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Sequence of the *pckA* Gene of *Escherichia coli* K-12: Relevance to Genetic and Allosteric Regulation and Homology of *E. coli* Phosphoenolpyruvate Carboxykinase with the Enzymes from *Trypanosoma brucei* and *Saccharomyces cerevisiae*

VERONICA MEDINA,[1] RENO PONTAROLLO,[2] DANIEL GLAESKE,[1] HENRY TABEL,[2] AND HUGHES GOLDIE[1]*

Departments of Microbiology[1] and Veterinary Microbiology,[2] University of Saskatchewan, Saskatoon, Saskatchewan, S7N 0W0, Canada

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The sequence of the *pckA* gene coding for phosphoenolpyruvate carboxykinase in *Escherichia coli* K-12 and previous molecular weight determinations indicate that this allosteric enzyme is a monomer of M_r 51,316. The protein is homologous to ATP-dependent phosphoenolpyruvate carboxykinases from *Trypanosoma brucei* and *Saccharomyces cerevisiae*. A potential ATP binding site was conserved in all three sequences. A potential binding site for the allosteric activator, calcium, identified in the *E. coli* enzyme, was only partially conserved in *T. brucei* and *S. cerevisiae*, consistent with the observation that the enzymes from the latter organisms were not activated by calcium. The published sequence of the *ompR* and *envZ* genes from *Salmonella typhimurium* is followed by a partial sequence that is highly homologous to *pckA* from *E. coli*. The order of these genes and the direction of transcription of the presumptive *S. typhimurium pckA* gene are the same as those in *E. coli*. The potential calcium binding site of the *E. coli* enzyme is conserved in the partial predicted sequence of the *S. typhimurium* phosphoenolpyruvate carboxykinase, consistent with the observation that calcium activation of the *S. typhimurium* phosphoenolpyruvase carboxykinase is very similar to that observed for the *E. coli* enzyme. A *pckA* mRNA transcript was observed in stationary-phase cells but not in logarithmically growing cells. The mRNA start site was mapped relative to the sequence of the *pckA* structural gene.

Phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49) catalyzes the first committed step of gluconeogenesis in *Escherichia coli*: the phosphorylation and decarboxylation of the Krebs cycle intermediate oxaloacetic acid to PEP (21). PEP carboxykinase is activated by calcium in *E. coli* (6). Although the physiological function of calcium activation is unknown, the mechanism is allosteric, since partial digestion with trypsin desensitizes the purified enzyme to calcium activation without changing other kinetic parameters. Furthermore, binding of the fluorescent calcium analog terbium is abolished by trypsin cleavage (6).

Expression of the *E. coli pckA* gene encoding PEP carboxykinase is regulated by cyclic AMP (5, 7). Also, transcription of *pckA-lacZ* fusions increases 100-fold in the stationary phase of growth by a mechanism that is independent of cyclic AMP (7). The function of this increased PEP carboxykinase synthesis may be to provide carbohydrates required for energy reserves after cessation of growth, since protease activity, Krebs cycle enzyme activities, and glycogen synthesis all increase in the stationary phase (5). The mechanism responsible for increased rates of *pckA* transcription in the stationary phase is unknown.

To learn more about the genetic regulation of PEP carboxykinase, the *pckA* gene was sequenced, an mRNA start site was mapped, and potential transcriptional regulatory sequences were identified. N-terminal sequences of the enzyme and of proteolytic fragments were used to confirm the identity of the protein product and the position of the translational start site. The molecular weight calculated from the amino acid sequence was used to confirm the monomeric structure of this allosteric enzyme. Comparisons with the amino acid sequences predicted from PEP carboxykinase genes from *Trypanosoma brucei* and *Saccharomyces cerevisiae* were made, and a sequence from the gene bank, which is likely to contain part of the *Salmonella typhimurium pckA* gene, was identified. PEP carboxykinases from these organisms were tested for possible regulation by calcium, and comparisons of their sequences with those of known calcium-binding proteins were made to identify potential functional domains.

MATERIALS AND METHODS

DNA sequence analysis. Fragments of the cloned *pckA* gene (8) were subcloned in M13 bacteriophages mp18 and mp19 and sequenced by the dideoxy-chain termination method (17). Data obtained were stored and analyzed by using the computer programs of Queen and Korn (20) and the FRAMESCAN program (22).

Peptide mapping. Partial digests were performed in the stacking gels of sodium dodecyl sulfate-polyacrylamide electrophoresis gels (3). PEP carboxykinase (5 μg) was incubated at 100°C for 2 min in sample buffer lacking 2-mercaptoethanol, cooled, loaded with 0.25 to 5 μg of *Staphylococcus aureus* V8 protease on an 8 to 18% polyacrylamide gradient-sodium dodecyl sulfate gel, and electrophoresed at 3 V/cm until stacking occurred. The power was turned off for 45 min to allow for digestion, and electrophoresis was completed at 10 V/cm (3).

Protein sequence analysis. The N-terminal sequence of purified PEP carboxykinase was determined by the Protein

* Corresponding author.

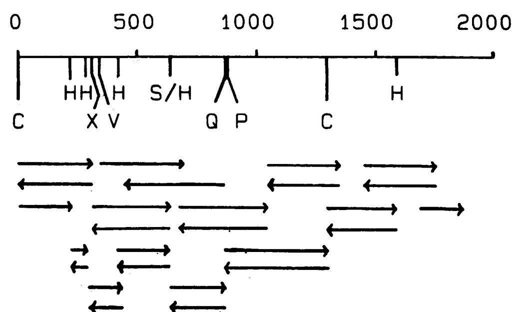

FIG. 1. Restriction map and sequencing strategy for the *pckA* gene. Arrows indicate the direction and extent of dideoxy sequencing of M13 clones. Both strands have been sequenced, including overlaps, for 1,768 bp. H, *Hin*cII; S/H, *Sal*I and *Hin*cII; C, *Cla*I; X, *Xho*I; V, *Eco*RV; Q, *Sph*I; P, *Pvu*II. *Sph*I and *Pvu*II overlap, starting with *Sph*I on the left.

Microsequencing Laboratory, Department of Biochemistry and Microbiology, University of Victoria, with an Applied Biosystems model 470A gas-phase sequencer.

Sodium dodecyl sulfate-polyacrylamide gels of peptide fragments of PEP carboxykinase were blotted onto Immobilon-P (polyvinylidine difluoride) membranes, stained with Coomassie blue, dried, and frozen at −20°C (16). Isolated peptides were cut out and sequenced as described above.

Transcript mapping. RNA was prepared (14) from *E. coli* MM294A(pHG26) (8) grown either to the midlog phase (1.5 h) or to the early-stationary phase (3.5 h) in LB medium (7). Probes were made by primer extension with M13 clones as templates (Fig. 1), annealed to the RNA in 3 M sodium trichloroacetate–50 mM piperazine-*N*,*N*′-bis(2-ethane sulfonic acid)–5 mM EDTA (pH 7), and subjected to S1 nuclease digestion (1). Products of sequencing reaction were cleaved with *Pst*I, which cuts 2 bp upstream from *pckA* in M13 mp18, necessitating a 2-bp correction in the start site (1). Sodium trichloroacetate crystals were prepared as described previously (18).

Enzyme preparations. PEP carboxykinase was purified to homogeneity (6) from French-pressed extracts of *E. coli* K-12 strain MM294A(pHG26), which expresses about 30-fold-elevated levels of this enzyme (8). Ticarcillin (100 μg/ml) was substituted for ampicillin for growth of the inoculum, and antibiotics were omitted from the final growth medium.

Extracts of *S. typhimurium* EMG39 (strain LT2, *trpE8*; ATCC 3582) were prepared as described previously for *E. coli* extracts (8), except no antibiotics were used.

T. brucei subsp. *brucei* strain Shinyanga was purified from infected blood of a BALB/c mouse by DEAE-cellulose chromatography (12) and sonicated in 0.25 M sucrose plus 20 mM Tris (pH 8) at 4°C for 10 s at 100 W with a Vibra-cell sonicator. A particulate fraction (11) was obtained by centrifuging at 30,000 × *g* for 30 min and resuspended in the same buffer, centrifuged again, and suspended in 20 mM Tris–1 mM EDTA (pH 7.5).

S. cerevisiae 262 was grown in yeast extract-peptone-dextrose medium plus 1% ethanol and harvested as described previously (26). Cells were then suspended in 20 mM Tris–1 mM EDTA (pH 7.5) and disrupted by two passages through a French pressure cell at 10,000 lb/in^2.

Assays. PEP carboxykinase activity was measured by the $^{14}CO_2$ exchange assay (6), and protein concentrations were measured by the method of Lowry et al. (15).

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been submitted to Genbank under accession no. M34605.

RESULTS

Sequence of PEP carboxykinase from *E. coli*. A 2.7-kb fragment of the *E. coli* K-12 chromosome apparently contains the *pckA* gene and its transcriptional control region, since plasmids containing this fragment complemented *pckA* mutants and overexpressed PEP carboxykinase activity. Also, the cloned *pckA* gene was regulated in a manner qualitatively similar to that used for the chromosomal *pckA* gene (8). M13 subclones representing 1,860 bp from this fragment were sequenced (Fig. 1). The sequence contained an open reading frame of 1,392 bp, preceded by a sequence that is potentially a strong ribosomal binding site (22) (Fig. 2). The predicted amino acid sequence matched the N-terminal amino acid sequences of purified PEP carboxykinase and of peptides generated by *S. aureus* V8 protease (Fig. 3; underlined in Fig. 2). Also, the FRAMESCAN program (22), calibrated with the codons of *E. coli* β-galactosidase, indicated that codon usage was very typical of *E. coli* throughout. Mean values of $\log[p/(1 - p)]$ were 9.4 for reading frame 1, −9.9 for reading frame 2, and −9.6 for reading frame 3 for a window of 33 codons; therefore, frameshift errors in the amino acid sequence due to DNA sequencing errors are unlikely.

The PEP carboxykinase sequence contains 464 amino acids, with 52 basic, 54 acidic, 46 aromatic, and 145 hydrophobic residues (aromatic plus Ile, Leu, Met, and Val). Results of hydrophobicity analyses (10) were typical of a soluble protein. The amino acid sequence was 54% identical to the sequence of PEP carboxykinase from *T. brucei* (19) and 42% identical to the sequence of PEP carboxykinase from *S. cerevisiae* (24) when the sequences were aligned according to similarity of amino acid residues (20) (data not shown). One of the highly conserved regions contained the consensus for an ATP binding site (Fig. 4).

A possible calcium binding site in the *E. coli* protein sequence (Fig. 1 and 4) has amino acid side chains containing oxygen atoms corresponding to positions 1, 3, 5, 7, and 9 of a typical calcium binding loop, with Glu at position 12 (9, 25). The potential calcium binding loop in the *E. coli* sequence contains 13 amino acids, similar to vitamin D-dependent intestinal calcium binding protein (9). *T. brucei* PEP carboxykinase has Lys instead of Glu at position 12, and the *S. cerevisiae* protein has Val at position 3 and Asn at position 12 of the region corresponding to the hypothetical calcium binding site (Fig. 4).

The last 829 bp of the *pckA* sequence is 48% identical to a sequence downstream of *ompR* and *envZ* in the published *S. typhimurium* sequence (13) (Fig. 5). The *S. typhimurium* sequence does not contain a complete open reading frame in this region, but an open reading frame was generated by assuming a total of six frame shifts due to sequencing errors, and an amino acid sequence 87% identical to residues 252 to 463 of the *E. coli* sequence was then generated (data not shown). The positions and orientations of these genes in *S. typhimurium* are the same as those determined by restriction mapping in *E. coli*: *pckA-envZ-ompR*, with *pckA* transcribed in the direction of *envZ* (8). The predicted *S. typhimurium* PEP carboxykinase sequence starts with GKT, part of the possible nucleotide binding site shown in Fig. 2, and is identical to the *E. coli* sequence in the hypothetical calcium binding site, except that Ile at position 4 of the *E. coli* sequence (Fig. 4) is changed to Val.

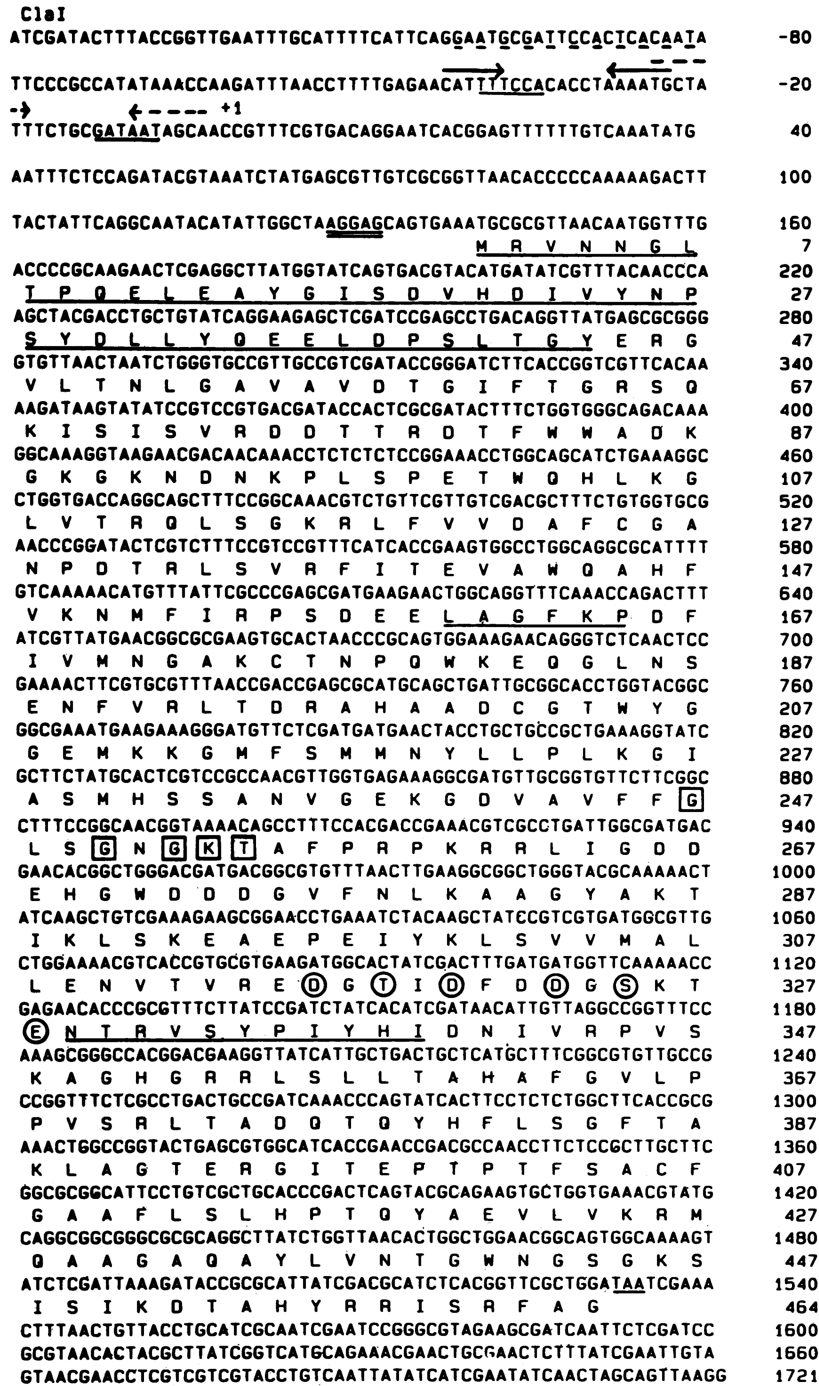

FIG. 2. DNA sequence of the *pckA* gene from *E. coli* K-12 (starting with the *Cla*I site at the left in Fig. 1) and amino acid sequence of its product, PEP carboxykinase. The DNA sequence is numbered from the transcriptional start site shown in Fig. 6. Sequences homologous to consensus sequences for promoters recognized by σ70 at −10 and −37 bp are underlined, and a potential binding site for cyclic AMP receptor protein is underlined with a broken line at −90 bp. Two interrupted inverted repeats −42 and −3 bp are indicated with solid and broken arrows. A potential ribosomal binding site is underlined twice. The amino acid sequence confirmed by N-terminal sequencing of PEP carboxykinase is underlined. Amino acids 14 to 18, 37 to 43, 160 to 165, and 329 to 339 (underlined) were also confirmed by partial N-terminal sequencing of peptides generated by *S. aureus* V8 protease (Fig. 3). Residues matching the consensus for an ATP binding site are boxed, and residues of a potential calcium binding site are circled (see Fig. 4).

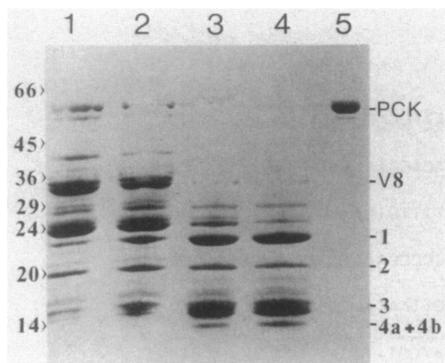

FIG. 3. Protein blot of a sodium dodecyl sulfate gel (8 to 18% acrylamide gradient) of *S. aureus* V8 protease digests of purified PEP carboxykinase. Molecular weight standards (in thousands) are shown at the left, and peptides are identified at the right. Amounts of protease added (lanes): 1, 0.25 μg; 2, 1 μg; 3, 5 μg; 4, 20 μg; 5, no protease.

Peptide map of PEP carboxykinase. Partial digestion of purified *E. coli* PEP carboxykinase with *S. aureus* V8 protease (Fig. 3) produced peptides representing most of the protein molecule. All five peptides sequenced had N-terminal sequences that followed one or two Glu residues, indicating that *S. aureus* V8 appears to cut preferentially after Glu when digests are performed in stacking gels. Peptide 1 (Fig. 3) represents most of the C terminus, from Asn-329 to the end, based on its size and its N-terminal sequence. Peptide 2 runs from Ala-14 to about position 158, where there are two consecutive Glu residues, and peptide 3 (which is well isolated in lane 1 of Fig. 3) runs from Leu-160 to about position 268. Peptide band 4 had two sequences that could readily be distinguished from peak heights in the sequencing data: 4a (major) and 4b (minor). Peptide 4a ran from Leu-37 to about position 158, and 4b ran from Leu-160 to about position 238.

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A

E. coli    231     G D V A V F F G L S G N G K T A F P R P K
T. brucei  215     . . . T C . . . . . . T . . .   T L S A D
Yeast      241     . . . T L . . . . . . T . . . . T L S A D

Consensus:                       G X X G X G K T

B

E. coli    314     R E D G T I D F D D G S K T E N T R
T. brucei  290     R R T H E . . . N . E . I C K . . .
Yeast      319     E K S Q V V . Y . . S . I . N . . .

Calmodulin^a              D K D G D G   T I T T K E
Intestinal Ca BP^b        A K E G D P N Q L S K E E
S. erythraeus Ca BP^c     D F D G N G   A L E R A D

                          1   3   5     7   9     12
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Fig. 4. Comparison of possible functional regions of PEP carboxykinases from *E. coli*, *T. brucei*, and *S. cerevisiae*. (A) Possible ATP binding sites, with the consensus sequence underneath (X, any amino acid). The consensus used is from Walker et al. (27) (GXXXXGKT), slightly modified to conform better with their data and with the sequences shown above. (B) Possible calcium binding site in the *E. coli* sequence, with homologous regions from *T. brucei* and *S. cerevisiae*. Examples of known calcium binding sites are listed below. Positions 1, 3, 5, 7, 9, and 12 of examples are labeled. a, Human calmodulin, loop I (9); b, vitamin D-dependent calcium binding protein, loop I (9); c, *Streptomyces erythraeus* calcium binding protein, loop I (24).

Effects of calcium on PEP carboxykinase activities. Activities of PEP carboxykinases from *E. coli*, *T. brucei*, and *S. cerevisiae* in the presence of 6 mM $MgCl_2$ or 1 mM $MnCl_2$ are shown in Table 1. PEP carboxykinase in crude extracts of *S. typhimurium* was activated by metals in a manner identical to that of the *E. coli* enzyme (data not shown). The magnesium concentration was slightly suboptimal, since all three PEP carboxykinases had about 60% of their respective maximum activities with 6 mM $MgCl_2$, but maximum activation by calcium of the *E. coli* enzyme (about sixfold) was observed at 6 mM $MgCl_2$ and 6 mM $CaCl_2$ (Table 1) (6). From the constants for binding of metals to different substrates in the assay mixture, it has been calculated that the free Ca^{2+} and Mg^{2+} concentrations are 0.93 and 2.1 mM, respectively, under these conditions (6). No activation of the PEP carboxykinases from *T. brucei* and *S. cerevisiae* by 6 mM $CaCl_2$ (Table 1) or by $CaCl_2$ concentrations of 0.1 or 20 mM (data not shown) was observed. We have also repeated these experiments with 1 and 40 mM $MgCl_2$, with similar results. All three PEP carboxykinases had optimum activity at 1 mM $MnCl_2$, and higher concentrations of manganese were inhibitory (data not shown). Calcium did not activate these enzymes in the presence of manganese (Table 1), as previously observed for the *E. coli* enzyme. We have also repeated these experiments with 0.2 mM $MnCl_2$ with similar results (data not shown).

Transcriptional start site. An mRNA was detected that was highly expressed in early-stationary-phase cells (lane S in Fig. 6) but not in log-phase cells (lane L), consistent with known transcriptional regulation of *pckA* (5, 7). The start site (+1 in Fig. 2) was preceded by a 5-out-of-6 match position at −10 and a 4-out-of-6 match near position −35 with the consensus sequence for promoters recognized by the 70,000-M_r subunit of RNA polymerase, σ70 (underlined in Fig. 2). A potential binding site for the cyclic AMP receptor protein (2) at positions −81 to −101 is underlined with a broken line in Fig. 2. The presence of such a sequence is consistent with the observed regulation of *pckA* by catabolite repression (5, 7). Two regions of interrupted dyad symmetry occur in the promoter region (indicated by arrows in Fig. 2).

DISCUSSION

The M_r of 51,316 for the *E. coli* PEP carboxykinase is consistent with estimates by gel filtration, sucrose gradient centrifugation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which indicate that the protein is a monomer of M_r 53,000 to 55,000 (6). Since PEP carboxykinase is activated allosterically by calcium, it may represent the only well-documented case of a monomeric, allosteric enzyme.

The extensive homology of PEP carboxykinases from *E. coli*, *T. brucei*, and *S. cerevisiae* may reflect the fact that all three enzymes are ATP-dependent enzymes (19) and may have similar mechanisms. The region surrounding the possible ATP-binding site is completely conserved (Fig. 4), suggesting that this sequence is important for function. None of these enzymes exhibits homology to GTP-dependent PEP carboxykinases from rats, chickens, or *Drosophila melanogaster* in the National Institutes of Health data base.

It is likely that the *S. typhimurium* sequence of the DNA fragment including the *ompR* and *envZ* genes (13) contains part of *pckA*, but it may have as many as six sequencing

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                                                                              3232
CGG AAA AAC CAC TCT CTC CAC CGA CCC GAA ACG TCG TCT GAT TGG CGA TGA TGA GCA CGG
 G   K   T   a   f   p   r       P   K   R   R   L   I   G   D   D   E   H   G
                                                                              3172
TTG GGA TGA CGA CGG CGT GTT CAA TTT TGA AGG CGG CTG CTA CGC CAA AAC CAT CAA ACT
 W   D   D   D   G   V   F   N       L   K   A   A  gY   A   K   T   I   K   L
                                                                              3112
GTC GAA AGA AGC GGA GCC AGA AAT TTA TCA TGC GAT TCG CCG TGA TGC GCT GCT GGA AAA
 S   K   E   A   E   P   E   I   Y       k   l   s   v   V  MA   L   L   E   N
                                                                              3052
CGT CAC CGT GCG CGA AGA CGG CAC CGT TGA TTT CGA CGA TGG TTC CAA AAC CGA AAA CAC
 V   T   V   R   E   D   G   T   i   D   F   D   D   G   S   K   T   E   N   T
                                                                              2992
CCG CGT CTC TTA CCC GAT CTA TCA TAT CGA TAA CAT CGT TAA GCC CGT GTC CAA AGC AGG
 R   V   S   Y   P   I   Y   H   I   D   N   I   V   r   P   V   S   K   A   G
                                                                              2932
TCA CGC CAC CAA GGT TAT CTT CCT GAC GGC GGA CCG TTC GGC GTG TTG CCG CCG GTT TCC
 H   a   p   R   L   S   L   L   T   A   h   a   F   G   V   L   P   P   V   S
                                                                              2872
CGT TTA ACC GCT AAC CAG ACG CAG TAC CAC TTC CTG TCA GGT TTC ACC GCT AAA CTG GCC
 R   L   T   A   d   Q   T   Q   Y   H   F   L   S   G   F   T   A   K   L   A
                                                                              2812
GGC ACC GAA CGC GGC GTC ACC GAA CCG ACC CCC ACC TTC TCC GCC TGC TTC GGC GCC GCG
 G   T   E   R   G   i   T   E   P   T   P   T   F   S   A   C   F   G   A   A
                                                                              2752
TTC CTG ACG CTG CAC CCA ACG CAG TAT GCT GAG GTG CTG GTG AAA CGT ATG CAG GCA GCA
 F   L   s   L   H   P   T   Q   Y   A   E   V   L   V   K   R   M   Q   A   A
                                                                              2692
GGC GCG CAG GCT TAT CTG GTT AAC ACC GGC TGG AAT GGC ACC GGC AAA CGT ATC TCC ATC
 G   A   Q   A   Y   L   V   N   T   G   W   N   G   s   G   K   s   I   S   I
                                                                              2632
AAA GAT ACG CGC GCA ATT ATC CGA CGC CAT TCT GAA CGG TTC TCT TGA TAA CGC GGA AAC
 K   D   T       A   h   y   R   R   l   S       R   F   a   g (END)
```

FIG. 5. Part of a published sequence, 3′ of *ompR* from *S. typhimurium* (STYENVZ; accession no. X12374; 3,291 to 2,332 bp), and the sequence of PEP carboxykinase from *E. coli* (Gly-252 to Gly-464, C terminus). Spacing of the *S. typhimurium* DNA sequence represents the predominant open reading frame, which matches the C-terminal sequence of the *E. coli* protein sequence. Spacing of the protein sequence reflects optimum alignment with codons. Regions potentially containing frame shifts or sequencing errors in the *S. typhimurium* DNA sequence are overligned. The postulated *S. typhimurium* partial protein sequence (generated by assuming six frameshifts) would be 213 amino acids—87% identical to the *E. coli* sequence shown—and would terminate with two consecutive stop codons.

errors (or frameshift mutations relative to the *E. coli* sequence; Fig. 6). It contained no open reading frame, and the predicted protein sequences were 87% identical when the presumptive errors were eliminated. Since *E. coli* and *S. typhimurium* PEP carboxykinases had very similar activation kinetics when assayed with different metals, one would expect a high degree of sequence homology.

PEP carboxykinases from *T. brucei* and *S. cerevisiae* were quite homologous to the *E. coli* enzyme, particularly in the region surrounding the possible calcium binding sites; however, they lacked a complete match to the consensus sequence (Fig. 4). Most calcium binding sites studied by X-ray crystallography have an α-helix–turn–α-helix structure with the oxygen ligands in the turn (Fig. 4) (9). A predicted secondary structure for this region of the *E. coli* PEP carboxykinase was predominantly α-helix–turn–β-sheet (unpublished observation), although such predictions are only 50% reliable for a given residue (4).

TABLE 1. PEP carboxykinase activities with different divalent metals

Metal salt concn (mM)			Sp act of PEP carboxykinase[a] (nmol of $^{14}CO_2$ exchanged min^{-1} mg^{-1})		
$MgCl_2$	$MnCl_2$	$CaCl_2$	*E. coli*[b]	*T. brucei*[c]	*S. cerevisiae*[d]
6			4,200	1.1	14
6		6	24,000	1.1	13
	1		6,100	2.5	14
	1	6	5,800	2.3	12

[a] Activity in the absence of metal ions was undetectable.

[b] Purified enzyme from *E. coli* K-12—similar relative effects of divalent metal ions were also obtained with crude extracts (data not shown).

[c] Particulate fraction (see Materials and Methods) containing *T. brucei* PEP carboxykinase activity.

[d] Total crude extract of ethanol-grown *S. cerevisiae* 262.

The absence of calcium activation for the *T. brucei* and *S. cerevisiae* PEP carboxykinases is consistent with the lack of complete calcium binding motifs in their sequences and provides further evidence that calcium activation of the *E. coli* enzyme is not due to an artifact in the enzyme assay for ATP-dependent PEP carboxykinase. The kinetics of calcium activation, desensitization of the enzyme to calcium activation with trypsin, and Tb^{3+} fluorescence binding curves have been reported previously (6). It would be useful to study the tertiary structure of this monomeric, allosteric enzyme and also to determine whether specific mutations to the DNA sequence coding for the potential calcium-binding domain can give rise to PEP carboxykinase molecules that are not activated by calcium.

Interrupted inverted repeats in the promoter region of *pckA* resemble binding sites for certain genetic regulatory proteins. Since it has been shown that increased transcription of *pckA* in the stationary phase is not correlated with a change in cyclic AMP levels (7), further study of the 5′ region will be necessary to determine the mechanism. Given the strong homology of the promoter to the consensus −10 and −35 sequences, one can speculate that the *pckA* promoter may be intrinsically quite active and regulated by a repressor during the log phase.

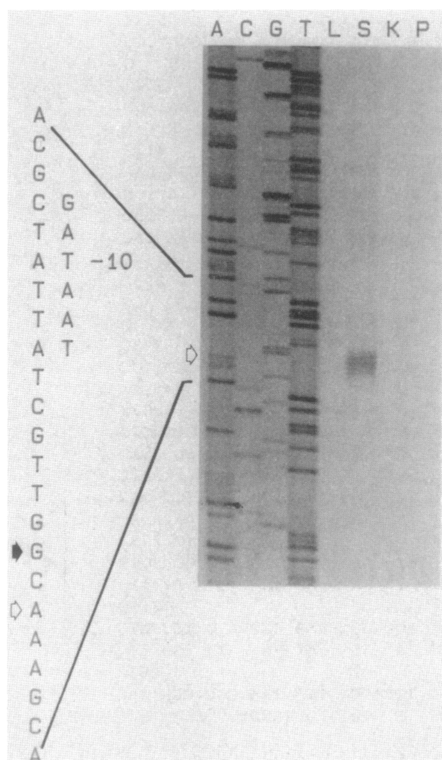

FIG. 6. S1 nuclease mapping of the *pckA* transcript. Lanes: A, C, G, T, DNA sequencing reactions cut with *Pst*I; L, RNA from cells in the logarithmic stage of growth; S, RNA from stationary-phase cells; K, control without labeled probe; P, probe without RNA. The open arrow indicates the position corresponding to the darkest transcript band. The closed arrow indicates the mRNA start site after a 2-bp correction for M13 DNA cut by *Pst*I. The sequence shown is the sense strand from sequencing reactions and the antisense sequence for a potential promoter sequence at −10 bp.

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