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Isolation and Characterization of Cloned cDNAs Encoding Human Liver Chlordecone Reductase

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ABSTRACT: Chlordecone (Kepone), a toxic organochlorine pesticide, undergoes bioreduction to chlordecone alcohol in human liver. This reaction is controlled by a cytosolic enzyme, chlordecone reductase (CDR), which may be of the aldo–keto reductase family of xenobiotic metabolizing enzymes [Molowa et al. (1986) *J. Biol. Chem. 261*, 12624–12627]. To further investigate the primary structure and expression of CDR, we screened a library of human liver cDNAs cloned in the expression vector λgt11 and isolated an 800 bp cDNA that directed synthesis of a fusion protein recognized by polyclonal anti-CDR antibodies. Using this cDNA as a probe, we screened two human liver cDNA libraries and found several 1.2-kb cDNAs which would code for a polypeptide with 308 residues (35.8 kDa). However, a similar full-length cDNA, possibly the transcript of a pseudogene, contained an in-frame nonsense codon. The deduced protein sequence of CDR showed 65% similarity to the primary structure of human liver aldehyde reductase and 66% similarity to the inferred protein sequence of rat lens aldose reductase. A search of GenBank revealed significant nucleotide similarity to a cDNA coding for bovine lung prostaglandin f synthase and to a partial cDNA coding for frog lens ρ-crystallin. Southern blot analysis of human genomic DNA displayed between 45 and 65 kilobases of DNA hybridizable to CDR cDNA and demonstrated several restriction fragment length polymorphisms among 26 individuals. Northern blot analysis of RNA from human, gerbil, rabbit, hamster, mouse, and rat livers disclosed hybridization with CDR cDNA only for the first three species. These same three species' livers contain CDR activity and one or more proteins immunoreactive with anti-CDR antibodies. RNA from adult but not fetal human liver, and from the human hepatoma cell-line Hep G2, contained major (1.6 kb) and minor (2.8 kb) species hybridizable to a CDR cDNA. The relative amounts of these RNAs varied markedly among nine subjects. From this initial description of the nucleotide sequence for a human carbonyl reductase, we conclude that CDR and several related enzymes are part of a novel multigene family involved in the metabolism of such xenobiotics as chlordecone and possibly endogenous substrates.

The aldo–keto reductases are a family of cytoplasmic enzymes that convert many xenobiotic aldehydes and ketones to their corresponding alcohols. These enzymes have been purified from several tissues and species and are characterized by their broad, overlapping substrate specifities and lack of inducibility (Bachur, 1976). The multiplicity and diversity of these enzymes have made it difficult to define their specific molecular forms. In spite of their ubiquitous presence in mammalian tissues, the precise physiological functions of the aldo–keto reductases remain to be established.

Several years ago, we studied workers exposed heavily to the toxic, organochlorine pesticide 1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one (chlordecone)[1] (Kepone). We proposed that the major pathway for metabolism of chlordecone was its bio-

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[1] Abbreviations: CDR, chlordecone reductase; bp, base pair(s); kb, kilobase(s); chlordecone, 1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one; chlordecone alcohol, 1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-ol.

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transformation to chlordecone alcohol in the liver. We purified the human liver enzyme, termed chlordecone reductase (CDR) (Houston et al., 1981), specific for the bioreduction of chlordecone in vitro (Molowa et al., 1986a). Like other aldo–keto reductases, this cytosolic, monomeric protein had a molecular mass of 37.4K, a pH optima of 7.5, and a requirement of NADPH as a cofactor (Molowa et al., 1986a). However, CDR was insensitive to well-known inhibitors of aldehyde and carbonyl reductases. Immunoblot analysis demonstrated that protein immunoreactive with anti-CDR antibodies paralleled CDR activity by its presence in liver cytosol from those species that produce chlordecone alcohol from chlordecone in vivo (human, rabbit, guinea pig, and gerbil), but not in other species incapable of making this metabolic conversion (mouse, rat, or hamster) (Molowa et al., 1986a).

As the next step in our efforts to better define this mysterious group of enzymes, we used recombinant DNA methods to examine the structure and expression of CDR. By isolating and characterizing cloned cDNAs to CDR, we now present evidence that CDR is species-specific, varies widely among individuals, and is only one of a multigene family of diverse enzymes.

Materials and Methods

Materials

Meloy Laboratories (Springfield, VA) provided a λgt11 cDNA library they constructed (Young & Davis, 1983a,b) from single adult human liver. An additional λgt11 human liver cDNA library also constructed from a single human liver was purchased from Clonetech Laboratories (Palo Alto, CA). The nitrocellulose filters used for the immunoscreening were purchased from Millipore Corp. (Bedford, MA); filters used with nucleic acid probes were purchased from NEN Research Products (Boston, MA); filters used for Northern, Southern, and Western blots were purchased from Bio-Rad (Richmond, CA). We purchased 3,3′-diaminobenzidine from Pfaltz and Bauer (Stamford, CT); goat anti-rabbit peroxidase was from ICN Immunobiologicals (Lisle, IL); deoxycytidine 5′-[α-^{32}P]triphosphate (4500 Ci/mmol) was from ICN Radiochemicals (Irvine, CA); nick translation kit, pUC 8 plasmid vector restriction enzymes, electrophoresis-grade agarose, and ultrapure urea were from Bethesda Research Laboratories, Inc. (BRL) (Gaithersburg, MD); M13mp18 and M13mp19 sequencing vectors were from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ); Sequenase sequencing kit was from U.S. Biochemical (Cleveland, OH); deoxyadenosine 5′-α-[^{35}S]thiotriphosphate (1400 Ci/mmol) was from NEN Research Products; autoradiography film was X-Omat AR from Kodak (Rochester, NY). Blood for leukocyte DNA isolation was obtained from normal volunteers who had given informed consent. Human liver specimens for RNA isolation were obtained at surgery from patients 9–11, 16–19, 22, and 23 under protocols for the Conduct of Human Research at the Medical College of Viginia and are described elsewhere (Molowa et al., 1986b; Wrighton et al., 1986; Watkins et al., 1985). All reagents used were of the highest grade commercially available from Sigma Chemical Co. (St. Louis, MO). A Zeiss scanning densitometer was used for quantitation of Northern blots (Molowa et al., 1986a).

Methods

Library Screening. Approximately 10^5 recombinants from the Meloy λgt11 human liver cDNA library were screened (Young & Davis, 1983b) with the use of rabbit antibodies directed against purified human liver CDR (Molowa et al., 1986a). Positive plaques were visualized by sequential addition of primary antibodies followed by incubation with goat anti-rabbit peroxidase and 3,3′-diaminobenzidine tetrachloride in hydrogen peroxide (Molowa et al., 1986a). Plaques giving positive signals were purified by multiple infections and screenings until all colonies reacted positively (Maniatis et al., 1982). The DNA from purified phage was isolated by the plate lysate method (Maniatis et al., 1982) and was excised by digestion with *Eco*RI. A cloned insert, termed MCDR1, was subcloned into a pUC8 plasmid followed by transformation into JM101 (Berger & Kimmel, 1987). After amplification of the recombinant plasmid, the insert was purified (Berger & Kimmel, 1987), radiolabeled to 10^8 dpm/μg with [α-^{32}P]dCTP by nick translation according to the supplier's instructions, and used as a probe for library screening, and for Northern and Southern blot analysis.

When the radiolabeled MCDR1 was used as a hybridization probe, 10^5 recombinants were screened from both the Meloy and Clonetech libraries. After replica screening (Maniatis et al., 1982), filters were prehybridized and then hybridized for 12 h in a solution of 50% formamide containing 100 μg/mL salmon testes DNA, 0.5% sodium dodecyl sulfate (SDS), 5× SSPE (1× SSPE = 150 mM NaCl and 11.5 mM monobasic sodium phosphate with 1.0 mM EDTA), and 5× Denhardt's solution [1× Denhardt's = 0.2 mg/mL each ficoll, poly(vinylpyrrolidone), and bovine serum albumin fraction V] solution. Filters were then washed twice with 2× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate at pH 7.5) for 5 min at 25 °C, with 2× SSC and 1% SDS for 30 min at 65 °C, and with 0.1× SSC for 30 min at 25 °C. After the filters were blotted dry, they were exposed to film for a minimum of 6 h at −70 °C with the use of two intensifying screens. When all plaques hybridized to the radiolabeled insert, phage DNA was prepared by means of the plate lysate method (Maniatis et al., 1982).

Sequence Analysis. Cloned cDNAs were isolated by *Eco*RI digestion of the phage DNA and then ligated into plasmid M13mp19 and used to transform JM101 (Berger & Kimmel, 1987). Recombinants were assayed for insertional inactivation of β-galactosidase (Berger & Kimmel, 1987). Positive subclones were analyzed for the presence of cloned cDNA by digestion of the double-stranded replicative form of the M13 vector (Birnboim & Doly, 1979). Single-stranded templates were prepared as suggested by the sequencing kit manufacturer, and their nucleic acid sequence was determined by using the method of Sanger et al. (1977) with [α-^{35}S]dATP as the radiolabel. Complete sequence analysis was performed by subcloning into M13mp18 fragments of *Bam*HI-, *Hin*dIII-, and *Pst*I-digested cDNA. In some instances, the sequence was determined with the use of dideoxyinosine triphosphate and deoxyinosine triphosphate to reduce potential regions of compression. The chain-terminated fragments were subjected to electrophoresis through a 6% polyacylamide gel for 2–6 h at 60 W. The gel was transferred to Whatman paper, dried for 3 h under vacuum at 80 °C, and exposed to film for at least 6 h at 25 °C. Each nucleotide position for MCDR1 and MCDR2 was confirmed by sequencing at least 3 times. The nucleotide sequences for CCDR12 and CCDR33 were determined by sequencing from both directions with the exception of a 100-base region between the internal *Eco*RI and *Pst*I sites which was sequenced 4 times in one direction. The alignment and analysis of the full-length cDNA and translated protein sequences was accomplished with the use of NUCALN, PRTALN, and GCG software running on a VAX computer (Wilbur & Lipman, 1983; Devereux et al., 1984).

Southern Blots. *Eco*RI-digested DNA from positive plaques was subjected to electrophoresis in 0.8% agarose and then denaturation in 1.5 M NaCl and 0.5 M NaOH for 1 h at 25 °C. Following neutralization with 1.5 M NaCl and 1 M Tris-HCl (pH 8.0) for 1 h at 25 °C, the DNA was transferred by capillary action to nitrocellulose incubated in 10× SSC and baked at 80 °C for 2 h in vacuo (Southern, 1975). The filter was prehybridized in a solution of 50% formamide containing 50 mM sodium phosphate (pH 6.7), 500 μg/mL salmon testes DNA, 5× SSC, 5% dextran sulfate, and 10× Denhardt's for 18 h at 42 °C and was then hybridized for 18 h at 42 °C with the ^{32}P-labeled MCDR1 in a solution of 50% formamide containing 20 mM sodium phosphate (pH 6.7), 100 μg/mL salmon testes DNA, 10% dextran sulfate, 5× SSC, and 1× Denhardt's solution (Church & Gilbert, 1984). The filter was washed twice in 2× SSC containing 0.1% SDS for 15 min at 25 °C followed by two final washes with 0.1× SSC containing 0.1% SDS for 30 min at 42° C. The filter was air-dried and exposed to film for 12 h. The same experiment was repeated for the genomic DNA blots except 10 μg of DNA obtained from the leukocytes (Bell et al., 1981) of volunteers was digested for 18 h at 65 °C with 30 units of the *Taq*I restriction endonuclease. The digested DNA was processed by electrophoresis through a 1.0% agarose gel at 20 mA for 18 h. After hybridization, the filter was washed at a final temperature of 50 °C. Hybridizing DNA on the filter was visualized by autoradiography for 12 h. Using the migration distance of the molecular weight standards, the size of hybridizing DNA bands was computed from a linear logarithmic regression equation.

Northern Blots. Total RNA was extracted from human, gerbil, hamster, rabbit, rat, and mouse liver samples by brief homogenation in 4 M guanidine isothiocyanate followed by centrifugation through a 1.76 g/mL CsCl cushion (Chirgwin et al., 1979). Forty micrograms of total RNA from nine patients and 35 μg of total RNA from other species were subjected to electrophoresis in a 1.0% agarose gel cast in 10 mM sodium phosphate and 1.1 M formamide at 30 V for 17 h (Thomas, 1980). The RNA was transferred to nitrocellulose in 20× SSC, prehybridized, and hybridized with labeled probe in a solution of 50% formamide containing 0.1% SDS, 200 mg/mL salmon testes DNA, 5× SSPE, and 5× Denhardt's solution. The blots were subjected to a final high-stringency wash with 0.1× SSC containing 0.1% SDS at 50 °C and then visualized by autoradiography for 6–12 h at −70 °C. Removal of hybridized probe required three high-stringency washes at 100 °C, as judged by autoradiography for 3 days. To normalize for the amounts of added and transferred RNA, the same filter was prehybridized and then hybridized with a radiolabeled chicken β-actin cDNA followed by a wash with 0.2× SSC containing 0.2% SDS for 30 min at 25 °C. The molecular weights of the hybridizing RNA species were calculated from a linear logarithmic regression equation based on the migration distance of rRNA standards. The integrated densities of the autoradiographic bands were quantitated on a scanning densitometer (Molowa et al., 1986a) and were expressed as arbitrary units relative to the values from a single patient.

Results

Library Screening and Sequence Analysis. A cDNA library constructed from a single human liver and cloned in the expression vector λgt11 was screened with polyclonal antibodies directed against purified human liver chlordecone reductase (CDR) (Molowa et al., 1986a). Eleven clones exhibiting strong positive signals were isolated and digested with *Eco*RI to release the cDNA insert. Each of these clones contained an 800 bp cDNA. One of these (MCDR1) was subcloned into a pUC plasmid vector, radiolabeled with [^{32}P]dCTP by nick translation, and used as a probe to screen the same λgt11 library for longer cDNA inserts (Figure 1). Of 40 plaques which exhibited strong positive signals, two were isolated and purified (MCDR2, MCDR3). Each contained a cDNA insert of the same size (1.1 kb) that produced an 800 bp and a 330 bp fragment upon digestion with *Eco*RI. The two *Eco*RI fragments from a single recombinant (MCDR2) were subcloned into an M13 vector, and their nucleic acid sequences were determined by the method of Sanger et al. (1977). The nucleotide sequence of the 800 bp *Eco*RI fragment from MCDR2 proved identical with that determined for MCDR1. The 330 bp fragment from MCDR2 contained a polyadenylation signal 20 bases 5′ to a poly(A)$_{20}$ tract. Computer-assisted analysis of the nucleotide sequence of MCDR2 revealed a continuous open reading frame of 909 nucleotides corresponding to a protein with 303 amino acids. However, MCDR2 lacked a putative translational start site which would initiate synthesis of a protein having the molecular mass observed for purified CDR (37.4K).

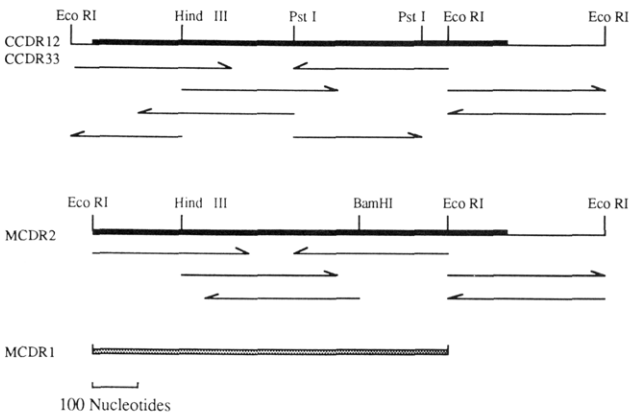

FIGURE 1: Sequencing strategy and partial restriction map for human chlordecone reductase cDNA. Shown are representations of human liver cDNA clones from the Clonetech (CCDR12, CCDR33) and Meloy (MCDR1, MCDR2) λgt11 libraries. The figure labeled MCDR1, spanning the two *Eco*RI sites of MCDR2, represents the first cDNA insert isolated from the Meloy library and was used as a hybridization probe in subsequent experiments. Restriction sites within the large *Eco*RI fragments were used for directional subcloning into an M13 sequencing vector. The wide lines represent coding regions, and the arrows beneath each insert indicate the origin, direction, and approximate distance each subclone was sequenced.

A second λgt11 human liver cDNA library constructed from a single individual by Clonetech, Inc., was screened with [^{32}P]MCDR1. Of 60 plaques exhibiting strong positive signals, 40 were purified and characterized by digestion with *Eco*RI. The cloned inserts of each of these 40 recombinant phage were of identical size and produced the same sized pair of fragments (850 plus 330 bp) upon digestion with *Eco*RI. However, none of these inserts contained a *Bam*HI restriction enzyme site as is found in both MCDR1 and MCDR2. When 10 of these 40 independently isolated and subcloned *Eco*RI fragments were partially sequenced, it became evident that there were at least 2 different groups of 5 each. A representative clone from each group (CCDR12 and CCDR33) was completely sequenced after directional subcloning into an M13 vector. Both CCDR12 and CCDR33 extended 50 nucleotides further in the 5′ direction than did MCDR2. Both cDNAs contained a polyadenylation signal upstream of a poly(A)$_{20}$ tract at the 3′ end. CCDR12 contained a continuous open reading frame of 924 bases, beginning with a putative translational start site 44 bases downstream from the most 5′ *Eco*RI site (Figure 2).

```
CCDR12   -43                                                                    cgatcccaaatatcagcgtgtagagctaaatgatggtcacttc   -1

           1  MetProValLeuGlyPheGlyThrTyrAlaProProGluValProArgAsnArgAlaValGluValThrLysLeuAlaIleGluAlaGly    30
CCDR12     0  ATGCCCGTATTGGGATTTGGCACCTATGCACCTCCAGAGGTTCCGAGGAACAGAGCTGTAGAGGTCACCAAATTAGCAATAGAAGCTGGC    89
MCDR2                    CC              G   G          T AA GT A    C      C GT      G           C  G

          31  PheArgHisIleAspSerAlaTyrLeuTyrAsnAsnGluGluGlnValGlyLeuAlaIleArgSerLysIleAlaAspGlySerValLys    60
CCDR12    90  TTCCGCCATATTGATTCTGCTTATTTATACAATAATGAGGAGCAGGTTGGACTGGCCATCCGAAGCAAGATTGCAGATGGCAGTGTGAAG    179
MCDR2          A              AC  G T

          61  ArgGluAspIlePheTyrThrSerLysLeuTrpCysThrPhePheGlnProGlnMetValGlnProAlaLeuGluSerSerLeuLysLys    90
CCDR12   180  AGAGAAGACATATTCTACACTTCAAAGCTTTGGTGCACTTTCTTTCAACCACAGATGGTCCAACCAGCCTTGGAAAGCTCACTGAAAAAA    269
MCDR2                                    A   A  C CA  G    G  T      G             G             T

          91  LeuGlnLeuAspTyrValAspLeuTyrLeuLeuHisPheProMetAlaLeuLysProGlyGluThrProLeuProLysAspGluAsnGly    120
CCDR12   270  CTTCAACTGGACTATGTTGACCTCTATCTTCTTCATTTCCCAATGGCTCTCAAGCCAGGTGAGACGCCACTACCAAAAGATGAAAATGGA    359
MCDR2           T                        A       T   G  T  G A            GAAGTGA C

         121  LysValIlePheAspThrValAspLeuSerAlaThrTrpGluValMetGluLysCysLysAspAlaGlyLeuAlaLysSerIleGlyVal    150
CCDR12   360  AAAGTAATATTCGACACAGTGGATCTCTCTGCCACATGGGAGGTCATGGAGAAGTGTAAGGATGCAGGATTGGCCAAGTCCATCGGGGTG    449
MCDR2          A  C    T                G              C              A

         151  SerAsnPheAsnCysArgGlnLeuGluMetIleLeuAsnLysProGlyLeuLysTyrLysProValCysAsnGlnValGluCysHisPro    180
CCDR12   450  TCAAACTTCAACTGCAGGCAGCTGGAGATGATCCTCAACAAGCCAGGACTCAAGTACAAGCCTGTCTGCAACCAGGTAGAATGTCATCCT    539
MCDR2          C         CA     T                                G                              G

         181  TyrLeuAsnGlnSerLysLeuLeuAspPheCysLysSerLysAspIleValLeuValAlaHisSerAlaLeuGlyThrGlnArgHisLys    210
CCDR12   540  TACCTCAACCAGAGCAAACTGCTGGATTTCTGCAAGTCAAAAGACATTGTTCTGGTTGCCCACAGTGCTCTGGGAACCCAACGACATAAA    629
MCDR2          T          A                                                T T            T    T   G AG

         211  LeuTrpValAspProAsnSerProValLeuLeuGluAspProValLeuCysAlaLeuAlaLysLysHisLysArgThrProAlaLeuIle    240
CCDR12   630  CTATGGGTGGACCCAAACTCCCCAGTTCTTTTGGAGGACCCAGTTCTTTGTGCCTTAGCAAAGAAACACAAACGAACCCCAGCCCTGATT    719
MCDR2          C            G        G  G  C              C           G     A  G     G

         241  AlaLeuArgTyrGlnLeuGlnArgGlyValValValLeuAlaLysSerTyrAsnGluGlnArgIleArgGluAsnIleGlnValPheGlu    270
CCDR12   720  GCCCTGCGCTACCAGCTGCAGCGTGGGGTTGTGGTCCTGGCCAAGAGCTACAATGAGCAGCGGATCAGAGAGAACATCCAGGTTTTTGAA    809
MCDR2                                                                          C      C     G G     G

         271  PheGlnLeuThrSerGluAspMetLysValLeuAspGlyLeuAsnArgAsnTyrArgTyrValValMetAspPheLeuMetAspHisPro    300
CCDR12   810  TTCCAGTTGACATCAGAGGATATGAAAGTTCTAGATGGTCTAAACAGAAATTATCGATATGTTGTCATGGATTTTCTTATGGACCATCCT    899
MCDR2                    T        G        CCA        C             GTG       T GAC C T    A  T   GCT G   CC

         301  AspTyrProPheSerAspGluTyr                                                                    308
CCDR12   900  GATTATCCATTTTCAGATGAATATTAGcatagagggtgttgcacgacatctagcagaaggccctgtgtgtggatggtgatgcagaggatg    989
MCDR2          A             T           A

CCDR12   990  tctctatgctggtgactggacacacggcctctggttaaatccctcccctcctgcttggcaacttcagctagctagatatatccatggtcc    1079

CCDR12  1080  agaaagcaaacataataaattttttatcttgaagt                                                          1113
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FIGURE 2: Nucleotide and deduced amino acid sequence of human liver chlordecone reductase. The complete nucleotide and deduced amino acid sequence of CCDR12 is shown with only the coding region nucleotide differences of MCDR2, a partial cDNA isolated from a different λgt11 human liver cDNA library. The sequence differences of CCDR33, an additional cDNA isolated from the same library as CCDR12, are indicated by boldface letters with differences occurring at positions 59, A → G; 215, C → G; 388, C → G; 832, T → A; and 885, C → G. Underlined are the *Eco*RI site (**GAATTC**) and polyadenylation signal (*aataaa*) for CCDR12 and CCDR33.

This segment would encode a protein of 308 amino acids with a calculated molecular mass of 35.8K. The latter value is in agreement with the estimated molecular mass of purified human liver CDR (37.4K) (Molowa et al., 1986a).

Sequence Comparisons. The nucleotide sequence of CCDR12 was 87% identical with that of the partial cDNA, MCDR2 (Figure 2), and their deduced protein sequences were 82% identical. CCDR12 and CCDR33 contain a TAG termination codon, whereas MCDR2 utilizes a TAA nonsense codon. The 2 full-length clones, CCDR12 and CCDR33, from the same library were nearly identical, differing by only 10 nucleotides throughout the entire length of the cDNA insert. Among 10 nucleotides which were different from those in CCDR12, CCDR33 contained 5 substitutions in the coding region (Figure 2) and 2 deletions in the 3′ untranslated region at positions 994 and 995. Furthermore, CCDR33 contained a C to G transversion at position 215 which converted a cysteine codon to an in-frame nonsense codon. CCDR12 and MCDR2 cloned from different livers share 82% inferred amino acid sequence identity. When the complete amino acid sequence of either CCDR12 or MCDR2 is aligned with that of human liver aldehyde reductase [determined by peptide sequencing (Wermuth et al., 1987)] or with that from rat lens aldose reductase cDNA (Carper et al., 1987), the identity is 43% and 50%, respectively.

A search of GenBank against the nucleotide sequence of CCDR12 revealed significant identity with a cDNA coding for bovine (*Bos taurus*) prostaglandin f synthase (Watanabe et al., 1988) and with a partial cDNA coding for ρ-crystallin from the lens of the European common frog (*Rana temporaria*) (Tomarev et al., 1984). Indeed, the cDNA structure of CCDR12 was 79% identical with that of prostaglandin f synthase in the coding region. Except for human liver aldehyde reductase, the amino acid sequences of these similar polypeptides (CCDR12, MCDR2, prostaglandin f synthase, and ρ-crystallin) can be aligned optimally without amino acid insertions or deletions while alignment of rat lens aldose reductase requires only one insertion at position 208 (Figure 3). Furthermore, a region encompassing amino acids 160–179 of CCDR12 is invariant for each of the inferred polypeptide sequences, while a region from amino acids 290 to 298 shows almost no similarity among these proteins. The summary of the computer-generated amino acid similarities, which allows conservative amino acid subtitutions based on the evolutionary

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ALYR      13    LI L  WKSEPGQVKA  --- Y LSV Y    C AI G  PEI E LKEDVGP KA P  EL V     N KHH ED E   RKT AD   E L
PGFS      16  I         E   KSE L A  F   V    V   H  Q      Q          T            NSL  EL R    K  QN
CCDR12     1  MPVLGFGTYAPPEVPRNRAVEVTKLAIEAGFRHIDSAYLYNNEEQVGLAIRSKIADG-SVKREDIFYTSKLWCTFFQPQMVQPALESSLKKLQLDYVDLY   99
MCDR2          P       A   KSK L AV        H     HV                                  SNSHR EL R    R   N
ALDR                               KV  DM Y    C QV Q  KE  V LQE LKEQ V   Q L IV       HDQS  KG CQKT SD     L
RHOC                                                                                          L R  RDVGM  L  F


ALYR           M W Y FER DN F  NAD TICY STHYKE  KAL ALVAK  VQAL L    S  IDD  SVASVR-- AVL        A NE IAH QARGLEVT
PGFS          II S VS    NKFV    S  L   S   CH   AL        T         HK   K                          E    H
CCDR12  100   LLHFPMALKPGETPLPKDENGKVIFDTVDLSATWEVMEKCKDAGLAKSIGVSNFNCRQLEMILNKPGLKYKPVCNQVECHPYLNQSKLLDFCKSKDIVLV   199
MCDR2          I   VSV    EVI       IL      C    A                  H L                       F  R
ALDR           I W TGF    PDYF L AS N  PSDT FVD  TA  QLV E  V A       PL I R           AV  I      T E  IEY HC G  VT
RHOC           M W VS   SGASD S KDKPF Y N   C    AL AR     VR L      R    R                    V    N  HSY


ALYR           YPLGSSD A-- R  DE     E  VL   E YG S  Q L  W V  K ICIP  ITPS  LQ  K  D TFSP E  Q NA  K W  I PMLTV G
PGFS           YA   A LLSE  NS N          I     Q    V     V         F KK  K  M   D E  P    AI      I  YDFQKGIG
CCDR12  200   AHSALGTQRHKLWVDPNSPVLLEDPVLCALAKKHKRTPALIALRYQLQRGVVVLAKSYNEQRIRENIQVFEFQLTSEDMKVLDGLNRNYRYVVMDFLMD-   298
MCDR2          Y    SH EEP                                                    Q V          E  AI      V  LTL IFAG
ALDR           Y P  SP-DRP AK ED S     RIKEI A YNK T QVLI FPI  NL  IP  VTPA  A  FK  D E SN   AT LSY   W VCALMSCAK
RHOC          TY V  SH DRN   LSL    D  I NKV A YN  S E  M FI  K I      FTPA  KQ LG    E KP    S ES D  LH GPFREVKQ


ALYR           KRVPRDAG  L   N P   323
PGFS                     E    E    323
CCDR12  299   --------HPDYPFSDEY  308
MCDR2                 P N         304
ALDR                   K    HA V  284
RHOC                   E   H      225
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FIGURE 3: Comparison of deduced amino acid sequences for human liver chlordecone reductase, bovine lung prostaglandin f synthase, frog lens ρ-crystallin, rat lens aldose reductase, and human liver aldehyde reductase. Amino acid sequence differences from CCDR12 are depicted for similar proteins: the full-length deduced sequence of bovine lung prostaglandin f synthase (PGFS) (Wantanabe, 1988); the primary structure of human liver aldehyde reductase (ALYR) (Wermuth, 1987); and the inferred partial sequences of MCDR2, rat lens aldose reductase (ALDR) (Carper, 1987), and frog lens ρ-crystallin (RHOC) (Tomarev, 1984). Underlined one-letter amino acid abbreviations in CCDR12 represent differences in the deduced sequence of CCDR33, with changes observed at position 72, C → nonsense; position 129, S → C; position 277, M → K; and position 296, L → V. Boldface one-letter codes denotes the beginning of the partial sequences for MCDR2 (P), rat lens aldose reductase (K), and frog lens ρ-crystallin (L). The dashed lines represent insertions necessary for optimal alignment with aldehyde reductase.

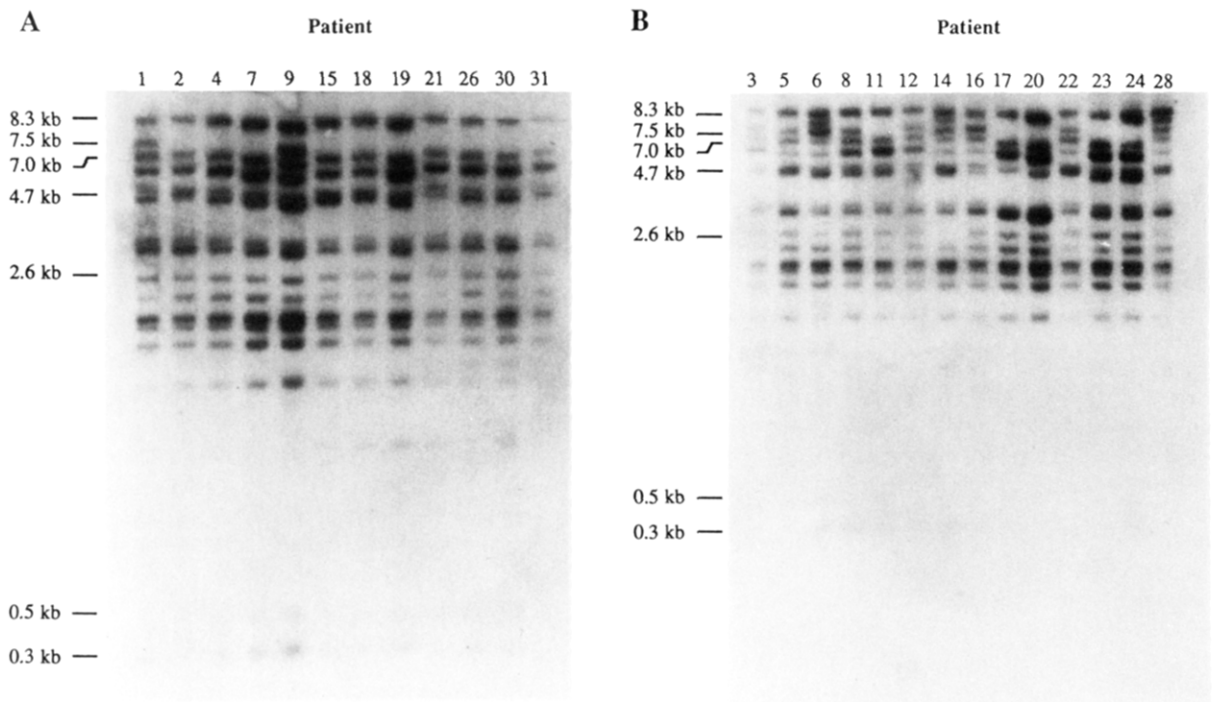

FIGURE 4: Southern blot analysis of human DNA restriction fragments for polymorphisms in the CDR gene. Ten micrograms of human genomic DNA was digested with *Taq*I at 65 °C for 18 h. After transfer to nitrocellulose, the filters were hybridized for 18 h with the radiolabeled probe, subjected to a high-stringency wash for 30 min at 50 °C, and exposed to film for 12 h at −70 °C (see Methods).

distances of peptides (Gribskov & Buress, 1986), is presented in Table I with calculated amino acid and cDNA sequence identities (Wilbur & Lipman, 1983).

Southern Analysis. Human leukocyte DNA from 26 individuals was digested with *Taq*I and was analyzed on Southern blots hybridized with [^{32}P]MCDR1 (Figure 4). Bands comprising 45–65 kb were visualized in patients 17 and 6, respectively, a finding which suggested the presence of multiple, related genes. In patients 6–9, the autoradiographic intensity for the 2.6-kb band was markedly reduced by comparison to the bands for the other patients which suggests the presence of at least two genes recognized by this cDNA probe.

Table I: Sequence Relationships of Human Liver Chlordecone Reductase to Similar Proteins

	CCDR12			
	nucleotide identity[a] (%)	protein identity[a] (%)	protein similarity[b] (%)	ref
MCDR2[c]	85	82	90	
bovine lung prostaglandin f synthase	73	74	85	Wantanbe et al. (1988)
frog lens *p*-crystallin[c]	54	59	76	Tomarev et al. (1984)
rat lens aldose reductase[c]	45	50	66	Carper et al. (1987)
human liver aldehyde reductase	NA[d]	43	65	Wermuth et al. (1981)

[a] Calculated from the alignments generated by NUCALN and PRTALN programs (Wilbur & Lipman, 1983). [b] Computed by Genetics Computer Group (Devereux et al., 1984) programs which determine similarity based on conservative amino acid substitutions (Gribskov & Buress, 1986). [c] Partial cDNA. [d] NA, nucleotide sequence not available.

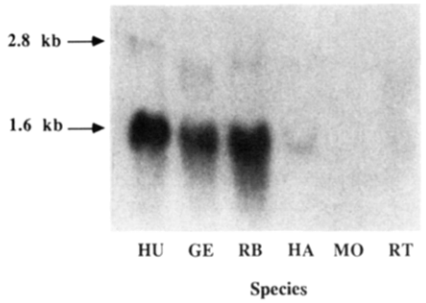

FIGURE 5: Northern blot analysis of liver RNA hybridized with human liver chlordecone reductase cDNA. Total liver RNA (35 μg) from human (HU), gerbil (GE), rabbit (RB), hamster (HA), mouse (MO), and rat (RT) was resolved in a 1.0% agarose gel containing 1.1 M formamide. After transfer to nitrocellulose, the filter was hybridized with [^{32}P]MCDR1 followed by a high-stringency wash and a 6-h autoradiographic exposure at −70 °C (see Methods).

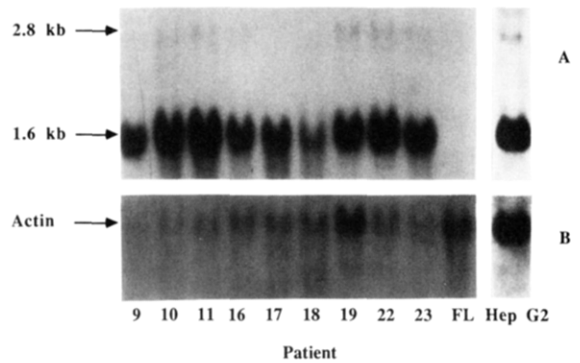

FIGURE 6: Northern blot of human liver RNA samples hybridized with CDR cDNA. Total RNA (40 μg) from nine patients (none exposed to chlordecone nor presented in Southern analysis) and fetal liver (FL) was separated by gel electrophoresis and transferred to nitrocellulose. This same procedure was used for analysis of total RNA (10 μg) from a hepatoma cell line (Hep G2) culture. The autoradiographs shown in panel A are [^{32}P]MCDR1-hybridized RNA subjected to an 18-h exposure at −70 °C after a high-stringency was at 50 °C for 30 min. The filter with adsorbed Hep G2 RNA was treated similarly except for a final wash at 25 °C and a 24-h exposure. The autoradiographs for the same filters hybridized with β-actin are shown in panel B. The relative density of the 1.6-kb mRNA from the nine individuals, as determined by scanning densitometry, was calculated from the ratio of the CDR to the β-actin signal density. For comparison, the densities were standardized with patient 23 arbitrarily equal to 1 and are as follows: patient 9, 2.7; patient 10, 4.3; patient 11, 3.1; patient 16, 0.4; patient 17, 0.2; patient 18, 0.2; patient 19, 0.1; patient 22, 1.0.

The pattern of hybridizing DNA among the 26 individuals displayed numerous restriction fragment length polymorphisms, and each patient displayed a minimum of 13 hybridizing restriction fragments.

Northern Analysis. When samples of total RNA from human, gerbil, rabbit, hamster, mouse, and rat liver were hybridized with [^{32}P]MCDR1, only the RNA from the first three species produced a strong hybridization signal (Figure 5). The size of the most intensely hybridizing major band (1.6 kb) in human liver is consistent with that of an mRNA having a typical poly(A+) tract of 400 nucleotides. In the human liver RNA sample, an additional, minor 2.8-kb band was observed. In gerbils and rabbits, the size of the major band was also approximately 1.6 kb, while the sizes of the minor bands were 2.4 and 2.6 kb, respectively. Northern analysis of liver RNA from nine different humans revealed a dramatic difference in signal density, as determined by scanning densitometry, for each hybridizing RNA species (Figure 6). After normalization of the RNA present on the filter by hybridization with β-actin, the difference in the density of hybridizing 1.6-kb RNA between patients (e.g., 10 and 19) was as much as 40-fold. There was no apparent correlation between the amounts of RNA hybridizing to MCDR1 and the age, sex, or medical history of the individual. Hybridization with MCDR1 was not detected in RNA from fetal liver (Figure 6) or from human lung (not shown). In addition, RNA from a hepatoma cell line (Hep G2) culture displayed a similar pattern of hybridization as did the human liver RNA samples (Figure 6).

Discussion

In our previous studies, we found it difficult to classify purified human liver CDR using classical biochemical criteria. Like other carbonyl reductases, CDR is a monomeric, cytosolic, and NADPH-dependent enzyme capable of reducing chlordecone, a carbonyl-containing xenobiotic, to its corresponding alcohol. However, CDR was inactive in reducing common carbonyl reductase substrates (Molowa et al., 1986a). Moreover, from studies of the distribution of CDR activity (Molowa et al., 1986c) and anti-CDR immunoreactive protein (Molowa et al., 1986a), it is clear that CDR is species and tissue specific, unlike the seemingly ubiquitous presence of classical aldo–keto reductases (Bachur, 1976). Further complicating the classification of CDR is the observation that CDR activity is induced in the liver of gerbils following their treatment with chlordecone (Molowa et al., 1986c) although it is commonly believed that aldo–keto reductases are not inducible (Bachur, 1976). Finally, the multiple carbonyl reductase activities in humans (Nakayama et al., 1985) (Wermuth, 1981) and other mammalian species (Felsted & Bachur, 1980) could reflect the presence of common or separate gene products.

In this report, we cloned three similar, but not identical, cDNA inserts coding for CDR. To find, even in a single liver, multiple transcripts related to a cDNA (MDCR1) encoding immunoreactive protein would not be unexpected because anti-CDR antibodies recognize two proteins in immunoblot analysis of human liver cytosol (Molowa et al., 1986a). Hence, the significant sequence differences between MDCR2 and CCDR12, each obtained from a different liver, suggest that these cDNAs may be derived from separate CDR genes. Furthermore, the large amount of genomic DNA apparently related to a CDR cDNA (Figure 4) is consistent with the

existence of several CDR genes or pseudogenes. One other related cDNA, CCDR33, was almost identical with CCDR12, except for the presence of an in-frame TGA (nonsense) triplet (Figure 2). If not the result of a cloning artifact, CCDR33 may represent a psuedogene transcript. This TGA triplet may also direct incorporation of selenocysteine into a CDR protein, as has been reported for several bacterial and mammalian (including human liver) proteins (Stadtman, 1987). However, no evidence yet proves that CDR is a selenoprotein. Furthermore, we recognize that the unequivocal identity of CCDR12 as CDR (against which the antibody was directed) requires a protein sequence identical with that predicted by the cDNA or the expression of the cDNA in an appropriate vector system. However, apparently due to a blocked N-terminus, attempts to sequence the purified protein failed to give amino-terminal sequence information, and no unique internal sequence fragments were obtained. Currently, our efforts are focused on demonstrating CDR activity from a suitable cell line transfected with a viral vector containing the CCDR12 cDNA.

Structural analysis of cloned cDNAs to CDR greatly helps define its relationship to other proteins. The deduced protein sequence of CDR demonstrates some similarity to that of human liver aldehyde reductase, a cytosolic enzyme with related catalytic activities (Wermuth et al., 1987). However, the active site of human aldehyde reductase has been found to contain the tetrapeptide sequence I-P-K-S (Mojana et al., 1987), a region also conserved in human aldose reductase. At the same relative position, the partially conserved tetrapeptide sequence (L-A-K-S) is contained in the deduced amino acid sequences for CDR, prostaglandin f synthase, and frog lens ρ-crystallin. Moreover, we were surprised to discover the overall sequence similarity between CDR and a frog lens structural protein, ρ-crystallin (Tomarev et al., 1984). This novel crystallin protein is reported to be distinct from other members of the crystallin supergene family, and yet it is the major protein product produced in a cell-free translation of frog lens mRNA (Tomarev et al., 1984). However, it is now clear that some taxon-specific crystallin genes are similar to genes for enzymes (Wistow & Piatogorsky, 1987). For example, chick δ-crystallins are related by sequence to human and yeast argininosuccinate lyase, turtle τ-crystallin to human and yeast enolases, and squid S_{III}-crystallin to rat glutathione *S*-transferase. Furthermore, a major lens structural protein (ϵ-crystallin), isolated from duck, shows lactate dehydrogenase activity (Wistow & Piatogorsky, 1987). Since the reported molecular weight of ρ-crystallin (35K) is the same as we have found for CDR, it is possible that the frog lens protein may function as a carbonyl reductase. Indeed, both CDR and ρ-crystallin are similar to a rat lens protein which exhibits aldose reductase activity (Carper et al., 1987).

CDR was also found to be related to the sequence for bovine lung prostaglandin f synthase (Watanabe et al., 1988). While this structural similarity raises the interesting possibility that prostaglandin metabolism is a physiological function for CDR, prostaglandins have been regarded as relatively poor substrates for human liver (Nakayama, 1985) and brain (Wermuth, 1981) carbonyl reductases. Indeed, bovine lung prostaglandin f synthase (Wantanbe et al., 1985) and human brain carbonyl reductase (Wermuth, 1981) demonstrated higher affinities and turnover rates for such xenobiotics as 9,10-phenanthrenequinone, as compared to prostaglandins. However, 9,10-phenanthrenequinone was not metabolized by CDR (Molowa et al., 1986a) nor was CDR mRNA detected in a human lung RNA sample. Thus, CDR is unique in its structure, function, and tissue distribution. Moreover, while xenobiotics, like chlordecone, and endogenous substrates, like prostaglandins, are useful to categorize aldo–keto reductases such as CDR and the structurally related proteins we have identified, the precise physiological function remains to be discovered.

Southern blot analysis of the human genome suggest that at least two genes are recognized by the CDR cDNA. Rat DNA may also contain at least two copies of gene sequences coding for lens aldose reductase (Nishimura et al., 1988). In light of the multiple homologous proteins found in different organs of different species, it seems likely that the aldo–keto reductases constitute a superfamily of genes. We found several restriction fragment length polymorphisms (RFLP) in human genomic DNA recognized by CDR cDNA (Figure 4). In future studies, we will investigate whether these RFLPs are linked to interindividual differences in the ability to metabolize chlordecone or other carbonyl-containing substrates. Previously, we found that among 32 persons heavily exposed to chlordecone, there was a 6-fold difference in rates of elimination of chlordecone (Cohn et al., 1978).

As judged by Northern blot analysis, only humans, gerbils, and rabbits contain readily detectable amounts of CDR mRNA in the liver (Figure 5). These results coincide with our previous studies of liver cytosol which showed that the same three species contained anti-CDR immunoreactive proteins and CDR activity in vitro (Molowa et al., 1986a). Moreover, RNA from human hepatoma cells (HepG2), but not from a human lung sample, was reactive with the cDNA for CDR, a finding consistent with our suggestion that expression of CDR in gerbil (Molowa et al., 1986a) and in man is confined to the liver. We also failed to find hybridizable CDR mRNA in a human fetal liver sample. While almost nothing is known about the developmental regulation of aldo–keto reductases, this observation suggests the intriguing possibility that expression of CDR is suppressed early in gestation. The widely variable expression of CDR mRNA was far greater than what we have observed in many of the same RNA samples when they were analyzed for a human liver cytochrome P-450, HLp (Molowa et al., 1986b). The variability in amounts of CDR mRNA (as much as 40-fold) is far greater than that observed for the amounts of anti-CDR immunoreactive protein in samples of human liver cytosol (6-fold) (Molowa et al., 1986a). Further studies are needed to examine such possible explanations as accumulation of CDR mRNA or formation of truncated, unstable protein products from an mRNA reflected in CCDR33.

Registry No. Chlordecone reductase, 102484-73-1; DNA (human liver clone CCDR12 chlordecone reductase messenger RNA complementary), 124316-17-2; chlordecone reductase (human liver clone CCDR12 reduced), 124316-13-8.

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