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Detection and Isolation of the Repressor Protein for the Tryptophan Operon of *Escherichia coli*

(β-galactosidase/transducing bacteriophage/cell-free extract/cyclic AMP/*lac* operon)

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ABSTRACT **DNA from a transducing bacteriophage carrying a fusion of the tryptophan and lactose operons of *E. coli* (λd*trp–lac*) has been used to direct cell-free synthesis of β-galactosidase (EC 3.2.1.23). Whereas normal *lac* operon (λd*lac*) DNA requires adenosine-3′:5′-cyclic monophosphate (cAMP) for β-galactosidase synthesis, *trp–lac* DNA is unaffected by cAMP. This difference in cAMP dependence verifies the presence of a cAMP-requiring promoter in the *lac* operon that has been removed from the *trp–lac* DNA. Synthesis with *trp–lac* DNA is controlled by the protein product of the tryptophan repressor gene (*trpR*). Synthesis in extracts of *trpR*$^-$ (repressor-negative) cells is progressively reduced by increased additions of extract from *trpR*$^+$ cells. No *trpR*$^-$ product repression is seen when β-galactosidase synthesis is programmed by normal *lac* DNA. This highly sensitive and specific assay has facilitated quantitation and partial purification of the *trp* repressor.**

The molecular mechanisms for the control of genes coding for biosynthetic functions remains to be elucidated. Control of amino-acid biosynthetic pathways in bacteria may be complex, and several different mechanisms may be involved (1). Expression of the tryptophan (*trp*) biosynthetic operon of *Escherichia coli* is repressed by tryptophan (or some product derived therefrom); this control is mediated by the *trpR* gene, which is genetically distant from the operon it controls (2). Genetic studies demonstrate that the product of the *trpR* gene is a cytoplasmic protein, produced in limited quantity within the cell; it performs no function indispensible to cell growth, and represents a specialized "negative control" regulatory protein—presumably, a "repressor" analogous to that of the *lac* operon (3).

Repression is also governed by an operator locus (*trpO*), lying at that end of the operon from which transcription begins (4). Since the intracellular tryptophan concentration is proportional to repression of the biosynthetic operon, it seems likely that tryptophan or a metabolic derivative of tryptophan serves as a "corepressor"; a possible regulatory role for Trp-tRNA or the activating enzyme appears unlikely (5, 6). The nature of the corepressor, and of the repressor mechanism itself, can only be established when the DNA–repressor interaction is studied in isolation. Toward this end, we have begun purification of the regulatory protein product of the *trpR* gene.

DNA-directed cell-free extracts for mRNA and protein synthesis have been most useful for study of mechanisms of gene regulation, particularly in the inducible operons *lac* (7), *ara* (8), and *gal* (9). Regulatory proteins have been detected, isolated, and characterized. We here report the use of this approach to study the repressible *trp* system, in which we have detected the repressing activity of the *trpR* gene product in crude and fractionated cell-free extracts. The sensitivity and specificity of this functional assay have allowed partial purification of the repressor protein.

METHODS

Bacterial Strains. Otherwise isogenic *trpR*$^+$ and *trpR*$^-$ derivatives of the strain previously used in studies of cell-free β-galactosidase synthesis (10) were obtained as follows. The parental strain (*E. coli* CA778, F Δ*lac trp tsx str A*) was transduced to *trp*$^+$ with a P1 lysate of a wild-type strain. A *thr*$^-$ auxotroph was obtained by ultraviolet-light mutagenesis, followed by penicillin enrichment. The *thr*$^-$ strain was tranduced to *thr*$^+$, using a P1 lysate of a strain that is *thr*$^+$ *trpR*$^-$ (W. J. Schrenk, manuscript in preparation). There was about 80% cotransduction of the *trpR*$^-$ allele with *thr*$^+$; *trpR*$^+$ and *trpR*$^-$ derivatives were identified among the *thr*$^+$ transductants by replica plating with 5-methyltryptophan, and verified by assays of tryptophan synthase (EC 4.2.7.20) (2).

Viral Strains. λd*lac* DNA was isolated from a doubly-lysogenic strain containing λh80 and λh80*lac*. λd*trp–lac* DNA was isolated from a doubly-lysogenic strain containing λh80 and λh80d*trp–lac*. Full details of the isolation of the λh80d*trp–lac* phage are described elsewhere (10). The DNA isolation procedures have been described (4).

Conditions for Cell-Free β-Galactosidase Synthesis and Assay. Except for slight modifications described herein, all procedures used for synthesis, enzyme assay, and preparation of bacterial extracts and DNA have been described in detail (10). The procedures for synthesis and assay are: The incubation mixture contains per ml: 44 μmol of Tris–acetate (pH 8.2); 1.37 μmol of dithiothreitol; 55 μmol of KAc; 27 μmol of NH_4OAc; 14.7 μmol of $Mg(OAc)_2$; 7.4 μmol of $CaCl_2$; 0.22 μmol each of 20 amino acids; 2.2 μmol of ATP; 0.55 μmol

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(each) of GTP, CTP, and UTP; 21 μmol of phosphoenolpyruvic acid; 0.5 μmol of cAMP when λd*lac* DNA is used, no cAMP added when λd*trp–lac* DNA is used; 100 μg of *E. coli* tRNA; 27 μg of pyridoxine·HCl; 27 μg of TPN; 27 μg of FAD; 11 μg of *p*-aminobenzoic acid; 27 μg of folinic acid. The above ingredients are incubated for 3 min at 37° with either λd*lac* DNA or λd*trp–lac* DNA, with shaking, before 6.5 mg of S-30 extract protein is added. Incubations with shaking are allowed to continue for 60 min at 37°. After synthesis is complete, a 0.2-ml aliquot is removed and mixed with 1.5 ml of assay buffer containing 0.53 mg of *O*-nitrophenyl-β-D-galactoside–0.1 M sodium phosphate (pH 7.3)–0.14 M 2-mercaptoethanol. After a suitable length of time (1–40 hr), the mixture is treated with one drop of glacial acetic acid, chilled, and centrifuged to remove the precipitate. The supernate is mixed with an equal volume of 1 M Na_2CO_3 and read against water in a 1-cm quartz cell at a wavelength of 420 nm. A zero-time value of 0.035 is subtracted from all readings. Duplicate analyses usually agree within 2%.

Partial Purification of trp Repressor. Cells of the *trpR*$^+$ strain described above are grown (10) and frozen at −70° in 6-mm thick sheets until ready for use. 200 Grams of paste are allowed to soften at 5° for 30 min. The paste is homogenized in 700 ml of buffer I [0.01 M Tris-OAc (pH 8.2)–0.01 M $Mg(OAc)_2$–0.06 M KCl–0.01 M 2-mercaptoethanol–5% glycerol]. The resulting suspension is centrifuged at 10,000 rpm for 30 min in a large Sorvall rotor. The supernatant is discarded, and the sediment is suspended in buffer I and recentrifuged, again discarding the supernatant. The sediment is suspended in 260 ml of buffer I, and the resulting suspension is lysed in a French pressure cell at pressures between 4000 and 8000 psi. The lysate is centrifuged at 10,000 rpm for 30 min in a large Sorvall rotor. The lysate is centrifuged an additional 3.5 hr at 30,000 rpm in a Spinco 30 rotor. The resulting supernatant is dialyzed overnight against buffer II [0.01 M KH_2PO_4–KOH (pH 7.7)–5% glycerol–0.7 mM dithiothreitol]. The solution is passed through a 600-ml DEAE-cellulose column (0.94 meq/g) previously equilibrated with buffer II. After the solution has passed into the column, the column is rinsed with another 2 liters of buffer II. The cellulose-bound protein is eluted with buffer II containing 0.25 M NaCl. The appropriate portion of the eluate is easily detected by its yellow color. The eluate is pooled, and 2 volumes of saturated $(NH_4)_2SO_4$ are added to precipitate the bulk of the protein, which is collected by centrifugation for 5 min at 10,000 rpm in a large Sorvall rotor. The sediment is suspended in 100 ml of buffer III [0.01 M KH_2PO_4–KOH (pH 6.4)–5% glycerol–0.7 mM dithiothreitol] and dialyzed overnight against the same buffer. A slight turbidity is removed from the solution by centrifugation for 5 min at 10,000 rpm in a large Sorvall rotor. The solution is passed through a 120-ml phosphocellulose column (Whatman P11, 7.4 meq/g), previously equilibrated with buffer III. After an additional 200 ml of buffer III containing 0.1 M KCl is passed through the column, 200 ml of buffer III containing 0.25 M KCl is added, and peak fractions (detected by 280-nm absorption) are collected, pooled, and dialyzed against buffer IV [0.01 M Tris–OAc (pH 8.2)–0.014 M $Mg(OAc)_2$–0.06 M KOAc–0.7 mM dithiothreitol–5% glycerol]. The resulting solution, containing about 40 mg of protein (in 30 ml), can be used directly or stored indefinitely in nitrocellulose tubes in liquid N_2.

RESULTS AND DISCUSSION

Trp-repression in a cell-free system

Anticipating difficulty in detecting a low level of *in vitro* enzyme synthesis for any of the enzymes normally associated with the *trp* operon, we have taken a novel approach to the study of *trp* regulation. To facilitate detection of the repressing effect of the *trpR* product in DNA-directed cell-free protein synthesis, we have used the previously developed system for β-galactosidase synthesis (10), using a unique transducing bacteriophage DNA template in which the β-galactosidase gene of the *lac* operon has been placed under *trp* control. Genetic transposition and deletion has fused the *lac* operon into the *trp* operon to yield the W1 fusion operon. This operon contains the *trp* promoter–operator, part of the *trp* E gene, and the *lac Z*, *y*, and *a* genes in that order. *In vivo* synthesis of β-galactosidase encoded by the *Z* gene in the W1 fusion is regulated by tryptophan in the presence of the *trpR*$^+$ product, and constitutive in the absence of this product (12); this synthesis results from a polycistronic mRNA molecule, whose synthesis commences at the *trp* promoter (13).

The DNA-directed cell-free system for β-galactosidase synthesis has been used in gene regulation studies for several years (10). This system comprises a cell-free extract of *E. coli* (S-30 extract), DNA from defective transducing phages carrying either the normal *lac* operon (λd*lac*) or the W1 *trp–lac* fusion (λd*trplac*), and those cofactors and substrates necessary for RNA and protein synthesis. The amount of β-galactosidase synthesized in the cell-free system is proportional to the amount of active DNA containing the *lac* operon present. Two bacterial strains were used as a source for S-30 preparation; these strains are isogenic, except for the *trpR*$^+$ allele in one and the *trpR*$^-$ allele in the other. Both strains carry deletions of the entire *lac* operon, so that any β-galactosidase observed in these extracts must represent *de novo* synthesis directed by exogenous bacteriophage DNA. The activities of λd*lac* DNA and λd*trp–lac* DNA were compared at all stages of this investigation.

When the *trpR*$^-$ S-30 is used, it is found that the λd*trp–lac* DNA (*trp–lac* DNA) is about 25% as active as the λd*lac* DNA (*lac* DNA) in β-galactosidase production. The *lac* DNA requires cAMP for β-galactosidase synthesis, but the *trp–lac* DNA is unaffected by cAMP. This difference in cAMP dependence verifies the presence of a normal cAMP-requiring promoter in the *lac* operon (7) that has been removed in the *trp–lac* DNA. The activity of *lac* DNA in *trpR*$^-$ and *trpR*$^+$ S-30 extracts is the same, within experimental error. In contrast, the activity of *trp–lac* DNA in *trpR*$^+$ S-30 is drastically reduced. The contrasting results with the two DNAs strongly suggest that the reduction is the result of specific repression by the *trp* repressor present in the *trpR*$^+$ S-30 extract. Further experiments were done in which the *trpR*$^-$ and *trpR*$^+$ S-30 extracts were mixed in various proportions. Some of these results are illustrated in Fig. 1. It can be seen that the yield of β-galactosidase when *lac* DNA is used is fairly constant over the entire range of S-30 concentrations. In contrast, when *trp–lac* DNA is used the yield of β-galactosidase drops to about 10% of its value in a pure R$^-$ S-30. Even at the highest concentrations of *trp* repressor, about 10% of the β-galactosidase synthesized cannot be repressed. This lack of complete repression at saturating concentrations of repressor has also been observed in the study of *lac* operon repression,

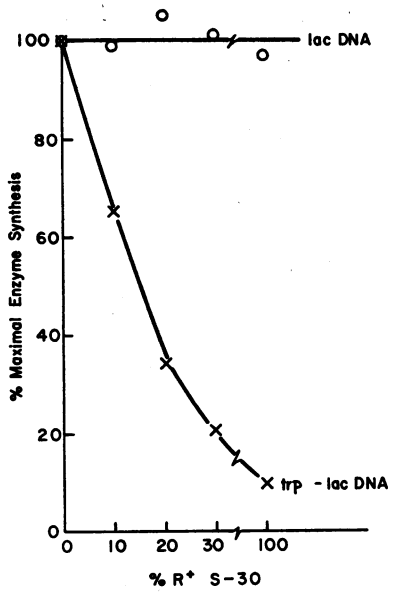

FIG. 1. β-Galactosidase synthesis as a function of S-30 composition. A series of experiments was performed in which S-30 extracts of different composition were used. Pure *trpR*⁻ S-30, pure *trpR*⁺ S-30, or mixtures thereof were used; the percentage of the *trpR*⁺ extract is indicated on the *abscissa*. λd*lac* and λd*trp–lac* DNA were used as indicated. The concentration of DNA is 0.78 nM. On the *ordinate*, 100% corresponds to 2×10^{-3} units/ml of β-galactosidase when λd*lac* DNA is used, and to 0.5×10^{-3} units/ml of β-galactosidase when λd*trp–lac* DNA is used (10).

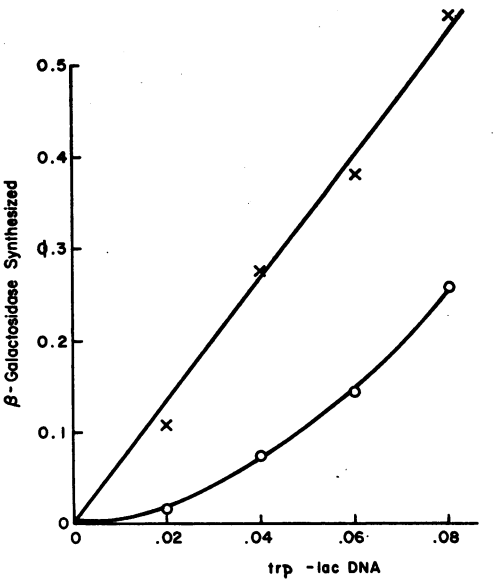

FIG. 3. β-Galactosidase synthesis as a function of λd*trp–lac* DNA concentration. Parallel experiments were executed with pure *trpR*⁻ S-30 (×——×) and with *trpR*⁻ S-30 with partially-purified repressor (54 μg/ml) added (O——O). The *abscissa* indicates DNA concentration in arbitrary units; 0.02 is equivalent to 0.78 nM λd*trp–lac* DNA in the incubation mixture. The *ordinate* indicates β-galactosidase activity in arbitrary units; 0.1 is equivalent to 2×10^{-3} units/ml (10).

and it is probably due to some initiation of RNA synthesis at points other than the usual promoter that cannot be repressed (14).

It should be remembered that the presence of repression in the DNA-directed cell-free system implies the presence of adequate corepressor, whether it be tryptophan, Trp-tRNA, or some other tryptophan derivative. Tryptophan and Trp-tRNA are of course present in the system, since protein is being synthesized.

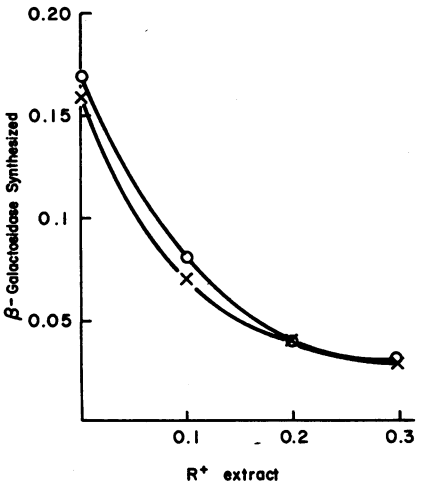

FIG. 2. β-Galactosidase synthesis as a function of added partially-purified *trp* repressor. In all experiments λd*trp–lac* DNA was used. ×——×: extract immediately before phosphocellulose treatment; O——O: phosphocellulose eluate. 0.1 Unit on the *abscissa* corresponds to 18 μg/ml of phosphocellulose eluate, and about 40 times this value for the untreated extract. The *ordinate* indicates β-galactosidase activity in arbitrary units; 0.1 is equivalent to 2×10^{-3} units/ml (10).

Partially purified tryptophan repressor

The concentration of tryptophan repressor estimated in crude extracts is very low (see below), making total purification a difficult task. Fortunately, many interesting experiments will in all probability not require total purification. Thus far we have achieved about 55-fold enrichment with respect to the total *E. coli* protein, and removed all the nucleic acid. The enrichment factor is based on the amount of repressor-containing extract required to produce 50% repression in the cell-free system. In essence, the purification procedure consists of removal of membranes and ribosomes from a crude lysate by high-speed centrifugation, removal of all nucleic acid by absorption of the protein and nucleic acid to DEAE-cellulose followed by selective desorption of the protein, and removal of some extraneous protein by fractionation on phosphocellulose. Most of the fractionation of protein and resulting enrichment occurs in the last step. It is interesting that the *trp* repressor interacts strongly with phosphocellulose, as do many proteins that bind to nucleic acid. There is no direct evidence that *trp* repressor binds to DNA.

The partially purified repressor has been studied for its inhibitory effect on β-galactosidase synthesis (see Fig. 2), using a pure *trpR*⁻ S-30 and various amounts of *trpR*⁺ extract taken before and after the phosphocellulose purification step. Increasing amounts of *trpR*⁺-containing extract lead to increased repression. Even though these extracts differ by about 40-fold in total protein concentration, they repress to

about the same extent; this result demonstrates a 40-fold purification on phosphocellulose.

In Fig. 3, the yield of β-galactosidase is determined as a function of the amount of *trp–lac* DNA in the cell-free system. When *trpR*⁻ S-30 is used, the amount of β-galactosidase synthesized is directly proportional to the amount of *trp–lac* DNA. In contrast, if the partially purified *trp* repressor is added to the *trpR*⁻ S-30, the synthesis of β-galactosidase is greatly reduced. The dependence on DNA concentration shows a function with increasing upward curvature, approaching the slope of the straight line for the experiment where no *trpR*⁺ extract is added. This result is consistent with the idea that as the amount of DNA is increased, less of the *trp* operator on the DNA is complexed with repressor, because of the limiting amount of repressor. When ordinary *lac* DNA is used, the amount of β-galactosidase synthesized is directly proportional to the amount of DNA, and the addition of *trpR*⁺ extract is without effect.

The amount of tryptophan repressor in a cell-free extract may be estimated

At 15% *trpR*⁺ S-30, about half of the maximum extent of represson is achieved when the concentration of *trp–lac* DNA is 0.78 nM, as in Fig. 1. Incomplete repression at this point could be due to either of two effects: a finite dissociation constant of the repressor–operator complex or a lack of sufficient repressor to complex all the tryptophan operator on the added *trp–lac* DNA. That the lack of sufficient repressor to complex the operator is the main effect producing incomplete repression at a low level of *trpR*⁺ S-30 was shown by studying the results obtained from adding more *trp–lac* DNA (see Fig. 3). Below saturating levels of S-30, the percent repression decreases the higher is the concentration of *trp–lac* DNA. From such results, it should be possible to roughly estimate the concentration of repressor in the S-30 extract. At 50% repression, where half the DNA is complexed with repressor, the total repressor concentration must be about half the DNA concentration. In the experiment reported in Fig 1, the DNA concentration is 0.78 nM (calculated from the concentration of added DNA and the molecular weight of λDNA, which is 30×10^6); therefore, the repressor concentration at 50% repression should be about 0.39 nM. In a pure *trpR*⁺ S-30 there would be about 2.6 nM repressor. The molecular weight of the repressor has been estimated (7) from its position of elution from Sephadex G-100 as 5.8×10^4. We know that there is about 6.5 mg/ml of total protein in the cell-free extract; from these numbers, we can calculate that about 0.002% of the protein in the S-30 is *trp* repressor. Our purest preparations of *trp* repressor are only about 0.2% pure based on these estimates. In a single cell, there would be about 10 molecules of repressor. This value is in accord with previous genetic data (3), and is about the same as the number of *lac* operon repressor molecules calculated for the whole cell (15). Because of the high binding constant between repressor and operator, and the fact that a normal cell only contains one *trp* operator per *E. coli* chromosome, it is not surprising that the concentration of the *trp* repressor is much lower than the concentration of a typical enzyme molecule.

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