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The molecular basis of the inherited deficiency of androsterone UDP-glucuronyltransferase in Wistar rats

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A major UDP-glucurnoyltransferase isoenzyme in rat liver (51 kDa), corresponding to androsterone glucuronidating activity, has been identified by immunoblot analysis. This isoenzyme is absent from Wistar rats exhibiting the low androsterone (LA) UDP-glucuronyltransferase activity phenotype. Northern blot analysis of total RNA from normal and androsterone glucuronidation deficient Wistar rats demonstrated that the mRNA encoding this protein was not synthesised. Differences in restriction fragment length observed on Southern blotting of genomic DNA from LA Wistar rats indicate that this inherited deficiency is the result of a deletion in the androsterone UDP-glucuronyltransferase gene.

Immunoblotting; Northern blotting; Southern blotting; genomic DNA; Restriction fragment length analysis

1. INTRODUCTION

Glucuronide formation is a major pathway in the conjugation and bio-elimination of a wide variety of endogenous and xenobiotic compounds [1]. These glucuronidation reactions have been determined to be catalysed by a number of closely related UDP-glucuronyltransferases (UDPGTs) [2]. Progress on the study of substrate specificity of purified hepatic microsomal enzymes has been recently summarised [3].

Matsui and Aoyagi [4] demonstrated that individual Wistar rats displayed variations in the biliary excretion of exogenously administered androsterone, and these animals could be divided into two groups on the basis of rate of glucuronidation of androsterone by hepatic microsomes [5]. Rats with high activity (HA) and low activity (LA) towards androsterone could be observed in Wistar, Wistar-King and Damyo strains but not in Long Evans or Sprague-Dawley strains.

We have also observed the existence of HA and LA rats in the Institute's colony and determined that the rate of androsterone glucuronidation can be some 36-fold greater in hepatic microsomes from HA when compared to LA rats [7] and also confirmed that UDP-glucuronyltransferases conjugating bilirubin, testosterone, oestradiol, 4-nitrophenol and 2-aminophenol are unaffected by this deficiency [7] as previously reported [6,8]. Recent purification work using HA and LA Wistar rats has indicated that this genetic variation is due to 90% reduction [9] or complete loss [10] of the UDP-glucuronyltransferase enzyme specifically catalysing the glucuronidation of androsterone.

Here we report an analysis of the molecular basis of this genetic deficiency using specific antibodies and cDNA. The results show that androsterone UDP-glucuronyltransferase is not present in hepatic microsomes, that the mRNA is not synthesised in liver and that a genetic difference can be observed by restriction fragment length analysis of genomic DNA.

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2. MATERIALS AND METHODS

Nitrocellulose was obtained from Anderman (London). Hybond N and [α-^{32}P]dCTP (3000 Ci/mol) were from Amersham (Amersham, Bucks). [9,11-^{3}H(N)]Androsterone was from New England Nuclear (Southampton); androsterone and UDP-glucuronic acid from Sigma (Poole, Dorset); oligonucleotide pd(N)$_6$ from Pharmacia (Uppsala) and Klenow fragment of DNA polymerase from Anglia Biotechnology (Essex). Restriction endonucleases *Eco*RI, *Hin*dIII and *Sac*I were purchased from Boehringer (London). Anti-sheep IgG (donkey) and peroxidase-anti-peroxidase (PAP) complex were kindly supplied by the Scottish Antibody Production Unit.

Male (200 g) Wistar rats were from the colony maintained in the Institute's animal unit.

2.1. *Assay of androsterone UDPGT activity*

Glucuronidation of androsterone catalysed by hepatic microsomes was measured as in [1]. Microsomal protein concentrations were determined by the biuret method [2] with bovine serum albumin (Boehringer) as standard. UDPGT activity is expressed as nmol/min per mg protein.

2.2. *Antibodies specific for UDPGTs*

Sheep anti-rat UDPGT antiserum was prepared using testosterone/4-nitrophenol UDPGT as antigen as described [13]. IgG was purified by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose [14].

2.3. *Immunoblot analysis*

SDS-polyacrylamide gel electrophoresis was carried out using 7.5% gels as described by Laemmli [15]. Proteins separated on SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose as described by Towbin et al. [16] and immunostained using sheep anti-rat UDPGT IgG. Immunoreactive polypeptides were visualised using the immunoperoxidase method and 4-chloro-1-naphthol as substrate [17].

2.4. *Preparation of radiolabelled cDNA*

Restriction fragments of cloned cDNAs were purified by agarose gel electrophoresis [7]. DNA fragments were ^{32}P-labelled by the method of Feinberg and Vogelstein [18]. Probes used were for class 3 cDNA *Sac*I/*Eco*RI (bases 1520–1728) and for androsterone UDPGT cDNA *Sac*I/*Eco*RI (1520–1885) (see [7]).

2.5. *Northern blot analysis*

Northern blotting of total liver RNA was as described previously [7] except that RNA was transferred by capillary blotting to Hybond N and then covalently bound to the membrane by UV irradiation as recommended by the manufacturers.

2.6. *Southern blot analysis*

Genomic DNA was prepared from frozen livers according to Frischauf et al. [19]. Restriction fragment length analysis of genomic DNA, Southern blotting and hybridisation were as described by Maniatis et al. [20], except that DNA was transferred to Hybond N and covalently bound to the membrane by UV irradiation.

3. RESULTS AND DISCUSSION

3.1. *Androsterone UDPGT activities in HA and LA Wistar rat liver microsome*

Rat liver microsomes were assayed for androsterone glucuronidating activity following optimal activation of the UDPGT enzyme with the detergent Lubrol PX. In the animals used here, the UDPGT activity towards androsterone was 1.78 ± 0.12 (n = 5) in HA rats and 0.03 ± 0.03 (n = 5) in LA rats. These results further demonstrate a 59-fold difference in specific activity of androsterone UDPGT in the hepatic microsomes from the two strains of Wistar rats.

3.2. *Examination of hepatic microsomes from HA and LA Wistar rats by SDS-polyacrylamide gel electrophoresis and immunoblot analysis*

Polyclonal anti-rat liver UDPGT antibodies specifically inhibited up to 85% of transferase activities towards bilirubin, testosterone, 1-naphthol, androsterone and 4-methylumbelliferone. Assays of these enzyme activities are believed to represent at least five different isoenzymes based on previous purification work. We have developed an immunoblot analysis which enables the identification of several UDPGTs which exhibit slightly differing mobilities and hence molecular masses (50–56 kDa) following SDS gel electrophoresis. Four of the specifically immunostaining bands

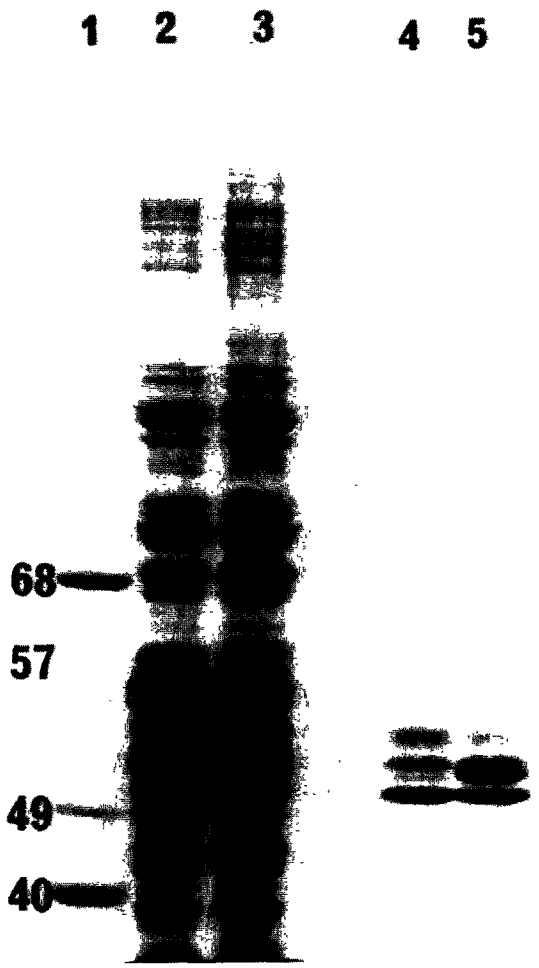

Fig.1. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of hepatic microsomes from LA and HA Wistar rats. Lanes: (1) protein standards, albumin (68 kDa), pyruvate kinase (57 kDa), fumarase (49 kDa) and aldolase (40 kDa). Microsomes (100 μg protein) from LA Wistar rat liver (lane 2) and HA rat liver (lane 3) and standards were stained with Coomassie blue. UDPGTs in the microsomes (10 μg protein) from LA rat liver (lane 4) and HA rat liver (lane 5), stained with anti-(rat liver)-UDPGT IgG. Chromogenic detection of specifically bound IgG was achieved by incubation with the immunoperoxidase system using 4-chloro-1-naphthol as substrate.

have been further characterised by an examination of the genetically deficient Gunn rat where bilirubin and phenol UDPGTs are absent [21], the use of renal and hepatic microsomes from xenobiotic-pretreated and developing animals [22], purified isoenzymes, and more specific antibodies [23]. The results of these studies are summarised and show that isoenzymes catalysing the glucuronidation of phenols or morphine (56 kDa), bilirubin (54 kDa), phenols (53 kDa), unknown (52 kDa), androsterone (51 kDa, see fig.1), unknown (51 kDa, see fig.1) and testosterone (50 kDa) are observed.

Immunoreactive UDPGT(s) at 51 kDa is (are) very low in LA rats and greatly elevated in HA rats indicating that androsterone UDPGT exhibits a molecular mass of 51 kDa following immunoblot analysis. The minor staining 51 kDa UDPGT protein observed in LA rat liver microsomes is present in HA rat liver microsomes before androsterone UDPGT activity has developed [24] (i.e. before 30 days of age [8]). Therefore, this unknown UDPGT

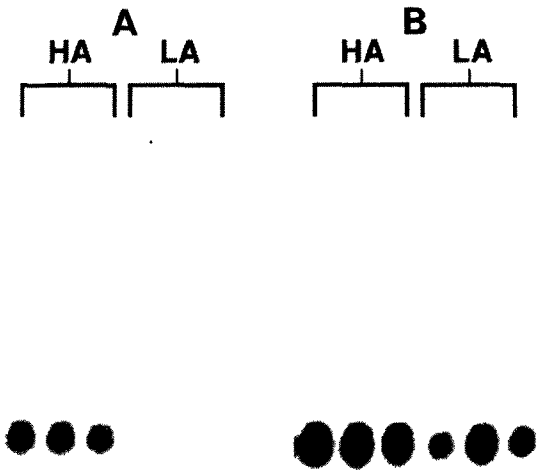

Fig.2. Analysis of total rat liver RNA from LA and HA Wistar rats. RNA (5 μg) from three different HA or LA rat livers was electrophoresed on a 1% agarose/formaldehyde gel, blotted onto Hybond N and then hybridised to ^{32}P-labelled androsterone UDPGT cDNA. The hybridised DNA was visualised by autoradiography (A). This cDNA was then removed from the blot by treatment with 10 mM Tris, pH 8, 1 mM EDTA at 68°C for 2 h, then autoradiographed to ensure the removal of the probe. The analysis was then repeated using the control probe, ^{32}P-labelled class 3 UDPGT cDNA (B).

is not androsterone UDPGT, and this conclusion is confirmed by Northern blot analysis described in section 3.3.

3.3. *Northern blot analysis of total RNA from HA and LA Wistar rat livers*

We have analysed total RNA from HA and LA Wistar rat livers using cloned cDNA probes specifically coding for androsterone UDPGT mRNA to determine whether the genetic defect is at the translational or transcriptional level. Fig.2 clearly shows that androsterone UDPGT mRNA (2.5 kb) is detectable in RNA extracts from HA rat livers, but not in RNA extracts from LA rat livers. Hybridisation to the same Northern blots with a cDNA probe, which encodes a different but unknown UDPGT [7], reveals equal labelling intensity in HA and LA rats and also indicates the specificity of this analysis. No androsterone UDPGT mRNA is detected in total RNA from 15-day-old rat livers (not shown), confirming that the minor immunoreactive 51 kDa polypeptide detected in LA rat liver microsomes is not androsterone UDPGT.

Androsterone UDPGT mRNA is not present in LA rat liver suggesting that the genetic lesion causes disruption in the transcription of genomic DNA or is due to a mutant or deleted gene.

3.4. *Restriction fragment length analysis of genomic DNA from HA and LA rat livers*

DNA isolated from HA and LA Wistar rat livers was incubated with two different restriction enzymes and the fragments were analysed by Southern blotting with cloned radiolabelled cDNA probes. The different restriction patterns observed when using DNA from HA or LA rats and two different restriction enzymes indicate the loss of predominant restriction fragments from LA rat DNA (fig.3A). The genetic lesion would appear to be a gene deletion rather than a point mutation. Blotting with class 3 UDPGT cDNA probes revealed a consistent pattern of radiolabelled restriction fragments (fig.3B), indicating the specificity of the lesion for the androsterone UDPGT gene.

We are currently determining the structure of the androsterone UDPGT gene to describe accurately the exact nature of this genetic deficiency.

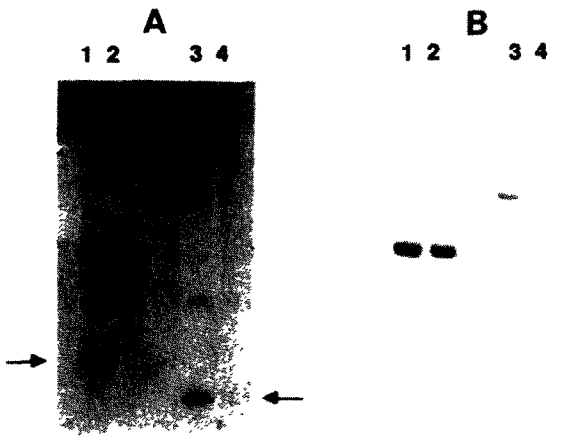

Fig.3. Southern blot analysis of liver genomic DNA from HA and LA Wistar rats. Genomic DNA (20 μg) was incubated with the restriction enzymes *Hin*dIII (lanes 1,2) or *Sac*I (lanes 3,4) and the restriction fragments (2–10 kb) separated on a 0.8% agarose gel. The DNA fragments were transferred to Hybond N and hybridised to the ^{32}P-labelled androsterone UDPGT cDNA (A). The hybridised cDNA was then stripped from the blot using 0.4 M NaOH at 42°C, then neutralised and reprobed with ^{32}P-labelled class 3 UDPGT cDNA (B, control probe). Hybridising DNA fragments were visualised by autoradiography of the blots for 3 days at −70°C. Both hybridisations were washed to the same stringencies.

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