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Isolation and identification of a cDNA clone coding for rat uroporphyrinogen decarboxylase

(heme biosynthetic pathway/porphyria/mRNA purification/hybrid-selected translation)

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ABSTRACT We have cloned and identified a DNA sequence complementary to the mRNA of uroporphyrinogen decarboxylase (UroDCase) from rat. This mRNA is a minor species (0.1%) of the total mRNA from anemic rat spleen. Poly(A)$^+$ mRNA was enriched for UroDCase mRNA to 20% purity by a very efficient procedure involving two successive steps of preparative gel electrophoresis under various denaturing conditions. cDNA prepared from partially purified UroDCase mRNA (1% purity) was cloned in the *Pst* I site of pBR322 by using the homopolymeric G-C tailing method. Primary screening of 500 clones from this cDNA library was performed with a cDNA probe complementary to highly purified mRNA for UroDCase (20% purity) and UroDCase cDNA clones were finally identified by hybrid-selected translation. The rat cDNA clones obtained hybridize to human UroDCase mRNA. This will permit the isolation of the corresponding human gene and molecular analysis of porphyria cutanea tarda, the commonest type of porphyria.

Uroporphyrinogen decarboxylase (UroDCase; EC 4.1.1.37) is a cytosolic enzyme that catalyzes the fifth step of heme synthesis—i.e., the sequential removal of the four carboxyl groups of the carboxymethyl side chains in uroporphyrinogen to yield coproporphyrinogen (1). This enzyme has several biological and clinical features that prompted us to isolate a DNA sequence complementary to its mRNA. (*i*) The synthesis of heme and globin moieties of hemoglobin are closely coordinated and it has been proposed that heme synthesis in early erythroid cells may initiate the development of hemoglobin synthesis by derepressing the synthesis of globin (2). Furthermore, coordinated induction of the enzymes of the heme biosynthetic pathway has been observed in cultured erythroleukemic cells, and thus these enzymes might be good markers of early erythroid differentiation (3). (*ii*) A genetically determined deficiency of UroDCase is suspected to be the primary lesion in two distinct forms of porphyria cutanea tarda (PCT), the commonest type of porphyria: in familial PCT, a form of PCT that is inherited as an autosomal dominant condition, the enzyme activity was reported to be reduced in all tissues investigated (4). In addition, it has been shown that immunoreactive enzyme and catalytic activity were decreased to a similar extent in erythrocytes from patients with familial PCT (5). In contrast, in sporadic PCT, the UroDCase deficiency seems to be restricted to the liver and, although an acquired nature of the enzyme defect cannot be excluded, a genetically determined predisposition is suspected since the presence of frequently associated factors does not appear to provide a sufficient explanation (6). If an inherited defect of UroDCase is present in the sporadic form of PCT, it must differ from the one that has been documented in the familial form and it is conceivable that liver and erythrocyte enzymes are in some manner under different genetic control. In addition, one recent report suggests that patients with hepatoerythropoietic porphyria, a rare but very severe porphyria, are, in fact, homozygous for the UroDCase defect found in familial PCT (7).

To approach these biological and clinical questions, we have undertaken a molecular analysis of mammalian UroDCase genes. In this paper, we described the isolation and characterization of a cloned cDNA sequence encoding rat UroDCase.

MATERIALS AND METHODS

DNA and Enzymes. Enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. Plasmid DNA was purified by the clear lysate procedure or by the acid/phenol method (8).

UroDCase Purification and Antibodies. Human UroDCase was purified to homogeneity from outdated erythrocytes as described by de Verneuil *et al.* (9) and an antiserum was raised in rabbit by repeated injections of this preparation. Specific antibodies were prepared by affinity chromatography of the IgG fraction using human UroDCase immobilized on glutaraldehyde-activated Ultrogel (10). Rat UroDCase was purified to homogeneity and crossreactivity of the anti-human UroDCase antibodies with rat UroDCase was established by their ability to immunoprecipitate the rat enzyme activity.

mRNA Isolation and Partial Purification of UroDCase mRNA. Total RNA was isolated from spleens of rats made anemic by phenylhydrazine injection or from the spleen of a thalassemic patient by the LiCl method (11). Poly(A)$^+$ RNA was selected on oligo(dT)-cellulose (type 3, Collaborative Research, Waltham, MA) (12) and rat mRNA was fractionated by gel electrophoresis. Preparative gel electrophoresis with continuous elution was performed by using an apparatus designed by W. G. Roskam (Heuros electrophoresis, Lunteren, Holland), as described by Brûlet and Roskam (13), except that 0.15% $NaDodSO_4$ was used in the electrophoresis and elution buffers and dextran T 40 was added as a carrier for ethanol precipitation instead of tRNA. Fractionation on methylmercury hydroxide/agarose gel is described in *Results*.

***In Vitro* Translation and Immunoprecipitation.** Cell-free translation was in micrococcal nuclease-treated rabbit reticulocyte lysate prepared according to Pelham and Jackson (14). The biological activity of mRNA preparations was measured by determining hot trichloroacetic acid-precipitable radioactivity above background. ^{35}S-labeled peptides were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis

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Abbreviations: UroDCase, uroporphyrinogen decarboxylase; bp, base pair(s); PCT, porphyria cutanea tarda.
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(15) and detected by fluorography. Immunoprecipitations were carried out according to Opperman *et al.* (16).

Preparation of cDNA Clones. Synthesis of double-stranded cDNA complementary to enriched poly(A)$^+$ RNA was accomplished essentially as described by Wickens *et al.* (17). Double-stranded cDNAs longer than 400 base pairs (bp) were selected by Sepharose 4B chromatography and inserted in the *Pst* I site of pBR322 by using the homopolymeric tailing and hybridization method according to Michelson and Orkin (18). The resulting hybrid molecules were used to transform *Escherichia coli* strain MC 1061, which was rendered competent for uptake of plasmid as described by Hanahan (19). Recombinant clones were stored frozen either individually in 96-microwell culture dishes containing freezing medium (20) or on nitrocellulose filters after high-density plating (21).

Identification of UroDCase cDNA Clones. Clones were screened on nitrocellulose filters with a single-stranded cDNA probe derived from poly(A)$^+$ RNA highly enriched for UroDCase sequences (22). Colony hybridization was done according to Grunstein and Hogness (23) as modified by Thayer (24). After hybridization and washing, positive colonies were visualized by exposing filters to Kodak AR5 x-ray films at −80°C for at least 48 hr with intensifying screens.

Individual clones selected in the primary screening were analyzed by positive selection. Hybrid-selected translation analysis of plasmid DNA was carried out according to Parnes *et al.* (25) with several modifications. DNA was isolated from 5-ml overnight cultures as described by Holmes and Quigley (26). Plasmid DNA (2 μg/20 μl) was heated to 100°C for 10 min and quickly chilled in ice. Then an equal volume of 1 M NaOH was added and the mixture was incubated at room temperature for 20 min. After neutralization with 1 M HCl, the DNA was pipetted onto nitrocellulose disks, which were air dried and baked for 2 hr at 80°C in a vacuum oven. Ten micrograms of anemic rat spleen mRNA was used for each hybridization. Translation was carried out in a 25-μl volume; 4 μl was applied directly to a $NaDodSO_4$/10% polyacrylamide gel and the remainder was immunoprecipitated with anti-UroDCase IgG.

RNA Blot Analysis. Size fractionation of RNA preparations on a denaturing methylmercury hydroxide/agarose gel was carried out according to Chandler *et al.* (27), except that samples were made up in 10 mM methylmercury hydroxide and applied onto a gel containing 1.3% agarose and 10 mM methylmercury hydroxide. After electrophoresis, the gel was washed before blotting sequentially in 100 ml of 10 mM phosphate buffer containing 1.1 M formaldehyde and 5 mM 2-mercaptoethanol for 40 min, then in the same buffer with 7 mM iodoacetate instead of 2-mercaptoethanol for 30 min, and finally in phosphate buffer with formaldehyde alone for 20 min (28). The RNA was then transferred onto a nitrocellulose filter (29) and hybridized to a nick-translated ^{32}P-labeled DNA probe (30). The filter was washed extensively before autoradiography.

RESULTS

Cell-Free Translation and Immunoprecipitation of Rat UroDCase. Rabbit antibody to human UroDCase strongly crossreacts with the rodent enzyme and immunoprecipitates rat UroDCase enzymatic activity, as shown in Fig. 1. This antibody was used for immunoprecipitation of neosynthetized UroDCase after cell-free translation of mRNA prepared from anemic rat spleens: $NaDodSO_4$/polyacrylamide gel electrophoresis of the immunoprecipitate revealed a single radioactive band corresponding to a polypeptide of the expected molecular weight (M_r 40,000). The size of this polypeptide differs slightly from the translation product obtained

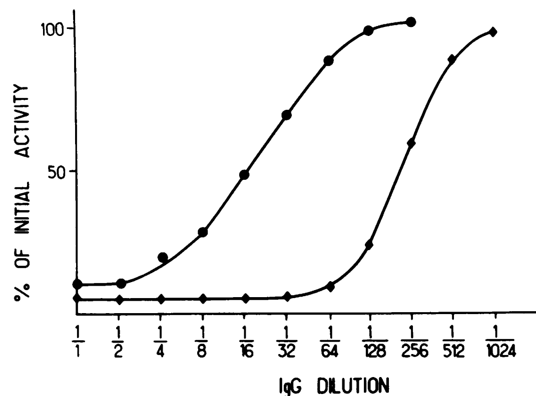

FIG. 1. Inactivation of human (◆) and rodent (●) UroDCase by a rabbit antibody prepared against the human enzyme.

when human mRNA is used as a template in cell-free synthesis assay but migrates exactly as does the pure rat UroDCase. Identification of this translation product as rat UroDCase was confirmed by immunological competition. Quantitation of the radioactivity incorporated into UroDCase allowed us to estimate that the corresponding mRNA represented about 0.1% of the total mRNA obtained from anemic rat spleens.

Partial Purification of UroDCase mRNA and Cloning of Its cDNA. Since UroDCase mRNA is a nonabundant species in anemic spleens from rats, partial purification of the specific mRNA was required to reduce the number of colonies that would have to be screened. This was performed by fractionation of poly(A)$^+$ RNA by using a high-resolution preparative gel electrophoresis with continuous elution. Fractions eluted from the first, nondenaturant, acrylamide gel were translated *in vitro* and the radioactive proteins were resolved on a $NaDodSO_4$/polyacrylamide slab gel (Fig. 2 *Upper*). Identification of the fractions containing UroDCase mRNA was done as follows. The fractions were pooled and the pool containing UroDCase mRNA was identified by specific immunoprecipitation of the translation products (data not shown). This pool was then divided into its individual fractions, and UroDCase was localized by analysis of the *in vitro* immunoprecipitated translation products (Fig. 2 *Lower*). UroDCase mRNA represented about 1% of the total mRNA present in five fractions, as estimated from incorporated [^{35}S]methionine. About half of this mRNA was used as a template to make double-stranded cDNA, which was then inserted into the *Pst* I site of pBR322. The recombinant DNA was used to transform *E. coli* strain MC 1061 and the tetracycline-resistant colonies were stored for further testing.

A portion of the UroDCase-enriched mRNA fraction was purified further by electrophoresis through a methylmercury hydroxide/agarose gel using the same preparative gel electrophoresis system. Agarose was used instead of acrylamide since methylmercury hydroxide inhibits acrylamide polymerization. Approximately 2 μg of partially purified mRNA was layered on top of a cylindrical methylmercury hydroxide/1.5% low-melting temperature agarose gel in sodium borate buffer (31) and electrophoresis was carried out overnight at 40 V. Aliquots of each fraction were pooled 5 by 5, precipitated with ethanol in the presence of 100 mM 2-mercaptoethanol, and translated *in vitro*. The translation products were analyzed by immunoprecipitation and fractions enriched for UroDCase mRNA were then individually tested. Fig. 3 shows the results of this secondary enrichment step. The specific mRNA was estimated to represent about 20% of total mRNA in the selected fractions and was used to derive a ^{32}P-labeled single-stranded cDNA probe. From 500 colonies that were screened with this probe, 10 gave a hy-

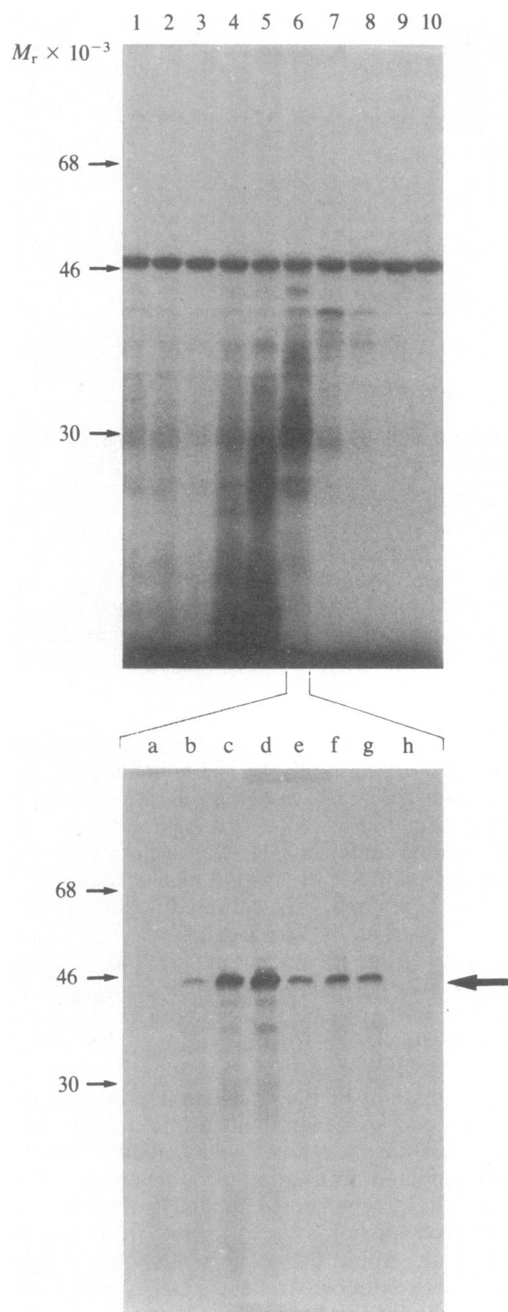

FIG. 2. Fractionation of mRNA on preparative gel electrophoresis. Poly(A)$^+$ RNA (150 μg) was fractionated on a 3% polyacrylamide gel and 100 fractions of 600 μl were collected. (*Upper*) Sixty microliters was removed from each fraction. Aliquots were pooled 10 by 10 and precipitated with ethanol. The mRNA contained in these 100 pooled fractions was then *in vitro* translated and the neosynthesized products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (lanes 1–10). The strong band having an apparent M_r of 47,000, visible in all of the lanes, is an artefact of cell-free translation with [^{35}S]methionine and the reticulocyte proteins synthesized from endogenous mRNA. (*Lower*) Immunoprecipitation of eight individual fractions from pool no. 6 with anti-UroDCase antibody (lanes a–h). The arrow on the right indicates the position of rat UroDCase.

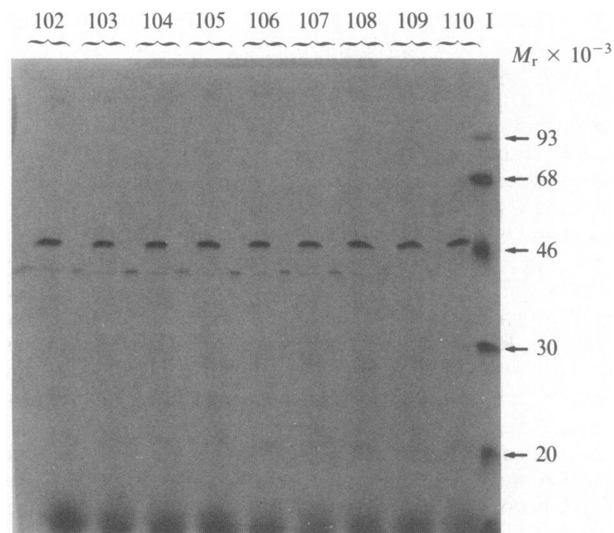

FIG. 3. Enrichment of mRNA for rat UroDCase by electrophoresis through a methylmercury hydroxide/agarose gel. The six individual fractions containing UroDCase mRNA were pooled and half of their mRNA content was fractionated on a 10 mM methylmercury hydroxide/1.5% agarose gel. One hundred fifty fractions of 600 μl were collected and UroDCase mRNA was found mainly in fractions 102–110. These fractions were individually precipitated with ethanol and resuspended in 10 μl of water. Twenty microliters was translated *in vitro* in 25 μl. For each fraction, the first channel is the immunoprecipitation of 20 μl of the translated products with anti-UroDCase antibody and the second channel is 4 μl of the total neosynthesized products. Lane I, ^{14}C-labeled molecular weight markers.

bridization signal and were submitted to further analysis.

Identification of UroDCase cDNA Clones. Candidates for UroDCase cDNA clones were analyzed by hybridization of anemic rat spleen mRNA to chimeric plasmid DNA immobilized on nitrocellulose filters followed by elution of the specifically hybridized sequence. Selected mRNAs were translated *in vitro*, and immunoprecipitation with specific antibodies against UroDCase was performed. The immunoprecipitates were then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. One recombinant plasmid, pUD19, reproducibly selected a rat mRNA sequence, the translation product of which had a M_r of 40,000 and was specifically immunoprecipitated by anti-UroDCase antibodies (Fig. 4). In contrast, the other recombinant DNAs selected in the primary screening did not produce any immunoprecipitable translation products. When human anemic spleen mRNA was used in the hybrid-selected translation assay, pUD19

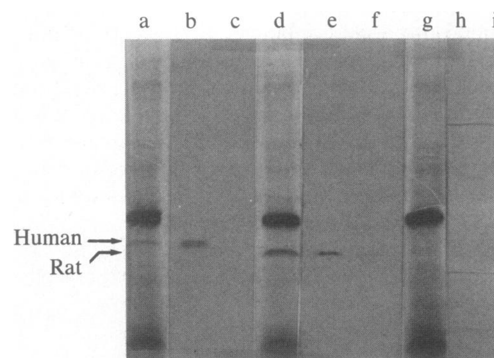

FIG. 4. Identification of cDNA sequences corresponding to rat UroDCase by positive hybridization selection. Human and rat UroDCase are indicated on the left. Lanes: a, translation products of plasmid pUD19-selected human mRNA; b, immunoprecipitation of an equal aliquot of cell-free translation products from selected human mRNA with anti-UroDCase IgG; c, same as in lane b but in the presence of 10 μg of unlabeled pure human UroDCase; d–f, same as in lanes a–c but with rat mRNA. Competition in lane f was demonstrated with pure rat UroDCase; g, translation products of rat mRNA hybridized to a recombinant plasmid containing an unidentified insert; h and i, immunoprecipitation and competition carried out as in lanes e and f, respectively.

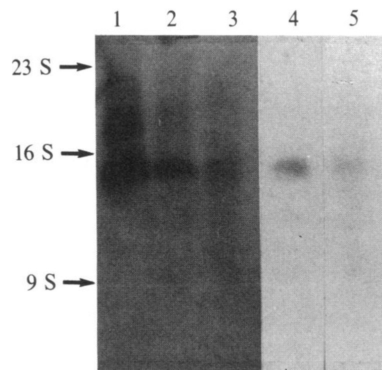

FIG. 5. Blot hybridization of labeled pUD19 DNA insert with total poly(A)$^+$-containing RNA from rat and human tissues. Lane 1, 5 μg of mRNA from anemic rat spleen; lane 2, 5 μg of mRNA from anemic human spleen; lane 3, 10 μg of mRNA from human reticulocytes; lane 4, 10 μg of mRNA from rat liver; lane 5, 10 μg of mRNA from rat kidney.

was able to select a mRNA sequence encoding human UroDCase according to the following criteria: (*i*) its *in vitro* translation product displayed the molecular weight of human UroDCase (M_r 43,000); (*ii*) this product was specifically immunoprecipitated by homologous anti-UroDCase antibody; (*iii*) the immunoprecipitation was efficiently prevented when performed in the presence of pure human enzyme (Fig. 4).

The identified plasmid pUD19, when digested with *Pst* I, generated an insert of 450 bp. Colony hybridization was carried out with this labeled DNA insert to screen the remaining 12,000 clones plated at high density. Seven recombinants gave a signal and were shown to contain UroDCase sequences upon hybridization selection and immunoprecipitation. The sizes of the DNA inserts ranged from 450 to 1350 bp.

RNA Analysis by Blot Hybridization. Total poly(A)$^+$-containing RNAs were extracted from various rat and human tissues and the presence of UroDCase sequences was analyzed by using a rat UroDCase cDNA clone as probe (Fig. 5). A hybridizing molecule of about 1500 bases was obtained for total rat spleen mRNA (lane 1). In rat adult liver and kidney, this RNA molecule has the same size but the signal is weaker, as expected for these nonerythroid tissues. The rat cDNA was capable of cross-hybridizing with UroDCase mRNA from human anemic spleens, suggesting that the corresponding human genes are accessible to mapping and cloning with this cDNA probe.

DISCUSSION

We report here the cloning of a cDNA sequence complementary to UroDCase mRNA from rat. Most genes encode mRNAs that are relatively minor species and cloning and screening of such cDNAs is often difficult. Preliminary experiments (32) have shown that the proportion of mRNA encoding UroDCase in human reticulocytes or fetal liver is always <0.05%. Knowing that antibody against human UroDCase crossreacts with the rat enzyme, we chose, as starting material, spleens of rats made anemic by phenylhydrazine injection. This tissue is very easy to obtain in large amounts and contains at least twice as much UroDCase mRNA as found in human fetal liver (our unpublished data). We designed a strategy involving a first enrichment of the mRNA preparation for UroDCase sequences before cloning in order to increase the representation of specific clones. A second purification step allowed us to prepare a cDNA probe highly enriched for UroDCase sequences for use in screening the partial cDNA bank obtained. Among the different techniques presently available for enrichment of a particular mRNA sequence, the highest resolution is most often obtained by performing successive gel fractionations under various denaturing conditions. We first performed polyacrylamide gel electrophoresis under conditions in which mRNA sequences migrate according to both their molecular weight and their secondary structure. Then, we used a methylmercury hydroxide/agarose gel. Methylmercury hydroxide is known to be one of the most powerful denaturing agents that can be added to agarose gels to obtain fractionation based only on RNA molecular weight. In both cases, to circumvent the difficulty of RNA recovery from the gel, we used a preparative gel electrophoresis system with continuous elution. After a 10-fold purification initially obtained by using polyacrylamide gel electrophoresis of poly(A)$^+$ RNA from anemic rat spleen, the methylmercury hydroxide/agarose gel electrophoresis resulted in a final 200-fold enrichment. The cDNA probe derived from this highly enriched mRNA allowed us to isolate a cDNA clone, pUD19, that selected a mRNA encoding rat UroDCase.

In the absence of a published amino acid sequence for rat or human UroDCase, the ultimate proof of clone identification cannot be obtained by DNA sequence analysis. The fidelity of the cDNA clones was provided by the fact that these recombinant plasmids were capable of selectively hybridizing with UroDCase mRNA obtained from rat or human tissues. The translated product encoded by the selected mRNA sequence was specifically immunoprecipitated by anti-UroDCase antibody and this immunoprecipitation was prevented by addition of pure UroDCase. Furthermore, the molecular weights of the translated products correspond to the expected ones. When blot hybridization was carried out with mRNA preparations from different tissues, the rat UroDCase cDNA was able to hybridize with RNA from anemic rat spleen, liver, and kidney. Hybridization signals were stronger with anemic rat spleen than with the two other nonerythroid tissues, suggesting that this enzyme is synthesized at a higher rate during erythropoiesis.

The length of rat UroDCase mRNA is 1500 nucleotides. This is compatible with the molecular weight of rat UroDCase (M_r 40,000). The eight UroDCase-specific clones have their inserted cDNA sequences fully excisable and their insert lengths range from 450 to 1350 bp. Thus, most or all of the protein-encoding sequence should be contained in these inserts. The fact that the antibody against human erythrocyte UroDCase precipitates rat UroDCase activity indicates that human and rat enzymes exhibit a certain degree of structural and sequence homology. In light of the observation that the rat cDNA clone cross-hybridizes with human UroDCase mRNA, it will be possible to isolate the human UroDCase gene by using the rat cDNA probe. The availability of human gene sequences for UroDCase will permit the analysis of the molecular basis of inherited PCT.

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