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Phenol UDP-glucuronosyltransferase deficiency in Gunn rats: mRNA levels are considerably reduced

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Humans with the hereditary disease Crigler–Najjar syndrome type I suffer severe, non-haemolytic unconjugated hyperbilirubinaemia, usually resulting in infant death, due to a deficiency of UDP-glucuronosyltransferase (UDPGT) [EC 2.4.17] activity towards bilirubin [1]. The congenitally hyperbilirubinaemic Gunn rat has been used as a model for human Crigler–Najjar syndrome type 1 as this rodent strain is also incapable of glucuronidating bilirubin [1]. The genetic lesion has been shown to be due to the absence of the bilirubin UDPGT enzyme protein in this mutant rat [2].

The glucuronidation of planar phenols such as 2-aminophenol, 1-naphthol and 4-nitrophenol has been shown to be impaired in the Gunn rat [2, 3] and also in some human Crigler–Najjar patients [4, 5]. Nagai *et al.* have reported that the defects in bilirubin and phenol glucuronidation in Gunn rats always co-segregate and suggested that these genes may be linked [6]. Therefore, the genetic deficiency of glucuronidation in the Gunn rat has been investigated using a rat phenol UDPGT cDNA as a molecular probe.

Materials and methods

Male Wistar rats and Gunn rats were from the colonies maintained in the Institute's Animal Unit. Congenic Wistar and Gunn rats [7] were obtained from the Catholic University of Leuven, Belgium. A full length rat phenol UDPGT cDNA (RKUG39) was isolated by low stringency cross-hybridisation to a radiolabelled human UDPGT [8] cDNA probe. RKUG39 was found to contain an identical coding region to that of the 4-NP UDPGT cDNA isolated by Iyanagi *et al.* [9] and was used as a hybridisation probe to analyse the phenol glucuronidation deficiency. The 3′ non-coding region of rat testosterone UDPGT cDNA [10] was used as a control probe. The cDNAs were labelled with [α-^{32}P]dCTP as described by Feinberg and Vogelstein [11]. Dot blots of a number of UDPGT cDNA clones were prepared as described by Jackson and Burchell [12] and hybridised with the phenol and testosterone UDPGT probes to demonstrate the specificity of the cDNA probes used. Total RNA was prepared from rat livers and transfer of total RNA from Wistar and Gunn rats to the nylon Hybond-N membrane was as described previously [13]. Slot blotting of total RNA from congenic Gunn rats was performed using a Hybri Slot™ manifold purchased from Bethesda Research Laboratories. Southern blotting of total genomic DNA prepared from Wistar and Gunn rats was achieved as described by Corser *et al.* [13]. Genomic DNA (20 μg) was digested with *Eco* RI or *Hin* dIII before Southern transfer. All blots were washed in 0.1 × SSPE at 68° for 1 hr before autoradiography at −70° using Fuji RX X-ray film. The Fuji RX X-ray film was preflashed prior to autoradiography to allow quantitation by laser densitometry scanning.

Results and discussion

Areas of strong nucleotide sequence identity exist between some UDPGT cDNAs which could cause cross-

hybridisation [12]. However, the diagnosis of the androsterone UDPGT deficiency with a cDNA probe demonstrated the ability to generate specific hybridisation probes for individual UDPGT mRNA species using cDNA fragments. The dot blot analysis (Fig. 1) also showed that with the use of appropriate restriction fragments and high stringency washes, UDPGT cDNAs can be effectively used as specific hybridisation probes.

The level of phenol UDPGT mRNA found in all Gunn rat tissues examined by Northern and slot blotting was significantly reduced down to 20% of normal levels (Figs 2 and 3). The size of the phenol mRNA in Gunn rats was identical to that of the functionally mature phenol UDPGT mRNA in Wistar rats and was inducible by 3-MC in both Wistar and Gunn rats. The phenol UDPGT mRNA was inducible by 3-MC in Gunn rats to near normal uninduced Wistar levels by pretreatment with 3-MC, but no equivalent rise in phenol UDPGT activity occurred (data not shown: see Refs. 3, 14) and the phenol UDPGT protein was undetectable by immunoblotting after induction as previously reported (data not shown: see Ref. 3).

R/APfd, R/APfd-j/+ and R/APfd-j/j rats show high, intermediate and basal levels of bilirubin and phenol UDPGT activity respectively [7, 14]. Hybridisation of the phenol UDPGT cDNA probe to slot blotted RNA prepared from livers of these rats revealed that the phenol UDPGT mRNA was expressed at high levels in the R/APfd rat, at intermediate levels (51%) in the heterozygous R/APfd-j/+ rat and low levels (23%) in the jaundiced R/APfd-j/j rat. This indicates that the normal and defective phenotypes are expressed at the molecular level in a co-dominant manner; although at the physiological level the jaundiced phenotype is recessive [1].

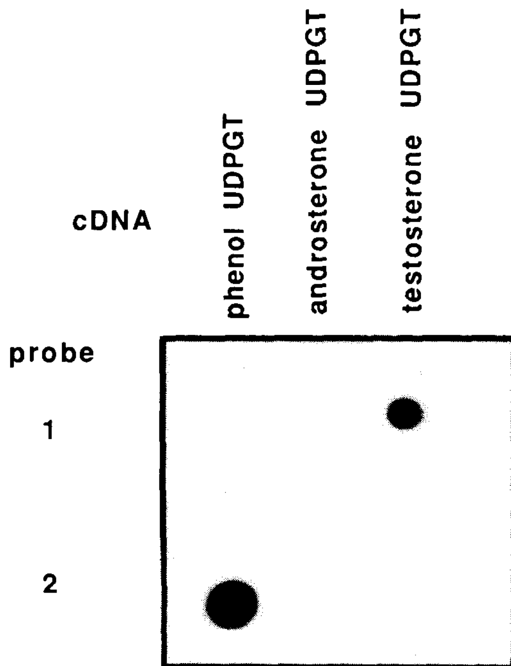

Fig. 1. Hybridisation specificity analysis of rat testosterone and phenol UDPGT cDNA probes. Dot blots of a 100 ng of rat phenol, androsterone and testosterone UDPGT cDNAs were prepared and hybridised with radiolabelled cDNA probes; probe 1, 3′ non coding region of rat testosterone UDPGT cDNA: probe 2, full length rat phenol UDPGT cDNA.

Testosterone UDPGT mRNA levels are not correlated with the three congenic genotypes as the amount of testosterone UDPGT mRNA in the R/APfd, R/APfd-j/+ and R/APfd-j/j rats is relatively constant. Thus the very slightly lowered levels of testosterone UDPGT activity observed in congenic Gunn rats [14] is probably due to the loss of activity of an isoenzyme with some overlapping substrate specificity towards testosterone.

Southern blotting of high molecular weight DNA isolated from Wistar rats and Gunn rats digested with *Eco* RI and *Hin* dIII using phenol UDPGT cDNA as a hybridisation probe identified two bands from Wistar and Gunn samples (see Fig. 4). No difference was observed at this level between the two strains indicating that no large rearrangement or gross deletion had occurred within the structural phenol UDPGT gene of Gunn rats, as previously suggested [3, 5]. The total size (<15kb) of the restriction fragments identified by RKUG39 on the Southern blot was consistent with the recognition of one gene by this cDNA [15] and further demonstrated the specificity of this cDNA as a hybridisation probe.

It has been reported that at least 3 different UDPGT isoenzymes are defective in the Gunn rat; bilirubin UDPGT, phenol UDPGT (Mr 53,000 kDa) [3, 4] and digitoxigenin monodigitoxoside UDPGT [16]. Three mechanisms have been proposed to explain the absence of multiple UDPGT isoenzymes in Gunn rats. Coughtrie *et al.* [3] and Burchell *et al.* [5] suggested that a homologous recombination event may have occurred between regions of two UDPGT genes resulting in deletion of parts of these genes. The data presented here indicate this is not the case. Roy Chowdhury *et al.* [17] proposed that a single defective UDPGT gene encoding two UDPGT activities by post-transcriptional modification or post-translational processing may be the cause of the Gunn rat mutation. This is unlikely as more than two isoforms are apparently defective in the Gunn rat; also deglycosylation of purified bilirubin UDPGT does not change the activity of this isoenzyme [5]. Nakata *et al.* [18] have suggested that UDPGT is an oligomeric protein and that combinations of various subunits modify the function of UDPGT. Incorporation of a defective subunit then gives rise to the mutant phenotypes. However the expression of individual UDPGT cDNAs [19] (including a rat phenol UDPGT cDNA: Jackson *et al.*, submitted) in tissue culture to yield catalytic enzyme activity indicates that if UDPGT is a holoenzyme it is composed of identical monomers rather than different subunits [19].

The results presented here and in the literature indicate that the UDPGT deficiencies in the Gunn rat may have resulted from a mutation that affected a number of isoenzymes. This mutation may cause lowered levels of phenol UDPGT mRNA and apparently prevented synthesis of a functionally active or immunologically detectable phenol UDPGT protein [3, 5, 13]. An analagous pleiotropic mutation has been previously reported which affects levels of several mRNA species and translation of specific mRNAs [20, 21]. This mutation also prevents the expression of bilirubin UDPGT activity [22].

One further point of interest was raised by the Northern blotting with the rat phenol UDPGT cDNA probe (Fig. 2). Relatively low levels of constitutive expression of rat kidney phenol UDPGT mRNA were observed consistent with lower levels of phenol UDPGT protein and activity in this tissue [23]. This result is different to the findings of Iyanagi *et al.* [9], who observed relatively high constitutive levels of kidney phenol UDPGT mRNA. The mRNA samples used in this report were prepared from male rats, whereas Iyanagi *et al.* [9] used female rats. Thus, this discrepancy may reflect sex differences. Certainly it has been reported that female rat kidney microsomal preparations glucuronidated 4-NP faster than similar samples from male rats [23] and that the inverse situation was

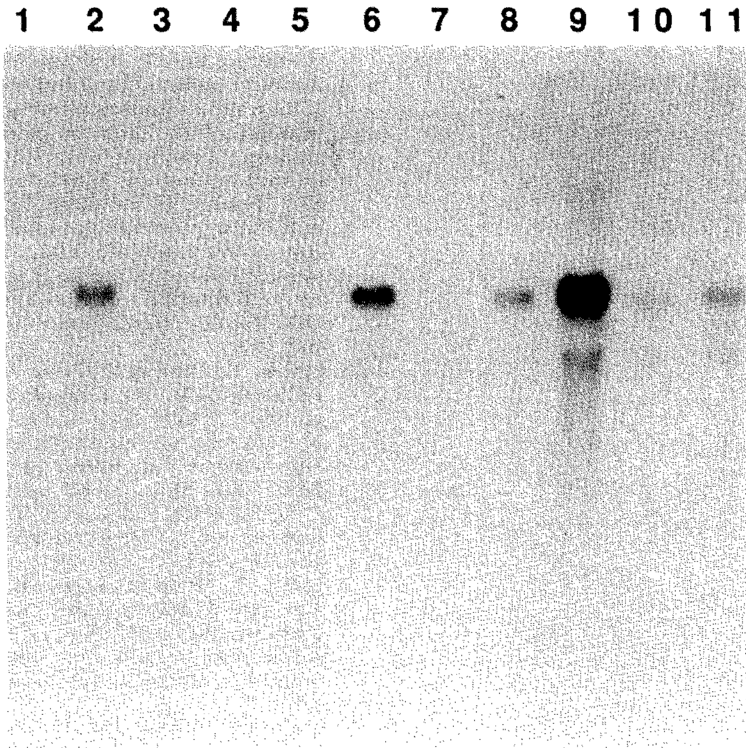

Fig. 2. Northern blot of total Wistar and Gunn rat RNA hybridised with a rat phenol UDPGT cDNA probe. Fifteen micrograms of total RNA was loaded in each lane. Lanes 2,8, untreated Wistar rat liver RNA; 9, Wistar rat liver RNA isolated after treatment with 3-MC; 1,3,10, untreated Gunn rat liver; 11, Gunn rat liver RNA isolated after treatment with 3-MC; 4, Wistar rat kidney RNA; 5 Gunn rat kidney RNA; 6, Wistar rat intestinal RNA; 7, Gunn rat intestinal RNA.

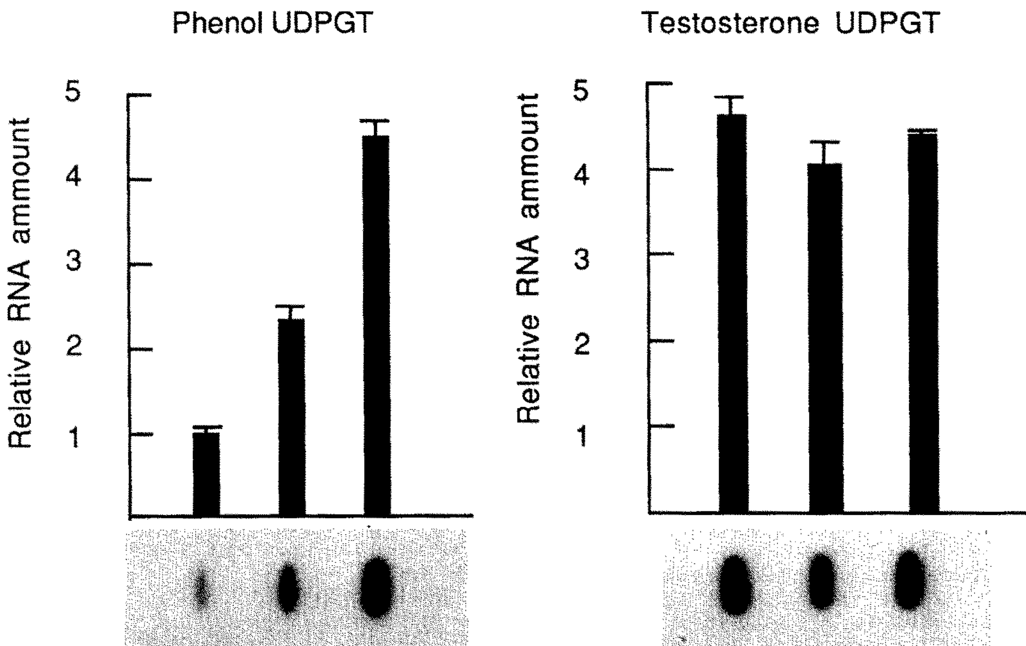

Fig. 3. Analysis of phenol UDPGT and testosterone UDPGT RNA levels in congenic R/APfd, R/Afd-j/+ and R/APfd-j/j rats. Five micrograms of total liver RNA isolated from R/APfd, R/APfd-j/+ and R/APfd-j/j rats was slot blotted in duplicate onto nitrocellulose and then hybridised to ^{32}P labelled phenol and testosterone UDPGT cDNA probes. The relative levels of hybridising mRNA were determined by laser densitometry. The data in the histogram shows the mean and range of analysis of RNA samples prepared from two different livers of each genotype.

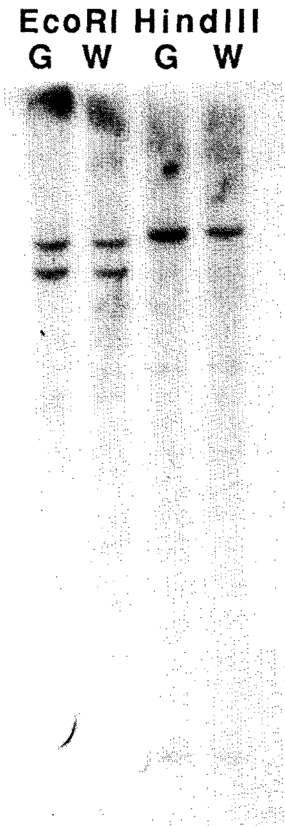

Fig. 4. Southern blot analysis of liver genomic DNA isolated from Wistar and Gunn rats. Total DNA isolated from Wistar rats (W) and Gunn rats (G) was digested with *Eco* RI or *Hin* dIII and transferred to Hybond N after agarose gel electrophoresis. The blot was then hybridised with the complete phenol UDPGT cDNA probe.

found in male and female rat liver microsomes using 2-aminophenol as substrate [24]. This type of reciprocal sex-linked tissue specific expression has been reported previously for 15 α-testosterone hydroxylase mRNA [25].

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Inducibility of rat liver cytochrome P-450IA1 (P-450c) mRNA during the partial inhibition of protein synthesis

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A labile protein, responsible for repressing the transcription of the cytochrome P-450IA1 gene, has been postulated on the basis of observations describing "superinduction" of gene expression when Hepa 1c1c7 cells are exposed to an inhibitor of protein synthesis, cycloheximide (CHX*) [1–3]. An attempt to observe this phenomenon *in vivo* was not successful, possibly as a result of cycloheximide-induced depression of labile cytochrome P-450IA1 gene *activator* proteins [4]. The protocol reported here attempts to bypass this obstacle by allowing for the interaction of these potential "activator" proteins with 3-methylcholanthrene (3-MC) prior to the partial inhibition of protein synthesis *in vivo*.

Materials and methods

Materials. Cycloheximide was purchased from the Sigma Chemical Co., St. Louis, MO; L-[4,5-^{3}H]leucine (122.5 Ci/mmol) and Nuclear Chicago Solvent (NCS) were from the Amersham Corp., Arlington Heights, IL; Nytran (0.45 μm) membranes were from Schleicher & Schuell, Inc., Keene, NH.

Some of the plasmids used in these studies were obtained from the following: pRSA57, a cDNA clone for rat serum albumin [5], was provided by Drs. James Bonner and Thomas Sargent; clone 46, a cDNA clone for mouse cytochrome P-450IA1 [6], was a gift from Drs. Daniel Nebert and Masahiko Negishi. All other materials have been described previously [7, 8].

Methods. Male 4-week-old Sprague–Dawley rats that were obtained from Sasco Inc., Omaha, NE, were provided Lab Chow (Ralston Purino Co.) and water *ad lib*. The rats were injected i.p. as follows: CHX (3.0 mg/kg body weight) was administered 2 and 6 hr after 3-MC (25 mg/kg body weight); labeled leucine was injected 8 hr after treatment with 3-MC and 1 hr before the animals were killed. This group was referred to as 3-MC/CHX. The 3-MC/NaCl group received 0.9% NaCl in place of CHX, while the CO/NaCl group received corn oil (the vehicle of 3-MC) and 0.9% NaCl in place of 3-MC and CHX respectively. The CO/CHX group was treated with corn oil and CHX.

Hepatic protein was isolated as described by Cook *et al.* [9]. The trichloroacetic acid-insoluble precipitate was redissolved in 6 vol. (w/v) of NCS at 50%. The incorporated radioactivity was determined directly using a Beckman LS-150 liquid scintillation counting system.

Total hepatic nuclear and cytosolic RNA were isolated essentially as described by Lamers *et al.* [10]. The application of RNA onto a Nytran membrane, prehybridization, hybridization, washing, and conditions for the removal of the probe were as specified by the membrane manufacturer. Radiolabeled DNA for use as hybridization probes was prepared by nick-translation [11]. The intensity of the hybridization signal was determined using an LKB model 2202 UltroScan laser densitometer and Gel Scan software. The intensity of hybridization, which is expressed in arbitrary units, refers to the integrated area of peak absorbance at 633 nm.

Results and discussion

Cycloheximide administration has been demonstrated to result in an inhibition of the incorporation of labeled precursor amino acids into hepatic protein *in vivo* [12]. In our studies, the inhibition of [^{3}H]leucine incorporation into hepatic protein is indicated in Table 1. A 50–70% inhibition of protein synthesis was maintained in the liver over the duration of the experiments (CO/NaCl vs CO/CHX and 3-MC/NaCl vs 3-MC/CHX). 3-Methylcholanthrene administration alone caused a 50% stimulation in the incorporation of [^{3}H]leucine into protein of the liver (3-MC/NaCl vs CO/NaCl).

The effects of 3-MC and CHX treatments upon the steady-state levels of cytochrome P-450IA1 mRNA are indicated in Fig. 1. The hybridization to pRSA57, a probe for the albumin gene, was used to control for differences in retention of RNA on the membrane, and for experimental artifacts. The administration of CHX to control rats, i.e. CO/NaCl vs CO/CHX, resulted in an average increase in steady-state cytochrome P-450IA1 mRNA of 2.1- and 6.2-fold in nuclear and cytosolic compartments respectively. Cycloheximide has been reported to stimulate hepatic nuclear RNA synthesis by 12 hr after treatment [13]. However, as demonstrated in Fig. 1, no marked effect on rat serum albumin mRNA levels was observed. The "general" stimulation of nuclear RNA synthesis, therefore, may be attributable to "specific" nuclear RNA subpopulations. A similar stimulation of cytochrome P-450IA1 mRNA levels

* Abbreviations: CHX, cycloheximide; 3-MC, 3-methylcholanthrene; NCS, Nuclear Chicago Solvent; and CO, corn oil.