PROPERTIES OF TWO REGULATING GENES FOR ALKALINE PHOSPHATASE

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ABSTRACT

GAREN, A. (University of Pennsylvania, Philadelphia) and H. ECHOLS. Properties of two regulating genes for alkaline phosphatase. J. Bacteriol. 83:297-300. 1962.—A constitutive mutation in one of the two regulating genes for alkaline phosphatase has two effects on the synthesis of the enzyme: repression of synthesis is blocked, and the cell’s capacity to produce the enzyme is reduced. The behavior of this type of mutation has been studied in heterozygotes. The results suggest that the product of this regulating gene functions as an inducer as well as a repressor.

The synthesis of the enzyme alkaline phosphatase in Escherichia coli K12 is repressed in a medium containing a high concentration of orthophosphate (Torriani, 1960). Repression of this enzyme is under the genetic control of two separate genes, R1 and R2 (Echols et al., 1961). A mutation in either of the genes can produce a constitutive mutant (i.e., one that is not repressible by high concentrations of orthophosphate). Both genes appear to be involved in the formation of a “repressor” for alkaline phosphatase, analogous to the role of the regulating gene, i, for b-galactosidase (Pardee, Jacob, and Monod, 1959). This conclusion is based on the observation that repressibility is dominant over constitutivity in heterozygous diploids which carry a constitutive gene, either cis or trans, to the active structural gene for alkaline phosphatase (Echols et al., 1961).

In a study of the synthesis of alkaline phosphatase by constitutive mutants, a difference was noted between the behavior of R1− and R2− mutants (Echols et al., 1961). It was found that, under conditions of nonrepression (low concentration of phosphate), most of the R1− mutants produced less enzymatic activity than either the R2− mutants or the original repres-
sible strain. This indicates that an R1− mutation can affect alkaline phosphatase synthesis in two ways: repression in high phosphate is prevented, and the cell’s capacity to synthesize the enzyme is reduced. The latter effect has not been observed with any of the R2− mutants, nor with all of the R1− mutants; one R1− mutant produces a full quota of enzymatic activity in a low concentration of phosphate, but is somewhat repressed in a high concentration of phosphate. (We shall refer to this mutant as the Rlb− type, and to the other R1− mutants as the R1α− type.) These findings are summarized in Table 1.

The effect of an R1α− mutation shows that the R1+ gene is involved not only in the repression of alkaline phosphatase synthesis but also in the determination of the maximum level of synthesis attainable. In the present report we examine the relationship between these two effects.

MATERIALS AND METHODS

Strains. The wild-type (R1+ R2+ P+) is the Hfr strain K10 of E. coli K12. The constitutive mutations are derived from the wild type in a single step, as described in an earlier report (Echols et al., 1961). The double-mutant strains, R1a− R2− and R1b− R2−, were prepared by crossing the constitutive mutations from the Hfr, in which they were originally isolated, into an F− strain. The F′ diploid strains were prepared by crossing F− strains with strain F′13 (originally isolated by Dr. Hirota); F′13 carries an episome, containing the R1 and P genes, which is transferred to an F− mating partner. The P− mutation incorporated into the F′ strains is from the mutant U8, which does not produce any protein that is related serologically to alkaline phosphatase (Leventhal, 1959; Garen, 1960).

Media. The media and conditions of culture were described in an earlier report (Echols et al., 1961).
TABLE 1. Alkaline phosphatase activity in repressible and nonrepressible strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzymatic activity:cell density ratio</th>
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<tbody>
<tr>
<td></td>
<td>Low phosphate concn</td>
</tr>
<tr>
<td>R1+ R2+ R+</td>
<td>8.6</td>
</tr>
<tr>
<td>R1a- R2- P+</td>
<td>3.7</td>
</tr>
<tr>
<td>R1b- R2+ P+</td>
<td>12</td>
</tr>
<tr>
<td>R1+ R2- P+</td>
<td>12</td>
</tr>
</tbody>
</table>

* The cultures were grown to maximum cell density in S medium (Echols et al., 1961) containing a growth-limiting concentration of glucose (0.025%) and a high concentration of phosphate, and were subcultured 100 times in two media: S medium with low glucose (0.025%) and high phosphate concentrations, and S medium with high glucose (0.025%) and low phosphate (5 X 10^-5 M KH2PO4) concentrations. The subcultures were grown to maximum cell density at 37 C with aeration; approximately the same maximum density was reached in both media. The optical density of each culture was read at 540 m, and a sample was prepared for enzyme assay by centrifuging, resuspending the cells in 0.1 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.4), and shaking the cell suspension with toluene. Enzyme assays were performed as described in the text. It should be noted that the activity (8.6 units) of the repressible strain R1+ R2+ in low phosphate concentration is equivalent to the activity (12 units) of the constitutive strains R1b- R2+ and R1+ R2-, since the repressible strain does not begin to produce the enzyme in large amounts until the terminal growth stage when conditions for nonrepression have been established in the culture.

RESULTS

It was first necessary to establish whether the reduced level of alkaline phosphatase activity observed with R1a- mutants was due to a reduced level of enzyme protein, or to an inhibitory effect on the functioning of the enzyme. This question was resolved by measuring the amount of enzyme protein produced by an R1a- mutant in a low phosphate concentration as compared with an R2- mutant and the wild-type repressible strain. As an indicator of protein synthesis, the incorporation of C14-proline into alkaline phosphatase was followed. The C14-proline was added to the cultures after conditions for nonrepression had been established, as indicated by the appearance of alkaline phosphatase activity in the wild-type culture. The cultures of the wild-type and R2- mutant incorporated, respectively, 6.4 and 7.2% of the cellular C14-proline into the purified alkaline phosphatase fraction; a culture of the R1a- mutant incorporated only 2.5% (Table 2). We can, therefore, adequately account for the reduced level of alkaline phosphatase activity obtained with R1a- mutants by the reduction in amount of enzyme synthesized.

TABLE 2. Synthesis of alkaline phosphatase under nonrepressed growth conditions*

<table>
<thead>
<tr>
<th>R genotype</th>
<th>Cellular C14-proline present in alkaline phosphatase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1+ R2+</td>
<td>6.4</td>
</tr>
<tr>
<td>R1+ R2-</td>
<td>7.3</td>
</tr>
<tr>
<td>R1a- R2+</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Cultures were grown to a density of 4 X 10^9/ml in TG medium (Echols et al., 1961) containing 1.6 X 10^-4 M KH2PO4, and were centrifuged and resuspended in an equal volume of TG medium without phosphate. After 90 min aeration at 37 C (at which time the standard R1+ R2+ culture had started to produce a high level of alkaline phosphatase activity), C14-proline was added and aeration continued for 2 hr. The cultures were centrifuged, resuspended in a medium of 0.05 M tris buffer (pH 7.4) plus 0.02 M MgSO4, and put through a French pressure cell to break open the cells. The suspension was assayed for C14 (to measure total cellular C14-proline), treated with deoxyribonuclease, and centrifuged for 60 min at 35,000 rev/min. The supernatant was treated with ribonuclease, heated to 75 C for 5 min, and dialyzed overnight at 4 C against 0.01 M tris (pH 7.4). The dialyzate was centrifuged at 10,000 rev/min for 20 min, and the supernatant was added to a column of diethylaminoethyl cellulose (DEAE); under these conditions alkaline phosphatase attaches to the column. Elution was carried out with a linear gradient of NaCl ranging from 0 to 0.2 M and buffered at pH 7.4 with 0.01 M tris. The samples were assayed for enzymatic activity, and those with activity were pooled and dialyzed against 0.01 M tris (pH 7.4). The dialyzate was chromatographed again on DEAE cellulose, and the samples were assayed for enzymatic activity and C14. The peaks obtained for enzymatic activity and C14 were superimposable. The samples with enzymatic activity were pooled and assayed for total C14. This value was used for the amount of C14-proline in alkaline phosphatase.
The marked difference in alkaline phosphatase synthesis between R1a⁻ and R2⁻ mutants raises the question of how a double mutant, carrying both R1a⁻ and R2⁻ mutations, behaves: is the phenotype that of an R1a⁻ or R2⁻ mutant? The results reported in Table 3 demonstrate the epistasis of the R1a⁻ gene over the R2⁻ gene.

The same question can be asked about a double mutant carrying both R1b⁻ and R2⁻ mutations. The R1b⁻ single mutant, in contrast to an R1a⁻ mutant, produces a high level of enzyme in a low phosphate concentration, but is partly repressed in a high phosphate concentration (Table 1). The double mutant is not repressed in a high phosphate concentration, as shown in Table 3. This result demonstrates the epistasis of the R2⁻ gene over the R1b⁻ gene.

We then examined the behavior of a partial diploid F' strain heterozygous for the Rl and P genes (R1⁺ P⁻/R1a⁻ P⁺ R2⁺). The key difference between this diploid and a haploid R1a⁻ strain is the presence in the diploid of an R1⁺ gene in a trans position relative to the P⁺ gene. It was shown previously (Echols et al., 1961) that the synthesis of alkaline phosphatase by a trans diploid is repressed in high phosphate. The point now in question is whether the synthesis in low phosphate concentrations corresponds to the reduced level of enzyme characteristic of an R1a⁻ strain or to the high level characteristic of an R1⁺ strain. A high level of enzyme is produced by the trans diploid (Table 4), equaling the level in the control F' strain, which has an R1⁺ gene on both its episome and chromosome. Thus, the two phenotypic characteristics of an R1⁺ gene, that of a high level of nonrepressed synthesis in a low concentration of phosphate and of repression in a high concentration of phosphate, are dominant in the trans diploid. This indicates that the expression of both char-

**TABLE 3. Alkaline phosphatase activity in double-mutant strains**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Enzymatic activity: cell density ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low phosphate concn</td>
</tr>
<tr>
<td>R1α⁻ R2⁻ P⁺</td>
<td>3.4</td>
</tr>
<tr>
<td>R1β⁻ R2⁻ P⁺</td>
<td>12</td>
</tr>
</tbody>
</table>

* The conditions for these experiments were the same as in Table 1.

**TABLE 4. Alkaline phosphatase activity in partial diploid strains**

<table>
<thead>
<tr>
<th>Diploid genotype</th>
<th>Enzymatic activity: cell density ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells grown in high phosphate concn</td>
</tr>
<tr>
<td>R1⁺ P⁻</td>
<td>R1α⁻ P⁺ R2⁺</td>
</tr>
<tr>
<td>R1⁺ P⁻</td>
<td>R1⁺ P⁺ R2⁺</td>
</tr>
</tbody>
</table>

* The conditions for these experiments were the same as in Table 1.

† This slightly high value for a repressed culture probably reflects the presence of 4% haploid R1α⁻ R2⁺ P⁺ segregants found upon plating the culture used for enzyme assay.

**DISCUSSION**

The present results show that one of the two regulating genes for alkaline phosphatase, the R1⁺ gene, specifies the formation of a substance which exercises two controlling functions: it is essential for both the realization of a high level of alkaline phosphatase synthesis (amounting to about 7% of the total cellular protein) under conditions of nonrepression, and for the repression of synthesis that occurs in a high concentration of phosphate. The R2⁺ regulating gene differs in this respect, being required only for repression and not for the high level of nonrepressed synthesis.

It is possible to explain the dual effect of the R1⁺ gene by the following scheme:

\[
\text{R1⁺ gene} \rightarrow \text{inducer} \rightarrow \text{R1⁺ gene} \rightarrow \text{repressor}
\]

The term "inducer" designates the substance required by the cell to achieve a high level of alkaline phosphatase synthesis. It is assumed that, in a high phosphate concentration, the inducer can be transformed into a repressor, and that this transformation requires a function controlled by the R2⁺ gene. The formation of the repressor results in a drastic reduction in the level of alkaline phosphatase synthesis.

This scheme can serve to explain all of the present observations. For an R1α⁻ mutant, we postulate that the mutation causes an alteration of the inducer that prevents its transformation...
into repressor and, at the same time, impairs its ability to function as an inducer. It is possible in this way to account for the epistasis of the Rtα− gene over the Rβ− gene in an Rtα− Rβ− double mutant (Table 3), since this strain should not be capable of forming a normal inducer. It is also possible to explain the dominance of the Rt+ gene in an Rt+ P−/Rtα− P+ heterozygote (Table 4), since the presence of an Rt+ gene in this strain should allow for the formation of a normal inducer and repressor.

The effect of an Rtβ− mutation (Table 1) can be attributed to another kind of alteration of the inducer, which partially blocks its transformation into repressor without impairing its ability to function as an inducer. This will explain the epistasis of the Rβ− gene over the Rtβ− gene in an Rtβ− Rβ− double mutant (Table 3), since the Rβ− mutation should prevent the formation of repressor. This explanation of alkaline phosphatase regulation differs from the model proposed for β-galactosidase (Pardee et al., 1959) in the assignment of an "inducer" function as well as a "repressor" function to the product of the Rt regulatory gene. This was done to account for the behavior of the Rtα− class of constitutive mutants, in which the capacity for alkaline phosphatase synthesis is reduced as a result of the mutation in the Rt gene. It is of interest that a constitutive β-galactosidase mutant has been isolated which produces a reduced amount of the enzyme with or without an inducer added to the growth medium (Monod and Jacob, personal communication). This indicates that a mutation in the regulating gene, i, can reduce the capacity for β-galactosidase synthesis, an effect similar to that of an Rtα− mutation on alkaline phosphatase synthesis.

ACKNOWLEDGMENTS

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LITERATURE CITED


