DNA-DIRECTED PEPTIDE SYNTHESIS, III. REPRESSION OF β-GALACTOSIDASE SYNTHESIS AND INHIBITION OF REPRESSOR BY INDUCER IN A CELL-FREE SYSTEM*

BY GEOFFREY ZUBAY, MURIEL LEDERMAN, AND JOANNE K. DEVRIES

DEPARTMENT OF BIOLOGICAL SCIENCES, COLUMBIA UNIVERSITY

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The operon model for regulation of enzyme synthesis was proposed by Jacob and Monod in 1961.[1, 2] The genetic elements of this control system consist of two parts: a locus, *i*, which directs the synthesis of a repressor, and a locus called the operon which contains the operator gene and associated structural genes. A messenger RNA molecule can be transcribed from the operon, which is believed to contain in one continuous polynucleotide chain the information for making the polypeptides encoded by the different structural genes. It is not known whether the messenger RNA contains a region corresponding to the operator site and, likewise, it is not known whether the repressor stops protein synthesis by interacting with the DNA, the transcribed mRNA, or a hybrid of both. The inhibiting effect of repressor is reversed by inducer which interacts directly with the repressor. Most available genetic and biochemical data favor the idea that repressors are proteins.[2] Confirmation of the repressor-operator model awaits a cell-free biochemical system in which the effect of the repressor on peptide synthesis can be directly demonstrated. In this paper such a system is described for the *lac* operon of *Escherichia coli.*

Materials and Methods.—E. coli strains: *r1F′:* This strain was derived from strain r1, described by Zipser and Newton,[3] by introduction of an episome. r1F′ has a deletion of most of the *Z* gene and carries two *i* genes, one on the chromosome and one on the episome. This strain was obtained from Dr. D. Zipser.

M107: This strain is F^-, i^-, Z^-, a63su$^+$, Sm^R, and was obtained from Dr. D. Zipser.

Z: This strain is HfrH, B_1^-, lysogenic for φ80 and φ80*lac*, and contains a temperature-sensitive repressor for bacteriophage induction. It was obtained from Dr. W. Gilbert.

21F′: This strain was derived from strain M15 described by Ullmann, Jacob, and Monod;[4] it has a deletion of about one third of the α portion of the *Z* gene. Strain 21F′ is i^- and carries that portion of the gene for the Z protein present in strain M15 on both chromosome and episome. It was obtained from Mrs. C. Michels.

Preparation of S-30 extract: The cell-free extract of *E. coli* used in all syntheses is prepared by a modification of the method of Nirenberg[5] described elsewhere.[6]

Preparation of DNA: A partially purified bacteriophage preparation was obtained from strain Z by a modification of the procedure of Kaiser and Hogness[7] as described elsewhere.[8] The DNA is prepared from the bacteriophage preparation by a minor modification of the procedure of Mandell and Hershey.[6, 9]

Incubation conditions for synthesis: All materials which require thawing are warmed to 5°C in a water bath. The incubation mixture contains per ml: 40 μmoles tris-acetate, pH 8.0; 4.5 μmoles 2-mercaptoethanol; 14.6 μmoles magnesium acetate; 7.3 μmoles $CaCl_2$; 0.2 μmole 19 C^{12}-amino acids; 2.0 μmoles ATP; 0.5 μmole each of GTP, CTP, UTP; 18 μmoles PEP trisodium salt; 50 μg PEP kinase; 0.1 μmole C^{14}-L-leucine; 200 μg φ80*lac* DNA; 50 μmoles potassium acetate; 100 μg tRNA; and 6500 μg protein as S-30 extract. All ingredients except the S-30 extract are mixed together and preincubated for 3 min at 37°C. After adding the S-30 extract, the reaction tubes are incubated at 37°C for 60 min. In experiments where partially purified repressor or IPTG inducer is added, these are mixed with the S-30 extract and the above procedures are followed.

Preparation of α complementation factor: Alpha complementation factor was prepared from strain 21F′ by a minor modification of the procedure of Ullmann, Jacob, and Monod.[4]

Assay of incubation mixture after synthesis: Normally, incubation mixtures are assayed for leucine incorporation and β-galactosidase activity. The turbid incubation mixture should be stirred before taking an aliquot for assay.

Assay for C^{14}-leucine incorporation: This assay is described in reference 6.

Assay for β-galactosidase activity: When the source of the S-30 extract is strain M107 or r1F, a 0.1-ml sample of the incubation mixture taken after the synthesis step is mixed with equal volume of α complementation factor. This is allowed to stand for 60 min at room temperature during which time complementation takes place leading to β-galactosidase activity. When the source of the S-30 extract is strain 21F′, 0.2-ml samples of the incubation mixture are taken directly after synthesis for the β-galactosidase assay. In either case, 1.5 ml of ONPG solution is added and the β-galactosidase assay carried out as previously described[10] except for a modification suggested by Dr. D. Zipser. At the end of the incubation with the substrate ONPG, 1 drop of glacial acetic acid is added to each tube to precipitate the protein, thus decreasing the background absorption and preventing errors due to turbidity. The tubes are quickly stirred and chilled in ice, then centrifuged in the cold for 15 min at 2000 × *g*. The supernatant is transferred to a clean tube and an equal volume of 1 *M* Na_2CO_3 is added. The optical density is determined at 420 mμ.

Preparation of partially purified repressor: All steps are carried out at 1° to 4°C. Buffer A is 0.01 *M* tris-HCl, pH 7.4, 0.01 *M* $MgCl_2$, 10^{-4} *M* EDTA, and 0.006 *M* 2-mercaptoethanol. Buffer B is buffer A plus 0.2 *M* KCl. Buffer C is 0.01 *M* tris-acetate, pH 7.8, 0.014 *M* magnesium acetate, 0.06 *M* potassium acetate, and 0.006 *M* 2-mercaptoethanol. Streptomycin sulfate is obtained from Eli Lilly and Sons. Frozen cells of strain r1F, 170 gm, are thawed and washed once in buffer B. The cells are resuspended in 226 ml of buffer B, chilled to 1°C and lysed in a French pressure cell (Aminco Industries) at 4000–8000 psi. Buffer B, 200 ml, is added to the lysate and the resulting solution is centrifuged at 15,000 × *g* for 20 min. To the supernatant is added 13.6 ml of 10% w/v streptomycin sulfate. After 15 min of stirring, the precipitate is removed by centrifugation at 15,000 × *g* for 7 min. This is called fraction I.

The pH of the supernatant is adjusted to 7.0 with 0.5 *M* NH_4OH, and saturated $(NH_4)_2SO_4$ at pH 7.4 is added to 35% saturation. After 15 min of stirring, the precipitate is collected by centrifugation at 30,000 × *g* for 10 min, resuspended in 13.6 ml buffer B, and dialyzed overnight against two 1-liter portions of buffer B. This is called fraction II.

Fraction II is then centrifuged for 90 min at 40,000 rpm in a #50 Spinco rotor, and the supernatant is dialyzed overnight against 1 liter of buffer A. This is called fraction III.

Fraction III is mixed with 0.75 vol of hard-packed DEAE cellulose which has been previously rinsed with buffer A. The mixture is gently shaken for 90 min and then poured into a 1.5-cm-diameter column, packed under 3 psi pressure and eluted with buffer B at a flow rate of 1 ml/min. During the elution, a brown band of protein appears on the column. This band is collected and dialyzed for 4 hr against 1 liter of buffer C and rapidly frozen in 0.5- to 1.0-ml aliquots. This is called fraction IV. Fraction IV is used only once after thawing.

The various fractions were assayed for their ability to bind IPTG by the dialysis equilibrium method as described by Gilbert and Müller-Hill.[11] C^{14}-IPTG of specific activity 25 μc/μmole was used at 1.1 × 10^{-7} *M*. Protein concentration was measured by the modified Lowry method.[12]

Results and Discussion.—Description of an assay system for gene activity: The *lac* operon of *E. coli* consists of an operator gene *O*, and three structural genes, *z* for β-galactosidase, *y* for permease, and *a* for thiogalactoside transacetylase, adjacent to the operator in that order. Since the β-galactosidase gene is adjacent to the operator and since both transcription and translation are believed to start near the operator, it was thought that evidence for the synthesis of the operator-proximal portion of the β-galactosidase polypeptide chain in the cell-free system would be useful as an assay for gene activity.

The detection of the operator-proximal fragment of β-galactosidase called α has been made possible by the technique of intracistronic complementation developed by Ullmann, Jacob, and Monod.[4] If the α fragment of β-galactosidase is mixed with

an extract containing a suitable complementing protein with a deletion in the α region, β-galactosidase activity results. Noncomplemented extracts of either α or the α-complementing protein show no such activity.

In previous studies[10] the α fragment was synthesized in a cell-free system containing a so-called S-30 extract from a lac^- strain of *E. coli* in the presence of ϕ80*lac* DNA and a variety of other components (see *Methods*). The *de novo* synthesis of α was detected after formation by adding an equal volume of suitably prepared extract from another strain containing a complementing protein which combines with the α fragment to produce active β-galactosidase. The amount of enzyme activity was quantitatively estimated by the standard assay procedure of determining the rate of digestion of the substrate ONPG.

In most of the present studies it has been convenient to carry out synthesis and complementation in one step. This is accomplished by using an S-30 extract prepared from strain 21F′ which carries the α-complementing protein. Under these conditions complementation takes place during the synthetic period. As before, the synthesis is completely dependent upon the presence of intact ϕ80*lac* DNA. A typical β-galactosidase assay of the product of this system is shown in Figure 1. The rate of digestion of substrate ONPG is linear for at least 45 hours. In further unpublished observations it has been found that a linear rate continues for over 80 hours, attesting to the stability of the enzyme. If 2 μg/ml of actinomycin D is added or if UTP and CTP are omitted in the synthesis step, no enzyme activity is produced and the peptide synthesis measured by C^{14}-leucine incorporation is reduced by more than 90 per cent. Since both of these factors are known to affect transcription rather specifically,[13] this can be taken as strong evidence that ϕ80*lac* DNA is directing the synthesis of α through a messenger RNA intermediate.

It should be emphasized that α has been defined by the genetic complementation test as the operator-proximal segment corresponding to one fifth to one quarter of the entire length of the β-galactosidase polypeptide chain. Since the entire chain is 1173 amino acids in length, this would make the α fragment somewhere between 200 to 300 amino acids in length. It seems likely that there is considerable polydispersity in the β-galactosidase polypeptide fragments synthesized in the cell-free system and that only a fraction of these are in the size range that gives effective complementation. The central concern here is that this fraction can be quantita-

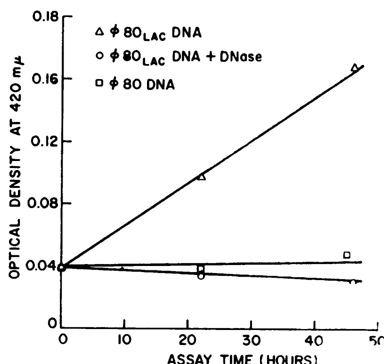

FIG. 1.—Digestion of ONPG substrate by standard incubation mixtures which contained 6500 μg protein/ml of S-30 extract from strain 21F′ and 200 μg/ml of ϕ80*lac* DNA, or of ϕ80 DNA, or of ϕ80*lac* DNA + 5 μg/ml DNase. Incubation conditions for synthesis and assay are described in *Methods*. The digestion of substrate as measured by the increase in optical density at 420 mμ occurs only when intact ϕ80*lac* DNA is present in the incubation mixture. The enzyme digests substrate at a linear rate for at least 45 hr. With either ϕ80*lac* DNA or ϕ80 DNA the gross leucine incorporation in this system is 2.4 $\pm$ 0.2 mμmoles. Addition of DNase lowers the incorporation by more than 90%.

tively estimated and that its formation requires the presence of the gene for β-galactosidase. Repression of the gene *in vitro* should be detectable by a lowering of the α formed. Before experiments aimed at demonstrating specific repression can be discussed, it is necessary to describe the results on the partial purification of repressor.

Description of partially purified repressor: Gilbert and Müller-Hill[11] were the first to develop a scheme for the purification of the *lac* operon repressor. This scheme is based on the hypothesis that repressor binds inducer. At various stages during purification it should be possible to assay for enrichment in repressor by measuring the binding to inducer. Binding measurements were made by the technique of equilibrium dialysis using C^{14}-IPTG. Gilbert and Müller-Hill were able to achieve some purification of repressor from a crude extract and to show that this IPTG binding substance was a protein. Our goal was to purify the repressor to a sufficient degree so that small amounts of it could be added to a cell-free system to specifically inhibit β-galactosidase synthesis. Partially purified repressor was made from strain r1F by a new procedure described in *Methods*. At various stages in the purification the preparation was characterized according to the method of Gilbert and Müller-Hill (see Table 1). When the same isolation method was used with strain M107 lacking repressor, the yields of protein in the various fractions were found to be about the same but no binding of IPTG was observed in the corresponding fractions II–IV.

TABLE 1

BINDING OF IPTG BY REPRESSOR-CONTAINING FRACTIONS ISOLATED FROM STRAIN r1F

Fraction	Total yield of protein (mg)	μμMoles IPTG bound/mg protein	Concn. of protein in binding expt., (mg/ml)	Excess bound as % of concn. outside dialysis sac
I	2640	—	—	—
II	880	0.84	22	122
III	210	3.94	7.0	126
IV	55	3.76	11	127

In Figure 2 the ultraviolet absorption spectrum of the purest material, fraction IV, is shown. The maximum is at 278 mμ. There is no indication of substance with an absorption maximum of 260 mμ, so that little if any nucleic acid is present. The absorption spectrum of fraction IV prepared from strains r1F and M107 is similar in this respect.

By combining the information on binding of IPTG obtained here with the estimates of Gilbert and Müller-Hill[11] for the intrinsic association constant for the repressor-inducer complex and the molecular weight of repressor, a rough estimate of the repressor concentration and purity of fraction IV was obtained. The intrinsic association constant, K_{RI}, for the repressor-inducer complex is 0.77×10^6. (Note: this is the reciprocal of the K_m which Gilbert and Müller-Hill refer to incorrectly as the binding constant.) If (R_T) is defined as the molar concentration of inducer binding sites, then $(R_T) = (R) + (RI)$ where (R) is the molar concentration of available binding sites and (RI) is the molar concentration of complexed binding sites. The equation for the intrinsic association constant is $K_{RI} = (RI)/(R)(I)$, where (I) is the molar concentration of free inducer.[14] K_{RI} is given, (I) is known from the concentration of IPTG outside the dialysis sac in the binding study,

(RI) is known from Table 1, and hence (R) and (R_T) can be calculated from the above equations. (R_T) is found to be 47 mμM for a solution of fraction IV containing 1 mg/ml of protein. Gilbert and Müller-Hill have estimated the molecular weight of repressor as 2×10^5 and believe that each repressor molecule contains two IPTG binding sites. Using this information it is calculated that the protein of fraction IV is 0.47 per cent repressor. Fraction IV prepared from strains r1F and M107 was used in subsequent studies.

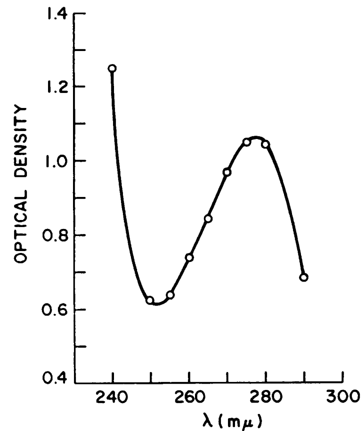

FIG. 2.—Ultraviolet absorption spectrum of the partially purified repressor, fraction IV, prepared from strain r1F in buffer C (see *Methods*).

Experiments designed to test the effect of repressor on synthesis in the cell-free system: The design of these experiments was predicated on the assumption that the inducer, IPTG, which is believed to function by interfering with the repressor-operator complex, should increase α synthesis in the cell-free system when repressor is present but should have no effect on synthesis when repressor is absent. In these experiments IPTG was used at a final concentration of 10^{-3} M since this concentration has been shown to give full derepression *in vivo* in mutants that cannot actively concentrate inducer inside the cell.[15] The synthesis of α was studied in standard incubation mixtures described above in which the S-30 extract contained or lacked repressor. The α formed when the S-30 extract was prepared from strain M107 (lacking repressor) was two to three times higher than when the S-30 extract was prepared from strain r1F (containing repressor). It seemed likely that the lower yield in the latter case was due to the presence of repressor. To test this, 10^{-3} M IPTG was added before synthesis and this increased the yield of α in the repressor-containing extract to the higher level. These results suggest that α synthesis is specifically repressible in the cell-free system and that the repression is largely eliminated in the presence of 10^{-3} M IPTG as it is *in vivo*.[15]

In the next series of experiments the effect of adding varying amounts of partially purified repressor to an incubation mixture otherwise lacking repressor was studied. In these studies S-30 extract from strain 21F′ was used and β-galactosidase activity was measured after the synthesis as already described. These results are presented in Figure 3. The amount of enzyme activity formed in the absence of both repressor and inducer is assigned the value 100. The repressor extract used was the previously characterized fraction IV prepared either from the repressor-carrying strain r1F or the repressor-lacking strain M107. The amount of repressor extract added is expressed as the fraction (fraction IV protein)/(S-30 extract protein). The repressor concentration of fraction IV prepared from strain r1F has already been estimated, and from the amount of fraction IV used it can be estimated that at the three concentrations of fraction IV studied, the repressor concentration is 2.6, 5.2, and 10.8 mμM. The amount of virus DNA added is 200 μg/ml in all the experiments. From the fact that the viral DNA molecular weight is 30×10^6 and that the DNA preparation contains approximately equal amounts of φ80 DNA and φ80*lac* DNA it can be calculated that the *lac* operator concentration

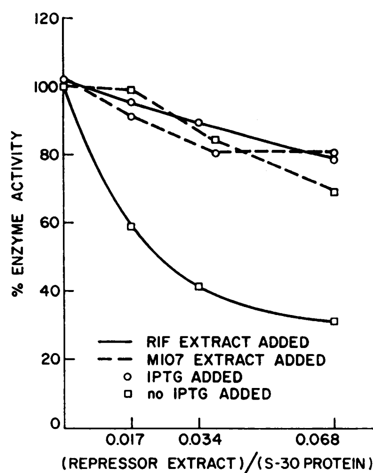

FIG. 3.—Amount of enzyme formed as a function of repressor concentration with or without 10^{-3} *M* IPTG. The enzyme activity is determined from the rate of digestion of ONPG; the amount of enzyme activity found when fraction IV and IPTG are absent is defined as 100%. The amount of fraction IV added is expressed as the ratio of protein from the added fraction IV to the protein in the S-30 extract. Except for the addition of fraction IV and IPTG the conditions for synthesis and assay are the same as those used in Fig. 1. All assays were run for 45 hr.

is 3.7 mμM. This gives a repressor/operator ratio at the three levels of repressor studied of 0.7, 1.4, and 2.8. Because of the number of approximations and assumptions that go into the calculation of the repressor concentration, these ratios should only be regarded as a rough estimate.

In Figure 3 it can be seen that the repressor-containing extract causes a pronounced lowering of enzyme formed to about 30 per cent of normal at the highest repressor concentration studied. The effect of adding repressor appears to be approaching an asymptotic limit. The addition of fraction IV from strain M107 which does not contain repressor causes some lowering of enzyme formed. Addition of 10^{-3} *M* IPTG is without effect on the latter system but it raises the level of enzyme formed in the system containing repressor so that the amount of enzyme formed in the two systems is the same. In all of the experiments reported the gross peptide synthesis was the same as reported in Figure 1. It appears that IPTG is completely reversing the effects of the *lac* operon repressor since the decrease that is not overcome by IPTG is common to fraction IV from both strains r1F and M107. It is concluded that repressor, whether normally present in the S-30 extract or added back to an S-30 extract after partial purification, can inhibit the synthesis of the α fragment of β-galactosidase and this inhibition can be reversed by 10^{-3} *M* IPTG, a specific inducer of the *lac* operon.

Summary.—Previously reported results on the synthesis of the operator-proximal segment of the β-galactosidase molecule in a cell-free system are confirmed and extended. Experiments are discussed that support the notion that the ϕ80*lac* DNA transmits the necessary information through a messenger RNA. That this system is inhibited by specific *i* gene-produced repressor can be demonstrated in two ways: (1) IPTG increases the synthesis two- to threefold in incubation mixtures containing repressor but not at all in incubation mixtures lacking repressor. (2) Partially purified repressor added back to an incubation mixture which does not contain repressor inhibits the synthesis of α. The inhibitory effect does not occur if the repressor is pretreated with 10^{-3} *M* IPTG.

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Abbreviations used: IPTG, isopropyl-1-thio-β-D-galactopyranoside; ONPG, o-nitrophenyl-β-D-galactopyranoside; φ80*lac*, φ80 virus containing the *lac* operon; ATP, CTP, GTP, and UTP, the 5′-triphosphates of adenosine, cytidine, guanosine, and uridine, respectively; PEP, phosphoenolpyruvate.

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