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Disruptions of Two Apparent rho-Independent Transcription Terminator Structures do not help in Enhancing the Expression of *aceK* in *E. coli*

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Abstract: Two apparent rho-independent transcription terminator structures within the coding sequence of *aceK* have been destroyed to access their roles in the differential expression between *aceA* and *aceK* in the glyoxylate bypass operon of *E. coli*. The effect of mutations on the expression of *aceK* was evaluated in two different ways: one by maxicell labeling and the other by *lacZ* fusion gene construction. The maxicell labeling experiment with the mutant operon clones has failed, like that of the wild type operon clone, to visibly show isocitrate dehrogenase (IDH) kinase/phosphatase, the product of *aceK*, on the autoradiogram of a protein gel. When the same mutations were introduced into an *aceK::lacZ* fusion gene to quantitatively evaluate the mutational effect, the activity of β-galactosidase in neither of the mutant versions of the fusion gene was elevated significantly enough to explain the degree of polarity observed in this region. Thus, we conclude that neither of these intragenic, apparent rho-independent transcription terminator structures, which have long been suspected as a major determinant in the downregulation of *aceK*, really act as a premature transcriptional terminator.
Key words: fusion gene, glyoxylate operon, isocitrate dehydrogenase, rho independent terminator, site-directed mutagenesis.

The enzymes of the glyoxylate bypass cycle allow *Escherichia coli* to grow on acetate by preventing the net loss of the acetate carbons as carbon dioxide in the Krebs cycle (Kornberg, 1966; Kornberg and Madsen, 1957). The enzymes unique to this cycle, isocitrate lyase, malate synthase, are induced during growth on acetate and are repressed if any preferred carbon source, such as glucose, is simultaneously present. Once induced, the flow of isocitrate through the glyoxylate bypass is regulated, in part, by the phosphorylation of isocitrate dehydrogenase (IDH), the Krebs cycle enzyme which competes with the isocitrate lyase (Holms *et al.*, 1971; Garnak *et al.*, 1979a, 1979b). The phosphorylation and dephosphorylation of IDH are catalyzed by IDH kinase/phosphatase which is a bifunctional enzyme. The genes for the metabolic and regulatory enzymes of this pathway reside in the same operon, whichmaps at 91 min on the *Escherichia coli* chromosome (Brice and Kornberg, 1968; Chung *et al.*, 1988; LaPorte and Chung, 1985; Maloy and Nunn, 1982). Isocitrate lyase and malate synthase are encoded by *aceA* and *aceB*, respectively, while IDH kinase/phosphatase is encoded by a single gene, *aceK*. The organization of this operon is *aceBAK*, with *aceB* being the most proximal gene to the promoter.

S1 nuclease mapping has indicated that this operon employs a single promoter during growth on acetate (Chung *et al.*, 1988). Although these genes are expressed from the unique operon promoter, the intracellular level of isocitrate lyase is more than 100-fold higher than that of IDH kinase/phosphatase. This is a striking example of differential expression within an operon, which is not uncommon in *Escherichia coli*. The polarity in the glyoxylate bypass operon was originally demonstrated when the specific activities of isocitrate lyase and IDH kinase/phosphatase in crude extracts were compared with those of the purified enzymes. Later it was confirmed by labeling the protein products expressed from an operon clone in the maxicells (Chung *et al.*, 1988). The nucleotide sequence of *aceK* and its flanking region, including the intergenic sequence between *aceA* and *aceK*, has been determined (Klumpp *et al.*, 1988). Several REP (repetitive extragenic palindromic) sequences have been found both in the intergenic region and in the coding sequence of *aceK*. They have been suggested as playing important roles in the differential expression between the two genes (Klumpp *et al.*, 1988).

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However, in the previous report, we showed that one of the intergenic REP sequences, which encompasses much of the intergenic region, plays only a small role in the downregulation of *aceK* (Chung *et al.*, 1993). Thus, in this study, we focused on the role of two apparent rho-independent transcription terminators, both of which reside in the coding sequence of *aceK*, in the differential expression between *aceA* and *aceK* of the glyoxylate bypass operon.

Materials and Methods

Enzymes and chemicals

Restriction enzymes and various DNA modifying enzymes were obtained from KOSKO. The kit for site-directed mutagenesis was the product of Boehringer Mannheim (U-DNA Mutagenesis Kit). [^{35}S]-methionine was purchased from Amersham and all other chemicals were the products of Sigma Chemical Co. (St. Louis, USA).

Recombinant DNA methods

Except where indicated, routine manipulations of plasmid DNA were accomplished using standard techniques as described by Maniatis *et al.* (1982).

Site-directed mutagenesis

The method described by Kunkel (1985) was adopted for site-directed mutagenesis. The *SphI/Hin*dIII fragment of *aceK* was subcloned into the M13mp18 vector. Uracil-containing single strand DNA was prepared from *E. coli* RZ 1032 (*dut, ung, thi, relA, spo*T1/F' *lys*A), which was previously infected with the recombinant phage containing the *aceK* insert. This template DNA was then annealed to the mutageneic oligonucleotides. Both of the 48mer oligonucleotides used had *Pst*I restriction endonuclease sites,and their sequences were:

5' AACCGACAGTACACGGAGTTCTGCAGCGTCTCCGCAATCTCGAAGCGC 3'
5' CCGTCTGGTGGATCGGCAGCCTGCAGCCCAATGTGCCGGAAGGTGTGA 3'

where the underlined bases represent the *Pst*I sites. The top one was used for mutagenesis of region I and the bottom one for region II shown in Fig. 1. Following extension and ligation steps, *E. coli* JM101 (*sup*E, *thi*, Δ(*lac*~*pro*AB)/F'*tra*D36, *pro*AB, *lacI*^{q}ZΔ15) was transformed with the ligation mixture. RF DNAs were isolated from the resulting individual plaques by the alkaline lysis method and desirable clones were identified by *Pst*I digesion.

Maxicell labeling

The maxicell technique was used to specifically label proteins produced from recombinant plasmids. The method employed was similar to that described by Sancar *et al.* (1979) with the following minor modifications. The strain used for this experiment, MM294Δ, was *rec*A but was not *uvr*A. Light activated repair of the chromosome was avoided by covering the cultures with aluminum foil following ultraviolet irradiation. In addition, cells surviving the irradiation were killed by the addition of D-cycloserine to 200 μg/ml two hours after the irradiation. Samples were subjected to SDS gel electrophoresis as described by Laemmli (1970). Molecular weight standards were myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000).

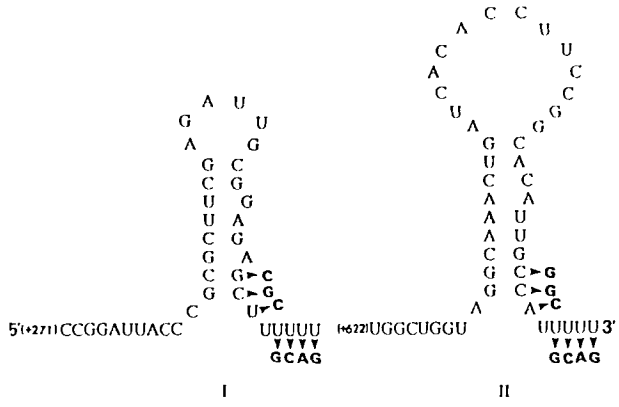

Fig. 1. Two apparent rho-independent transcription terminator structures residing in the coding sequence of *aceK*. The base substitutions upon mutagenesis are shown by the arrow heads.

Measurement of enzyme activities

β-galactosidase was assayed at 37℃ using permeabilized cells as described by Miller (1972). The activity of β-lactamase was determined by the method of O'Callghan *et al.* (1968) using cephalosporin as the substrate. The reaction was monitored by observing the decrease in absorbance at 252 nm.

Results and Discussion

To clarify the roles of the two potential rho-independent transcription terminator structures which reside around 300 bp and 650 bp downstream from the start codon of *aceK*, site-directed mutagenesis was employed. The effect of mutations on the expression of *aceK* was then evaluated in two different ways; one by maxicell labeling procedure and the other by fusion gene constructions.

Construction of mutant versions of operon clone

The palindromic sequences which can form secondary structures when transcribed have been suggested as playing important roles in differential expression in many bacterial operons either by interfering with the translation of downstream genes or by selectively stabilizing the mRNA segment upstream from these elements (Newbury *et al.*, 1987). Several such secondary structures have been found within the coding sequence of *aceK*, including the two shown in Fig. 1, as well as in the intergenic region between *aceA* and *aceK* (Klumpp *et al.*, 1988). Those residing in the intergenic region, however, do not seem to contribute significantly to the polarity in expression between the two genes, since deletion of one of the biggest repetitive extragenic palindromic (REP) sequences, which includes most of the intergenic region, has failed to enhance the expression of *aceK* (Chung *et al.*, 1993). Thus, in this study, we focused on the two intragenic palindromic sequences within *aceK*, which are located around 300 bp and 650 bp downstream from the start codon of *aceK*. We had good reasons to suspect these two structures as major determinants for the downregulation of *aceK*. First of all, as shown in Fig. 1, both of the sequences reveal characteristics of the rho-independent transcription terminator: a GC rich stem-loop followed by a string of uracil. Another reason for suspecting them was the fact that the 3'ends of the major species of the glyoxylate operon message have been mapped in these regions (Chung, 1995). To clarify their roles, the two potential terminator structures were destroyed by site-directed mutagenesis. Specifically, for each structure, two bases at the end of the stem and the immediate following thymidine residues were replaced with the sequence shown in the Experimental part. Each of the mutageneic oligonucleotides used in this replacement harbored a *PstI* site. Since no *PstI* site existed in the wild type sequence used for mutagenesis, the deliberate introduction of this restriction enzyme site made the identification procedure of the mutants easier. Mutagenesis was performed onto two different kinds of *aceK* insert in the M13 vector. One of the *aceK* inserts was derived from pCL8, which retains the full length operon clone on pBR322. Only one of the two target regions was mutagenized at a time to make independent mutants for region I and II. Acquiring a new *PstI* site was the criteria for successful mutagenesis in either case. For the positive clones, final confirmation was made through nucletide sequencing by the Sanger dideoxy method. The mutant versions of the insert were then moved back to the parent plasmid pCL8 by replacing the *SphI/HindIII* fragment of pCL8 with those of the mutated inserts from the M13 vector (Fig. 2). The desirable clones were identified again by digestion with *PstI* and designated as pLC7 and pLC8 for the mutant in region I and II, respectively. As shown in Fig. 3, in contrast to a single band from the wild type clone pCL 8, which comes from the single site of *PstI* in the pBR 322 vector, both of the mutants produced an extra

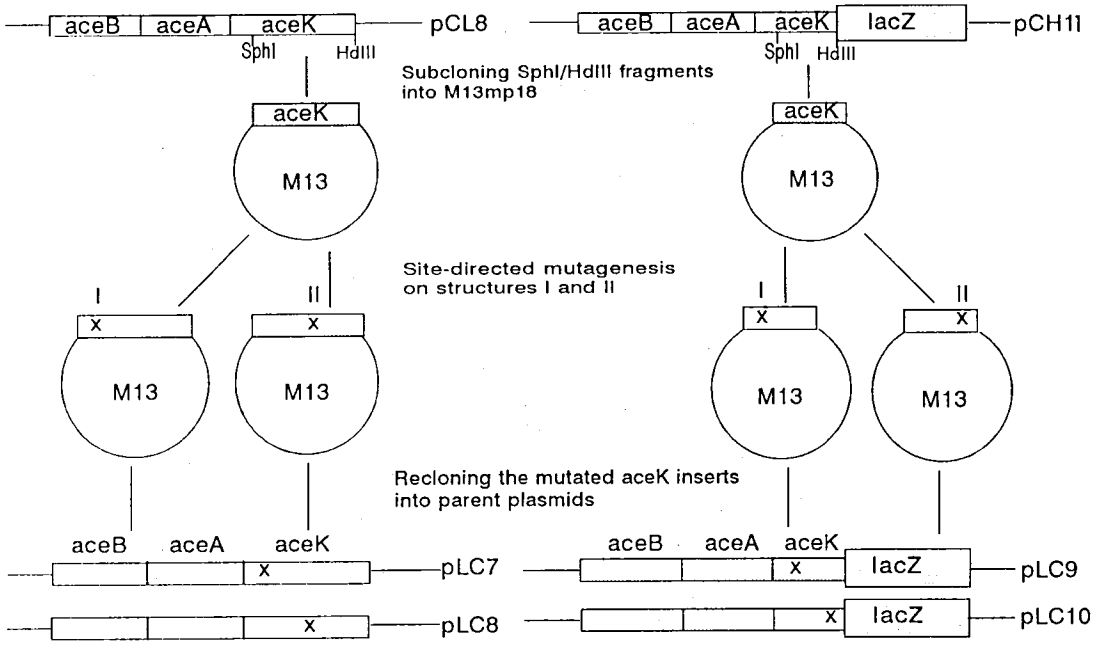

Fig. 2. Strategy for evaluating the effect of disruptions of the structures I and II. Using pLC7 and pLC8, the mutational effect was evaluated by maxicell labeling procedure and the pLC9 and pLC10 were used to quantitatively measure the mutational effect by determining β-galactosidase activity.

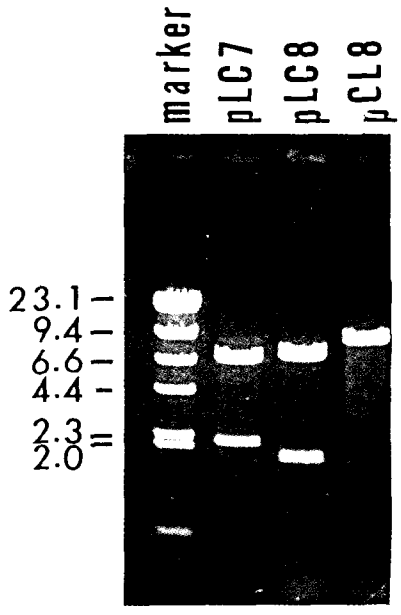

Fig. 3. Construction of two mutant versions of operon clone, pLC7 and pLC8. The sequences I and II were site-directed mutagenized independently on a *Sph*I/*Hin*dIII *ace*K fragment in M13 and the mutated *ace*K inserts were moved back to the parent clone pCL8 to produce pLC7 and pLC8. To confirm the mutagenesis and recloning, pLC7 (mutant in the structure I), pLC8 (mutant in the structure II) and the wild type clone pCL8 were digested with *Pst*I and fractionated on a 1% agarose gel.

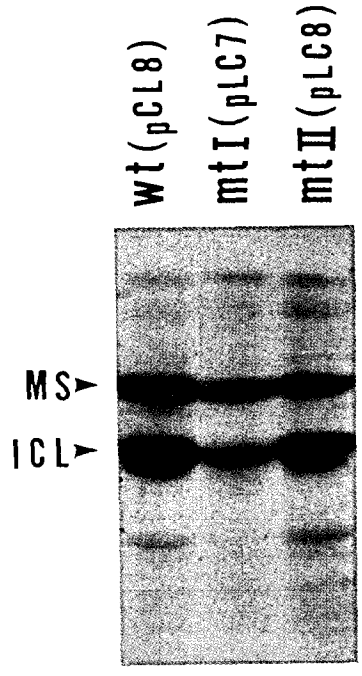

Fig. 4. Protein products expressed from maxicells harboring pLC 7, pLC8 and the wild type clone pCL8. Cells harboring the plasmids were irradiated with UV and the proteins expressed from the surviving plasmids were labeled with [^{35}S]-methionine. MS: malate synthase (the product of *ace*B); ICL: isocitrate lyase (the product of *ace*A).

band. The sizes of the bands well agreed with the generations of new *Pst*I sites at region I and II, thus confirming both the mutagenesis and the subcloning. These mutant versions of the operon clone were then used, as described below, to visibly compare the expression level of *ace*K with that from the wild type.

Protein products expressed from the mutant operon clones

To visibly observe the effect of disruption of the potential tamscription terminators, only the plasmid-born proteins were labeled with ^{35}S-methionine using the maxicell procedure. When the mutant operon clones, pLC7 and pLC8, were expressed from maxicells and the labeled proteins were fractionated and visualized by autoradiography, the isocitrate dehydrogenase kinase/phosphatase, which has a molecular weight of 66 kDa, was not visible from either clone (Fig. 4). The pattern of expression of the three operon genes was identical to that of the wild type. Thus, at least judged from the levels of the proteins on the gel, neither of the structures seems to work as a premature transcriptional terminator.

Quantitative measurement of the effect of mutations

Although the maxicell labeling procedure described above was successful in demonstrating at large that the two palindromic sequences within *ace*K do not play a major role in differential expression, it didn't allow measurement of a subtle change of *ace*K expression, if any, from the mutants. Thus a different approach was employed to quantitatively measure mutational effects. For this purpose, mutagenesis was carried out onto another version of the *ace*K fragment in the M13 vector. The insert also came as a *Sph*I/*Hin*dIII fragment, but it was derived from pCH11, which was one of the *ace*K::*lac*Z fusion genes produced previously (Chung *et al.*, 1993). Since the reporter gene *lac*Z used here had no transcriptional or translational initiation signals of its own, the expression of *lac*Z was totally dependent on the upstream gene segment, in this case, on the *ace*K part. This particular fusion gene had the fusion point at 763 bp downstream from the start codon of *ace*K, which means that both of the target secondary structures survived in the truncated *ace*K part of the fusion gene. Therefore, pCH11 was a suitable fusion gene for quantitative evaluation of the effect of mutations of region I and II. After mutagenesis was normally carried out onto the *Sph*I/*Hin*dIII insert derived from pCH11, the recombinant M13 plasmids were isolated and digested with *Pst*I to screen mutants. Here again, the final confirmation for the mutagenesis was by nucleotide sequencing. The mutant *ace*K fragments from the M13 vector were then used to substitute for

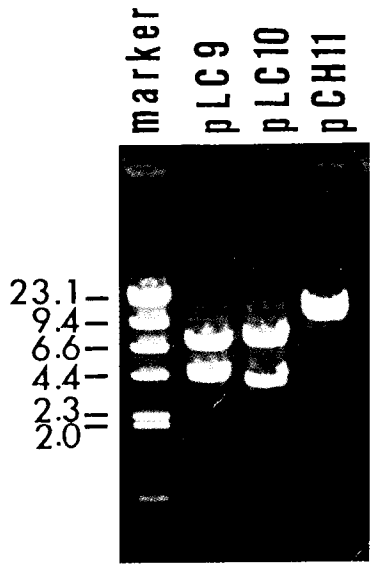

Fig. 5. Construction of two mutant versions of an *aceK*::*lacZ* fusion gene plasmid. An *aceK* insert originally derived from an *aceK*::*lacZ* fusion plasmid pCH11 was employed for site-directed mutagenesis of the structures I and II. The mutated *aceK* inserts were then transferred to the parent plasmid pCH11 to produce pLC9 and pLC10. pLC9 (mutant in the structure I, pLC10 (mutant in the structure II) and the wild type fusion plasmid pCH11 were digested with *Pst*I.

the corresponding wild type sequence in pCH11 (Fig. 2). Restriction digestion analysis to confirm the constructions of these mutant vesions of this *aceK*::*lacZ* fusion plasmid is shown in Fig. 5. The wild type fusion gene gave a single band upon *Pst*I digestion, while the desirable clone with mutation in region I or II produced two fragments with appropriate sizes. Two of them were selected and designated pLC9 and pLC10, of which the former has the mutation in sequence I and the latter in sequence II. These mutant fusion gene plasmids were separately introduced into a β-galactosidase-deficient *E. coli* strain JM101 and the expression of the fusion genes was induced by growing the cultures in the presence of 2% sodium acetate. Relative β-galactosidase activities expressed from these fusion genes are shown in Table 1. For compensation of the plasmid copy number, the activity of β-lactamase was determined for each culture and used to correct the relative activity of β-galactosidase. Although disruption of structure II resulted in a two-fold increase in the expression of *aceK*, it was still far from being comparable to that of *aceA*. Therefore, we conclude that the two apparent rho-independent transcription terminators within the coding sequence of *aceK* do not act as true terminators *in vivo*. We previously suggested that the major glyoxylate operon RNAs be produced either by premature transcription termination or posttranscriptional trimming back by exonucleases. The failure to enhance the expression of *aceK* here after rendering the structures inactive to act as rho-independent terminators thus enhances the possibility of their role as barriers to the action of the putative exonucleases. However, we do not preclude their possible role as rho-dependent terminators either, because the mutations introduced here didn't disrupt the whole secondary structures per se. Rather the mutations rendered the structures inactive as rho-independent terminators. Thus,further studies will be needed to determine whether the differential expression still occurs in the *E. coli* strains defective in rho function.

Table 1. Effect of disruptions of the apparent terminator structures I and II on the expression of *aceK* from *lacZ* fusion genes

Plasmid	Relevant genotype	Relative β-galactosidase activity
pCH13	*aceA*::*lacZ*	100
pCH11	*aceK*::*lacZ*	0.7
pLC9	$aceK^{mI}$::*lacZ*	0.2
pLC10	$aceK^{mII}$::*lacZ*	1.5

The superscripts mI and mII indicate that the *aceK* parts of these fusion genes have mutations in the structure I and II, respectively.

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References

Brice, C. G. and Kornberg, H. L. (1968) *J. Bacteriol.* **96**, 2185.

Chung, T., Klumpp, D. J. and LaPorte, D. C. (1988) *J. Bacteriol.* **170**, 386.

Chung, T., Resnik, E., Stueland, C. and LaPorte, D. C. (1993) *J. Bacteriol.* **175**, 4572.

Chung, T. (1995) *Mol. Cells* **5**(2), 140.

Gamak, M. and Reeves, H. C. (1979a) *Science* **203**, 11.

Gamak, M. and Reeves, H. C. (1979b) *J. Biol. Chem.* **254**, 7915.

Holms, W. H. and Bennett, P. M. (1971) *J. Gen. Microbiol.* **65**, 57.

Klumpp, D. J., Plank, D. W., Bowdin, L. J., Stueland, C. S., Chung, T. and LaPorte, D. C. (1988) *J. Bacteriol.* **170**, 2763.

Kornberg, H. L. (1966) *Biochem. J.* **99**, 1.

Kornberg, H. L. and Madsen, N. B. (1957) *Biochim. Biophys. Acta* **24**, 651.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci.* USA **82**, 488.
Laemmli, U. K. (1970) *Nature* **227**, 680.
LaPorte, D. C. and Chung, T. (1985) *J. Biol. Chem.* **260**, 15291.
Malloy, S. R. and Nunn, W. D. (1982) *J. Bacteriol.* **149**, 173.
Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Newbury, S. F., Smith, N. H., Robinson, E. C., Hiles, I. D. and Higgins, C. F. (1987) *Cell* **48**, 297.
O'Callaghn, C. H., Muggleton, P. W. and Ross, G. W. (1968) in Antimicrobial Agents and Chemotheraphy (G. Hobby, ed.) p. 57, American Soc. of Microbiology.
Sancar, A., Hack, A. M. and Rupp, W. D. (1979) *J. Bacteriol.* **137**, 692.