Coordinated, Differential Expression of Two Genes through Directed mRNA Cleavage and Stabilization by Secondary Structures

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Metabolic engineering and multisubunit protein production necessitate the expression of multiple genes at coordinated levels. In bacteria, genes for multisubunit proteins or metabolic pathways are often expressed in operons under the control of a single promoter; expression of the genes is coordinated by varying transcript stability and the rate of translation initiation. We have developed a system to place multiple genes under the control of a single promoter and produce proteins encoded in that novel operon in different ratios over a range of inducer concentrations. RNase E sites identified in the Rhodobacter capsulatus puf operon and Escherichia coli pap operon were separately placed between the coding regions of two reporter genes, and novel secondary structures were engineered into the 5′ and 3′ ends of the coding regions. The introduced RNase E site directed cleavage between the coding regions to produce two secondary transcripts, each containing a single coding region. The secondary transcripts were protected from exonuclease cleavage by engineered 3′ secondary structures, and one of the secondary transcripts was protected from RNase E cleavage by secondary structures at the 5′ end. The relative expression levels of two reporter genes could be varied up to fourfold, depending on inducer concentration, by controlling RNase cleavage of the primary and secondary transcripts. Coupled with the ability to vary translation initiation by changing the ribosome binding site, this technology should allow one to create new operons and coordinate, yet separately control, the expression levels of genes expressed in that operon.

In prokaryotes, expression of genes for multistep pathways or for production of multisubunit proteins is often controlled by regulating the posttranscriptional processing of a polycistrionic mRNA containing the coding regions for all the enzymes in that pathway or all subunits in a multisubunit protein. This type of control eliminates the need for multiple promoters of different strengths for each gene. To produce enzymes at appropriate levels, the cell balances translation efficiency with the rate that the message is inactivated by RNases.

These nucleases can be classified into two categories by comparing their mechanisms of action and role in mRNA decay. Two endonucleolytic activities are responsible for bulk mRNA processing—RNase III and RNase E (3, 10). RNase III cleaves mRNA at a weak consensus sequence within double-stranded regions of an RNA (9, 31). This indicates the role of RNase III in RNA processing, as it can cleave stem regions of RNA secondary structures (30, 31). RNase E is responsible for bulk inactivation of mRNA (14). The nuclease scans the mRNA transcript in a 5′-to-3′ direction and cleaves within AU-rich segments (1, 23, 24, 34). It is generally recognized that RNase E requires a free 5′ end to bind to the mRNA before scanning the transcript for cleavage sites (11–13, 15). The two exonucleases responsible for bulk mRNA degradation into mononucleotides are RNase II and polynucleotide phosphorylase (25). These enzymes degrade mRNA in a processive 3′-to-5′ direction, which indicates their role in degradation rather than inactivation (1).

For the majority of mRNA species, the initial cleavage within a transcript functionally inactivates the mRNA and is followed by 3′-to-5′ exonuclease activity (2). It has been shown that hairpin structures at the 3′ end protect the mRNA from degradation by exoribonucleases (1, 10, 26), and hairpins at the 5′ end protect from initial inactivation by endoribonucleases (11–13). Although there are a number of commercial products that contain 3′ hairpins to protect mRNA from exonucleases, there have been few attempts to design 5′ hairpins and recognition sites for endonucleases to alter mRNA stability and affect gene expression (2, 6, 7, 12, 13).

An expression system was developed that allows for the introduction of synthetic DNA cassettes into the region between the transcription and translation start sites of a gene of interest. Upon transcription, the 5′ end of the mRNA corresponding to the synthetic cassette forms a hairpin that protects the mRNA from endonucleolytic cleavage (6, 7). Depending on the structure of the inserted hairpin, the hairpin-containing mRNA exhibits half-lives between 3 and 10 times that of the mRNA with no hairpin, resulting in increases in mRNA and protein levels (8). These results indicate that it is possible to engineer mRNA stability as an additional means of controlling gene expression.

In this work, we constructed an expression system that allows one to vary the expression levels of two genes, both of which are under the control of the same promoter, by introducing DNA cassettes encoding mRNA secondary structures and RNase cleavage sites at various locations in the operon. The design for this decoupled, dual-gene expression system was derived from two native systems: the puf operon of Rhodobacter capsulatus (1, 21, 22) and the pap operon of Escherichia coli (5, 29). The expression system was tested with a combination of
mRNA secondary structures at the 5’ and 3’ ends of the two coding regions and putative RNase E cleavage sites between the coding regions to determine if it is possible to independently vary expression of the two genes. Northern blotting and primer extension analysis indicate that the primary transcript was cleaved into two secondary transcripts, each containing a coding region, when a putative RNase E site was placed between the coding regions. Northern blotting and enzyme assays indicate that expression of the two genes was effectively decoupled and that the relative expression levels of the two genes varied with the mRNA secondary structures and RNase E sites in the operon.

### MATERIALS AND METHODS

**Bacterial strains, media, chemicals, and enzymes.** E. coli DH10B (Gibco BRL) was used for all cloning steps. E. coli JS4 (Bio-Rad) was used for Northern blot analysis and enzyme assays (Table 1).

Luria-Bertani (LB) medium was made as described by Maniatis et al. (32). C medium (18) was supplemented with 3.4% glycerol, 1.6% Casamino Acids, and micronutrients (28). Ampicillin, used at a concentration of 100 μg/ml, and arabinose were purchased from Fisher Scientific. Diethyl pyrocarbonate and rifampin were obtained from New England Biolabs. DNA sequencing kits were purchased from USB.

**T4 DNA ligase and High-Fidelity PCR enzyme mix** were obtained from Roche. F. coli Turbo polymerase. Primer 13 (5′-GAGAA-3′) was designed to amplify XhoI restriction site

**Transcription vector (SP6/T7 promoters); multicloning site within pGFPuv** were digested with High-Fidelity PCR enzyme mix. All PCRs were performed with 20 μM Tris-HCl (pH 8.3), 30 mM KCl, 1.5 mM MgCl2, 200 μM dNTPs, 0.5 μM primers, and 2.6 U of enzyme. Cycle times and temperatures followed those suggested by the enzyme manufacturer. All plasmids used in this study are described in Table 1.

**Plasmid construction.** The base dual-gene plasmid containing both gfp and lacZ (p50g) was constructed in several cloning steps. Primers used in each PCR step were synthesized by Genemed Synthesis, Inc. DNA amplification was performed with High-Fidelity PCR enzyme mix. All PCRs were performed with 20 mM Tris-HCl (pH 8.3), 30 mM KCl, 1.5 mM MgCl2, 200 μM deoxynucleoside triphosphates (dNTPs), 300 μM primers, and 2 μl of template DNA. DNA sequencing kits were purchased from USB.

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coli DH10B. The transformed cells containing the plasmid with the cassette insert were screened using a restriction site within the cassette.

**Enzyme assays.** To determine β-galactosidase and green fluorescent protein (GFP) activities, C medium was inoculated into a stock culture of E. coli 744 containing the various plasmids at an OD_{600} of 0.016 and grown at 30°C. At an OD_{600} of 0.20, samples were removed and placed on ice. β-Galactosidase assays were performed as described previously (6, 27). β-Galactosidase activity is reported in Miller units. GFP activity was determined by measuring the relative fluorescence of a 2-ml sample in a VersaFluor fluorometer (Bio-Rad). This fluorescent reading was divided by the OD_{600} reading for the sample to obtain the GFP specific activity.

**Northern blot analysis.** Northern blot analysis was performed to determine transcript stabilities. C medium was inoculated with a stock culture of E. coli 744 containing the various plasmids to an OD_{600} of 0.20 and grown at 30°C. At an OD_{600} of 0.05, arabinose was added to a final concentration of 0.1%. At an OD_{600} of 0.20, rifampin was added to a final concentration of 2 mg/ml. RNA extraction was performed on samples as described previously at an OD_{600} of 0.20 (6).

The lacZ and gfp probes were synthesized by digesting pTC01 and pCS01 with PvuII and Ncol. The fragments were separately combined with SP6 polymerase and radiolabeled [α-32P]CTP (Amersham) and nonradiolabeled ribonucleotide triphosphates (Promega). Prior to the Northern blotting, all equipment was treated with 3% hydrogen peroxide and all solutions were treated with dimethyl pyrocarbonate. Northern blot analysis was conducted as described previously (2). Labeled probe was added to the hybridization solution at a concentration of 10^6 cpm/ml. Membranes were hybridized with 5 ml of the probe hybridization solution. The membranes were visualized using a PhosphorImager (Molecular Dynamics). The intensities were recorded using IPLab Gel software and were plotted as a function of time for several different exposure times to ensure linearity of the band intensity with exposure time. Northern blots were repeated at least twice on samples to ensure reproducibility. Uncertainties in half-life calculations were determined according to the method described by Taylor (33).

**Half-life calculations.** Half-lives of the various transcripts were determined according to the following model:

\[
\frac{d[P]}{dt} = k_{p}[X] - k_{d}[P]
\]  

(1)

\[
\frac{d[S_1]}{dt} = k_{d}[P] - k_{d}[S_1]
\]  

(2)

\[
\frac{d[S_2]}{dt} = k_{d}[P] - k_{d}[S_2]
\]  

(3)

where [P] is the concentration of the primary transcript, [S_1] and [S_2] are the concentrations of the two secondary transcripts, k_d is the decay constant for the primary transcript, k_{d1} and k_{d2} are the decay constants of the secondary transcripts, and k_{p}[X] is the rate of synthesis of the primary transcript. This model assumes that the process of the primary transcript is the only source of the secondary transcripts and that decay of the transcripts is proportional to the concentrations of the transcripts present. In the experiments reported here, k_{d}[X] is set equal to zero, because transcription is stopped with rifampin. Thus, the half-life of the primary transcript can be calculated from the simplified form of equation 1.

We determined the half-lives of the secondary transcripts using two different methods and assumptions. The first method assumes that k_{d1} is much larger than k_{d1} and k_{d2} or that the primary transcript is degraded fast relative to the secondary transcripts. With this assumption, the source term for the secondary transcripts can be calculated from the simplified form of equations 2 and 3. The assumptions made in the first method are correct only when no message is being produced (that is, for stable secondary transcripts and relatively unstable primary transcripts). The second method used to determine half-lives of the secondary transcripts makes no assumptions about the stability of the primary transcript and solves the equations using forward differences. This method provides more accurate half-life values for secondary transcripts arising from primary transcripts that have approximately the same half-life.

**Primer extension analysis.** An oligonucleotide, RT-primer 1 (5'-CAGCGGG ATCTGCAATGTCGT3'), was synthesized (Genemed Synthesis, Inc.) to bind 50 nucleotides downstream of the suspected RNase E cleavage site location. RT-primer 1 was labeled at its 5' end with [γ-32P]ATP (ICN Biochemicals) using T4 polynucleotide kinase. The labeled oligonucleotide, at a concentration of 10^6 cpm/μl, was annealed to 25 μg of the RNA isolated from the cellular extract (6). RNA samples were taken at an OD_{600} of 0.20. Primer extension was performed using avian myeloblastosis virus reverse transcriptase and 5 μg of total RNA in 50 mM KCl, 12 mM DTT, 3 mM spermidine, 10 mM MgCl_2, and 1.1 mM dNTPs. The reactions were stopped after 30 min with an 8 M urea–0.1% sodium dodecyl sulfate stop buffer–denaturing of the samples following the locations of the stop bands, a DNA sequencing ladder was generated by annealing the labeled oligonucleotide at a concentration of 5 × 10^6 cpm/μl to 5 μg of the correspond-

ing denatured plasmid. Plasmid DNA was denatured in a 0.2 M NaOH–0.2 mM EDTA solution at 37°C for 30 min. Denatured DNA was neutralized by adding sodium acetate to a final concentration of 0.3 M. The dideoxynucleotide (Sanger) method of sequencing was performed using Sequenase Kit Version 2.0. Protocol for sequencing followed that provided by the manufacturer. These samples were separated in individual lanes on a denaturing 7 M urea–6% polyacrylamide gel in Tris-borate-EDTA (Stratagene CastAway Sequencing System). This 16- by 7-in. gel was run for 1.5 h at 40 W and then dried for 30 min. The sequence was read from the gel by exposing it to film for an appropriate amount of time and developing the film. The film was scanned into Adobe Photoshop using ViaScan software. Because the amount of RNA that could be added to the reactions was limited, the efficiency of the primer extension reaction from the total RNA present in the samples, it was not possible to vary the intensity of the primer extension bands. Therefore, bands for primer extension and DNA sequencing differed greatly in intensity, and the images were adjusted separately. Image intensities were adjusted, first to read the sequence ladders. The intensities of the bands in the lanes containing the three reverse transcription reactions were adjusted equally to visualize the bands in these lanes. These increased intensity lanes were placed next to their corresponding DNA sequencing ladder and lower intensity lanes on the original image for comparison.

Cleave locations were determined where reverse transcriptase would no longer extend the transcript. To ensure that the stop bands were not a result of secondary structure, the procedure was repeated with 10 ng of RNA synthesized in vitro. In vitro-synthesized RNA was obtained by digesting pCS02 with PvuII. The fragments were combined with SP6 RNA polymerase and ribonucleotide triphosphates.

**RESULTS AND DISCUSSION**

**Design of dual- and single-gene systems.** A synthetic operon containing two genes was designed to allow introduction of secondary structures and RNase cleavage sites in the form of DNA cassettes at various locations in the operon and easy evaluation of the effects of these changes on gene expression. The system was constructed in a high-copy-number plasmid that has an arabinose-inducible araBAD promoter (P_{BAD}) and ampicillin resistance marker (Fig. 1A). The gfp gene was placed upstream of the lacZ gene. Unique restriction sites were placed at the 5' end of each untranslated region for the introduction of DNA cassettes that, when transcribed, would form secondary structures. These sites were placed out of range of the ribosome binding sites so that secondary structures would not interfere with translation efficiency. To ensure that there would be no differences in translation initiation, the regions between the unique restriction sites and the translation start sites were identical. The restriction sites at the 5' end of gfp allow one to introduce the hairpin at the very beginning of the transcript, as it has been reported that more than five unpaired bases at the 5' end of a transcript will relieve the stability enhancements of the hairpins (4). Hairpins at the 3' end of lacZ protect the mRNA against degradation by 3'-to-5' exonucleases and terminate transcription.

The decay characteristics of lacZ are known from previous work (6, 7). An understanding of the decay characteristics for gfp alone was required. By removing the lacZ coding region from the construct described above, an expression system with the gfp gene alone was developed. This single-gene system had 5' restriction sites for the insertion of DNA cassettes and the same transcription terminators positioned at the 3' end of gfp.

**Design of DNA cassettes.** Two DNA cassettes encoding previously designed hairpin structures (HP17 and HP4) were inserted into the single-gene system (Fig. 1). These DNA cassettes were inserted sequentially into the dual-gene system. A DNA cassette encoding a 3' hairpin for gfp and containing an intercistronic region with a putative RNase E site immediately 5' of lacZ was inserted into the dual-gene plasmid p50g, making plasmid p60g. This cassette was based on the sequence between the pufQ and pufB genes of R. capsulatus, which has been shown to contain an RNase E site (16). The second DNA cassette encoded a 5' hairpin for lacZ and was inserted immediately downstream of the putative RNase E site, making plas-
The design of this hairpin was based upon HP4 (8). The third DNA cassette encoded a known RNase E site found between the papA and papB regions of the E. coli pap operon (29) and was inserted to replace the first intercistronic region, making plasmid p70gHP4l (Fig. 1).

**Transcript stability analysis.** Northern blot analysis performed on the single-gene gfp systems (p50gDL, p50HP4gDL, and p50HP17gDL) revealed that the half-life of the gfp transcript was unaffected by 5' hairpins (Table 2). This result indicates that gfp is not susceptible to inactivation by an endoribonuclease, such as RNase E. In contrast, similar hairpins placed at the 5' end of lacZ significantly affected its stability (6).

Northern blot analysis conducted on the dual-gene systems revealed useful qualitative and quantitative information. No stable intermediate transcripts were detected for p50g1 when probed with either gfp or lacZ (Fig. 2B and 3B). The primary transcript containing the lacZ and gfp coding regions (Fig. 2B and 3B, band a) was slightly larger (by the length of gfp) than the lacZ-only transcript of pHP4l (Fig. 2A, band b). As there was no 5' hairpin to protect the primary transcript, it was rapidly degraded. As there were no internal hairpins (5' to lacZ), no stable secondary transcripts resulted or can be seen. The primary transcript had a short half-life of approximately 2 min, since the 5' end of this transcript was not protected by secondary structures (Table 2).

The results of the Northern blot analysis for p50g1 can be compared to those obtained with p60gHP4l. The blots for this system revealed bands for both the primary and secondary transcripts for lacZ and gfp (Fig. 2C and 3C), indicating that after the initial RNase cleavage between the genes, the resulting transcripts were protected at vulnerable ends from RNases. The lacZ secondary transcript of p60gHP4l (Fig. 2C, band b) was darker and more distinct than that of p50g1 (Fig. 2B, band b). Given the size of the bands, cleavage must occur approximately upstream of the lacZ 5' hairpin. The 3' hairpin for gfp protects against exonuclease activity. (Note that the secondary gfp transcript from p60gHP4l is smaller than that from p50gΔ1 because it lacks the 5' end of lacZ and the 3' hairpins on p50gΔ1.) The half-life of the primary transcript was approxi-

**TABLE 2. Primary and secondary transcript half-lives for tested plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primary transcript</th>
<th>Secondary transcript (lacZ)</th>
<th>Secondary transcript (gfp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p50g1</td>
<td>2.0 (±0.1)</td>
<td>3.5 (±0.2) [2.9 (±0.2)] b</td>
<td>2.9 (±0.1) [2.9 (±0.1)]</td>
</tr>
<tr>
<td>p50gΔ1</td>
<td>8.0 (±0.3)</td>
<td>6.9 (±0.4) [5.4 (±0.4)]</td>
<td>3.9 (±0.4) [3.1 (±0.4)]</td>
</tr>
<tr>
<td>p50HP4gΔ1</td>
<td>7.6 (±0.3)</td>
<td>3.5 (±0.2) [2.9 (±0.2)] b</td>
<td>2.9 (±0.1) [2.9 (±0.1)]</td>
</tr>
<tr>
<td>p50HP17gΔ1</td>
<td>7.5 (±0.5)</td>
<td>3.5 (±0.2) [2.9 (±0.2)] b</td>
<td>2.9 (±0.1) [2.9 (±0.1)]</td>
</tr>
<tr>
<td>p60gHP4l</td>
<td>3.5 (±0.2)</td>
<td>3.5 (±0.2) [2.9 (±0.2)] b</td>
<td>2.9 (±0.1) [2.9 (±0.1)]</td>
</tr>
<tr>
<td>p70gHP4l</td>
<td>4.0 (±0.2)</td>
<td>6.9 (±0.4) [5.4 (±0.4)]</td>
<td>3.9 (±0.4) [3.1 (±0.4)]</td>
</tr>
</tbody>
</table>

a Values for half-lives are given as mean values and standard errors obtained by method 1.
b Values in brackets are mean values and standard errors obtained by method 2.
The half-lives of the lacZ and gfp secondary transcripts were approximately 3.5 and 3 min, respectively, from method 1 and 2.9 min for both from method 2 (Table 2). Note that the half-lives for the gfp secondary transcript from p60gHP4l and p70gHP4l were significantly shorter than those for the transcripts of p50gDl, p50HP4gDl, and p50HP17gDl. The secondary gfp transcripts of p60gHP4l and p70gHP4l carry smaller hairpins 3’ of gfp, in contrast to p50gDl, p50HP4gDl, and p50HP17gDl, which carry the large hairpins. The differences in the hairpins at the 3’ end could significantly affect 3’-to-5’ processing and thus stability.

Northern blot analysis for p70gHP4l showed qualitative trends similar to those of p60gHP4l. The half-lives of the primary transcript and the gfp secondary transcript were similar (within experimental error) to those for p60gHP4l (Table 2). In contrast, the half-life of the lacZ secondary transcript was approximately 7 min according to method 1 for determining half-lives and 5.4 min according to method 2, nearly double that for p60gHP4l. The higher stability of the lacZ secondary transcript in p70gHP4l may be the result of the putative RNase E site in p70gHP4l being more susceptible to cleavage by E. coli RNase E than the one in p60gHP4l (17).

The stability analysis for these systems is complicated by the fact that the lacZ secondary transcript is close in size to the primary transcript and runs directly beneath the primary band on the gel, making the two bands appear as one longer band on the Northern blot. The break between the two bands can be determined by comparing it to the single-gene blots (Fig. 2A and 3A).

**Primer extension analysis.** To confirm that the mRNAs of p60gHP4l and p70gHP4l were cleaved between the two coding regions and that two secondary transcripts were present, the nuclease cleavage site location between the two genes in p60gHP4l and p70gHP4l was determined using primer extension analysis (Fig. 4). For p60gHP4l, cleavage occurred approximately 10 bases from the base of the hairpin stem 5’ of the lacZ coding region in the mRNA (indicated by band 2 in Fig. 4A). For p70gHP4l, cleavage occurred approximately 5 bases from the base of the hairpin stem 5’ of the lacZ coding region in the mRNA (band 2 in Fig. 4A). For p50gDl, no band appeared on the gel, indicating that cleavage does not occur between the two coding regions. This analysis indicates that the system is working as designed and suggests that a stable secondary transcript containing lacZ is formed when the primary transcript of p60gHP4l and p70gHP4l is cleaved by an RNase. Presumably this RNase is RNase E, as the regions were designed with previously identified RNase E sites. However, the actual identity of the RNase responsible for the processing is not critical. What is critical is that cleavage occurs between the two coding regions, thereby stabilizing the coding regions for the downstream gene. The higher stability of the lacZ secondary transcript from p70gHP4l than that for the transcript from p60gHP4l may be due to the number of the 5’ unpaired bases. The p60gHP4l lacZ secondary transcript contained approximately 10 unpaired bases, whereas the p70gHP4l transcript had approximately 5 unpaired bases. It has been shown that having more than 5 unpaired bases at the 5’ end of a transcript alleviates the protective effect of the secondary structure (4). Cleavage closer to the hairpin 5’ of lacZ results in a more stable lacZ transcript and more β-galactosidase produced from p70gHP4l than from p60gHP4l.

Note that the larger bands (indicated by the numeral 1 in Fig. 4A) in the primer extension lanes for p60gHP4l and p70gHP4l are due to reverse transcriptase stopping at the secondary structure 3’ of the gfp coding region; p50gDl lacks this
secondary structure and this band. It should also be pointed out that the intensity of this band, resulting from the secondary transcript by the addition of 5′-terminal base pairing of hairpins. This stabilization results in a 9 stem-loop for longevity protection against further immediate RNase cleavage. While larger than the lacZ secondary transcript by the addition of gfp at the 5′ end, the primary transcript of p50gl is not protected from RNase attack by any 5′ hairpins. This stabilization results in more 5′-galactosidase-to-GFP activities for p70gHP4l and p60gHP4l relative to the same for p50gl, (β-gal/GFP)_{p70/β-gal} and (β-gal/GFP)_{p60/β-gal} were consistently 3.5 and 1.7, respectively (Fig. 5C). The ratios of the lacZ secondary transcript half-life to gfp secondary transcript half-life (based on values obtained using method 1 of the half-life calculations) for these systems were 1.8 and 1.2, respectively. (The secondary transcripts of p50gl were nonexistent.) This can be compared to the ratios for the values obtained using method 2 of the half-life calculations, 1.7 and 1.0, respectively. As the only difference between these two constructs was the putative RNase E site, this result may reflect the relative efficiency with which RNase E cleaved the RNase E site (16, 29). Indeed, the results of the enzyme assay indicate that p70gHP4l contains an RNase site upstream of the lacZ gene that is more susceptible to cleavage by an RNase than that in p60gHP4l. The secondary lacZ transcripts, and to some extent the gfp transcripts, from p60gHP4l and p70gHP4l are more stable than the primary transcript of p50gl. Cleavage by an RNase directly 5′ of the 5′ hairpin of lacZ results in a secondary transcript with hairpins at the 5′ and 3′ ends to protect against further immediate RNase cleavage. While larger than the lacZ secondary transcript by the addition of gfp at the 5′ end, the primary transcript of p50gl is not protected from RNase attack by any 5′ hairpins. This stabilization results in more 5′-galactosidase-to-GFP activities for p60gHP4l and p70gHP4l than from p50gl. These results show that this technology can be used to differentially control the stabilities of secondary transcripts, resulting in differential gene expression. More importantly, the relative ratios of the enzymes produced can be affected with this type of control.

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