

Nucleotide Sequence of *aceK*, the Gene Encoding Isocitrate Dehydrogenase Kinase/Phosphatase

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Received 28 December 1987/Accepted 8 March 1988

In *Escherichia coli*, the phosphorylation and dephosphorylation of isocitrate dehydrogenase (IDH) are catalyzed by a bifunctional protein kinase/phosphatase. We have determined the nucleotide sequence of *aceK*, the gene encoding IDH kinase/phosphatase. This gene consists of a single open reading frame of 1,734 base pairs preceded by a Shine-Dalgarno ribosome-binding site. Examination of the deduced amino acid sequence of IDH kinase/phosphatase revealed sequences which are similar to the consensus sequence for ATP-binding sites. This protein did not, however, exhibit the extensive sequence homologies which are typical of other protein kinases. Multiple copies of the *REP* family of repetitive extragenic elements were found within the intergenic region between *aceA* (encoding isocitrate lyase) and *aceK*. These elements have the potential for combining to form an exceptionally stable stem-loop structure ($\Delta G = -54$ kcal/mol [ca. -226 kJ/mol]) in the mRNA. This structure, which masks the ribosome-binding site and start codon for *aceK*, may contribute to the downshift in expression observed between *aceA* and *aceK*. Another potential stem-loop structure ($\Delta G = -29$ kcal/mol [ca. 121 kJ/mol]), unrelated to the *REP* sequences, was found within *aceK*.

For *Escherichia coli*, adaptation to growth on acetate requires the induction of the enzymes of the glyoxylate bypass, isocitrate lyase and malate synthase (26, 27). This pathway allows the net synthesis of cellular constituents from acetate because it bypasses the CO₂-producing steps of the Krebs cycle. Once induced, this pathway is regulated, at least in part, through the phosphorylation of isocitrate dehydrogenase (IDH), the Krebs cycle enzyme which competes with isocitrate lyase (3, 17, 18, 21, 54). This phosphorylation site has been identified as serine 113 (5, 51). During growth on acetate, ca. 70% of the IDH is maintained in the inactive, phosphorylated form (4, 30, 31), forcing isocitrate through the glyoxylate bypass (32, 41). Mutant strains which fail to express IDH kinase/phosphatase are usually unable to grow on acetate, indicating that the phosphorylation of IDH is essential for growth on this carbon source (31).

The phosphorylation and dephosphorylation of IDH are catalyzed by a bifunctional enzyme, IDH kinase/phosphatase. This protein is expressed from a single gene, *aceK* (28), and yields a single band on sodium dodecyl sulfate electrophoresis (28, 29). These observations support the proposal that IDH kinase and IDH phosphatase reside on the same polypeptide. The physical association of opposing regulatory activities is not unique to IDH kinase/phosphatase. Other bifunctional regulatory proteins have been identified in mammals (14), plants (6), and microorganisms (7, 16, 42).

In addition to regulation mediated by the phosphorylation of IDH, the glyoxylate bypass is regulated at the level of gene expression. Using genetic techniques, Maloy and Nunn demonstrated that the genes which encode isocitrate lyase (*aceA*) and malate synthase (*aceB*) are expressed from the same operon and that *aceB* is proximal to the promoter (35). We used a similar approach to show that the gene encoding IDH kinase/phosphatase (*aceK*) is also in this operon, downstream from *aceA* (31). Thus, the organization of this operon is:

aceB aceA aceK
PO

where PO indicates the promoter/operator sequences. We have recently confirmed this organization by cloning the glyoxylate bypass operon and determining its functional map (10). Although *aceA* and *aceK* are expressed from the same promoter, the cellular level of isocitrate lyase is 100- to 1,000-fold greater than that of IDH kinase/phosphatase (10, 31).

In this paper, we report the nucleotide sequence of *aceK* and of the *aceA-aceK* intergenic region. We have also examined the deduced amino acid sequence of IDH kinase/phosphatase. While this manuscript was under review, the nucleotide sequence of *aceK* was presented by Cortay et al. (11). The nucleotide sequence which we have determined is generally in agreement with that presented by Cortay et al. However, as discussed below, the amino acid sequences of IDH kinase/phosphatase deduced from these nucleotide sequences differ between amino acid residues 92 and 165.

MATERIALS AND METHODS

Materials. Radioactive nucleotides were obtained from New England Nuclear Corp. DNA modification enzymes were products of New England BioLabs, Inc., or Bethesda Research Laboratories, Inc. Affi-Gel Blue was purchased from Bio-Rad Laboratories. All other reagents were the purest grades available.

Growth media and bacterial strains. L broth contained 1% tryptone (Difco Laboratories), 1% NaCl, 0.5% yeast extract, and, when appropriate, 200 μ g of ampicillin per ml. The minimal medium was the MOPS (morpholinepropanesulfonic acid)-based medium described by Neidhardt et al. (40) containing 1% glucose, the required amino acids, and, where indicated, 100 μ g of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) per ml.

E. coli MM294A (*endA thi hsdR recA*) was obtained from D. E. Koshland, Jr. *E. coli* JM101 [*supE gyrA thi Δ (lac*

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proAB) F' *traD36 proAB lacI^qΔM15*] was obtained from J. Messing.

Recombinant DNA procedures. Except as noted, plasmids and bacteriophage were manipulated by standard procedures (36).

An in-frame gene fusion between *aceA* (encoding isocitrate lyase) and *lacZ* (encoding β-galactosidase) was constructed to identify the translational reading frame of *aceA*. Plasmid pCL8 carries the glyoxylate bypass operon cloned between the *Bam*HI and *Hind*III sites of pBR322 (10). This plasmid was cleaved at the 3' end of the operon with *Hind*III and digested for various periods of time with BAL 31. After repair with the Klenow fragment of DNA polymerase I, *Hind*III linkers were attached. The sample was then cleaved with *Sal*II (which cuts only in the vector), and the insert was cloned between the *Sal*II and *Hind*III sites of pBR322. DNA was isolated from individual transformants, and the endpoints of the deletions were estimated by restriction mapping. The *lacZ* cassette from plasmid pMC1871 (8) was then introduced into the *Hind*III site. This cassette contains the structural gene for β-galactosidase but is missing the ribosome-binding site and the first eight codons. Plasmids in which the cassette was fused with *aceA* in the correct orientation and translational reading frame were identified on X-Gal-glucose plates. Expression of the fusion protein was confirmed by the assay of permeabilized cells for β-galactosidase (39).

Nucleotide sequencing. Nucleotide sequencing was performed by the dideoxy chain termination method of Sanger and Coulson (47) essentially as described by Messing (38). Several regions were also sequenced by the chemical cleavage method of Maxam and Gilbert (37).

For dideoxy chain termination sequencing, *aceK* was cloned into two M13 variants, M13mp19 (43) and M13tg130 (24). Plasmids pDL7 and pDL8 carry *aceK* and have been described previously (28). The insert from pDL8 was cloned between the *Hinc*II and *Hind*III sites in M13mp19. The opposite orientation of *aceK* was achieved by cloning the *Eco*RI-*Hind*III fragment from pDL7 into M13tg130. Nested deletions were introduced into these phage by the method of Yanisch-Perron et al. using exonuclease III and exonuclease VII (55).

Compilation and analysis of the DNA sequencing data were performed using programs developed by Intelligenetics, Inc.

Protein purification. IDH was purified by a modification of the method of Garnak and Reeves (18) from *E. coli* Y1090 harboring pTK509, a multicopy plasmid which overexpresses the gene encoding IDH (31). Phospho-IDH was prepared in vitro using purified IDH kinase/phosphatase (29). After the reaction, IDH kinase/phosphatase and dephospho-IDH were removed by chromatography on Affi-Gel Blue.

The purification of IDH kinase/phosphatase has been described previously (29). This protein was purified from *E. coli* Y1090 harboring pDL9, a plasmid which expresses *aceK* (28).

Amino acid analysis and sequence determination. IDH kinase/phosphatase was reduced and carboxymethylated by the method of Lundell and Howard (33), using [³H]iodoacetate. After dialysis against 50 mM Tris chloride (pH 7.5), the sample was digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (added to a total of 4% of the total protein) for 18 h at 30°C. Tryptic peptides were separated by reverse-phase high-pressure liquid chromatography on a Brownlee Labs C-8 RP300 column, using a linear

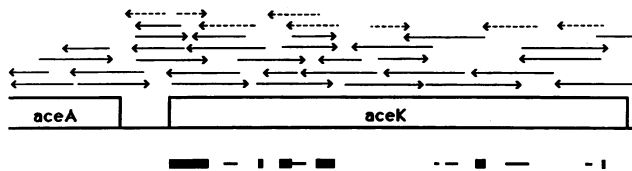


FIG. 1. Sequencing strategy. The boxes represent the open reading frames of *aceA* and *aceK*. The nucleotide sequence was determined by the dideoxy method of Sanger and Coulson (47) (solid arrows) and the chemical cleavage method of Maxam and Gilbert (dashed arrows) (37). To confirm the amino acid sequence of IDH kinase/phosphatase deduced from the nucleotide sequence of *aceK*, the amino acid sequences of the N terminus and of several tryptic peptides were determined (thick lines below *aceK*). Several additional tryptic peptides were identified by amino acid analysis (thin lines below *aceK*).

gradient from 0 to 50% acetonitrile in 0.1% trifluoroacetic acid (20). Amino acid analysis of the isolated peptides was performed on a Beckman 6300 amino acid analyzer after 24 h of hydrolysis at 110°C in 6 N HCl containing 1% (wt/wt) phenol. Sequence analysis of selected peptides and of the intact protein was performed by automated Edman degradation using either a Beckman model 890M or an Applied Biosystems model 470A protein sequencer.

RESULTS AND DISCUSSION

Nucleotide sequence of *aceK*. The sequence of *aceK*, the gene encoding IDH kinase/phosphatase, was determined using the strategy shown in Fig. 1. This gene consists of a single open reading frame of 1,734 base pairs (bp) preceded by a probable Shine-Dalgarno ribosome-binding site (Fig. 2). The length of this open reading frame and its location within the insert are in good agreement with the fine map of *aceK* which was determined by deletion analysis (28). The amino acid sequences of the N terminus and of several tryptic peptides isolated from IDH kinase/phosphatase were determined to confirm the sequence of the protein deduced from the nucleotide sequence of *aceK*. Several additional peptides were identified by amino acid analysis. Comparison of the N-terminal amino acid sequence with that deduced from the nucleotide sequence of *aceK* indicated that the formylated methionine had been removed after translation, leaving the proline in position 2 as the N-terminal residue.

While this manuscript was under review, the sequence of *aceK* was presented by Cortay et al. (11). The nucleotide sequence which we have determined is in general agreement with that presented by these authors. However, the deduced amino acid sequences of IDH kinase/phosphatase determined from these nucleotide sequences differ between amino acid residues 92 and 165 due to differences in the assignment of the translational reading frame. In our hands, the dideoxy chain termination method of sequencing (the method employed by Cortay et al.) left a number of ambiguities in the corresponding region of the gene. We employed the Maxam-Gilbert method of sequencing to resolve these ambiguities. In addition, we have identified three tryptic peptides from this region of the protein: peptides 115 to 120 and 142 to 155 were identified by amino acid sequencing, and peptide 156 to 163 was identified by amino acid analysis. These results support our assignment of the translational reading frame.

Sequence similarities with other protein kinases. Comparison of the deduced amino acid sequence of IDH kinase/phosphatase with the amino acid sequences of other protein serine/threonine kinases, using the GENALIGN algorithm

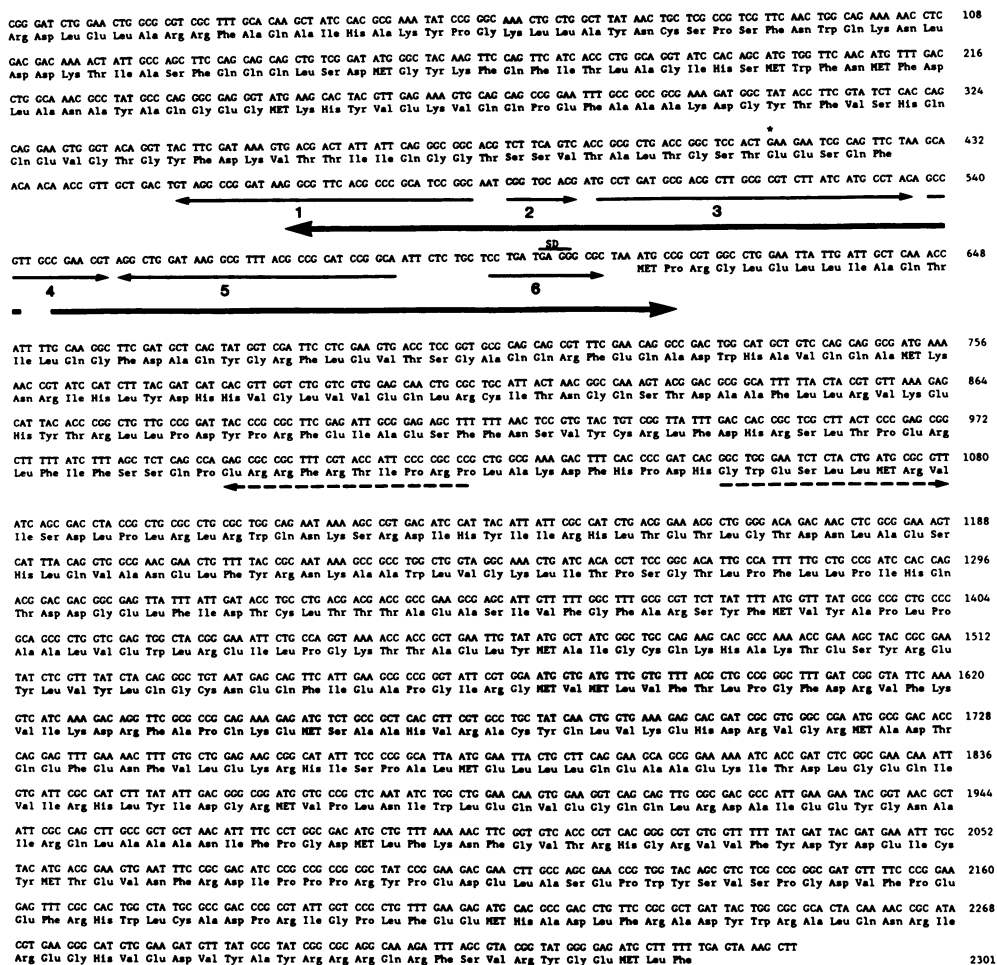


FIG. 2. Nucleotide sequence of *aceK* and the deduced amino acid sequence of IDH kinase/phosphatase. A portion of the C-terminal sequence of isocitrate lyase is given starting at position 1. The deduced amino acid sequence for IDH kinase/phosphatase begins at position 612. The probable Shine-Dalgarno ribosome-binding site is indicated as "SD." REP sequences are marked by thin arrows, with the length indicating full or partial elements. Since REP sequences can occur in either orientation, a leftward arrow indicates that the best match to the consensus sequence is on the transcribed strand while a rightward arrow indicates that it is on the untranscribed strand. The REP elements are numbered to aid comparison with Fig. 4. The heavy arrows below the *aceA-aceK* intergenic region indicate the dyad symmetry responsible for the potential stem-loop structure shown in Fig. 5A. The dashed arrows indicate the palindromic sequence within *aceK*. The junction site of the *aceA-lacZ* fusion whose sequence is presented in Fig. 5 is indicated with an asterisk.

from Intelligenetics, failed to identify significant sequence similarities. However, the more rigorous ALIGN algorithm identified several regions of IDH kinase/phosphatase which exhibit some degree of similarity with the sequences of these other protein kinases. The most significant of these regions is shown in Fig. 3 along with the corresponding regions of the gamma subunit of rabbit skeletal muscle phosphorylase kinase (46), bovine lung cyclic GMP-dependent protein kinase (50), and the catalytic subunit of bovine heart cyclic AMP-dependent protein kinase (48). IDH kinase/phosphatase matches one or more of these sequences at 10 of the 28 residues shown and matches 4 of the 6 residues which are conserved in all three proteins. Of particular note is the apparent conservation of the "invariant lysine" (indicated by the arrow in Fig. 3) which has been found in all serine/threonine and tyrosine kinases sequenced to date. These sequences have been proposed to form at least part of the ATP-binding sites of these protein kinases, a suggestion which is supported by the ability of the ATP analog 5'-fluorosulfonylbenzoyl adenosine to modify the invariant lysine in several of these proteins. Of course, it remains to be

determined whether the sequence similarity of the corresponding region of IDH kinase/phosphatase denotes a functional homology.

Sequence homology has been a characteristic of all protein kinases whose primary structures have been determined

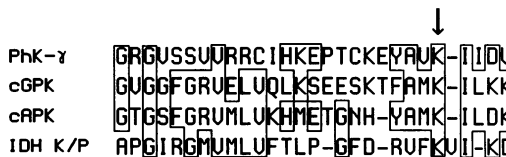


FIG. 3. Sequence similarity between IDH kinase/phosphatase and other protein kinases. Amino acid residues 315 to 340 of IDH kinase/phosphatase (IDH K/P) are aligned with residues 25 to 53 of the gamma subunit of rabbit skeletal muscle phosphorylase kinase (PhK-γ), residues 366 to 393 of bovine lung cyclic GMP-dependent protein kinase (cGPK) and residues 50 to 76 of the catalytic subunit of bovine heart cyclic AMP-dependent protein kinase (cAPK). The arrow indicates the invariant lysine, which has been found in all serine/threonine and tyrosine kinases sequenced to date (23).

previously (23). The strength of this correlation is perhaps best illustrated by the success with which sequence homology has been used to predict the protein kinase activity of the products of genes for which only the nucleotide sequence was available (9, 45). In contrast, although IDH kinase/phosphatase does appear to exhibit some sequence similarities with other protein kinases, this similarity is clearly not extensive. IDH kinase/phosphatase may not exhibit the characteristic sequence homologies typical of other protein kinases because these other protein kinases are of eucaryotic origin. Sequence homology has, however, been found to extend across the eucaryote-procaryote boundary for other gene families (e.g., GTP-binding proteins; 13). Regardless of the precise reasons underlying this lack of extensive homology, it is apparent that extensive sequence homology is not a universal property of protein kinases.

We have also compared the nucleotide sequence of *aceK* and the deduced amino acid sequence of IDH kinase/phosphatase with sequences in the National Institutes of Health and European Molecular Biology Organization data bases by using the IFIND algorithm from Intelligenetics. No significant homology was detected with any of the sequences in those data bases.

Is *aceK* the product of gene duplication? Enzymes catalyzing related reactions have, in many cases, been shown to exhibit substantial sequence similarity, suggesting that they arose from a common ancestor. Gene duplication has, for example, been proposed as a mechanism for the evolution of biosynthetic pathways (22). Consistent with this proposal, the enzymes catalyzing the sequential reactions in the biosynthesis of methionine in *E. coli* exhibit a high degree of sequence similarity (2). The association of IDH kinase and IDH phosphatase on the same polypeptide led to the proposal that *aceK* might have arisen from the duplication of a primordial kinase gene (29). Duplication could then have been followed by gene fusion, the process which has been suggested to be generally responsible for the generation of multifunctional proteins (12). Duplication and fusion appears to have been the pathway by which a variety of other bifunctional proteins were generated (see references 15, 34, and 44). An imperfect duplication of the primordial kinase gene might allow water to gain access to one of the active sites, providing a basis for the phosphatase reaction. An attractive feature of this model is that it provides a variety of alternative explanations for the absolute requirement of IDH phosphatase reaction for ATP or ADP (29). We have tested this model by using the GENALIGN algorithm from Intelligenetics. No internal sequence homology was detected, indicating that IDH kinase/phosphatase probably did not arise by such a mechanism.

Potential secondary structure in the *aceK* mRNA. Examination of the sequences upstream of *aceK* revealed the presence of several members of the *REP* (repetitive extragenic palindromic) family of genetic elements (Fig. 2 and 4). *REP* elements, whose functions are unknown, are characterized by a high degree of sequence homology (19, 49). These sequences have been identified in the untranslated regions of a variety of operons and genes in *E. coli*. The sequences upstream of *aceK* include three elements which are homologous with the full consensus sequence and two elements which match only the left half of the consensus (Fig. 4). The Shine-Dalgarno ribosome-binding site is included in one of these partial elements. A third partial *REP* element matches the left half of the consensus sequence at 9 of 14 positions, but is missing the other 5 bases. Although *REP* elements exhibit a high degree of sequence homology,

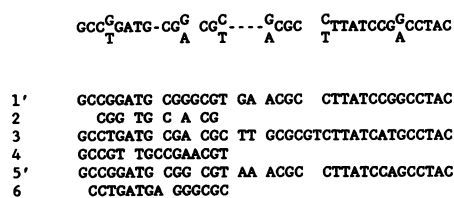


FIG. 4. *REP* sequences upstream of *aceK*. The inverse complements of elements 1 and 5 are shown to simplify comparison. The consensus sequence for *REP* elements is given at the top of the figure.

their organization in the untranslated regions of *E. coli* genes is rather variable. *REP* elements are often present as single copies but may also occur in clusters. The most complex cluster found by Stern et al. was downstream of *sucB* and consisted of four full elements and no partial elements (49). The cluster of *REP* elements found upstream of *aceK* is thus notable as one of the most complex observed to date.

Although several *REP* elements had been identified upstream of *aceK*, it was not clear whether these sequences were in *aceA* or in the intergenic region. To delimit the intergenic region, we constructed a gene fusion between *aceA* and *lacZ*. The deletion which was introduced during the construction of this fusion produced a 90% reduction in isocitrate lyase activity, consistent with an endpoint within *aceA* (10). The observation that this fusion gene expressed β -galactosidase activity indicated that *lacZ* had been fused in the same translational reading frame as *aceA*. The sequence of the *aceA-lacZ* junction was determined, and the reading frame of *aceA* could then be identified by reference to that of *lacZ* (Fig. 5). The endpoint of the deletion was 14 bp upstream of the translational termination codon of *aceA* (Fig. 2). Examination of the other two reading frames revealed termination codons upstream of this junction, confirming the assignment of the translational reading frame. The intergenic region extends 181 bp upstream of *aceK* and includes the *REP* elements.

The *REP* sequences upstream of *aceK* have the potential for producing a variety of secondary structures within the mRNA. The structure with the greatest predicted stability is shown in Fig. 6A. The sequences included in this structure are indicated in Fig. 2 by heavy arrows. This stem-loop structure has a calculated free energy (52, 53) of -54 kcal/mol (ca. -226 kJ/mol). The sequences making up the stem include the Shine-Dalgarno ribosome-binding site and the translational initiation codon of *aceK*. An additional region of dyad symmetry was identified within *aceK* (Fig. 2 and 6B). These sequences, which are unrelated to the *REP* sequences, begin 386 bp downstream of the translational initiation site of *aceK*. Formation of this potential stem-loop structure in the mRNA has a predicted free energy of -29 kcal/mol (ca. -121 kJ/mol).

Although *aceA* and *aceK* are expressed from the same promoter, the cellular level of isocitrate lyase is 100- to 1,000-fold greater than that of IDH kinase/phosphatase (10, 31). The potential stem-loop structure in the *aceA-aceK* intergenic region might contribute to this downshift in expression by masking the ribosome binding site and the initiation codon, thus inhibiting translation initiation. Kliensky et al. have presented evidence that a similar mechanism is responsible for the fivefold downshift in expression observed between *uncE* and *uncF* of *E. coli* (25). However, we have recently tested this model for the glyoxylate bypass operon by using oligonucleotide-directed dele-

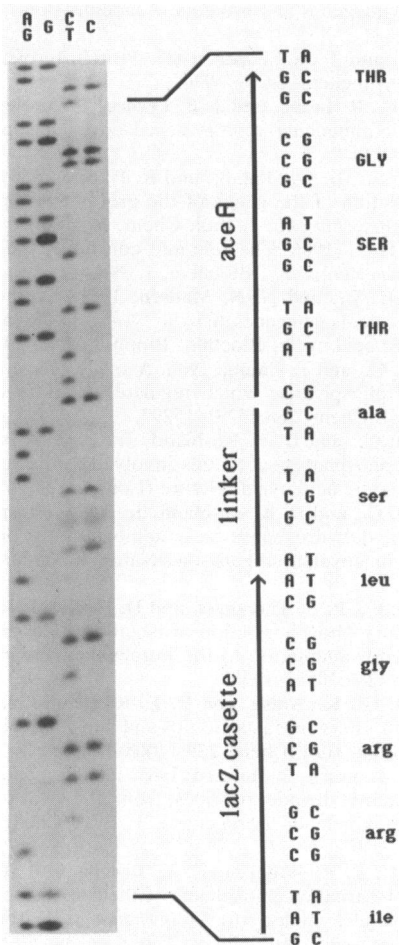
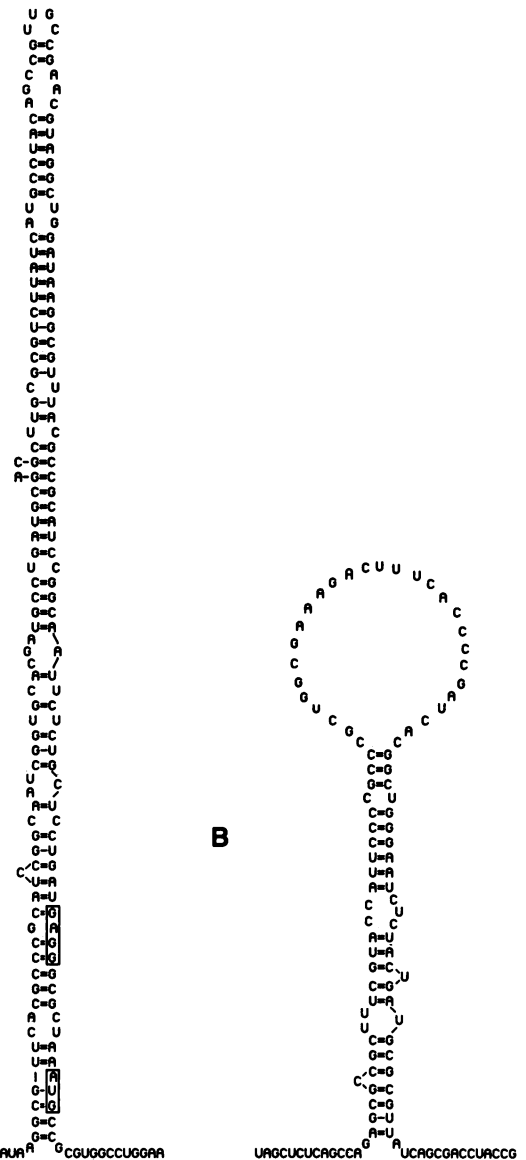


FIG. 5. Nucleotide sequence of the *aceA-lacZ* junction. An in-frame *aceA-lacZ* fusion gene was constructed by insertion of the *lacZ* cassette from pMC1871 into a *Hind*III site at the 3' end of a truncated allele of *aceA* (see the text). The resulting plasmid was cleaved with *Bam*HI, which cuts downstream of the *Hind*III site in the polylinker of the *lacZ* cassette, and was then end labeled with T4 polynucleotide kinase. The sample was then cleaved with *Pvu*II, and the appropriate fragment was purified by electrophoresis. The sequence of the *aceA-lacZ* junction was determined by the method of Maxam and Gilbert. The deduced amino acid sequence from isocitrate lyase is shown in capital letters. The site of the fusion between *aceA* and the *Hind*III linker is indicated by an asterisk in Fig. 2.

tion mutagenesis to remove the sequences which comprise this potential structure. This deletion had little effect on the expression of IDH kinase/phosphatase, indicating that the stem-loop structure had not inhibited translational initiation in the native operon. Deletion of these sequences from the *aceA-aceK* intergenic region did, however, produce a two-fold decrease in the expression of isocitrate lyase (unpublished results). This latter result is consistent with the suggestion that the stem-loop structures in the *aceA-aceK* intergenic region and within *aceK* could differentially stabilize the *aceA* region of the primary transcript (possibly by acting as barriers to exonucleolytic digestion) and thus be at least partially responsible for the downshift in expression observed between *aceA* and *aceK* (10). A similar mechanism has been proposed to contribute to polarity within the *rxcA* operon of *Rhodospseudomonas capsulata* (1).



A

B

FIG. 6. Potential secondary structure in the *aceK* message. The SEQ program from Intelligenetics, Inc., was used to predict potential secondary structure formed from the sequence given in Fig. 2. Although several variations were possible, only the most stable structures are shown. (A) A stem-loop structure predicted to occur in the *aceA-aceK* intergenic region. The sequences which form this structure are indicated by the heavy arrows in Fig. 2. The calculated free energy of formation is -54 kcal/mol (ca. -226 kJ/mol). (B) A stem-loop structure predicted to form within *aceK*. The stem begins at position 998, 386 bp downstream of the translational start site for IDH kinase/phosphatase, and is indicated by the dashed arrows in Fig. 2. The calculated free energy for the formation of this structure is -29 kcal/mol (ca. -121 kJ/mol).

ACKNOWLEDGMENTS

We thank James Howard, John Lipscomb, and Daniel Geraghty for helpful discussions, Dr. Howard for assistance with amino acid analysis and sequencing, and Dawn Hall for technical assistance.

This work was supported by Public Health Service grant GM33927 from the National Institutes of Health.

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