Takano, E., Yumoto, N., Kannagi, R., & Murachi, T. (1984b) *Biochem. Biophys. Res. Commun. 122*, 912–917.
Takano, E., Kitahara, A., Sasaki, T., Kannagi, R., & Murachi, T. (1986a) *Biochem. J. 235*, 97–102.
Takano, E., Maki, M., Hatanaka, M., Mori, H., Zenita, K., Sakihama, T., Kannagi, R., Marti, T., Titani, K., & Murachi, T. (1986b) *FEBS Lett. 208*, 199–202.
Tayler, J. W., Ott, J., & Eckstein, F. (1985a) *Nucleic Acids Res. 13*, 8765–8785.
Tayler, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985b) *Nucleic Acids Res. 13*, 8749–8763.
Towbin, H., Staehelin, T., & Gordon, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Vieira, J., & Messing, J. (1987) *Methods Enzymol.* (in press).
Wartell, R. M., & Reznikoff, W. S. (1980) *Gene 9*, 307–319.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.
Yoshikawa, M., Mizukami, T., Omori, K., Sasaki, R., & Chiba, H. (1981) *Agric. Biol. Chem. 45*, 177–183.
Zakut-Houri, R., Oren, M., Bienz, B., Lavie, V., Hazum, S., & Givol, D. (1983) *Nature (London) 306*, 594–597.

Conservation of Primary Structure in the Lipoyl-Bearing and Dihydrolipoyl Dehydrogenase Binding Domains of Mammalian Branched-Chain α-Keto Acid Dehydrogenase Complex: Molecular Cloning of Human and Bovine Transacylase (E2) cDNAs†

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ABSTRACT: The subunit structures and conservation of the dihydrolipoyl transacylase (E2) components of bovine and human branched-chain α-keto acid dehydrogenase complexes were investigated by Western blotting, peptide sequencing, and cDNA cloning methods. Rabbit antiserum prepared against the sodium dodecyl sulfate (SDS) denaturated bovine E2 subunit recognized the inner E2 core, and the first hinge region of the E2 chain, but failed to react with the lipoyl-bearing domain as determined by Western blot analysis. The lack of antigenicity in the lipoyl-bearing domain was confirmed with antibodies directed against the native E2 component. A human E2 cDNA (1.6 kb) was isolated from a human liver cDNA library in λgt11 with a combination of the above anti-native and anti-SDS-denatured E2 immunoglobulin G's as a probe. The fidelity of the human E2 cDNA was established by nucleotide sequencing which showed the determined peptide sequences of the amino terminus and tryptic fragments of bovine E2. A bovine E2 cDNA (0.7 kb) was also isolated from a bovine liver cDNA library in λZAP with the human E2 cDNA as a probe. Northern blot analysis using the human E2 cDNA probe showed that E2 mRNAs in bovine liver and human kidney mesangial cells are 3.3 and 4.6 kb in size, respectively. Primary structures derived from human and bovine E2 cDNAs show leader sequences including the initiator methionine and the homologous mature peptides consisting of complete lipoyl-bearing and dihydrolipoyl dehydrogenase (E3) binding domains and two hinge regions. In addition, the human E2 cDNA contains a portion of the inner E2 core sequence, a 3′-untranslated region, and a poly(A+) tail. Deduced amino acid sequences of the mammalian E2's were compared with those of *Escherichia coli* transacetylase and transsuccinylase and bovine kidney transacetylase. The results indicate a high degree of conservation in the sequence flanking the lipoyl-attachment site and in the E3-binding domain. Models are presented to discuss implications for the conserved structure–function relationship in the lipoyl-bearing and E3-binding domains of α-keto acid dehydrogenase complexes.

The mammalian branched-chain α-keto acid dehydrogenase (BCKAD)[1] complex catalyzes the oxidative decarboxylation of α-keto acids that are derived by transamination from the branched-chain amino acids: valine, leucine, and isoleucine. This mitochondrial multienzyme complex is both structurally and mechanistically analogous to pyruvate and α-ketoglutarate dehydrogenase complexes (Reed et al., 1980). The BCKAD complex consists of three catalytic components: a branched-

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[1] Abbreviations: BCKAD, branched-chain α-keto acid dehydrogenase; E1, branched-chain α-keto acid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoyl dehydrogenase; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; HPLC, high-performance liquid chromatography; TBS, 50 mM Tris-HCl, pH 8.1/150 mM NaCl; SSC, 15 mM sodium citrate, pH 7.4/150 mM NaCl; kb, kilobase pair(s); bp, base pair(s); TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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chain α-keto acid decarboxylase or E1, a dihydrolipoyl transacylase or E2, and a dihydrolipoyl dehydrogenase or E3 (Pettit et al., 1978; Heffelfinger et al., 1983; Chuang et al., 1984). The enzyme complex also contains two regulatory enzymes, a specific kinase (Odessey, 1982; Lau et al., 1982; Paxton & Harris, 1982) and a specific phosphatase (Damuni et al., 1984; Fatania et al., 1983), that modulate activity of the complex through a phosphorylation–dephosphorylation cycle.

The BCKAD complex is organized around a structural core consisting of E2 subunits to which E1, E3, the specific kinase, and presumably the phosphatase are attached through noncovalent interactions (Pettit et al., 1978; Cook et al., 1985; Fatania et al., 1983). The E2 subunit has been shown to contain an inner E2 core and lipoyl-bearing domain that are linked by an exposed hinge region (Chuang et al., 1985; Hu et al., 1986). The inner E2 core of the BCKAD complex is highly assembled with apparent octahedral symmetry (Chuang et al., 1985). The lipoyl-bearing domain appears to be folded and contains a single lipoyl residue (Hu et al., 1986). Limited proteolysis with trypsin results in a release of the lipoyl-bearing domain without affecting the assembly of the inner E2 core (Heffelfinger et al., 1983; Chuang et al., 1985; Hu et al., 1986). Similar domain structures exist in the transacetylase of pyruvate dehydrogenase complex from *Escherichia coli* (Hale & Perham, 1979; Bleile et al., 1979) and bovine heart (Bleile et al., 1981) and in the transsucinylase of α-ketoglutarate dehydrogenase complex from *E. coli* (Perham & Roberts, 1981; Pettit et al., 1973; Wagenknecht & Frank, 1984) and bovine kidney (Oliver & Reed, 1982).

The transacetylase (Stephens et al., 1983) and transsuccinylase (Spencer et al., 1984) genes of *E. coli* have been cloned. The deduced primary structure shows that the lipoyl-bearing region of the *E. coli* transacetylase consists of three repeated and homologous lipoyl-bearing domains of about 100 amino acids, with each domain lipoylated (Stephens et al., 1983; Packman & Perham, 1984). The domain structure of the *E. coli* transacetylase has been confirmed and extended by the protein engineering experiments of Guest et al. (1985). Substantial structural homologies exist between *E. coli* transacetylase and *E. coli* transsuccinylase subunits, with the latter containing a single 100-residue lipoyl-bearing domain (Spencer et al., 1984; Packman & Perham, 1987). The E3-binding domain of *E. coli* transsuccinylase has recently been isolated and identified by Packman and Perham (1986). The E3-binding domain is located between the lipoyl-bearing and inner-core domains. These folded functional units are linked in order by flexible hinge regions (Packman & Perham, 1986). Despite these advances in bacterial systems, little is known concerning the primary structures of mammalian branched-chain E2's.

In the present study, we investigated the primary structure and conservation of the E2 component of the mammalian BCKAD complex. The antigenic regions of the E2 chain were mapped by Western blotting with antiserum prepared against native or SDS-denatured E2 of bovine liver. The amino-terminal sequence and sequences of tryptic fragments of the bovine BCKAD complex were determined by peptide sequencing. Moreover, cDNAs encoding human and bovine E2 were isolated. Nucleotide sequences of these E2 cDNAs predict lipoyl-bearing and E3-binding domains, as well as hinge regions in the mammalian E2's. Comparisons of primary structures reveal extensive conservation in the lipoyl-bearing and E3-binding domains among the mammalian BCKAD, *E. coli* pyruvate, and *E. coli* α-ketoglutarate dehydrogenase complexes. Implications for the conserved structure–function relationship are discussed.

Experimental Procedures

Materials. ^{125}I-Protein A, [γ-^{32}P]ATP, [α-^{32}P]dCTP, and [α-^{35}S]dATP were obtained from Amersham Corp. All other chemicals and biochemicals were of the highest purity commercially available. Sequencing and nick translation kits and restriction enzymes were from Bethesda Research Laboratories.

Protein Purification and Peptide Sequence Analysis. The BCKAD complex was purified from bovine liver mitochondria as described previously (Chuang et al., 1984, 1985). To purify E2 for N-terminal sequencing, 250 μg of purified BCKAD complex (6.4 units/mg of protein) was separated into its polypeptide components by reverse-phase HPLC (Waters) (Crabb & Hanstein, 1985) on a C_{18} Synchropak RP-P column. The elution gradient was as follows: 0–10 min, 0% acetonitrile; 10–20 min, 0–45% acetonitrile; 20–70 min, 45–55% acetonitrile. The absorbance peak at 214 nm corresponding to E2 was identified by SDS–polyacrylamide gel electrophoresis of the peak samples. To purify tryptic peptides of E2, 2 mg of purified BCKAD complex was dissociated into E2 and E1 components as described previously (Chuang et al., 1985). The isolated E2 (250 μg) was digested with 2.5 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin in 100 mM $NaHCO_3$ and 5 mM dithioerythritol for 4 h at 37 °C. Tryptic E2 peptides were separated by reverse-phase HPLC as described above with the following elution gradient: 0–10 min, 0% acetonitrile; 10–50 min, 0–40% acetonitrile. Individual peptides were purified for sequencing by subsequent elutions at shallower gradients. Amino-terminal sequence analysis was carried out by the manual Edman sequencing method developed by Tarr (1986). Peptide sequencing of bovine E2 tryptic fragments was performed either by the manual method or on an Applied Biosystems Model 470A sequencer according to the manufacturer's procedures.

Radiolabeling and Isolation of Bovine E2. To radiolabel the lipoyl residue on the E2 subunit, the bovine BCKAD complex was subjected to reductive acylation with α-keto[U-^{14}C]isovalerate as a substrate (Hu et al., 1986). E1 and E2 components were separated by Sepharose 4B column chromatography in 1 M NaCl at pH 8.0 (Chuang et al., 1985).

Preparation of E2 Screening Antibody. Native and SDS-denatured E2 enzymes in complete Freund's adjuvant were used to raise rabbit polyclonal antibodies. Serum samples were collected 1 week after a booster injection of E2 antigen in incomplete Freund's adjuvant. Prior to screening expression libraries with the E2 antisera, endogenous *E. coli* titers were removed. Briefly, equal aliquots of native and SDS-denatured E2 antisera were pooled and passed through a CNBr-activated Sepharose column linked with *E. coli* lysate. Unbound IgG was precipitated with ammonium sulfate (30–50% saturation), resuspended, and incubated with a 10-fold (w/w) excess of *E. coli* lysate in 10 mM Tris, pH 7.5, containing 0.2% NaN_3 at 4 °C for 16 h. Immunoprecipitates were spun down, and the supernatant was incubated with an additional 10-fold excess of *E. coli* lysate for 16 h at 4 °C. Soluble anti-E2 IgG was collected by centrifugation and used for screening the cDNA expression library in λgt11.

Screening of a cDNA Library. The human liver cDNA library (a gift from Dr. Savio Woo of Baylor College of Medicine) was screened with the combined rabbit antisera raised against native and SDS-denatured E2 component of the BCKAD complex according to Huynh et al. (1985). Recombinants (1.1×10^6 pfu) were plated at a density of 40 000 pfu

per 15 cm diameter Petri dish. Nitrocellulose filters saturated in 10 mM isopropyl β-D-thiogalactopyranoside (IPTG) were overlaid on each plate for 2 h at 37 °C and then at 4 °C overnight. The filters were washed with 50 mM Tris-HCl, pH 8.1, containing 150 mM NaCl (TBS) twice, each wash for 10 min, and then blocked with 5% (w/v) milk in TBS for 30 min. Combined native/SDS-denatured E2 rabbit antisera were diluted at 1:1000 in TBC containing 5% dry milk and 0.05% sodium azide and used to incubate the nitrocellulose filters for 2 h at room temperature. After thorough rinsing with TBS, the positive clones were revealed by autoradiography, with ^{125}I-protein A (sp act. 30 mCi/mg) at a concentration of approximately 10^6 cpm/filter. Phage producing positive signals were picked and replated at a lower density until all the plaques on one plate produced positive signals.

Elution of Fusion Protein Bound Antibody and Western Blotting. Fusion protein from λhE2-1 was induced by overlaying lysed *E. coli* Y1090 cells infected at high density with λhE2-1 clone with nitrocellulose filters impregnated with IPTG for 2 h at 37 °C. The filters were washed with TBS and blocked with 5% milk in TBS. The filters were then incubated with E2 antisera at room temperature for 2 h. Unbound antibodies were removed by washing in TBS. Specifically bound antibodies were eluted as described (Litwer & Danner, 1985). Western blot analysis of trypsin-digested BCKAD complex was carried out as described previously (Hu et al., 1986). ^{125}I-Protein A (30 mCi/mg) or horseradish peroxidase linked second antibody was used to detect anti-E2 titers bound to the immunoreactive subunit and tryptic fragments.

Screening of a Bovine Liver cDNA Library for E2 Clones. Total RNA was prepared from fresh bovine liver slices as described by Chirgwin et al. (1979), and poly(A+) RNA was selected on oligo(dT)–cellulose (Maniatis et al., 1982). A λZAP phage cDNA library was constructed by Stratagene, San Diego, CA., using the purified bovine liver poly(A+) RNA. To screen for bovine E2 cDNA, 750000 recombinants were grown in *E. coli* BB4 cells on NZY agar plates at 37 °C for 8 h. After overnight refrigeration, duplicate plaque lifts were performed with nitrocellulose filters. Filters were successively incubated in 0.5 M NaOH/1.5 M NaCl for 2 min, 1.5 M NaCl/0.5 M Tris, pH 8.0, for 8 min, and 0.2 M Tris, pH 7.5, containing 2.0× SSC for 30 s. After air-drying, the filters were baked under vacuum at 80 °C for 2 h. They were then preincubated in hybridization solution N [10% dextran sulfate, 40% deionized formamide, 4× SSC, 7 mM Tris, pH 7.4, 1× Denhardt's solution (Denhardt, 1966), 20 μg/mL salmon sperm DNA] for 1 h at 42 °C. After preincubation, the denatured and nick-translated (Maniatis et al., 1982) human E2 cDNA insert was added. Hybridizations were performed at 42 °C for 16 h. Filters were then washed 3 times with 0.1× SSC containing 0.1% SDS at 55 °C (15 min each wash), air-dried, and exposed at −70 °C overnight with Kodak-X-Omat AR film. Plaques that produced positive signals were purified by further subscreening. The same procedure was used to isolate other bovine E2 clones with a bovine E2 cDNA (bE2-1) as a probe.

Restriction Analysis and DNA Sequencing Strategy. Plaque-purified phage DNA from the positive λgt11 clone (λhE2-1) was digested with *Eco*RI restriction enzyme and subcloned into the *Eco*RI site of a pTZ19 plasmid vector. The recombinant pTZ19 was grown in JM 109, and a partial restriction map of the λhE2-1 insert was constructed. The λhE2-1 insert was subcloned into the *Eco*RI site of an M13mp18 phage vector for nucleotide sequencing. Ordered serial deletions from the 5′ → 3′ end of both strands of the insert in the RF M13 recombinant were produced with exonuclease III as described by Henikoff (1984). After ligation, *E. coli* JM 101 was transformed with the deleted recombinants. Single-stranded DNA templates prepared from recombinant plaques were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (1977). The λhE2-1 sequence was confirmed by the Sequenase method (U.S. Biochemicals). Single-stranded DNA of the bovine E2 cDNAs (λbE2-1 and -5) was prepared by infecting transformed *E. coli* XL1-Blue cells carrying the clone in the Bluescript plasmid with helper phage (R408). The single-stranded DNA was isolated and sequenced as described for the human (λhE2-1) clone.

Northern Blot Analysis. Poly(A+) RNA of fresh bovine liver was prepared by the method of Chirgwin et al. (1979). Poly(A+) RNA from human kidney mesangial cells was kindly provided by Dr. Hanna Abboud. RNA concentrations were determined by absorbance at 260 nm. Poly(A+) RNA was denatured in formaldehyde/formamide and resolved by 0.8% agarose gel electrophoresis in the presence of formaldehyde. RNA was transferred onto nitrocellulose and the filter baked for 2 h at 80 °C under vacuum. Prehybridization was performed in hybridization solution N for 2 h at 42 °C. Nick-translated λhE2-1 was added and hybridization was performed at 42 °C for 16 h. The Northern blot was washed 3 times with 2× SSC containing 0.1% SDS at 55 °C (15 min each wash), dried, and subjected to autoradiography at −70 °C for 72 h with Kodak-X-Omat AR film.

RESULTS

Specificities of Anti-E2 Antisera. To map antigenic regions of the E2 chain, antisera against E2 were produced in rabbits with either native or SDS-denatured E2 as an antigen. Segments of the E2 chain recognized by the antisera were analyzed. This was carried out by digesting E2, which was radiolabeled at the lipoyl residue, with increasing concentrations of trypsin. The digests were subjected to Western blotting with either anti-native or anti-SDS-denatured E2 as a probe. Bound anti-E2 titers were detected by blotting with the peroxidase-linked second antibody, followed by color development. Figure 1A shows that the anti-native E2 reacts with E2 (lane 1) and tryptic fragments L_1, L_2, and L_3 (lanes 2–4). Figure 2 shows different regions of the bovine E2 chain recognized by the anti-native and the anti-SDS-denatured E2. The domain structure shows the previously identified trypsin-sensitive sites (L_1 to L_5) (Hu et al., 1986), the lipoyl-bearing domain, and the putative E3-binding domain inferred from alignments with *E. coli* transsuccinylase and *E. coli* transacetylase (see Discussion). On the basis of the model shown in Figure 2, fragments L_1, L_2, and L_3 which contain the radiolabeled lipoyl residue are generated by sequential tryptic cleavages (from L_1 to L_3 sites toward the amino terminus) of the E2 chain. Radioactive fragments L_4 and L_5, representing L_4 and L_5 sites to the amino terminus (Figure 2), respectively, were also present in the digest as detected by autoradiography (data not shown) but failed to bind anti-native E2. Thus the results in Figure 1A indicate that the anti-native E2 rabbit serum recognizes the region between L_1 and L_4 sites of the E2 chain (Figure 2). The antiserum prepared against SDS-denatured E2 reacts with fragments L_1, L_2, and L_3 (lanes 1–4) as well as fragments A and B (lanes 4 and 5) (Figure 1B). Fragments A and B result from an ordered tryptic digestion (from A to B site) of the inner E2 core (Figure 2). The data in Figure 1B show that the anti-SDS-denatured E2 serum recognizes a broad segment of the E2 chain including the entire inner E2 core and the region between L_1 and L_4 sites (Figure 2).

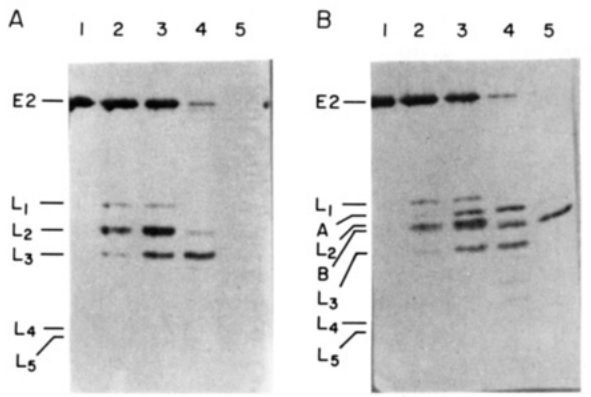

FIGURE 1: Western blots of isolated E2 and its tryptic fragments with rabbit anti-E2 antisera as probes. The isolated E2 was radiolabeled at the lipoyl residue by reductive acylation (see Experimental Procedures) and digested with increasing concentrations of trypsin at 0 °C (on ice) for 45 min. The digestion was terminated by the addition of 4 mM TLCK. The tryptic digests were subjected to SDS–polyacrylamide gel electrophoresis (12% gel) followed by Western blotting analysis. Rabbit antiserum (1:1000 dilution) prepared against native (panel A) or SDS-denatured (panel B) E2 component was used as a probe. Bound antibodies were detected by peroxidase staining (Hu et al., 1986). To each lane, 2 μg of radiolabeled E2 was applied. For panels A and B, the trypsin/E2 ratio (w/w) is as follows: lane 1, no trypsin; lane 2, 1/1000; lane 3, 1/100; lane 4, 1/10; lane 5, 1/1. Immunoreactive tryptic fragments L_1, L_2, L_3, A, and B are indicated. The location of radiolabeled but nonimmunoreactive L_4 and L_5 fragments are also shown.

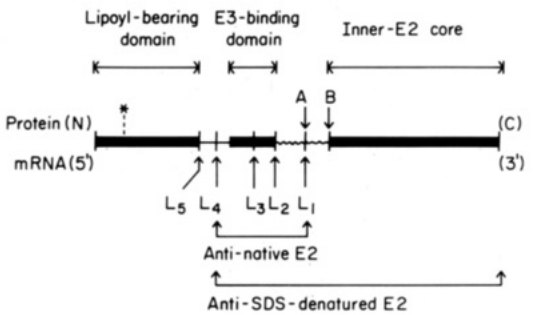

FIGURE 2: Regions of the transacylase (E2) chain recognized by rabbit antisera against native and SDS-denatured E2 protein. Closed boxes represent the folded lipoyl-bearing and E3-binding domains and the inner E2 core. Zigzag and solid lines refer to the first and second hinge regions, respectively, connecting the above folded domains (see Discussion). The A and L_1 sites are identical. Regions of the E2 chain recognized by anti-native E2 and anti-SDS-denatured E2 rabbit antisera as deduced from results shown in Figure 1 are indicated by arrowed brackets. The asterisk indicates the position of the lipoyllysine residue. The precise positions of the folded domains and tryptic sites have not been determined experimentally.

The anti-SDS-denatured E2 did not bind fragments L_4 and L_5, similar to that observed with anti-native E2. The results confirm that the lipoyl-bearing domain is not antigenic.

Isolation and Immunochemical Identification of the Human E2 cDNA Clone. To disclose the primary structure of mammalian E2, we isolated an E2 cDNA from a human liver cDNA library in λgt11. Approximately 1.1×10^6 recombinant λgt11 phage were screened with the antiserum raised against native and SDS-denatured E2 enzyme. Thirteen positive clones were picked from which only one (λhE2-1) remained positive after plaque purification. Titers bound to the fusion protein produced by the λhE2-1 clone were analyzed by incubating the anti-E2 sera with λhE2-1 lysate immobilized on a nitrocellulose filter. The specifically bound IgG was eluted from the filter and used to probe E2 and its tryptic fragments by Western blotting. Figure 3 shows that the eluted antibody binds specifically to E2 (lane 1) and tryptic fragments L_1, L_2, and L_3 (lanes 2–4). Fragment L_1 exists in a trace amount and is only marginally visible by the Western blot analysis. The results indicate that the λhE2-1 insert contains the sequence coding for the first hinge region and the E3-binding domain that are located between L_1 and L_4 sites (Figure 2).

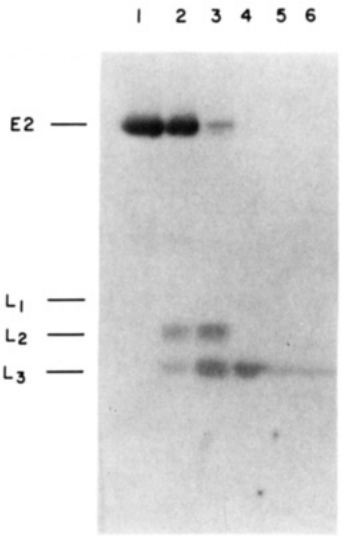

FIGURE 3: Subunit and tryptic fragments of the BCKAD complex recognized by the λhE2-1-lysate-selected rabbit IgG's. The purified bovine liver BCKAD complex (12 μg/lane) was digested with increasing concentrations of trypsin at 0 °C (on ice) for 60 min. The digestion was terminated by the addition of TLCK to a final concentration of 2 mM. The complex and its tryptic fragments were subjected to Western blotting using rabbit IgG's selected by the λhE2-1 lysate as a probe. Bound antibodies were detected by ^{125}I-protein A (0.1 μCi/mL). The trypsin/complex ratio (w/w) was as follows: lane 1, no trypsin; lane 2, $1/10^4$; lane 3, $1/10^3$; lane 4, $1/10^2$; lane 5, 1/10; lane 6, 1/1.

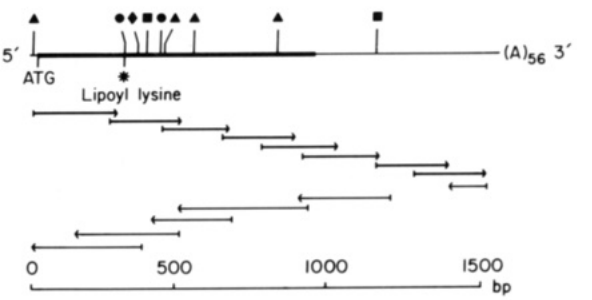

FIGURE 4: Restriction map and sequencing strategy of the human hE2-1 cDNA. The solid box and line represent coding and noncoding regions, respectively. Restriction enzymes used are *Pst*I (▲), *Hin*dIII (○), *Xba*I (♦), and *Acc*I (■). The codon encoding the putative lipoyllysine residue is indicated (*). Ordered 5′ → 3′ serial deletions of both strands of the insert in the RF M13 recombinant were produced with exonuclease III as described under Experimental Procedures. Arrows indicate the orientation and region of the hE2-1 insert sequenced for each single-stranded DNA template.

Restriction Map and Nucleotide Sequence of the Human E2 cDNA. A restriction map and sequencing strategy for the hE2-1 cDNA are presented in Figure 4. The hE2-1 clone has a length of 1557 bp (Figure 5). The longest open reading frame codes for a polypeptide containing 315 amino acid residues starting from nucleotide 14 and ending at nucleotide 958. This open reading frame is followed by a 3′-untranslated region of 562 bp. Two polyadenylation signals of the type ATTAAA are found 35 and 14 bp upstream of the start of the poly(A+) tail.

The derived amino acid sequence of the hE2-1 clone (Figure 5) was confirmed by peptide sequencing of the amino-terminal region and two tryptic fragments of purified bovine E2 (Table

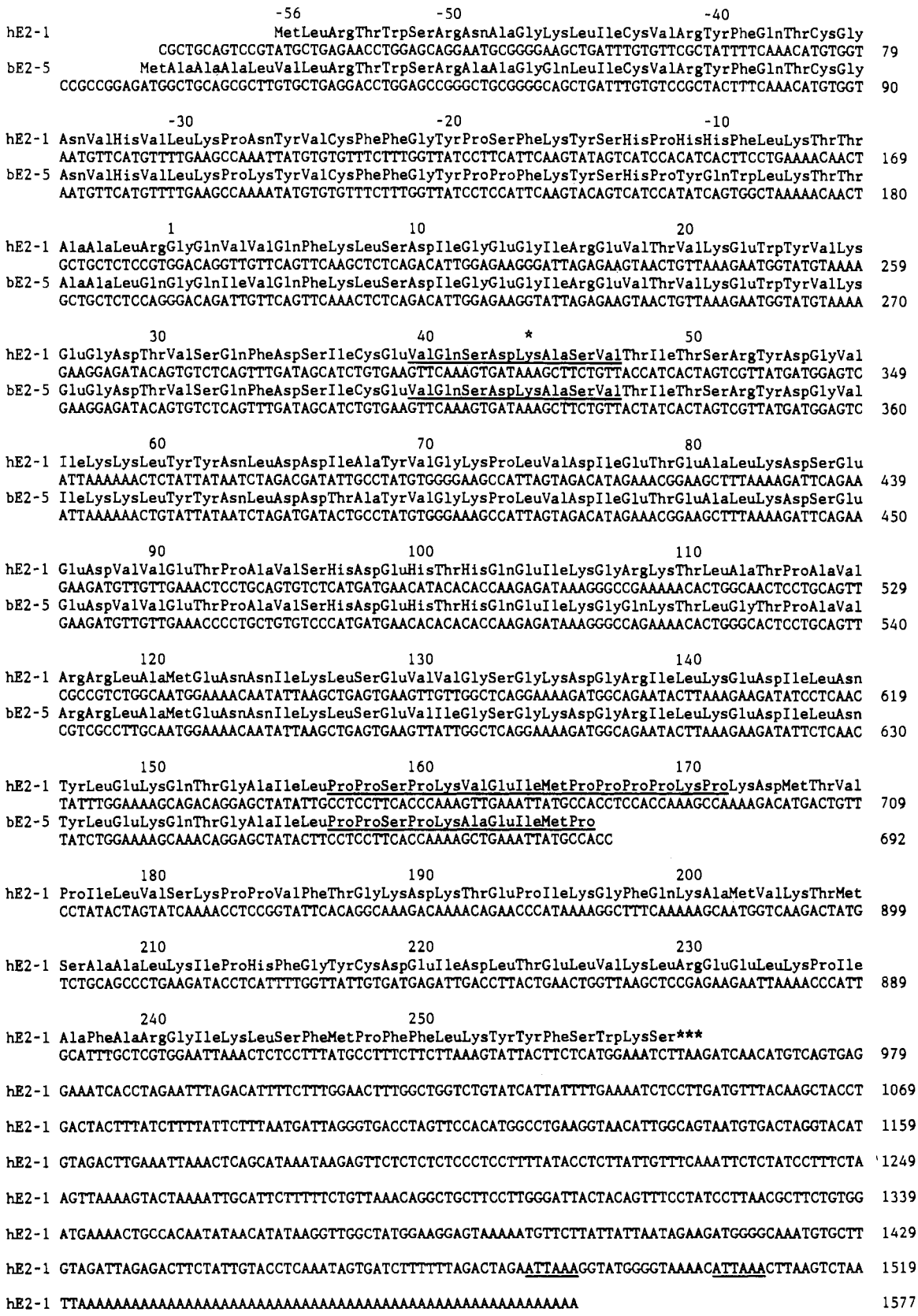

FIGURE 5: Nucleotide and deduced amino acid sequences of the human (hE2-1) and bovine (bE2-5) E2 cDNAs. The numbers at the end of each row are ordinates for nucleotide sequences of hE2-1 and bE2-5 cDNAs. The numbers above each row refer to the positions of amino acids derived from both human and bovine E2 cDNAs. The amino-terminal glycine (residue 1) was identified by peptide sequencing of bovine E2. The initiator methionine of the putative human and bovine E2 leader peptides is at position −56 and −61, respectively. The putative lipoyllysine (*) residue at position 44 is indicated. The conserved immediate lipoyl-bearing sequence (residues 40–47), the proposed first E2 hinge region (residues 157–171), and the two polyadenylation signals (ATTAAA) are underlined.

I). Complete matches are observed between predicted and analyzed amino acid sequences with the exception of two conservative substitutions between human and bovine E2 at residues 3 (Ile_{bovine} to Val_{human}) and 162 (Ala_{bovine} to Val_{human}). Comparison of the determined amino-terminal sequence of bovine E2 (residues 1–16) (Table I) with the derived amino

Table I: Protein Sequences Determined for the Amino Terminus and Tryptic Peptides of Bovine E2

peptide[a]	sequence[b]	residue no. in the deduced sequence[c]
E2	Gly-Glu-Ile-Val-Glu-Phe-Lys-Leu-Ser-Asp-Ile-Gly-Glu-Gly-Ile-Arg	1–16
I	Ile-Leu-Lys-Glu-Asp-Ile	139–144
II	Ala-Glu-Ile-Met-Pro	162–166

[a] The E2 subunit was isolated by subjecting 250 μg of the purified bovine BCKAD complex (6.4 units/mg of protein) to reverse-phase HPLC as described under Experimental Procedures. The purified E2 was identified by SDS–polyacrylamide gel electrophoresis of the peak samples. Tryptic E2 peptides were produced by digesting 250 μg of isolated bovine E2 with 2.5 μg of trypsin in 100 mM $NaHCO_3$ and 5 mM dithioerythritol for 4 h at 37 °C. Tryptic fragments were purified by HPLC as also described under Experimental Procedures. [b] Amino-terminal sequence of E2 and sequence of tryptic peptide I were determined by the manual Edman sequencing method. The sequence of tryptic peptide II was analyzed by the gas-phase sequencing method. [c] Amino acid sequence deduced from the bovine cDNA sequence is shown in Figure 5.

acid sequence for human E2 (Figure 5) indicates that the mature human E2 protein starts at residue 57 of the open reading frame. The human pre-E2 protein therefore contains a leader sequence of 56 amino acids. A lipoic acid attachment site is present at Lys-44 of the mature protein. The mature E2 polypeptide coded for by the hE2-1 clone has a deduced M_r of 27 596.

Isolation and Identification of Bovine E2 cDNA. A bovine E2 cDNA clone (λbE2-1) was initially isolated from a bovine liver cDNA library in λZAP with the human E2 insert (hE2-1) as a probe (see Experimental Procedures). The initial bovine E2 cDNA (λbE2-1) has a size of 0.7 kb and contains the nucleotide sequence encoding the amino-terminal protein sequence shown in Table I. The bE2-1 insert was used as a probe to screen the same λZAP library of bovine liver cDNA. A second bovine E2 cDNA clone (λbE2-5) was isolated by this method. Nucleotide sequencing showed that the bovine bE2-5 cDNA (692 bp) contains an open reading frame coding for 227 amino acids including the starting methionine, but no stop codon (Figure 5). The glycine residue at the amino terminus (residue 62) was determined by peptide sequencing of bovine E2 (Table I). Complete matches were observed between the determined amino-terminal sequence and sequences of the two tryptic peptides of bovine E2 (Table I) and the corresponding amino acid sequences deduced from the bE2-5 cDNA (Figure 5). Thus, the derived primary structure encoded by the bE2-5 cDNA consists of a complete leader sequence of 61 amino acids and a mature peptide of 166 residues (Figure 5). A putative lipoyllysine residue also occurs at position 44, identical with that observed in the deduced human E2 amino acid sequence (Figure 5).

Northern Blot Analysis of Human and Bovine mRNA. Figure 6 shows results of Northern blots of poly(A+) RNA prepared from bovine liver and human kidney mesangial cells with the human E2 cDNA (hE2-1) as a probe. A single species of E2 mRNA (3.3 kb) was detected in the bovine poly(A+) RNA (lane 1), whereas a doublet of 4.6 kb was observed in the human mRNA preparation (lane 2).

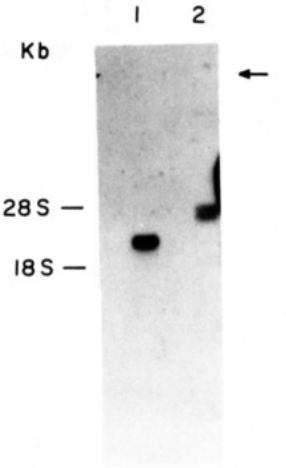

FIGURE 6: Northern blot analysis of bovine and human mRNA. Poly(A+) RNA prepared from bovine liver and human renal mesangial cells was denatured and electrophoresed on a 0.8% agarose gel. The separated RNA was transferred to nitrocellulose paper and probed with the nick-translated human hE2-1 cDNA. Lane 1, RNA (10 μg) from bovine liver; lane 2, RNA (5 μg) from human renal mesangial cells. The arrow indicates the origin of application.

DISCUSSION

This is the first report of deduced primary structures for the lipoyl-bearing and the E3-binding domains of mammalian BCKAD complexes. Evidence was presented previously that the E2 subunit of the human BCKAD complex had been cloned (Litwer & Danner, 1985). The structural genes that encode individual subunits of the BCKAD complex of *Pseudomonas putida* have recently been isolated (Sykes et al., 1987). The bovine E2 cDNA (bE2-5) in the present study was identified by the nucleotide sequence of the insert which correctly predicts the determined protein sequence of the amino-terminal region and tryptic fragments of bovine E2. The fidelity of the human E2 clone (λhE2-1) reported here was established by the following lines of evidence: (1) Antibodies selected by the IPTG-induced lysate of λhE2-1 specifically bound to the lipoyl-bearing and E3-binding domains and the first hinge region of bovine E2 (Figure 2). (2) A bovine E2 cDNA (bE2-1) was isolated from a λZAP library with the human E2 cDNA as a probe. (3) The deduced amino acid sequence of human hE2-1 includes the amino-terminal sequence and two tryptic fragment sequences of bovine E2 determined by peptide sequencing. (4) About 99% identity in the deduced amino acid sequences is observed between human and bovine E2.

The deduced primary structure of human E2 cDNA begins with an initiator Met in a span of 56 amino acids that is not present in the mature bovine E2 (Figure 5). This putative E2 leader peptide has features common to most mitochondrial-targeting sequences (von Heijne, 1986). It contains periodically spaced basic amino acids (Arg, Lys, and His), is rich in branched-chain residues (Val and Leu) and hydroxylated residues (Ser and Thr), and has no acidic residues. The first 15 residues of the human pre-E2 polypeptide are proposed to form a surface-seeking amphiphilic α-helix which is involved in the targeting of pre-E2 to mitochondria. By use of the normalized consensus hydrophobicity scale of Eisenberg (Eisenberg et al., 1984), the hydrophobic moment (μ_H) of this region is calculated to be 8.75, and the maximal hydrophobicity (H_{max}) of six adjacent residues on a face of the helix is 5.57 (residues −56, −55, −52, −48, −45, and −44). These values satisfy the criteria for a surface-seeking peptide ($\mu_H \geq 7.3$ and $H_{max} \geq 4.5$) (von Heijne, 1986). The putative amino-terminal glycine of human E2 is identical with that of the bovine E2 located by peptide sequencing of the latter. The data indicate that cleveage occurs between Arg(−1) and Gly(+1) during import of the human precursor E2 into mitochondria. A single lipoyllysine residue is located at position 44 of the mature human E2 chain. These results support our previous finding

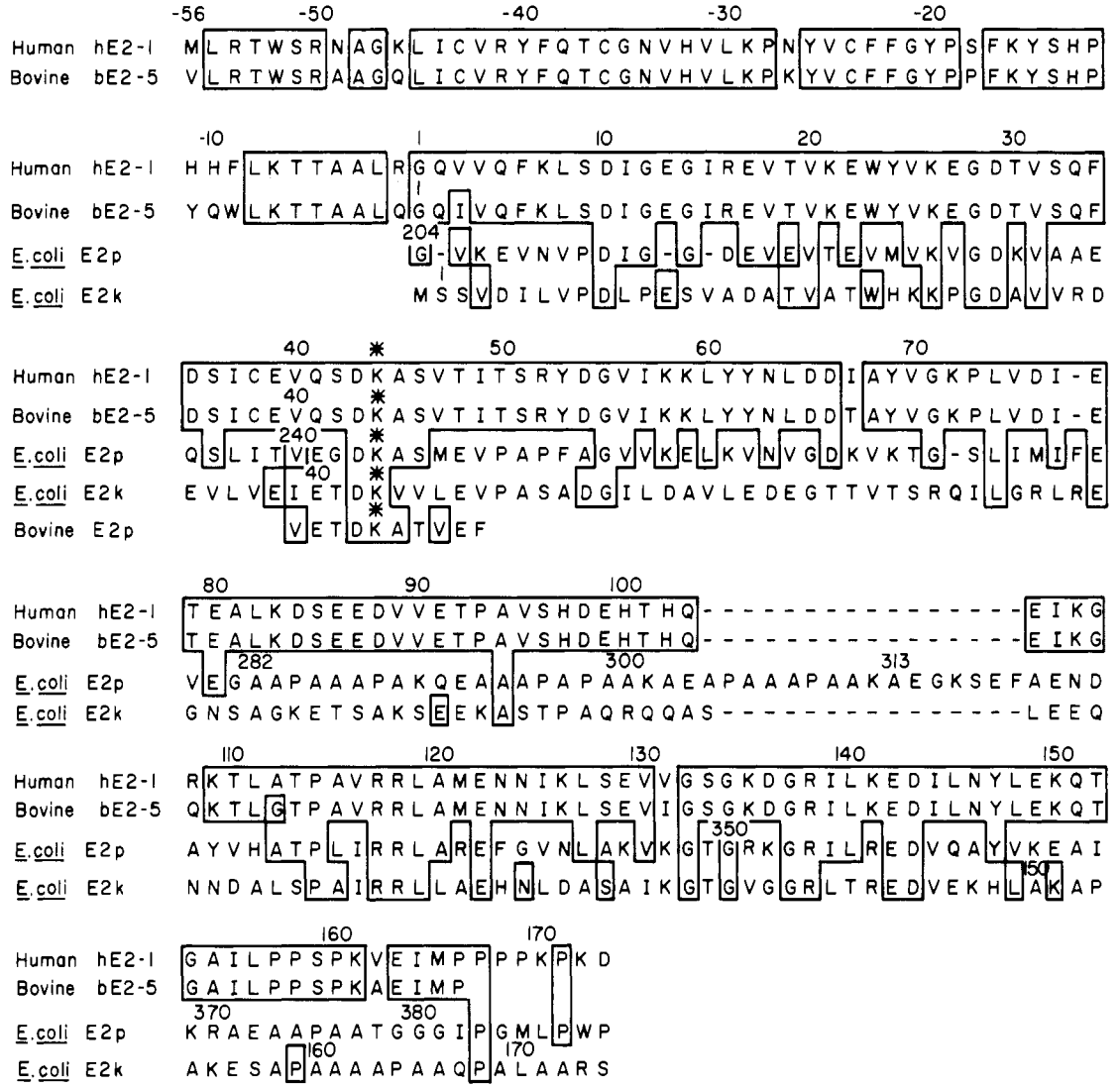

FIGURE 7: Comparisons of the amino acid sequences of the lipoyl-bearing domains and hinge regions among five different E2 proteins. Partial deduced primary structures of human (hE2-1) and bovine (bE2-5) E2 (the present study), *E. coli* transacetylase (E2p) (*lip* 3 domain) (Stephens et al., 1983), *E. coli* transsuccinylase (E2k) (Spencer et al., 1984; Packman & Perham, 1987), and the lipoyl-attachment site sequence of the bovine kidney transacetylase (E2p) (Bradford et al., 1987) are aligned for maximal homology. The boxed residues are those which are identical with those of human E2. The numbers of amino acid residues except bovine kidney E2p are shown above each protein sequence. Broken lines are the gaps created to achieve maximal alignment with the other proteins. The putative and determined (*E. coli* and bovine E2p) lipoyllysine residues (*) are indicated. The immediate lipoyl-bearing region is located between residues 40 and 47 of the human E2 sequence.

that the bovine E2 contains one lipoyllysine residue per polypeptide chain as concluded from reductive acylation experiments (Hu et al., 1986).

To assess possible sequence conservation as well as to further define the domain structure of mammalian E2's, the deduced amino acid sequence of human E2 is compared with those of bovine E2 (the present study), *E. coli* transacetylase (E2p) (Stephens et al., 1983), *E. coli* transsuccinylase (E2k) (Spencer et al., 1984; Packman & Perham, 1987), and bovine kidney transacetylase (E2p) (Bradford et al., 1987) (Figure 7). The boxed residues are those which are identical with those of human E2 derived from the cDNA sequence. As shown in Figure 7, the amino acid sequences in the immediate lipoyl-bearing region (underlined residues 40–47 of human E2) are identical for human and bovine E2's. Bovine and *E. coli* (*lip*3 domain) transacetylases are as similar to each other in the immediate lipoyl-bearing region (five of eight residues being identical) as they are to human E2. The Asp residue immediately before the putative lipoyllysine (Lys*) residue is completely conserved among all five proteins. A consensus sequence for the lipoyl-attachment site (residues 40–47 of human E2) can be derived: branched-chain residue–Gln/Glu–hydroxylated residue–Asp–Lys*–aliphatic residue–hydroxylated residue–aliphatic residue. It is noteworthy that the putative lipoyllysine residue is located 44 residues from the amino terminus of human and bovine E2, 43 residues from the putative amino terminus of *E. coli* transsuccinylase, and 40 residues from the amino-terminus equivalent (residue 204) of the *lip*3 domain of *E. coli* transacetylase (Figure 7). The data indicate that the sequence around the lipoyl-attachment site is highly conserved among these lipoyl-containing proteins. These conserved sequences may indicate regions which play an important role in the attachment of the lipoyl moiety to the E2 chain by the holoenzyme synthetase. On the other hand, the E3-binding domains of *E. coli* transacetylase (residues 316–372) and *E. coli* transsuccinylase (residues 101–156) are highly homologous and align well with the segment between residues 101 and 156 of human and bovine E2's (Figure 7). The latter region is likely to be the E3-binding domain of mammalian E2's (Figure 2). The sequence conservation in this region is possibly important for the folding of a correct tertiary structure and maintaining specific residues involved

in the interaction with the E3 component, which is common to all three mammalian α-keto acid dehydrogenase complexes. The BCKAD complex binds E3 loosely, and the latter tends to dissociate from the E2 core during enzyme purification (Pettit et al., 1978). An investigation into the E3-binding domain of bovine E2 may provide a basis for its lower affinity for E3 compared with other lipoyl-containing proteins.

The deduced primary structures (Figure 5) show the presence of two hinge regions in human and bovine E2's. The first hinge region connecting the E3-binding domain and the inner E2 core (Figure 2) is located between residues 157 and 171 of mammalian E2's. The region is characteristically rich in proline and is a candidate for a flexible random-coil structure. This segment aligns well with the Pro/Ala-rich hinge region (residue 150–172) of *E. coli* transsuccinylase (E2k) and the Pro/Gly-rich region (residues 372–387) of *E. coli* transacetylase (Packman & Perham, 1987). Although their functions are yet to be demonstrated experimentally, these aligned segments could confer the conformational flexibility of the "lipoate swinging arm" (Oliver & Reed, 1982; Perham et al., 1981; Perham & Roberts, 1981) which participates in intramolecular couplings (Bates et al., 1977; Collins & Reed, 1977; Cate et al., 1980) and interactions with E1 and E3 components (Oliver & Reed, 1982). A second potential hinge region is located between residues 73 and 91 of human and bovine E2's (Figure 7). The presence of this hinge region is supported by our limited proteolysis results (Hu et al., 1986), which showed that fragment L_4 (Figure 2) is unstable and readily converted to fragment L_5 by the removal of the hinged segment between L_4 and L_5 sites (tentatively Lys-83 and Lys-72, respectively, Figure 7). The second hinge appears to be less exposed than the first one, as a much higher trypsin concentration is required to produce fragment L_5 compared to fragments L_2 and L_3 (Figure 2) (Hu et al., 1986). The second hinge region of mammalian E2 is rich in the helix-breaking Glu and lines up with the Glu/Ala-rich hinge (residues 85–100) of the *E. coli* transsucinylase (Packman & Perham, 1986). The latter two regions are comparable to the long Pro/Ala-rich regions between the three lipoyl-bearing domains and between the *lip*3 and the E3-binding domains in the *E. coli* transacetylase (Packman et al., 1984; Packman & Perham, 1986). All the evidence thus far indicates that the latter three Pro/Ala-rich hinges are flexible and are the source of the sharp resonances observed in the NMR spectrum of the *E. coli* pyruvate dehydrogenase complex (Perham et al., 1981; Radford et al., 1986). The conformational mobility of the second hinge regions would facilitate interactions between the lipoyl-bearing domain and the E3 component of α-keto acid dehydrogenase complexes. Indeed, the fluorescence data by Grande et al. (1980) have shown that E3 bound to the pyruvate dehydrogenase complex is highly mobile. The amino acid sequences in the first and second hinges of mammalian E2's are substantially different from those of the *E. coli* transacetylase and transsucinylase, notably in the absence of Pro/Ala sequences. This suggests that the specific sequence is not important as long as it provides the needed flexible secondary structure. The pyruvate and α-ketoglutarate dehydrogenase complexes from bovine heart show the same family of sharp resonances in the NMR spectrum (Wawrzynczak et al., 1981). The data support conformational mobilities in the hinge regions of mammalian E2.

The human E2 cDNA (hE2-1) reported here appears to contain only a portion of the inner E2 core domain. This is based on the fact that the calculated molecular weight (10109) of the putative inner E2 core (residues 172–259, Figure 5) is much lower than that (M_r = 22000) of fragment B (Figure 2) of bovine E2. The molecular weights of human E2 (M_r 51000) (Ono et al., 1987) and bovine E2 (M_r 52000) (Hu et al., 1986) were similar, as judged by SDS–polyacrylamide gel electrophoresis. The data suggest that the inner E2 cores (fragments B) are similar in size between bovine and human E2's. The presence of the lipoyl moiety causes an overestimation of the sizes of lipoyl-bearing peptides by the electrophoretic method (Bleile et al., 1981; Guest et al., 1985). However, this situation does not apply to the inner E2 core since it is free of the lipoyl residue. The mechanism by which the incomplete inner-core sequence arose in the human hE2-1 clone is currently under investigation. Comparison of *Spe*I restriction fragments from hE2-1 and a full-length bovine E2 clone suggests that a deletion may have occurred in the inner-core sequence of the hE2-1 clone (T. A. Griffin, K. S. Lau, and D. T. Chuang, unpublished results). The nucleotide sequencing of the full-length bovine E2 cDNA is in progress, which upon its completion should shed light on the precise mechanism and location of the possible deletion in the human E2 cDNA clone. Palindromic sequences were suggested to be involved in a deletion inside a cDNA clone of human liver UDPglucuronosyltransferase (Jackson et al., 1987).

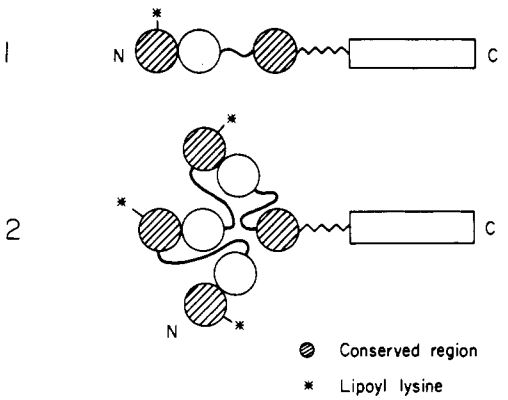

FIGURE 8: Topology of homologous regions of the lipoyl-bearing domains of α-keto acid dehydrogenase complexes. Model 1 depicts the structure of mammalian branched-chain E2 and *E. coli* transsuccinylase. Model 2 depicts the structure of *E. coli* transacetylase. The conserved regions of the lipoyl-bearing (left) and E3-binding (middle) domains of these proteins are indicated by shaded circles. The open circles denote nonconserved regions of the lipoyl-bearing domains. The inner E2 cores are indicated by rectangles. The first flexible hinge regions between the inner E2 cores and the E3-binding domains are indicated by zigzag lines. The solid lines represent the second hinge regions that connect the E3-binding and lipoyl-bearing domains (models 1 and 2) and the internal hinge regions linking the three tandemly repeated lipoyl-bearing domains of the *E. coli* transacetylase (model 2). Lipoyllysine residues are denoted by asterisks. Letters N and C refer to amino and carboxy termini, respectively.

Figure 8 illustrates the topology of the homologous regions of mammalian and bacterial lipoyl-bearing and E3-binding domains. The models show the positions of the flexible hinge regions between the E3-binding domain and inner E2 core (first hinge, zigzag line) and between the lipoyl-bearing and the E3-binding domains (second hinge, solid line). The lipoyl-bearing domains of mammalian E2 and *E. coli* transsuccinylase (model 1) consist of two conserved regions (shaded circles) that are separated by a nonconserved segment (open circle) and the second hinge region (solid line). The homologous amino-terminal region contains the lipoyl-attachment site (asterisk), whereas the conserved middle region represents the E3-binding sequence. The second hinge provides the conformational mobility required to bring the lipoyl residue close to the E3 component for reactions. The lipoyl-bearing domain of *E. coli* transacetylase (model 2, Figure 8) has three

homologous lipoyl-attachment regions (Stephens et al., 1983) that are joined in series by long internal (second) hinges (solid lines). We propose that these long internal hinges function by bringing each of the three lipoyl-attachment domains into alignment with the single E3-binding domain to produce structural units which function in a manner similar to that of model 1.

The availability of cDNAs for the E2 components of the mammalian BCKAD complexes will allow investigations into mitochondrial import, lipoic acid attachment, complex assembly, and structure–function relationships of these proteins at the molecular level. These cDNAs will also be useful probes for studying the mechanism of induction of the BCKAD complex during differentiation of 3T3-L1 preadipocytes into adipocytes (Chuang et al., 1983). Extensive homologies and the conserved structure–function relationship among the lipoyl-bearing and E3-binding domains strongly suggest that the structural cores of α-keto acid dehydrogenase complexes have evolved from a common genetic origin. This possibility is being investigated by the isolation of the mammalian E2 genes with these cDNA probes.

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REFERENCES

Bates, D. L., Danson, M. J., Hales, G. Hooper, E. A., & Perham, R. N. (1977) *Nature (London) 268*, 313–316.

Bleile, D. M., Munk, P., Oliver, R. M., & Reed, L. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4385–4389.

Bleile, D. M., Hackert, M. L., Pettit, F. H., & Reed, L. J. (1981) *J. Biol. Chem. 256*, 514–519.

Bradford, A. P., Howell, S., Aitken, A., James, L. A., & Yeaman, S. J. (1987) *Biochem. J. 245*, 919–922.

Cate, R. L., Roche, T. E., & Davis, L. C. (1980) *J. Biol. Chem. 255*, 7556–7562.

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

Chuang, D. T., Hu, C.-W. C., & Patel, M. S. (1983) *Biochem. J. 214*, 177–181.

Chuang, D. T., Hu, C.-W. C., Ku, L. S., Niu, W.-L., Myers, D. E., & Cox, R. P. (1984) *J. Biol. Chem. 259*, 9277–9284.

Chuang, D. T., Hu, C.-W. C., Ku, L. S., Markovitz, P. J., & Cox, R. P. (1985) *J. Biol. Chem. 260*, 13779–13786.

Collins, J. H., & Reed, L. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4223–4227.

Cook, K. G., Bradford, A. P., & Yeaman, S. J. (1985) *Biochem. J. 225*, 731–735.

Crabb, J. W., & Hanstein, W. G. (1985) *Biochem. Int. 11*, 255–263.

Damuni, Z., Merryfield, M. L., Humphreys, J. S., & Reed, L. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4335–4338.

Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun. 23*, 641–652.

Eisenberg, D., Schwarz, E., Komaromy, M., & Wall, R. (1984) *J. Mol. Biol. 179*, 125–142.

Fatania, H. R., Patston, P. A., & Randle, P. J. (1983) *FEBS Lett. 158*, 234–238.

Grande, H. J., Visser, A. J. W. G., & Veeger, C. (1980) *Eur. J. Biochem. 106*, 361–369.

Guest, J. R., Lewis, H. M., Graham, L. D., Packman, L. C., & Perham, R. N. (1985) *J. Mol. Biol. 185*, 743–754.

Hale, G., & Perham, R. N. (1979) *FEBS Lett. 105*, 263–266.

Heffelfinger, S. C., Sewell, E. T., & Danner, D. J. (1983) *Biochemistry 22*, 5519–5522.

Henikoff, S. (1984) *Gene 28*, 351–359.

Hu, C.-W. C., Griffin, T. A., Lau, K. S., Cox, R. P., & Chuang, D. T. (1986) *J. Biol. Chem. 261*, 343–349.

Huynh, T. V., Young, R. A., & Davis, R. W. (1985) in *DNA Cloning: A Practical Approach* (Glober, D. M., Ed.) Vol. I, pp 49–78 IRL, Oxford and Washington, DC.

Jackson, M. R., McCarthy, L. R., Harding, D., Wilson, S., Coughtrie, M. W. H., & Burchell, B. (1987) *Biochem. J. 242*, 581–588.

Lau, K. S., Fatania, H. R., & Randle, P. J. (1982) *FEBS Lett. 144*, 57–62.

Litwer, S., & Danner, D. J. (1985) *Biochem. Biophys. Res. Commun. 131*, 961–967.

Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Odessey, R. (1982) *Biochem. J. 204*, 353–356.

Oliver, R. M., & Reed, L. J. (1982) in *Electron Microscopy of Proteins* (Harris, J. R., Ed.) Vol. 2, pp 1–48, Academic, London.

Ono, K., Hakozaki, M., Nishimaki, H., & Kochi, H. (1987) *Biochem. Med. Metab. Biol. 37*, 133–141.

Packman, L. C., & Perham, R. N. (1986) *FEBS Lett. 206*, 193–198.

Packman, L. C., & Perham, R. N. (1987) *Biochem. J. 242*, 531–538.

Packman, L. C., Hale, G., & Perham, R. N. (1984) *EMBO J. 3*, 1315–1319.

Paxton, R., & Harris, R. A. (1982) *J. Biol. Chem. 257*, 14433–14439.

Perham, R. N., & Roberts, G. C. K. (1981) *Biochem. J. 199*, 733–740.

Perham, R. N., Duckworth, H. W., & Roberts, G. C. K. (1981) *Nature (London) 292*, 474–477.

Pettit, F. H., Hamilton, L., Munk, P., Namihira, G., Eley, M. H., Willms, C. R., & Reed, L. J. (1973) *J. Biol. Chem. 248*, 5282–5290.

Pettit, F. H., Yeaman, S. J., & Reed, L. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4881–4885.

Radford, S. E., Lane, E. D., & Perham, R. N. (1986) *Biochem. Soc. Trans. 14*, 1231–1232.

Reed, L. J., Pettit, F. H., Yeaman, S. J., Teague, W. M., & Bleile, D. M. (1980) *Proc. FEBS Meet. 60*, 47–56.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Spencer, M. E., Darlison, M. G., Stephens, P. E., Duckenfield, I. K., & Guest, J. R. (1984) *Eur. J. Biochem. 141*, 361–374.

Stephens, P. E., Darlison, M. G., Lewis, H. M., & Guest, J. R. (1983) *Eur. J. Biochem. 133*, 481–489.

Sykes, P. J., Burns, G., Menard, J., Hatter, K., & Sokatch, J. R. (1987) *J. Bacteriol. 169*, 1619–1625.
Tarr, G. E. (1986) in *Methods of Protein Microcharacterization* (Shively, J. E., Ed.) pp 155–194, Humana, Clifton, NJ.
von Heijne, G. (1986) *EMBO J. 5*, 1335–1342.
Wagenknecht, T., & Frank, J. (1984) *Biochemistry 23*, 3383–3389.
Wawrzynczak, E. J., Perham, R. N., & Roberts, G. C. K. (1981) *FEBS Lett. 131*, 151–154.

Evidence for a Polypeptide Segment at the Carboxyl Terminus of Recombinant Human γ Interferon Involved in Expression of Biological Activity

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ABSTRACT: A panel of 18 murine monoclonal antibodies was raised in BALB/c mice to the full-length, 146 amino acid residue recombinant human γ interferon (rHuIFN γ-A). Two monoclonal antibodies, designated 47N3-6 and 30N47-1, were purified from ascites tumors and further characterized. Antibody 47N3-6 neutralized both the antiviral and antiproliferative activities of rHuIFN γ-A. Both Western blotting and enzyme-linked immunosorbent assays indicated that antibody 47N3-6 could bind to rHuIFN γ-A as well as to a genetically engineered truncated form lacking the first three amino-terminal residues (rHuIFN γ-D) but did not recognize a genetically engineered variant terminating at residue 131 (rHuIFN γ-B). This antibody also demonstrated binding to a 15 amino acid residue oligopeptide, designated F-1, corresponding to residues 132–146 at the carboxyl terminus of rHuIFN γ-A. Chemical cleavage of peptide F-1 with cyanogen bromide produced two fragments that were separated by reversed-phase high-pressure liquid chromatography. Dot-blot analysis indicated that antibody 47N3-6 could bind to a fragment, $KRKRSQH_{se}$, derived from residues 132–137 of rHuIFN γ-A, but could bind only weakly to the cyanogen bromide fragment corresponding to residues 138–146. It was consistent with these results that antibody 47N3-6 demonstrated binding to a form lacking the five carboxyl-terminal amino acids (rHuIFN γ-D′) but did not bind to a synthetic polypeptide corresponding to residues 138–146. Peptide F-1 exhibited neither antiviral nor antiproliferative activity, and it did not antagonize the antiviral activity of rHuIFN γ-A. However, the specific antiviral and antiproliferative activities of rHuIFN γ-B were 3–4-fold lower than the corresponding values for rHuIFN γ-A. In distinction, antibody 30N47-1, which neutralized neither antiviral nor antiproliferative activity, was observed to bind to three forms of rHuIFN γ (γ-A, -D, and -B) but not to peptide F-1. These data suggest that a portion of the carboxyl terminus of rHuIFN γ-A and, in particular, residues 132–137 may be a component of a critical domain required for the expression of its biological activity.

HuIFN[1] γ, also designated immune or type II IFN, is a product of antigen-sensitized T-lymphocytes that expresses potent antiviral, anticellular, and immunomodulatory activities [for reviews, see Epstein (1981), Georgiades et al. (1984), and Trinchieri and Perussia (1955)]. It has a low primary structural homology with type I (α and β) IFNs (Gray & Goeddel, 1982; Gray et al., 1982) and can further be distinguished from type I IFNs by differences in antigenic determinants (deLey et al., 1980) and a variety of physicochemical properties, including molecular weight, isoelectric point, and sensitivity to low pH (Yip et al., 1981; Gray et al., 1982; Gray & Goeddel, 1983). The cell surface receptors through which the biological properties of type I and type II IFNs are mediated are also distinct (Branca & Baglioni, 1981; Littman et al., 1985; Joshi et al., 1984). It is significant that a number of biological properties appear to be more effectively mediated by HuIFN γ than type I IFNs, including monocyte/macrophage activation (Pace et al., 1983) and enhanced expression of class II major histocompatibility antigens (Wong et al., 1983). The application of recombinant DNA technology has resulted in the availability of large quantities of rHuIFN γ for conducting Phase I/II clinical trials on its utility as an antineoplastic agent (Kurzrock et al., 1985; Bennett et al., 1986; Kleinerman et al., 1986).

Limited information is available on the relationship of the structure of rHuIFN γ to its biological activity. Rinderknecht et al. (1984) have reported that biologically active natural HuIFN γ lacks the first three amino-terminal amino acids (Cys-Tyr-Cys) and is heterogeneous at the carboxyl terminus, as evidenced by the detection of six different carboxyl-terminal amino acids. Although these authors did not measure the antiviral activity of each of the six truncated variants of rHuIFN γ, in which up to 16 amino acids were removed from the carboxyl terminus, Rose et al. (1983) have reported that a rHuIFN γ variant truncated at position 131 retained full

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[1] Abbreviations: IFN, interferon; HuIFN, human interferon; rHuIFN γ, recombinant human γ interferon; HuIFN α, human α interferon; PBS, phosphate-buffered saline; EMEM, Eagle's minimum essential medium; Tris, tris(hydroxymethyl)aminomethane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBS, Tris-buffered saline; EMC, encephalomyocarditis; IU, international units; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine, aminopterin, and thymidine; IgG, γ immunoglobulin.

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