Studies on the genetic linkage of bilirubin and androsterone UDP-glucuronyltransferases by cross-breeding of two mutant rat strains

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Gunn rats, which have defects in bilirubin and 4-nitrophenol UDP-glucuronyltransferases (GT), were crossed with LA Wistar rats with a defect in androsterone GT. The F_1 hybrids showed normal GT activities towards androsterone, bilirubin and 4-nitrophenol, demonstrating that Gunn and LA ('low activity') Wistar rats inherit a homozygous dominant trait for androsterone GT and bilirubin GT respectively. The F_2 progeny showed four different combinations of bilirubin and androsterone GT activities: defects in both GT activities, a single defect in bilirubin GT activity, a single defect in androsterone GT activity and two normal GT activities. They were segregated in the approximate ratio of 1:3:3:9, which is compatible with Mendel's Principle of Independent Assortment. These results provide evidence that androsterone GT and bilirubin GT are located on different chromosomes. In the F_2 generation, defective bilirubin and 4-nitrophenol GT activities were not segregated, indicating that these two mutant genes are closely linked on the same chromosome.

INTRODUCTION

Glucuronidation represents one of the major conjugation reactions and is catalysed by UDP-glucuronyltransferase (UDP-GT), which transfers glucuronic acid from UDP-glucuronic acid to xenobiotics and endogenous compounds. Evidence for the existence of several GT isoenzymes has been provided by purification (Burchell, 1981; Falany & Tephly, 1983; Roy Chowdhury *et al.*, 1986) and by studies of cDNA sequences (Jackson & Burchell, 1986; Iyanagi *et al.*, 1986; Mackenzie, 1987).

Genetic deficiencies of GT isoenzymes are known in several strains of rats. Gunn (1938) described a mutant strain of Wistar rats which exhibits unconjugated hyperbilirubinaemia. The jaundice is inherited as an autosomal recessive trait. It was reported subsequently that Gunn rats lack the ability to glucuronidate bilirubin (Lathé & Walker, 1957), owing to the absence of bilirubin GT (Scragg *et al.*, 1985). Gunn rats are a good animal model for the study of human Crigler–Najjar syndrome and have been extensively studied (Dutton, 1980). Gunn-rat colonies appear to be genetically different from each other, except for the jaundice locus, because of their cross-breeding with various rat strains. In fact their 4-nitrophenol GT activity is controversial, and two Gunn-rat populations appear to exist: one with low and one with high 4-nitrophenol GT activity (Dutton, 1980). The Gunn rats used in the present study were initially obtained from Albert Einstein College of Medicine, New York, NY, U.S.A. and were cross-bred with Wistar–Imamichi rats. These Gunn rats have black pigment on their heads and a black stripe on their backs, similar to the Long–Evans strain. The occurrence of a similar type of hooded Gunn rats was described previously (Yeary & Grothaus, 1971; Leyten *et al.*, 1986). The

Gunn rats lacked bilirubin GT activity and showed low 4-nitrophenol GT and normal androsterone GT activities (Nagai *et al.*, 1987).

Another mutant rat strain which has a defect in androsterone GT activity was reported previously (Matsui *et al.*, 1979). GT activities towards bilirubin, testosterone and 4-nitrophenol were normal in this strain. The genetic expression of the low androsterone GT activity is inherited in an autosomal recessive fashion (Matsui & Watanabe, 1982). Purification of androsterone GT from the high (normal)-activity (HA) and the low-activity (LA) Wistar rats has provided evidence that androsterone GT protein was absent in LA Wistar rats (Matsui & Nagai, 1986). The genetic deficiency of androsterone GT appears to be due to the deletion of androsterone GT gene in LA Wistar rats (Corser *et al.*, 1987).

Six or more forms of GT isoenzymes with partially overlapping substrate specificities exist in the rat liver (Siest *et al.*, 1987). However, very little is known about the chromosomal organization of the GT gene family. To clarify the linkage relationships for GT genes, we obtained recombinant rat strains by one outcross of Gunn rats with LA Wistar rats, followed by brother–sister inbreeding. Analysis of their GT activities provides evidence that bilirubin and androsterone GT genes are located on different chromosomes and that defective bilirubin GT and 4-nitrophenol GT genes are closely linked with each other.

MATERIALS AND METHODS

Materials

[9,11- ^3H]Androsterone (sp. radioactivity 53.3 Ci/mmol) was purchased from New England Nuclear Corp., Boston MA, U.S.A. Androsterone and bilirubin

Abbreviations used: GT, glucuronyltransferase; LA, low activity, and HA, high (i.e. normal) activity, of androsterone glucuronidation.

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were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 4-Nitrophenol was purchased from Wako Pure Chemical Industries Tokyo, Japan. Triton X-100 was obtained from Tokyo Kasei Kogyo Co., Tokyo, Japan. *N*-Nitrodiethylamine was prepared as described previously (Matsui *et al.*, 1984). UDP-glucuronic acid disodium salt and UDP-glucuronic acid trisodium salt were obtained from Boehringer, Mannheim, Germany, and Yamasa Shyoyu Co. Ltd., Tokyo, Japan, respectively. All other reagents were of analytical grade.

Animals

Gunn rats were initially supplied to Sankyo Co., Shizuoka, Japan, by Albert Einstein College of Medicine New York, NY, U.S.A., in 1964. A cross of male Gunn rats (genotype *jj*) with female Wistar-Imamichi rats (+ +) gave rise to F_1 hybrids (*j* +). The F_1 hybrids were inter-crossed and subsequently in-crossed to select jaundiced animals (*jj*). Gunn rats were identified as icteric offspring by matings of male homozygotes (*jj*) and female heterozygotes (*j* +). Wistar rats were classified in homozygous HA (genotype *HaHa*) and LA (*haha*) Wistar rats in terms of hepatic androsterone GT activity (Matsui & Watanabe, 1982). In the present study the offspring (*haha*) from mating of LA Wistar rats were used.

Male Gunn rats (*HaHa/jj*) were crossed with female LA Wistar rats (*haha*/+ +). Hepatic GT activities of F_1 hybrids (*Haha/j* +) were assayed by partial hepatectomy at 60 days of age or by decapitation at 150 days of age, as described previously (Matsui & Watanabe, 1982). The F_2 generation was obtained by matings of F_1 hybrids at 4 weeks after the operation. Hepatic GT activities of the F_2 generation were determined by partial hepatectomy at 60–80 days of age.

Preparation of microsomal fractions

Animals were allowed free access to food and water before being hepatectomized or decapitated. The liver was homogenized in ice-cold 0.25 M-sucrose in 0.1 M-Tris/HCl buffer, pH 7.4, with a Teflon/glass homogenizer to give a 20% (w/v) homogenate. Microsomal fractions were obtained by differential centrifugation (2000 *g* for 10 min, 16000 *g* for 45 min and 105000 *g* for 60 min). The microsomal pellets were resuspended in 0.25 M-sucrose/0.1 M-Tris/HCl buffer, pH 7.4. The microsomal protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme assay

GT activities towards androsterone and 4-nitrophenol were assayed by a modification of the method described previously (Matsui & Nagai, 1986; Nagai *et al.*, 1987). The standard incubation medium contained 0.17 mM-[³H]androsterone (13 nCi) or 0.36 mM-4-nitrophenol, 2 mM-UDP-glucuronic acid, 10 mM-MgCl₂ and 0.1 M-Tris/HCl buffer, pH 7.4, in a total volume of 0.5 ml. The incubation was carried out for 20 min at 37 °C. GT activity towards bilirubin was assayed by the method described by Heirwegh *et al.* (1972). To activate GT activities, 0.02% Triton X-100, 10 mM-*N*-nitrodiethylamine, 10 mM-*N*-nitrodiethylamine, 0.02% Triton X-100 or digitonin was added to the incubation medium as described previously (Matsui *et al.*, 1984; Nagai *et al.*, 1987).

RESULTS AND DISCUSSION

In a previous paper (Nagai *et al.*, 1987), we compared hepatic GT activities of Gunn and LA Wistar rats. LA Wistar rats had very low androsterone GT activity and showed high bilirubin and 4-nitrophenol GT activities. Gunn rats lacked bilirubin GT activity and had high androsterone GT activity. In Gunn rats, native and Triton X-100-activated 4-nitrophenol GT activities were 20–30% of those of LA Wistar rats. When the assay was performed in the presence of *N*-nitrosodiethylamine or *N*-nitrodiethylamine, the low 4-nitrophenol GT activities in Gunn rats were increased to the high levels shown by LA Wistar rats. The combined addition of Triton X-100 and *N*-nitrosodiethylamine or *N*-nitrodiethylamine provided equally maximal activation of these enzymes activities. The restoration of 4-nitrophenol GT activity by *N*-nitrosodiethylamine or *N*-nitrodiethylamine appears to be typical for many Gunn-rat populations (Dutton, 1980).

In order to clarify the linkage relationships for androsterone, bilirubin and putative 4-nitrophenol GT genes, male Gunn rats were crossed with female LA Wistar rats. Table 1 shows the analysis of 24 F_1 hybrids in respect of GT activities towards bilirubin, 4-nitrophenol and androsterone. GT activities obtained by partial hepatectomy at 60 days of age and by decapitation at 150 days of age were not significantly different from each other. Thus partial hepatectomy at 60 days of age provides a good means for unequivocal classification of GT activities. All the F_1 hybrids showed normal GT activities towards bilirubin, 4-nitrophenol and androsterone. These results indicate that Gunn rats inherit the homozygous dominant genotype (*HaHa*) for androsterone GT, whereas LA Wistar rats inherit the homozygous dominant genotype (+ +) for bilirubin GT.

When F_1 hybrids were crossed with each other, the recessive traits reappeared in the F_2 generation. Table 2 shows the analysis of GT activities towards bilirubin, androsterone and 4-nitrophenol in 135 F_2 offspring. There were four different combinations of bilirubin and androsterone GT activities. These combinations were defects in both GT activities, a single defect in bilirubin GT activity, a single defect in androsterone GT activity and two normal GT activities. They were segregated in the ratio of 9:27:24:75, which is not significantly different from the expected ratio of 1:3:3:9 ($\chi^2 = 0.2229$, $0.98 > P > 0.95$). This ratio is compatible with Mendel's Second Law, the Principle of Independent Assortment, and provides evidence that bilirubin and androsterone GT genes are located on different chromosomes.

In the F_2 generation, low 4-nitrophenol GT and defective bilirubin GT were not segregated, and we were unable to detect any recombinant types due to the crossing-over of chromosomes. These mutant genes are consequently located on the same chromosome. It is known that 4-nitrophenol is glucuronidated by several GT isoenzymes (Roy Chowdhury *et al.*, 1986). Scragg *et al.* (1985) reported that bilirubin GT and possibly phenol (2-aminophenol and 1-naphthol as substrates) GT were not present in Gunn rats. Furthermore, Coughtrie *et al.* (1987) suggested that the defective phenol GT was due to the absence of 3-methylcholanthrene-inducible phenol GT. To explain the defect (or mutation) of bilirubin and phenol GT isoenzymes, three types of mechanisms have

Table 1. Hepatic UDP-GT activities in F₁ hybrids (*Haha/jj*+) derived from crosses between male Gunn rats (*HaHa/jj*) and female LA Wistar rats (*haha/++*)

Each value is the mean $\pm$ S.D. for F₁ hybrids at 60 days of age (six males and 18 females) and at 150 days of age (three females) as described in the Materials and methods section. Abbreviation: NEA, *N*-nitrodiethylamine.

Substrate	Treatment	Time (days) ...	UDP-GT activity (nmol of glucuronide/min per mg of protein)		
			60 (hepatectomy)		150 (decapitation)
			Male	Female	Female
Bilirubin	Native		0.21 $\pm$ 0.08	0.20 $\pm$ 0.10	0.32 $\pm$ 0.12
	Digitonin		0.48 $\pm$ 0.17	0.49 $\pm$ 0.27	0.86 $\pm$ 0.06
4-Nitrophenol	Native		2.18 $\pm$ 0.56	1.29 $\pm$ 0.72	1.17 $\pm$ 0.18
	Triton X-100		7.76 $\pm$ 0.95	5.60 $\pm$ 1.50	7.44 $\pm$ 2.59
	NEA		4.38 $\pm$ 0.59	3.15 $\pm$ 0.91	2.56 $\pm$ 0.19
	Triton X-100 + NEA		17.33 $\pm$ 2.42	14.63 $\pm$ 2.47	15.68 $\pm$ 1.57
Androsterone	Native		0.44 $\pm$ 0.11	0.36 $\pm$ 0.10	—
	Triton X-100		1.56 $\pm$ 0.47	1.41 $\pm$ 0.37	2.50 $\pm$ 0.89

been proposed. (1) A crossing-over between homologous regions of the two GT genes may result in the simultaneous deletion of these genes (Coughtrie *et al.*, 1987). (2) A single defective GT gene may result in the defects of the two GT isoenzymes, where the two GT isoenzymes are generated from a single gene through post-transcriptional or post-translational modification (Roy Chowdhury *et al.*, 1987). (3) The two isoenzymes may share a common mutant subunit derived from a single defective gene (Nakata *et al.*, 1975). However, further study is required to clarify the molecular basis of the deficiency in GT isoenzymes.

Our Gunn rats have black pigment on their heads and a black stripe on their backs. All the F₁ hybrids showed

a coat colour similar to that of Gunn rats. In the F₂ generation, the hooded rats showed the black pigment on their heads with several variations in the black stripe, and the albino and hooded rats were segregated in the ratio of 31:104, which is not significantly different from the expected ratio of 1:3 ($\chi^2 = 0.299$, $0.7 > P > 0.5$). This ratio is compatible with Mendel's First Law, the Principle of Segregation, and confirms that the albino colour trait is inherited in an autosomal recessive fashion (Castle, 1947). In the F₂ generation, albino rats were found as follows: four in the rats with defects in both bilirubin and androsterone GT activities, five in the rats with a defect in bilirubin GT activity, six in the rats with a defect in androsterone GT activity, and sixteen in

Table 2. Genetic expression of hepatic UDP-GT activities in the F₂ generation derived from crosses between Gunn (*HaHa/jj*) and LA Wistar (*haha/++*) rats

Each value is the mean $\pm$ S.D. for F₂ offspring at 60–80 days of age as described in the Materials and methods section. 'Treatment...' indicates the activator(s) used for the enzyme assay. Abbreviations: ND, not detectable; NEA, *N*-nitrodiethylamine.

UDP-GT activity (nmol of glucuronide/min per mg of protein)							
		Substrate...	Bilirubin	Androsterone	4-Nitrophenol		Genotype
Sex	No. of animals	Treatment...	Digitonin	Triton X-100	Triton X-100	Triton X-100 + NEA	
Male	7		ND	0.12±0.05	1.82±0.68	11.81±1.37	haha/jj
Female	2		ND	0.02, 0.13	1.72, 1.40	12.69, 13.77	
Total	9		ND	0.11±0.05	1.76±0.61	12.12±1.37	
Male	14		ND	1.65±0.60	1.99±0.57	11.88±2.14	HaHa/jj or Haha/jj
Female	13		ND	1.71±0.78	1.78±0.49	12.48±1.13	
Total	27		ND	1.68±0.69	1.89±0.54	12.17±1.75	
Male	11		0.62±0.21	0.15±0.04	7.29±1.90	13.70±1.44	haha/+ + or haha/j +
Female	13		0.47±0.23	0.08±0.03	5.17±1.58	11.68±2.69	
Total	24		0.54±0.23	0.11±0.05	6.14±2.03	12.60±2.42	
Male	36		0.43±0.17	1.92±0.62	6.89±2.28	13.42±2.35	HaHa/+ + , HaHa/j + , Haha/+ + or Haha/j +
Female	39		0.47±0.25	1.60±0.78	4.95±1.69	11.67±2.13	
Total	75		0.45±0.21	1.75±0.72	5.88±2.22	12.51±2.40	

the rats with high bilirubin and androsterone GT activities. These results provide evidence that albino- and hooded-coat-colour genes are not linked with bilirubin GT (Castle & King, 1940) and androsterone GT genes.

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