Isolation, Nucleotide Sequence, and Expression of a cDNA Encoding Pig Citrate Synthase†

Claudia T. Evans, Daniel D. Owens, Balazs Sumegi, Gyula Kispal, and Paul A. Srere*
Pre-Clinical Science Unit, Veterans Administration Medical Center, and Department of Biochemistry, University of Texas Southwestern Medical Center, 4500 South Lancaster Road, Dallas, Texas 75216
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ABSTRACT: Citrate synthase is a key enzyme of the Krebs tricarboxylic acid cycle and catalyzes the stereospecific synthesis of citrate from acetyl coenzyme A and oxalacetate. The amino acid sequence and three-dimensional structure of pig citrate synthase dimers are known, and regions of the enzyme involved in substrate binding and catalysis have been identified. A cloned complementary DNA sequence encoding pig citrate synthase has been isolated from a pig kidney λgt11 cDNA library after screening with a synthetic oligonucleotide probe. The complete nucleotide sequence of the 1.5-kilobase cDNA was determined. The coding region consists of 1395 base pairs and confirms the amino acid sequence of purified pig citrate synthase. The derived amino acid sequence of pig citrate synthase predicts the presence of a 27 amino acid N-terminal leader peptide whose sequence is consistent with the sequences of other mitochondrial signal peptides. A conserved amino acid sequence in the mitochondrial leader peptides of pig citrate synthase and yeast mitochondrial citrate synthase was identified. To express the pig citrate synthase cDNA in *Escherichia coli*, we employed the inducible T7 RNA polymerase/promoter double plasmid expression vectors pGP1-2 and pT7-7 [Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074–1078]. The pig citrate synthase cDNA was modified to delete the N-terminal leader sequence; then by use of a synthetic oligonucleotide linker, the modified cDNA was cloned into pT7-7 immediately following the initiator Met. A glutamate-requiring (citrate synthase deficient), recA⁻ *E. coli* mutant, DEK15, was transformed with pGP1-2 and then pT7-7PCS. pT7-7PCS complemented the *E. coli* gltA mutation. Following induction, the expressed pig citrate synthase protein was estimated to be about 9% of the total cell protein. Expression of the pig citrate synthase cDNA in *E. coli* yielded a functionally active enzyme that comigrated on sodium dodecyl sulfate–polyacrylamide gels with the purified pig heart protein.

Citrate synthase, which catalyzes the condensation of acetyl coenzyme A and oxalacetate to form citrate, has a central role in aerobic energy production and metabolite interconversions by catalyzing the initial step of the tricarboxylic acid (TCA)[1] cycle (Krebs & Lowenstein, 1960). Kinetic studies on the pig enzyme have described the catalytic mechanism and stereospecific course of the reaction [see Srere (1975), Beeckmans (1984), and Wiegand and Remington (1986) for reviews]. The amino acid sequence (Bloxham et al., 1981, 1982) and the three-dimensional structures of the pig heart citrate synthase dimers with and without substrate ligands (Remington et al., 1982; Wiegand et al., 1984) also have been determined, and regions of the enzyme involved in substrate binding and catalysis have been identified.

In order to define the role of specific amino acids in the reaction mechanism and structure of citrate synthase by applying current molecular biological techniques, including oligonucleotide site-directed mutagenesis, a DNA clone encoding pig citrate synthase was required. This paper reports the identification of a full-length cDNA clone encoding pig citrate synthase from a λgt11 pig kidney cDNA library. The clone was completely sequenced, and the amino acid sequence of the protein, including the mitochondrial leader peptide, was determined. The derived amino acid sequence from the cDNA confirmed the complete amino acid sequence determined on pig heart citrate synthase (Bloxham et al., 1981, 1982). A conserved seven amino acid sequence in the mitochondrial leader peptides of the pig and yeast citrate synthases was observed proximal to the N-termini of the mature processed proteins. We report further on the expression of the pig citrate synthase cDNA in a glutamate-requiring *Escherichia coli* mutant deficient in its own bacterial citrate synthase.

Experimental Procedures

Materials. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Boehringer Mannheim, or Bethesda Research Laboratories; *E. coli* DNA polymerase I large (Klenow) fragment was obtained from Bethesda Research Laboratories; other DNA-modifying enzymes were purchased from New England BioLabs or Boehringer Mannheim. All enzymes were used according to manufacturer's recommendations. Radionucleotides, [^{32}P]-ATP (3000 Ci/mmol), [^{35}S]dCTP (1400 Ci/mmol), and ^{125}I protein A (47 mCi/mg) were obtained from New England Nuclear. Protein molecular weight standards were obtained from Bio-Rad. All other chemicals used were of the highest purity available.

RNA Isolation and cDNA Synthesis. Total cellular RNA was extracted from adult pig tissues by a modification (Ilaria et al., 1985) of the guanidinium thiocyanate method (Chirgwin et al., (1979). Poly(A+) RNA was isolated by two cycles of oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). A pig kidney λgt11 cDNA library was constructed by a modification of the method described by Huynh et al. (1985).

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* Address correspondence to this author at the Veterans Administration Medical Center, 4500 S. Lancaster Rd., Dallas, TX 75216.

[1] Abbreviations: kb, kilobase(s); bp, base pair(s); PCS, pig citrate synthase; YCS, yeast citrate synthase; SDS, sodium dodecyl sulfate; TCA, tricarboxylic acid; pfu, plaque-forming unit(s); SSC, 0.15 M NaCl containing 15 mM sodium citrate.

Double-stranded cDNA synthesis, blunt-end *Eco*RI linker addition, chimeric bacteriophage formation with *Eco*RI-digested λgt11, in vitro bacteriophage packaging, and amplification of the resultant cDNA library were performed as described previously (Gubler & Hoffman, 1983; Young & Davis, 1983a,b; Watson & Jackson, 1985). The phage were titered in *E. coli* Y1090, and recombinants were identified on X-gal indicator plates. The resultant pig kidney λgt11 cDNA library contained 1.04×10^6 plaque-forming units (pfu) and was 72% recombinant.

Screening of Bacteriophage λgt11 cDNA Library. The pig kidney λgt11 cDNA library was plated in its entirety on 30 × 150 mm plates (30000 phage/plate), and bacteriophage clones containing pig citrate synthase sequences were identified by hybridization with a synthetic oligonucleotide probe (59-mer). The probe was constructed on an Applied Biosystems Model 380A solid-phase DNA synthesizer for a portion of the known pig citrate synthase amino acid sequence using codons based upon a bovine usage bias. The screening for recombinant phage that contained cDNA inserts encoding citrate synthase followed the modification (Ullrich et al., 1984) of the colony hybridization method (Benton & Davis, 1977). After overnight growth of the library, replica filters were prepared on Colony/Plaque Screen nylon membranes and prehybridized at 55 °C according to standard procedures (Maniatis et al., 1982). ^{32}P-Labeled 59-mer was added to the hybridization solution, and the filters were incubated at 45 °C overnight. Filters were washed (45 °C, 1 × SSC, 1% SDS) until the overall radioactivity on the filters was reduced to background. The filters then were dried and subjected to autoradiography. Plaques corresponding to autoradiographic signals were isolated and purified under identical conditions through three subsequent platings at low plaque density. Plaque-purified clones were amplified by the plate lysate method (Maniatis et al., 1982) to a titer of about 1×10^{12} pfu/mL and still produced colorless plaques on X-gal plates, indicating that the amplified clones remained recombinant. In addition, each individually amplified clone was rescreened and hybridized positively with the ^{32}P-labeled 59-mer oligonucleotide probe.

Size of the Cloned Pig Citrate Synthase cDNA. DNA was prepared from the phage produced from plate lysates of the plaque-purified clones (Maniatis et al., 1982). DNA (1 μg) from each clone was cut with *Eco*RI, and the inserts from the restriction enzyme digestion mixture were end-labeled with [^{32}P]dATP by using the Klenow fragment of DNA polymerase I and run on a 2% agarose gel. A *Hin*dIII digest of λ phage DNA was used for molecular weight markers. The size of the Largest cDNA inserts was estimated to be 1.5 kilobases (kb).

Subcloning. The *Eco*RI fragments from each of the recombinant phage were subcloned into the corresponding restriction site of pBR322 by ligating the fragments to the complementary ends of phosphatase-treated pBR322 under standard conditions and transforming *E. coli* RRI by described methods (Maniatis et al., 1982). Three recombinant plasmids with 1.4–1.5-kb inserts were designated pPCS4, pPCS7, and pPCS8.

RNA Hybridization. Poly(A+) RNA (10 μg), isolated as described above, was fractionated on formaldehyde denaturing gels as described (Maniatis et al., 1982). Best results were obtained by using 1.25% agarose gels run at 15 V for 16 h. λ DNA (1 μg), cut with the restriction endonuclease *Hin*dIII and treated exactly as RNA, was used as a marker for size calibration. Following electrophoresis, the RNA was blotted onto Zeta-Probe (Bio-Rad) and hybridized with a ^{32}P-labeled cDNA insert from pPCS4 (Thomas, 1980). Filters were washed under stringent conditions (65 °C, 0.1 × SSC, 1% SDS).

DNA Sequence Determination.[2] The cDNA inserts and respective restriction fragments were purified (Maniatis et al., 1982) and subcloned into the M13mp18 and M13mp19 vectors (Messing et al., 1981). The nucleotide sequences of the DNA fragments were obtained by standard ^{35}S dideoxy sequencing techniques (Sanger et al., 1977).

Expression of the Pig Citrate Synthase cDNA in E. coli. The plasmids pGP1-2 and pT7-7 were a gift from S. Tabor (Harvard University). pGP1-2 contains the T-7 RNA polymerase gene under the control of the inducible λP_L promoter and the gene for the temperature-sensitive λ repressor cI857. The second plasmid, pT7-7, contains the T7 RNA polymerase promoter *ϕ10* as well as a strong translation initiation region prior to the polylinker.

A 1395 bp *Sph*I–*Eco*RI fragment containing the pig citrate synthase cDNA sequence that encodes the mature form of the enzyme with the mitochondrial leader peptide sequences deleted was prepared by digestion of the previously cloned 1473 by *Eco*RI fragment from pBR322-PCS. The fragment was purified and ligated in the presence of excess synthetic *Nde*I–*Sph*I linker to pT7-7 that was digested with *Nde*I and *Eco*RI. *E. coli* strain HB101 was transformed with this ligation mixture. The plasmid pT7-7PCS was identified in an ampicillin-resistant transformant and contained an insert of the correct size and orientation.

E. coli strain DEK15 (K. Walsh, MIT) contains a point mutation in the gltA gene encoding *E. coli* citrate synthase. This strain was transformed sequentially with pGP1-2 and pT7-7PCS or pT7-7 by the $CaCl_2$ procedure (Maniatis et al., 1982). Transformants were selected on LB plates containing 50 μg/mL kanamycin and 50 μg/mL ampicillin at 30 °C. Doubly kanamycin- and ampicillin-resistant colonies were isolated that contained both pGP1-2 and either pT7-7PCS or pT7-7. Individual colonies were tested for their ability to grow in the absence of glutamate at 30 °C in the presence of kanamycin and ampicillin.

To express the pig citrate synthase cDNA in vivo, cells were grown in LB broth containing 50 μg/mL each kanamycin and ampicillin at 30 °C. At a cell density of 0.5 OD_{600} unit, the temperature of the cultures was rapidly shifted to 42 °C for 15 min. The cells were allowed to grow an additional 15 min at 37 °C. Cells were harvested, washed, frozen, and thawed at room temperature twice, then sonicated for 10 s at 4 °C, 50% power, and pulsed, setting 5 on a Model W225R sonicator (Heat Systems–Ultrasonics, Inc.). The cell debris was removed by centrifugation and the supernatant solution assayed for protein and citrate synthase activity.

Immunoblot Analysis. Immunoblot analysis of PCS was performed by using PCS-specific antiserum that was precleared of *E. coli* recognizing IgGs by adsorption to Sephadex Macrobeads to which a crude lysate of *E. coli* DEK15 proteins was covalently coupled. Procedures for transfer of proteins from SDS–polyacrylamide gels to nitrocellulose and for the processing of nitrocellulose blots have been described previously (DeBlas & Cherwinski, 1983).

Other Methods. Citrate synthase activity was measured by the method of Srere et al. (1963). Protein determination was performed by the method of Bradford (1976). SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970).

[2] The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank.

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                585                 615                 640
cDNA    AAGTACTGGGAGTTGATTTATGAAGACTGTATGGATCTGATCGCAAAGCTACCTTGTGT
        !!!!!!!!!!!! !!!! !! !! !!!!! !!!!! !!!!!!!! !!!!! !! !! !!
Probe   AAGTACTGGGAGCTGATCTACGAGGACTGCATGGACCTGATCGCCAAGCTGCCCTGCGT

Protein LysTyrTrpGluLeuIleTyrGluAspCysMetAspLeuIleAlaLysLeuProCysVal
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FIGURE 1: Oligonucleotide probe for pig citrate synthase. A 59-base oligonucleotide sequence was constructed for amino acid residues 166–185 of the pig citrate synthase amino acid sequence. A comparison of the probe sequence to the actual cDNA sequence shows 80% homology.

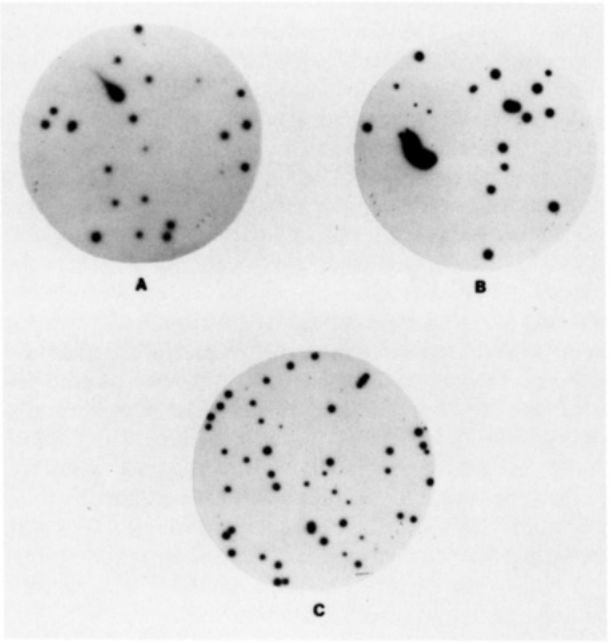

FIGURE 2: Plaque-purified λ recombinants that hybridized to ^{32}P-labeled 59-mer pig citrate synthase oligonucleotide probe. (A) λPCS4; (B) λPCS7; (C) λPCS8.

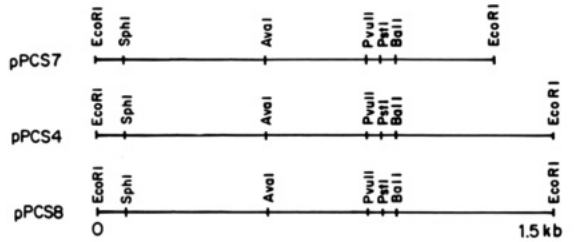

FIGURE 3: Restriction endonuclease maps of the cDNA inserts that hybridized to the 59-mer pig citrate synthase oligonucleotide probe. Restriction sites were determined by a combination of single, partial, and double enzyme digests of pPCS4, pPCS7, and pPCS8.

RESULTS

Identification of Phage Containing cDNA Sequences Complementary to the mRNA Encoding Pig Citrate Synthase. After the λgt11 pig kidney cDNA library was screened, 10 recombinant clones were selected that hybridized to the synthetic pig citrate synthase oligonucleotide probe (Figure 1). Three recombinants (Figure 2) contained cDNA inserts that cross-hybridized and were approximately 1.5 kb in size. Restriction maps (Figure 3) indicated that these three cDNA clones contained identical restriction sites. Southern blot analysis (Southern, 1975) of the restriction endonuclease digests further indicated that the three cDNAs were related to each other in the region homologous to the oligonucleotide probe.

Size of mRNA Species Complementary to the pPCS4 Insert. Since the three cDNAs were structurally related, the clone containing the largest insert, pPCS4, was selected for further analysis. The mature form of pig citrate synthase is a protein of 437 amino acids and must be encoded by mRNAs

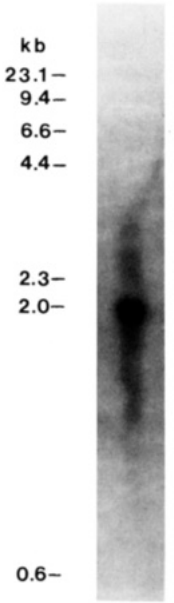

FIGURE 4: RNA blot analysis of pig citrate synthase mRNA. Poly(A+) RNA from pig liver (5 μg) was separated on a 1.25% denaturing agarose gel, electroblotted onto Zeta-Probe, and hybridized with a ^{32}P-labeled 1.5-kb cDNA insert from pPCS4.

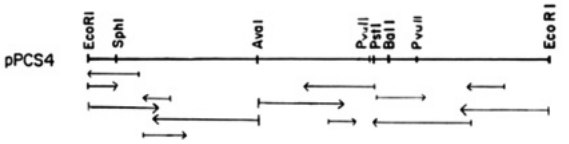

FIGURE 5: Sequencing strategy of pPCS4. The entire nucleotide sequence of pPCS4 was determined in both orientations by the dideoxy chain termination method. Arrows indicate the direction and extent of sequencing reactions.

at least 1.32 kb in size. To determine the size of the mRNA encoding pig citrate synthase, RNA blot hybridization was performed with the cloned cDNA pPCS4 (Figure 4). An mRNA, approximately 2.0 kb in size, hybridized strongly to the cloned cDNA insert and also was observed in blots of RNA isolated from pig heart and pig kidney. This result suggests that pig citrate synthase mRNAs contain at least 0.68 kb of 3′ and 5′ noncoding sequences.

DNA Sequence of pPCS4. The DNA sequence of pPCS4 was determined by the strategy shown in Figure 5. The complete DNA sequence contains one long open reading frame (1395 bp) that encodes pig citrate synthase (Figure 6). The derived amino acid sequence is identical with that determined for the purified enzyme and predicts the presence of a 27 amino acid N-terminal mitochondrial leader peptide. The 5′ and 3′ ends of a second, somewhat smaller clone, pPCS7, also were sequenced. The 5′ sequence of pPCS7 was identical with pPCS4 while the 3′ end of pPCS7 lacked 150 nucleotides including the 3′ noncoding and C-terminal regions of pig citrate synthase.

Southern analysis indicated that a region proximal to the unique *Ava* site (Figure 3) hybridized to the synthetic oligonucleotide probe. During sequencing, it was found that the oligonucleotide probe sequence was 80% homologous to the authentic cDNA sequence of this region (Figures 1 and 5).

The primary amino acid sequence of pig citrate synthase has been compared to those derived for the yeast mitochondrial, yeast cytosolic, and *E. coli* forms of the enzyme (Rosenkrantz et al., 1986). The two yeast citrate synthases show a high degree of homology at both the amino acid and DNA levels. It also was shown that the amino acid sequence of the

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        10         20         30         40         50         60
CCTGCCATGGCCTTACTCACTGCGGCCGCCCGGCTCTTCGGAGCCAAGAATGCATCCTGT
     MetAlaLeuLeuThrAlaAlaAlaArgLeuPheGlyAlaLysAsnAlaSerCys

        70         80        ↓90        100        110        120
CTTGTTCTTGCTGCCCGGCATGCCAGTGCTTCTTCCACGAACTTAAAAGACATTTTGGCT
LeuValLeuAlaAlaArgHisAlaSerAlaSerSerThrAsnLeuLysAspIleLeuAla

       130        140        150        160        170        180
GACCTTATACCTAAGGAGCAAGCCAGAATTAAGACCTTCAGGCAGCAACACGGCAACACC
AspLeuIleProLysGluGlnAlaArgIleLysThrPheArgGlnGlnHisGlyAsnThr

       190        200        210        220        230        240
GTGGTGGGCCAAATCACTGTGGACATGATGTATGGTGGCATGAGAGGCATGAAGGGATTG
ValValGlyGlnIleThrValAspMetMetTyrGlyGlyMetArgGlyMetLysGlyLeu

       250        260        270        280        290        300
GTGTATGAAACATCGGTTCTTGATCCTGATGAGGGCATCCGTTTCCGAGGCTACAGTATC
ValTyrGluThrSerValLeuAspProAspGluGlyIleArgPheArgGlyTyrSerIle

       310        320        330        340        350        360
CCTGAATGCCAGAAAATGCTGCCCAAGGCTAAGGGTGGGGAAGAACCGCTGCCAGAGGGC
ProGluCysGlnLysMetLeuProLysAlaLysGlyGlyGluGluProLeuProGluGly

       370        380        390        400        410        420
TTATTTTGGCTGCTGGTAACTGGACAGATCCCAACAGAGGAGCAGGTGTCTTGGCTCTCA
LeuPheTrpLeuLeuValThrGlyGlnIleProThrGluGluGlnValSerTrpLeuSer

       430        440        450        460        470        480
AAAGAGTGGGCAAAGAGGGCAGCTCTGCCTTCCCATGTGGTCACCATGCTGGACAACTTT
LysGluTrpAlaLysArgAlaAlaLeuProSerHisValValThrMetLeuAspAsnPhe

       490        500        510        520        530        540
CCCACGAATCTACACCCCATGTCTCAGCTCAGTGCAGCCATTACAGCCCTCAACAGTGAA
ProThrAsnLeuHisProMetSerGlnLeuSerAlaAlaIleThrAlaLeuAsnSerGlu

       550        560        570        580        590        600
AGTAACTTTGCCCGAGCATATGCAGAGGGTATCCACCGAACCAAGTACTGGGAGTTGATT
SerAsnPheAlaArgAlaTyrAlaGluGlyIleHisArgThrLysTyrTrpGluLeuIle

       610        620        630        640        650        660
TATGAAGACTGTATGGATCTGATCGCAAAGCTACCTTGTGTTGCAGCAAAGATCTACCGG
TyrGluAspCysMetAspLeuIleAlaLysLeuProCysValAlaAlaLysIleTyrArg

       670        680        690        700        710        720
AATCTCTACCGGGAAGGCAGCAGTATTGGGGCCATTGATTCTAAACTGGACTGGTCCCAC
AsnLeuTyrArgGluGlySerSerIleGlyAlaIleAspSerLysLeuAspTrpSerHis

       730        740        750        760        770        780
AATTTCACCAACATGTTAGGCTATACTGATGCTCAGTTCACGGAGCTCATGCGCTTGTAC
AsnPheThrAsnMetLeuGlyTyrThrAspAlaGlnPheThrGluLeuMetArgLeuTyr

       790        800        810        820        830        840
CTCACCATCCACAGTGACCATGAAGGTGGCAATGTAAGTGCTCATACCAGCCATTTGGTG
LeuThrIleHisSerAspHisGluGlyGlyAsnValSerAlaHisThrSerHisLeuVal

       850        860        870        880        890        900
GGCAGTGCCCTTTCAGACCCCTACTTGTCCTTTGCAGCAGCCATGAATGGGCTGGCAGGG
GlySerAlaLeuSerAspProTyrLeuSerPheAlaAlaAlaMetAsnGlyLeuAlaGly

       910        920        930        940        950        960
CCCCTACATGGGCTGGCAAATCAGGAAGTGCTTGTTTGGCTGACACAGCTGCAGAAGGAA
ProLeuHisGlyLeuAlaAsnGlnGluValLeuValTrpLeuThrGlnLeuGlnLysGlu

       970        980        990       1000       1010       1020
GTCGGCAAAGATGTGTCAGATGAGAAGTTACGAGACTACATCTGGAATACACTCAACTCA
ValGlyLysAspValSerAspGluLysLeuArgAspTyrIleTrpAsnThrLeuAsnSer

      1030       1040       1050       1060       1070       1080
GGACGGGTTGTCCCAGGCTATGGCCACGCAGTACTAAGGAAGACTGATCCACGATATACC
GlyArgValValProGlyTyrGlyHisAlaValLeuArgLysThrAspProArgTyrThr

      1090       1100       1110       1120       1130       1140
TGTCAAAGAGAGTTTGCTCTGAAACACCTGCCTCATGACCCCATGTTTAAGCTGGTTGCT
CysGlnArgGluPheAlaLeuLysHisLeuProHisAspProMetPheLysLeuValAla

      1150       1160       1170       1180       1190       1200
CAGCTGTACAAGATTGTGCCCAATGTCCTCCTGGAACAGGGCAAGGCTAAGAATCCTTGG
GlnLeuTyrLysIleValProAsnValLeuLeuGluGlnGlyLysAlaLysAsnProTrp

      1210       1220       1230       1240       1250       1260
CCCAATGTGGATGCTCACAGTGGGGTGCTGCTCCAGTACTATGGCATGACGGAGATGAAC
ProAsnValAspAlaHisSerGlyValLeuLeuGlnTyrTyrGlyMetThrGluMetAsn

      1270       1280       1290       1300       1310       1320
TACTACACAGTCCTGTTCGGGGTATCACGGGCACTGGGTGTATTAGCACAGCTCATCTGG
TyrTyrThrValLeuPheGlyValSerArgAlaLeuGlyValLeuAlaGlnLeuIleTrp

      1330       1340       1350       1360       1370       1380
AGCCGAGCCTTAGGCTTCCCTCTAGAGAGGCCCAAGTCCATGAGCACAGACGGTCTGATA
SerArgAlaLeuGlyPheProLeuGluArgProLysSerMetSerThrAspGlyLeuIle

      1390       1400       1410       1420       1430       1440
AAACTTGTGGACTCTAAGTGAGGGTGAAACTGCAGACTGGGAAGAAACTAACTACCAGAA
LysLeuValAspSerLysEnd

      1450       1460       1470
AAGGGAGGGATCTTTAAAAAAAAAAAAAAAAA
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FIGURE 6: Nucleotide sequence of PCS4 and predicted amino acid sequence for its product, pig citrate synthase. Amino acid residues 1–27 constitute the mitochondrial transit peptide. The cleavage site that generates the mature protein is designated by the arrow. The peptide region used in design of the oligonucleotide probe is underlined.

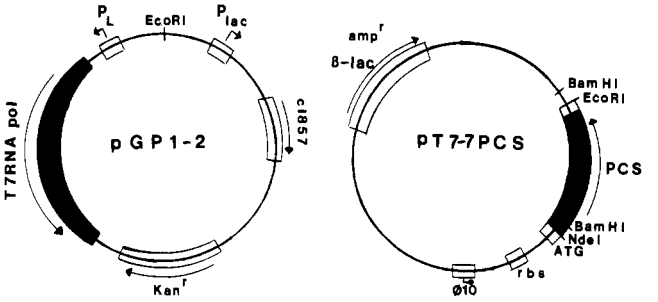

FIGURE 7: Coupled T7 RNA polymerase/promoter system. All cells contain pGP1-2 (left) which expresses T7 RNA polymerase. The structure of this plasmid (7200 bp) has been described (Tabor & Richardson, 1985). Cells also contain pT7-7PCS (3800 bp) which contains the T7 RNA polymerase promoter, ribosomal binding sequence, polylinker, and the modified pig citrate synthase cDNA inserted in-frame following the initiator Met. Control cells contain pT7-7 without inserted cDNA.

mature citrate synthase protein isolated from pig mitochondria is 55% homologous to either of the yeast enzymes (Rosenkrantz et al., 1986). When the cDNA sequence is pig citrate synthase was compared to the DNA encoding the yeast mitochondrial citrate synthase, the two DNA sequences showed 67% homology in the 1.4 kb region encoding the mature proteins, further indicating that these two sequences have been conserved.

From the sequence of the pig citrate synthase cDNA, the amino acid sequence of the mitochondrial leader peptide was determined (Figure 6). This peptide shows a predicted α-helical structure composed predominantly of hydrophobic amino acids. The basic amino acids, Arg and Lys, occur within the sequence and are separated by hydrophobic residues. When arranged in a helical wheel, the three positively charged residues lie on one side of the α helix which is consistent with the amphipathic structures of other known mitochondrial leader peptides (Douglas et al., 1986). The mitochondrial leader sequence of the yeast citrate synthase is known (Suissa et al., 1984) and was compared to the leader sequence for pig citrate synthase. When the two leader peptides were analyzed, they showed a conserved amino acid sequence, Leu-X-Ala-Arg-His-X-Ser, proximal to the N-terminus of the mature processed protein. This sequence is conserved at the DNA level (50% homologous) and is not found in the leader sequence of the cytosolic form of yeast citrate synthase (Rosenkrantz et al., 1986; Kim et al., 1986).

Expression of Pig Citrate Synthase in E. coli. The expression system shown in Figure 7 consists of two compatible plasmids, pGP1-2 and pT7-7PCS. pGP1-2 was described previously (Tabor & Richardson, 1985) and provides for the expression of T7 RNA polymerase. Cells transformed with pGP1-2 were selected on the basis of their expression of kanamycin resistance at 30 °C. T7 RNA polymerase was induced at 42 °C by derepression of the P_L promoter via inactivation of the cI857 repressor. pT7-7PCS contains the gene for ampicillin resistance, the T7 RNA promoter, *ϕ10*, a ribosomal binding sequence adjacent to the promoter, and the pig citrate synthase cDNA inserted in frame following the initiator methionine codon, ATG. In transformants cotransfected with pGP1-2 and pT7-7PCS, heat induction of T7 RNA polymerase results in transcription from the *ϕ10* promoter and the expression of pig citrate synthase.

DEK15 is an *E. coli* strain that carries a point mutation in the gltA locus encoding *E. coli* citrate synthase, and therefore is unable to grow on minimal acetate media. When DEK15 cells were cotransfected with pGP1-2 and pT7-7PCS, transformants not only grew in the presence of kanamycin and

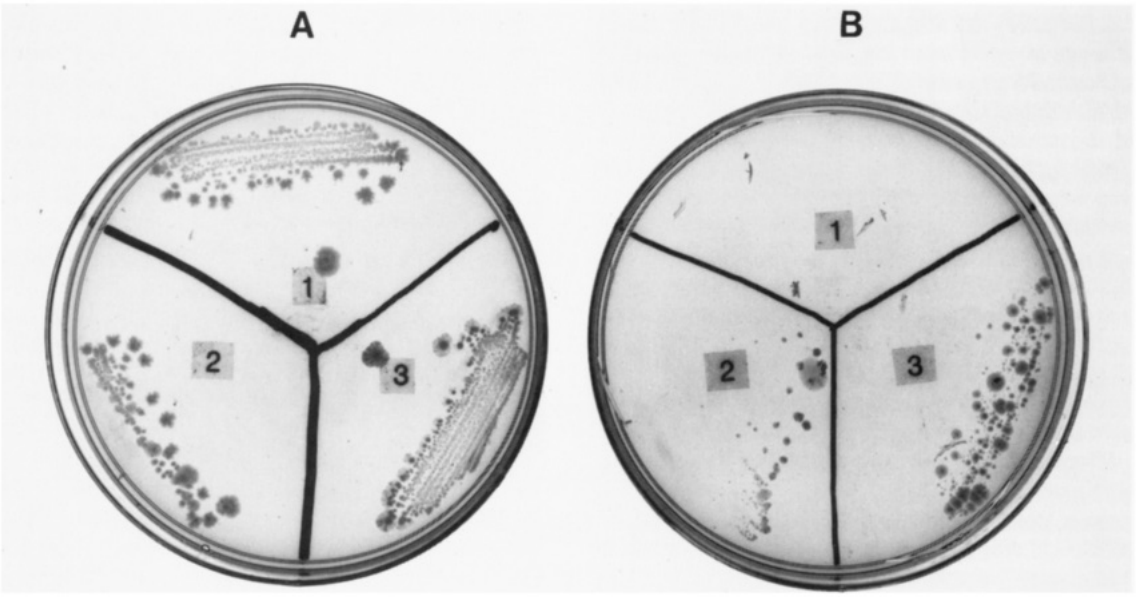

FIGURE 8: Complementation of the gltA mutation occurring in *E. coli* DEK15 by pT7-7PCS. Individual colonies were streaked on either minimal glutamate plates (A) or minimal acetate plates (B) and grown at 30 °C for 48 h. (1) DEK15/pGP1-2/pT7-7); (2 and 3) independent colonies/DEK15/pGP1-2/pT7-7PCS.

ampicillin but also were able to grow on minimal acetate media (Figure 8). On the other hand, when DEK15 cells were cotransfected with pGP1-2 and the control plasmid pT7-7 without the inserted pig citrate synthase cDNA, these transformants were unable to grow on minimal acetate media (Figure 8). Therefore, the pig citrate synthase cDNA was able to complement the gltA mutation occurring in *E. coli*.

With this expression system, we have directed the overproduction of pig citrate synthase in *E. coli*. Log-phase cells were heat induced at 42 °C for 15 min and then incubated at 37 °C for an additional 15 min as described under Experimental Procedures. Crude cell proteins were isolated and assayed for citrate synthase activity. No detectable citrate synthase activity was observed in DEK15/pGP1-2 transformants transfected with control pT7-7, while cells containing the pT7-7PCS plasmid contained approximately 5.7 ± 0.3 units of citrate synthase/mg of protein. Total cell proteins (50 μg) from DEK15/pGP1-2/pT7-7 and DEK15/pGP1-2/pT7-7PCS were separated on duplicate 10% polyacrylamide–SDS gels. The proteins were either stained with Coomassie blue or electrotransferred to nitrocellulose and reacted with rabbit anti-pig citrate synthase antibody and ^{125}I protein A (Figure 9A,B). As seen in Figure 9A, following heat induction, the DEK15 cells containing the pT7-7PCS plasmid expressed a unique protein, M_r 50000, that comigrated with authentic pig heart citrate synthase. In addition, the autoradiogram of the immunoblot shows that a single immunoreactive protein, M_r 50000, comigrates with authentic pig heart citrate synthase and is induced in *E. coli* DEK15 cells that contain pT7-7PCS (Figure 9B). When the gels were scanned for protein (Hoefer GS300 scanning densitometer with a GS350 data system) the induced pig citrate synthase represented 9% of the total *E. coli* cell protein.

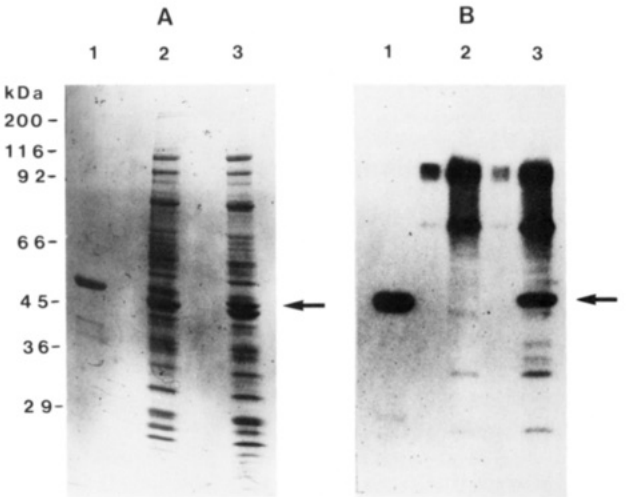

FIGURE 9: Expression of pig citrate synthase cDNA by T7 RNA polymerase. *E. coli* DEK15/pGP1-2/pT7-7 control cells (lane 2) and DEK15/pGP1-2/pT7-7PCS cells (lane 3) were grown to log phase, OD_{600} = 0.5, at 30 °C. The temperature of the cultures was rapidly shifted to 42 °C for 15 min and then maintained at 37 °C for an additional 15 min. Total cell proteins were prepared as described under Experimental Proceedures, and 50 μg was separated by SDS gel electrophoresis. (A) Coomassie-stained gel; (B) immunoblot analysis. Lane 1 is authentic pig heart citrate synthase.

Discussion

We have described the isolation of a cloned cDNA insert encoding pig citrate synthase. Approximately 1×10^6 recombinant phage from a λgt11 pig kidney library were screened using a synthetic oligonucleotide for pig citrate synthase. Three clones were identified that contained cDNA inserts, 1.5 kb in size, which cross-hybridized and shared common restriction sites.

To characterize the clones further, the cDNA insert from pPCS4 was completely sequenced. The predicted amino acid sequence from the pig kidney cDNA clone was identical with the sequence determined for the pig heart enzyme (Bloxham et al., 1981, 1982). This result is consistent with the previous observation which showed that rat kidney, rat brain, and rat heart citrate synthase were immunologically and kinetically similar (Matsuoka & Srere, 1973). An open reading frame was identified that encodes the complete pig citrate synthase protein including the mitochondrial leader peptide. Recent data from gene fusion experiments using synthetic signal peptides (Gillespie et al., 1985; Allison & Schatz, 1986) and producing hybrid proteins (Emr et al., 1986; Horwich et al., 1984; Hurt et al., 1984, 1985; Nguyen et al., 1986, 1987) have established that the information for mitochondrial targeting and translocation of nuclear-coded precursor proteins is con-

tained predominantly within the amino-terminal leader sequence. Presumably, translocation of proteins into the mitochondria is mediated by an import receptor located on the surface of the organelle that recognizes common structural characteristics contained within the signal peptides (Hurt & Van Loon, 1986; Singer et al., 1987). Mitochondrial signal peptides share certain similarities, including a net positive charge and an overall composition of polar residues interspersed by short stretches of hydrophobic amino acids (Epand et al., 1986; Roise et al., 1986). These characteristics confer amphiphilic properties to mitochondrial signal peptides and also are shared by the mitochondrial leader peptide for pig citrate synthase.

Although a large number of mitochondrial signal peptides now have been sequenced and structural similarities exist, little sequence similarity has been found among the peptides. We have found an amino acid sequence, Leu-X-Ala-Arg-His-X-Ser, that occurs in the leader peptide sequences of pig citrate synthase and yeast mitochondrial citrate synthase. In both cases, this sequence occurs proximal to the N-terminus of the mature processed protein. In addition, the sequence is conserved at the DNA level and is not found in the leader sequence of the cytosolic form of yeast citrate synthase. It is possible that this sequence is required for the correct mitochondrial processing of the signal peptide.

The amino acid sequence of the mature pig citrate synthase also is homologous to yeast citrate synthase (Bloxham et al., 1981, 1982; Rosenkrantz et al., 1986; Suissa et al., 1984). The data presented in this paper indicate that the observed homology is conserved at the DNA level. From X-ray crystallographic and amino acid data on pig citrate synthase, the amino acid residues involved in substrate binding and catalysis have been identified (Remington et al., 1982; Wiegand et al., 1984). The active site is composed of amino acid residues from both polypeptide chains of the dimeric enzyme. In addition, each monomer contains a large and small domain with the active site lying in a cleft between the two. Acetyl-CoA interacts with pig citrate synthase residues 314–322, as well as Arg-46, Arg-164, Arg-324, and Asp-375. Citrate interacts with His-238, His-274, His-320, Arg-329, Arg-401, Arg-421, and Asp-375. These residues also are conserved in yeast citrate synthase (Rosenkrantz et al., 1986) and account, in part, for the observed amino acid and DNA homologies between the two enzymes. It is now possible to test functionally the importance of these amino acids in the catalytic mechanism and structure of pig citrate synthase by performing oligonucleotide site-directed mutagenesis of the cDNA encoding pig citrate synthase.

For specific amino acid substitutions in pig citrate synthase by site-specific mutagenesis, we have overexpressed the pig citrate synthase cDNA in *E. coli* using double plasmid expression vectors (Tabor & Richardson, 1985). The expression of pig citrate synthase is under the control of the T7 RNA polymerase promoter, while transcription of T7 RNA polymerase is controlled by the λP_L promoter and cI857 repressor. We modified the pig citrate synthase cDNA by removing the DNA segment encoding the N-terminal leader sequence. Therefore, likely problems with subsequent removal of the unusual leader peptide by *E. coli* signal peptidases were circumvented. The pig citrate synthase cDNA was expressed in a gltA mutant of *E. coli*, DEK15, deficient in its own bacterial citrate synthase. The mammalian enzyme was able to complement the mutation as evidenced by the ability of transformants to grow on minimal acetate media. The production of the expected pig citrate synthase in *E. coli* was demonstrated by the following: (1) the presence of enzyme activity; (2) unique protein staining; (3) immunologic specificity; and (4) molecular weight determination. By densitometry and integration of the protein gels, it was demonstrated that, following heat induction, transformed cells expressed approximately 9% of total protein as pig citrate synthase. Previously, large-scale production of *E. coli* citrate synthase in *E. coli* from the cloned *E. coli* gltA gene was reported (Duckworth & Bell, 1982; Bloxham et al., 1983; Robinson et al., 1983). Likewise, overexpression of BCY1, the regulatory subunit of yeast cyclic AMP dependent protein kinase in *E. coli*, was reported (Johnson et al., 1987). Functional expression of the citrate synthase genes from *Rickettsia prowazekii* (Wood et al., 1983) and *Coxiella burnetti* (Heinzen & Mallavia, 1987) also was demonstrated. For the first time, this paper reports the functional expression and the overproduction of the mammalian citrate synthase in *E. coli*. Other mammalian and bacterial proteins similarly have been expressed in *E. coli* but to levels of 0.03–0.10% of total protein (Jaussi et al., 1987; Graziano et al., 1987; Dunn, et al., 1987). Since we are able to overexpress the pig citrate synthase cDNA, the functional importance of specific amino acids in the catalytic mechanism and structure of the enzyme can be evaluated by performing site-directed mutagenesis. Such studies are in progress.

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REFERENCES

Allison, D. S., & Schatz, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9011–9015.

Aviv, M., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Beeckmans, S. (1984) *Int. J. Biochem. 16*, 341–351.

Benton, W. D., & Davis, R. W. (1977) *Science (Washington, D.C.) 196*, 180–182.

Bloxham, D. P., Parmelee, D. C., Kumar, S., Wade, R. D., Ericsson, L. H., Neurath, H., Walsh, A., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5381–5385.

Bloxham, D. P., Parmelee, D. C., Kumar, S., Walsh, K. A., & Titani, K. (1982) *Biochemistry 21*, 2028–2036.

Bloxham, D. P., Herbert, C. J., Ner, S. S., & Drabble, W. T. (1983) *J. Gen. Microbiol. 129*, 1889–1897.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

DeBlas, A. L., & Cherwinski, H. M. (1983) *Anal. Biochem. 133*, 214–219.

Douglas, M. G., McCammon, M. T., & Vassarotti, A. (1986) *Microbiol. Rev. 50*, 166–178.

Duckworth, H. W., & Bell, A. W. (1982) *Can. J. Biochem. 60*, 1143–1147.

Dunn, R. J., Hackett, N. R., McCoy, J. M., Chao, B. H., Kimura, K., & Khorana, H. G. (1987) *J. Biol. Chem. 262*, 9246–9254.

Emr, S. D., Vassarotti, A., Garrett, J., Geller, B. L., Takeda, M., & Douglas, M. G. (1986) *J. Cell Biol. 102*, 523–533.

Epand, R. M., Hui, S.-W., Argan, C., Gillespie, L. L., & Shore, G. C. (1986) *J. Biol. Chem. 261*, 10017–10020.

Gillespie, L. L., Argan, C., Taneja, A. K., Hodges, R. S., Freeman, K. B., & Shore, G. C. (1985) *J. Biol. Chem. 260*, 16045–16048.

Graziano, M. P., Casey, P. J., & Gilman, A. G. (1987) *J. Biol. Chem. 262*, 11375–11381.

Gubler, U., & Hoffman, B. J. (1983) *Gene 25*, 263–269.
Heinzen, R. A., & Mallavia, L. P. (1987) *Infect. Immun. 55*, 848–855.
Horwich, A. L., Fenton, W. A., Williams, K. R., Kalonsek, F., Krans, J. P., Doolittle, R. F., Konigsberg, W., & Rosenberg, L. E. (1984) *Science (Washington, D.C.) 224*, 1068–1074.
Hurt, E. C., & van Loon, A. P. G. M. (1986) *Trends Biochem. Sci. (Pers. Ed.) 11*, 204–207.
Hurt, E. C., Pesold-Hurt, B., & Schatz, G. (1984) *EMBO J. 3*, 3149–3156.
Hurt, E. C., Muller, U., & Schatz, G. (1985) *EMBO J. 4*, 2061–2068.
Huynh, T. V., Young, R. A., & Davis, R. W. (1985) in *DNA Cloning* (Glover, D. M., Ed.) Vol. 1, pp 49–78, IRL, Oxford.
Ilaria, R., Wines, D., Pardue, S., Jamison, S., Ojeda, S. R., Snider, J., & Morrison, M. R. (1985) *J. Neurosci. Methods 15*, 165–174.
Jaussi, R., Behra, R., Giannattasio, S., Flura, T., & Christen, P. (1987) *J. Biol. Chem. 262*, 12434–12437.
Johnson, K. E., Cameron, S., Toda, T., Wigler, M., & Zoller, M. J. (1987) *J. Biol. Chem. 262*, 8636–8642.
Kim, K.-S., Rosenkrantz, M. S., & Guarente, L. (1986) *Mol. Cell. Biol. 6*, 1936–1942.
Krebs, H. A., & Lowenstein, J. M. (1960) in *Metabolic Pathways* (Greenberg, D. M., Ed.) Academic, New York.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Matsuoka, Y., & Srere, P. A. (1973) *J. Biol. Chem. 248*, 8022–8030.
Messing, J., Crea, R., & Seeburg, P. H. (1981) *Nucleic Acids Res. 9*, 309–321.
Nguyen, M., & Shore, G. C. (1987) *J. Biol. Chem. 262*, 3929–3931.
Nguyen, M., Argan, C., Lusty, C. J., & Shore, G. C. (1986) *J. Biol. Chem. 261*, 800–805.
Remington, S., Wiegand, G., & Huber, R. (1982) *J. Mol. Biol. 158*, 111–152.
Robinson, M. S., Eason, R. A., Danson, M. J., & Weitzman, P. D. J. (1983) *FEBS Lett. 154*, 51–54.
Roise, D., Horvath, S. J., Tomick, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327–1334.
Rosenkrantz, M., Alam, T., Kim, K.-S., Clark, B. J., Srere, P. A., & Guarente, L. P. (1986) *Mol. Cell. Biol. 6*, 4509–4515.
Sanger, F., Nicklen, S., & Coulson, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Singer, S. J., Maher, P. A., & Yafee, M. P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1015–1019.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Srere, P. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 57.
Srere, P., Brazil, H., & Gonen, L. (1963) *Acta Chem. Scand. 17*, S129–S134.
Suissa, M., Suda, K., & Schatz, G. (1984) *EMBO J. 3*, 1773–1781.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074–1078.
Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201–5205.
Ullrich, A., Berman, C. H., Dull, T. J., Gray, A., & Lee, J. M. (1984) *EMBO J. 3*, 361–364.
Watson, C. H., & Jackson, J. F. (1985) in *DNA Cloning* (Glover, D. M., Ed.) Vol. 1, pp 79–88, IRL, Oxford.
Wiegand, G., & Remington, S. J. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 97–117.
Wiegand, G., Remington, S., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 174*, 204–219.
Wood, D. O., Atkinson, W. H., Sikorski, R. S., & Winkler, H. (1983) *J. Bacteriol. 155*, 412–416.
Young, R. A., & Davis, R. W. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194–1198.
Young, R. A., & Davis, R. W. (1983b) *Science (Washington, D.C.) 222*, 778–782.