.1 mM CaCl$_2$, 1 mM MgSO$_4$, 0.4% Casamino acids, 1% glycerol, and thiamin at 2 μg/ml. Restriction endonucleases were obtained from Bethesda Research Laboratories. T4 DNA ligase was obtained from Amersham. Agarose gel electrophoresis was performed according to the procedures of Sharp *et al.* (10). Fragments were isolated from the gels by the method of Schmitt and Cohen (11).

Construction of Plasmids. Phage M13mp7 was used to isolate a *Bam*HI/*Cla* I fragment containing some of the sequence coding for the NH$_2$-terminal region of β-galactosidase, beginning at amino acid 6 (Fig. 1). The plasmid pRC23, which contains the phage λ P_L promoter, was cut with restriction endonucleases *Cla* I and *Bam*HI. The large *Cla* I/*Bam*HI fragment from the plasmid was isolated and ligated to the small *Bam*HI/*Cla* I fragment containing part of the *lacZ* as described above (Fig. 1). Ligations were performed in 66 mM Tris·HCl, pH 7.5/5 mM MgCl$_2$/5 mM dithiothreitol/1 mM ATP for 16 hr at 15°C. DNA concentration was between 1 and 10 nM and phage T4 DNA ligase was used at 300 units/ml. The result was a plasmid, pBD4/*lacZ*'-(rev), containing a *lacZ* gene fragment in reverse orientation with respect to the P_L promoter. This plasmid was transfected into *E. coli* 294 harboring the compatible plasmid pRK248*c*Its containing the temperature-sensitive λ repressor *c*Its (7–9). Little or no anti-mRNA would be synthesized at 30°C. At 42°C, anti-*lacZ* mRNA would be synthesized at high levels.

Growth of Bacteria. *E. coli* 294(pRK248*c*Its) harboring pRC23 or pBD4/*lacZ*'(rev) was grown in 10 ml of M9 medium containing 1% glycerol overnight at 30°C. To start the experiment, two flasks containing 150 ml of fresh M9 medium (1% glycerol) were inoculated with the *E. coli* containing pRC23 and another two flasks containing the same medium

Abbreviation: IPTG, isopropyl β-D-thiogalactoside.
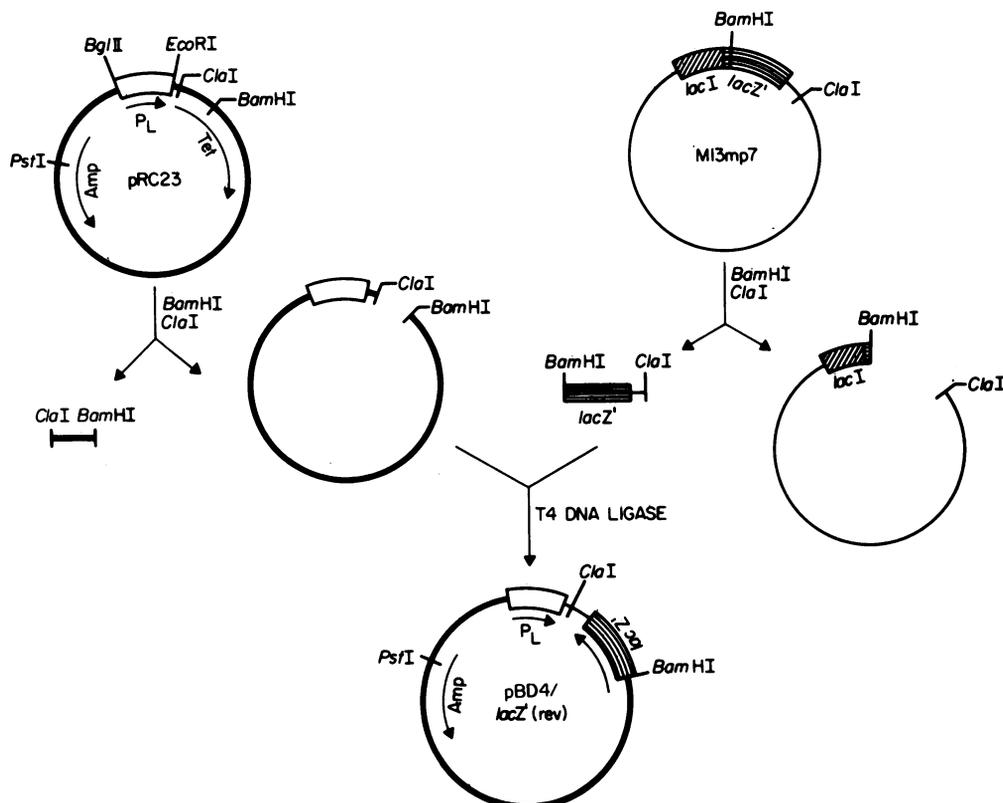
FIG. 1.   Construction of pBD4/*lacZ*'(rev) from pRC23 and M13mp7. Details are given in the text. The portion of the *lacZ* gene contained in the *Bam*HI/*Cla* I fragment from M13mp7 represents nucleotides 19–440 of *lacZ*, with the initiator AUG of the *lacZ* gene taken as position 1. This fragment is designated *lacZ*'. Amp, ampicillin; Tet, tetracycline.

were inoculated with the *E. coli* containing pBD4/*lacZ*'-(rev). These were grown at 30°C until the optical density at 600 nm reached about 0.5. At this point, isopropyl β-D-thiogalactoside (IPTG) was added to one flask of each of the cultures to a final concentration of 1 mM. Aliquots of each of the four cultures were removed and placed at 0°C at the starting time of induction (0 min). The cultures were then placed in a 42°C water bath and shaken vigorously. Aliquots from each of the four cultures were taken at 15, 30, 60, 90, and 120 min and placed at 0°C until assayed a short time later.

Enzyme Assays. Assays for β-galactosidase, lactose permease, transacetylase, and alkaline phosphatase were performed as described (12–15). Enzyme concentrations were determined from the linear portions of the curves in all cases. The specific procedures are given below.

Assay of β-galactosidase. β-Galactosidase was measured as described (12) with minor modifications. Samples of bacterial cultures were diluted with Z buffer (50 mM sodium phosphate, pH 7.0/10 mM KCl/1 mM MgSO$_4$/50 mM 2-mercaptoethanol) into a total volume of 1.1 ml. Two drops of chloroform and one drop of 0.1% sodium dodecyl sulfate were added, after which tubes were swirled on a Vortex mixer for 10 sec. The tubes were then placed in a water bath for 5 min at 30°C. A 1-ml sample was then added to a cuvette. The reaction was started by adding 0.2 ml of *o*-nitrophenyl β-D-galactoside (4 mg/ml) and mixed for a few seconds. The cuvette was then placed in a Beckman DU-8 spectrophotometer which was programmed to determine changes in absorbance at 420 nm per min.

Assay of lac *carrier protein.* Lactose permease was assayed as described (12, 13) with modifications as noted below. Aliquots of bacterial suspensions were centrifuged at 10,000 × *g* for 5 min. The pellets were washed with 2 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 10

mM MgSO$_4$ and resuspended in 0.2 ml of the same buffer. In each of four tubes, 50 μl of the bacterial suspension was added, after which they were incubated at 26°C for 5 min. With a Hamilton syringe, 2 μl of 10 mM [^{14}C]methyl β-D-thiogalactopyranoside (New England Nuclear; 22.5 mCi/ml; specific activity 25.6 Ci/mmol; 1 Ci = 37 GBq) was added to each of the four tubes for 0, 15, 30, and 60 sec. The reaction was stopped by the addition of 3.5 ml of 0.1 M potassium phosphate buffer, pH 5.5, containing 100 mM LiCl. The solution was filtered on a 25-mm 0.45-μm Amicon filter (no. 041255) and washed with 3.5 ml of the same solution. The radioactivity on the filter was then measured in 10 ml of Bray's solution in a scintillation spectrometer.

Assay of thiogalactoside transacetylase. Aliquots of bacterial suspension were centrifuged at 10,000 × *g* for 5 min. The pellets, resuspended in 250 μl of 50 mM Tris·HCl, pH 7.8, were assayed for thiogalactoside transacetylase as reported (14). The suspensions were sonicated twice for 15 sec on ice. The extracts were heated at 70°C for 5 min and centrifuged in a Microfuge for 15 min. Each supernatant was transferred to a fresh tube. The reaction was started by mixing 0.5 ml of a solution consisting of 50 mM Tris·HCl at pH 7.8, 2 mM EDTA, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM acetyl-CoA, and 50 mM IPTG with 200 μl of the bacterial extracts. The solution was transferred to a cuvette, which was placed in a Beckman DU-8 spectrophotometer that was programmed to calculate changes in absorbance at 412 nm as a function of time.

Assay of alkaline phosphatase. Aliquots of bacterial suspensions were centrifuged at 10,000 × *g* for 5 min. The pellets were washed in 2 ml of 0.1 M Tris·HCl, pH 7.4. After centrifugation, the pellets were resuspended in 2 ml of the same buffer. The assay consisted of mixing 0.5 ml of the bacterial suspension with 1 ml of 1 M Tris·HCl, pH 8.8, and incubating at 37°C for 5 min. The reaction was started by the

Genetics: Pestka *et al.*

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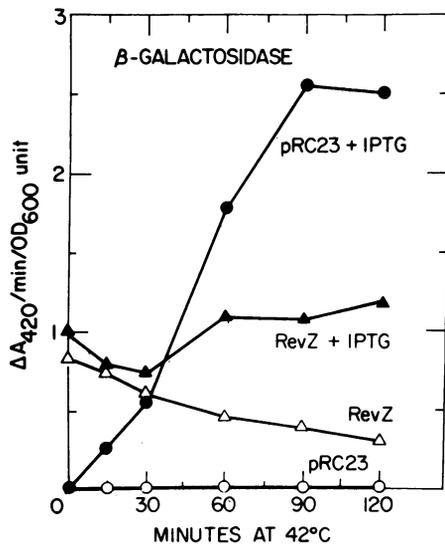
FIG. 2. β-Galactosidase as a function of time after cells were transferred to 42°C in the presence or absence of IPTG. β-Galactosidase levels in cells containing plasmid pRC23 or pBD4/*lacZ'*(rev) are designated by curves labeled pRC23 and RevZ, respectively.

addition of 0.5 ml of 0.04 M *p*-nitrophenyl phosphate and the mixture was incubated at 37°C. At 30, 60, and 120 min, 0.7 ml was centrifuged in a Microfuge for 5 min and the supernatant was transferred to a fresh tube. The absorbance at 420 nm was measured in a spectrophotometer and the change per min was determined from the readings at different times.

RESULTS

When *E. coli* 294 containing the parental plasmid pRC23 was induced at 42°C with IPTG, there was a rapid rise in synthesis of β-galactosidase (Fig. 2). When *E. coli* 294 with the plasmid containing the reverse orientation was induced, the rise in β-galactosidase synthesis on induction with IPTG was essentially prevented. The higher uninduced level of β-galactosidase in the presence of the plasmid carrying the *lacZ* fragment was due to titration of the *lac* repressor by the *lacZ* segment containing the secondary repressor binding site (refs. 16 and 17; also see discussion below). In the absence of IPTG, induction of the P_L promoter at 42°C decreased the endogenous level of β-galactosidase in *E. coli* 294/pBD4/*lacZ'*(rev).

To determine if the anti-*lacZ* segment also prevented syn-
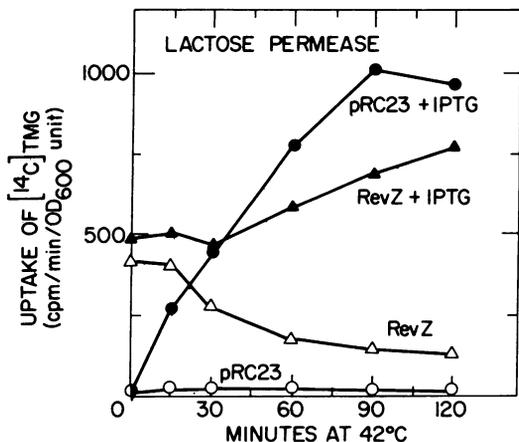
FIG. 3. Lactose permease as a function of time after cells were transferred to 42°C in the presence or absence of IPTG. Curves are labeled as in Fig. 2. TMG, thiomethyl galactoside.
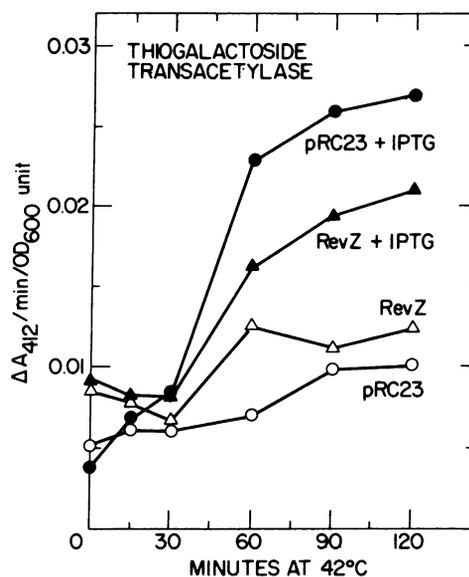
FIG. 4. Thiogalactoside transacetylase as a function of time after cells were transferred to 42°C in the presence or absence of IPTG. Curves are labeled as in Fig. 2.

thesis of lactose permease and thiogalactoside transacetylase encoded by the same polycistronic message, these enzymes were also assayed in the same series of cultures with and without induction with IPTG (Figs. 3 and 4). The permease and transacetylase were induced concomitantly with β-galactosidase; however, their synthesis was not inhibited as much as that of β-galactosidase by anti-*lacZ* mRNA (Figs. 3 and 4; Table 1). Nevertheless, the same pattern of inhibition was seen as with β-galactosidase. The synthesis of an enzyme, alkaline phosphatase, encoded by another mRNA was not affected either by IPTG or by the anti-*lacZ* mRNA (Fig. 5). Thus, in all cases, the presence of the plasmid containing *lacZ* in the reverse orientation was sufficient to inhibit the synthesis of β-galactosidase, permease, and transacetylase.

When induction of *E. coli* 294 containing plasmid pRC23 or pBD4/*lacZ'*(rev) was performed with IPTG at 30°C, β-galactosidase in both cultures increased to about the same levels (data not shown). Under these conditions, there was no inhibition of β-galactosidase synthesis in *E. coli* 294 containing the plasmid pBD4/*lacZ'*(rev), as expected, since no reverse transcript is synthesized at 30°C, where the P_L promoter remains repressed.

Table 1. Induction of enzymes of *lac* operon in presence or absence of reverse *lacZ*

Enzyme	Induction by IPTG, %		Inhibition of synthesis by *lacZ'*(rev), %
	Control	*lacZ'*(rev)	
β-Galactosidase	100	2	98
Lactose permease	100	20	80
Thiogalactosidase transacetylase	100	45	55

The induction of each enzyme at 90 min after the addition of IPTG was taken from the data of Figs. 2–4. Background values at 0 min in the absence of IPTG were subtracted from the enzyme levels at 90 min to determine the level of induction. Control cells contained plasmid pRC23. The cells with the segment of the *lacZ* gene oriented in the reverse direction with respect to the P_L promoter contained plasmid pBD4/*lacZ'*(rev). With the level of increase (induction by IPTG) in enzyme taken as 100% in the control cells, the increase in the cells containing pBD4/*lacZ'*(rev) is shown as a percentage of the values in the control cells.
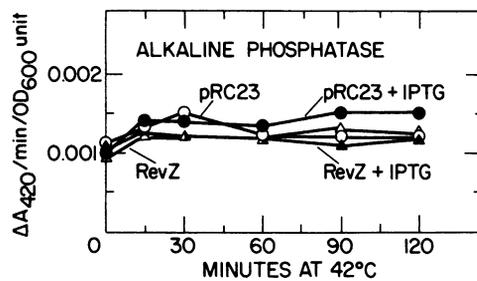
FIG. 5. Alkaline phosphatase as a function of time after cells were transferred to 42°C in the presence or absence of IPTG. Curves are labeled as in Fig. 2.

DISCUSSION

The presence of anti-*lacZ* mRNA can be controlled by the λ phage P_L promoter. Under conditions in which no anti-*lacZ* mRNA is made, β-galactosidase, lactose permease, and transacetylase are induced by IPTG. When anti-*lacZ* mRNA is present, the syntheses of all three enzymes produced from the same polycistronic mRNA are inhibited, whereas the synthesis of alkaline phosphatase, produced from a different mRNA template, is not affected. It is thus clear that anti-mRNA can be generated and can function in intact *E. coli*. Although the syntheses of all the enzymes made on the polycistronic *lac* mRNA are inhibited by the anti-*lacZ* mRNA, inhibition of the synthesis of permease and transacetylase, whose coding regions are distal to the coding region of β-galactosidase, occurs to a smaller degree than inhibition of β-galactosidase synthesis (Table 1). There is a polarity to the inhibition in that permease synthesis is inhibited 80%, whereas synthesis of transacetylase, whose coding region is most distal to that of β-galactosidase, is inhibited only 55%. Ribosomes initiating within polycistronic mRNAs (18) at the permease and transacetylase initiation codons must account for this lower degree of inhibition, and these observations support direct reinitiation at AUG codons corresponding to these enzymes.

The presence of the plasmid containing the *lacZ'* region in reverse orientation with respect to the P_L promoter increases the level of β-galactosidase. The plasmid appears to be titrating the repressor protein even though there is no *lac* operator segment present. This is consistent with the results of Reznikoff *et al.* (16), who reported the existence of a secondary binding site for *lac* repressor within the operator-proximal third of the *lacZ* gene. This site was precisely defined by Gilbert *et al.* (17) to be located at position 357–391 of the *lacZ* gene, starting from the initiator codon, ATG, of *lacZ* taken as position 1. The segment of the *lacZ* gene incorporated in pBD4/*lacZ'*(rev) represents positions 19–440 and thus encompasses this secondary binding site for *lac* repressor. When this secondary binding site was deleted from the plasmid pBD4/*lacZ'*(rev), there was no titration of repressor and thus no increase in β-galactosidase in the absence of IPTG compared to control cells. Nevertheless, β-galactosidase synthesis induced by IPTG was inhibited 97% upon induction of the anti-*lacZ* mRNA fragment at 42°C (unpublished data).

The use of anti-mRNA that can be induced within cells provides a potent mechanism by which specific transcripts can be translationally inactivated. This procedure should provide a convenient and rapid method to determine the function of proteins or other RNAs within cells. The techniques should be applicable to eukaryotic cells with appropriate vectors and regulatory elements. During the completion of these initial experiments, Izant and Weintraub (19) reported the use of anti-mRNA in mammalian cells. Indeed, it is possible that anti-mRNA transcripts have physiological roles in cells, as suggested by Simons and Kleckner (20) and by Mizuno *et al.* (21). We hope to be able to determine the optimal size and region for targeting by anti-mRNA segments in the near future. As hybrid-arrested translation has been used *in vitro* to screen recombinant DNA clones, anti-mRNA recombinants may be employed *in vivo* to screen and select for phenotypically altered cells lacking single functions. The method may be applicable to screen for recombinants that inhibit gene products in diploid cells where simultaneous insertional inactivation of genes is not feasible.

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