QTL analysis and localization of genes involved in the variation of cholesterol levels in the rat

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Cover design: Ger Timmermans

CIP-gegevens koninklijke bibliotheek, Den Haag

Bonné, Anita

QTL analysis and localization of genes involved in the variation of cholesterol levels in the rat
Utrecht: Universiteit Utrecht, Faculteit der Diergeneeskunde
Thesis Utrecht University. –with references.- With summary in Dutch
ISBN 90-393-3080-8
Keywords: rat, cholesterol, quantitative trait loci

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QTL analyse en lokalisatie van genen betrokken bij de variatie van cholesterol concentraties in de rat.

(met een samenvatting in het Nederlands).

Proefschrift

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 12 september 2002 des middags om 2.30 uur.

door

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Geboren op 8 augustus 1973 te Roermond.

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Printed by PrintPartners IPSKAMP, Enschede, 2002.

The author of this thesis is highly indebted to the following organizations for their financial support:

* Stichting Proefdier en Maatschappij, The Netherlands
* Hope Farms, The Netherlands

Voor Robert, pap en mam

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Chapter I

General Introduction

The rat

The rat (*Rattus norvegicus)* is frequently used as a model in biomedical research. Most of the studies are performed with outbred strains (e.g. Wistar or Sprague Dawley), but for genetic studies inbred strains are often more appropriate. The first inbred strains were developed in the beginning of the century.[1-6] At present more than 150 inbred strains are available.[7]

In this thesis inbred strains and inbred derived populations (F_2 progenies or recombinant inbred strains) have been used for studying genetic factors that are involved in cholesterol metabolism. The rat is widely used as an animal model for those studies. A search in the public database of Medline revealed that until now more than 18.000 articles have been published in which the rat was used for cholesterol related studies. However less than 2% of these publications dealt with genetic aspects, and in only nine of these papers a quantitative trait locus (QTL) was reported to be associated with a specific part of the chromosome.[8-16] From studies in mouse and man it is known that there are many genes involved in cholesterol metabolism. In these species more genes have been mapped to chromosomes. Some of the major genes and gene-families, involved in cholesterol metabolism are briefly highlighted here.

Fatty acid binding proteins (FABPs):

Mammalian FABPs were discovered in the early 1970s. These proteins that are able to bind long-chain fatty acids *in vitro* are abundant cytoplasmic proteins.[17,18] *In vivo* the FABP family expresses a large number of tissue specific homologues, each of which has its own unique function.[19] Several categories of functions have been proposed for the FABPs. These can be described as (i) modulation of specific enzymes of lipid metabolic pathways (ii) maintenance of cellular membrane fatty acid levels (iii) regulation of the expression of fatty acid-responsive genes.[20,21] The role of the FABPs in intracellular lipid metabolism suggests that these proteins are involved in controlling cholesterol homeostasis. There is also evidence that the FABPs play a role in cellular fatty acid transport.[19] The function of these proteins as intracellular transporters originally derived from the assumption that hydrophobic long-chain fatty acids would require a transport protein for transit in an aqueous environment such as the cytoplasm. However, we now know that long-chain fatty acids are fully capable of intermembrane transfer in the absence of any binding protein.[19]

Seven *Fabp* genes (*Fabp1-Fabp7*) have been mapped in mouse. In rat, *Fabp1*, *Fabp2* and *Fabp3* are located on chromosome 4, 2 and 5.[22-26] Plasma membrane Fabp (*Fabp-pm*) is mapped to rat chromosome 19.[24]

Sterol regulatory binding factors (SREBFs):

The metabolism of sterol and fatty acid metabolism is interdependent. This has been demonstrated in the studies of sterol regulation using mutants of Chinese hamster ovary cells.[27] Links in the mechanism of cholesterol and fatty acid metabolism were solved with the purification and cloning of the transcription factors, SREBF-1 and SREBF-2, and the adipocyte determination differentiation factor (ADD-1), the rat homologue of human SREBF-1c.[28-31] In man SREBF-1a and 1c are derived from different transcription start sites in the promoter of the *Srebf-1* gene and SREBF-2 is transcribed from a separate gene.[32,33] SREBFs are global regulators of cholesterol homeostasis and bind to sterol response elements in the promoters of the LDL receptor, 3-hydroxy-3-methylglutaryl-CoA synthase, farnesyl pyrophosphate synthase and squalene synthase.[29,30,34-37] The unique feature of SREBF regulation is the conversion from a membrane bound inactive form in sterol replete cells to a soluble nuclear-localized form when cells are depleted of sterols.[38]

Recently Sakakura and co-workers[39] described that the SREBFs are involved in the entire pathway of the cholesterol synthesis by increasing gene expression. In the rat the *Srebf-1* gene has been mapped to chromosome 10 and it was suggested that this gene is a positional candidate for a quantitative trait locus controlling liver cholesterol levels.[40] *Srebf-2* has not been mapped yet in rat, but based on homology with regions of human chromosome 22 and mouse chromosome 15, the gene can be expected to be located on rat chromosome 7.

ATP binding cassette (ABC) transporters:

ABC transporters are primary active transporters, i.e. they bind their substrate and move it through the membrane, using ATP to overcome the substrate concentration gradient.[41] It has been suggested that ABC transporters are involved in atherosclerosis.[42-45] ABC1 is one of the largest lipid transporters. In humans, it has been shown that mutations in the ABC1 gene result in familial high density lipoprotein deficiency. The complete absence of a functional gene results in Tangier disease, in which HDL is nearly absent, due to rapid degradation of HDL precursors.[41] Interestingly, ABC8 is up regulated if macrophages are incubated with acetylated LDL.[46] Therefore there may be other ABC transporters linked to lipid transport and atherosclerosis. More than 40 *ABC* genes have been mapped in human and mice, but in the rat only 8 *ABC* genes have been mapped so far.

Lipoprotein lipase (LPL):

The role of LPL is two fold. One is in the assimilation of triglyceride fatty acids into tissues, the other in regulating plasma concentrations of lipoprotein classes. The role LPL plays in the stepwise degradation of triglyceride-rich lipoproteins consists of the sequential lipolysis of phospholipid, triglyceride, diglyceride and monoglyceride. For the regulation of plasma lipoprotein concentration, LPL acts on natural substrates, as chylomicrons and very low density lipoproteins. *In vitro*, lipolysis of VLDL, in the presence of HDL_3, results in the formation of HDL_2, which suggests a metabolic relationship between HDL_3 and HDL_2. The HDL_2 subfraction results from a transfer of VLDL surface constituents to HDL_3, which further suggests a role for LPL in regulating not only plasma VLDL but also HDL_2 concentrations.[47] Plasma HDL can be separated by centrifugation into HDL_2 and HDL_3 ; the latter particles are smaller, contain less cholesteryl ester, and have a lower apoA-I/apoA-II ratio than HDL_2.[48] In rat this gene has been mapped to chromosome16.[16]

Hepatic Lipase (HL):

Following the initial hydrolysis of chylomicrons and VLDL triglyceride by LPL at capillary surfaces of peripheral tissues, further modification of the particles occurs through the action of HL, a triglyceride lipase originating in the liver. This hepatic lipase has the capability of hydrolyzing a variety of substrates, e.g. monoglycerides, triglycerides and phospholipids.[49,50] In contrast to LPL, HL does not require an apolipoprotein activator, and its activity is not inhibited by high NaCl concentrations or by protamine.[51] The low catalytic activity of HL with lipoproteins *in vitro*, and the lack of correlation between HL activities and lipoprotein triglyceride concentrations *in vivo* strongly suggest that the activity of HL is not a rate-limiting step in removal of plasma triglyceride.[52] This gene is located on rat chromosome 8.[24,53,54]

Endothelial-derived lipase (LIPG):

Jaye *et al*. [55] identified a new member of the triglyceride lipase family, endothelial lipase (EL or LIPG). The corresponding gene locus was named *Lipg*.[56] Comparative sequence analysis of *Lpl* and *Lipg* revealed significant homologous regions, but also regions that define the specificity of each of these two enzymes.[56] EL does not have triglyceride hydrolytic activity, but possesses phospholipase activity *in vitro*.[57] Interestingly, overexpression of EL by an adenovirus vector, reduced plasma HDL cholesterol concentrations in mice, suggesting that EL may modulate HDL metabolism.[55] Hirata and co-workers[58] suggested that EL in endothelial cells is regulated and claimed that EL is expressed in endothelial cells of the arterial vessel wall. They concluded that EL plays a significant role in regulating lipid metabolism in the vessel wall.

Lecithin:cholesterol acyl transferase (LCAT):

In contrast to LPL and HL, which appear to function at sites on cell surfaces, LCAT is an enzyme that circulates in plasma. It is responsible for the formation of most of the cholesterol esters found in plasma. LCAT acts by transferring fatty acids from lecithin to the hydroxyl group of cholesterol and is essential for the HDL mediated net transport of cholesterol from peripheral tissues to the liver. The exact role of LCAT in the transfer and metabolism of surface components is yet fiercely understood. In the rat, this gene has been assigned to chromosome 19.[16]

Mapping of genes and quantitative trait loci (QTLs)

The basic methodology for mapping genes and QTLs involved in the metabolism of cholesterol is based on crosses between inbred strains that differ in a (quantitative) trait (e.g. serum cholesterol levels). An F_1 generation is produced by reciprocal matings of the parental strains. This is a genetically identical population except for the X-linked genes, although the F_1-animals are heterozygous for many genes, including the QTLs responsible for the trait of interest. By intercrossing animals of the F_1 generation a genetically heterogeneous F_2 offspring is formed. The genetic heterogeneity can be visualized by genotyping genetic markers.[59] Genetic markers based on simple sequence length polymorphisms (SSLPs or microsatellites) or on amplified fragment length polymorphisms (AFLP markers) have been proven to be excellent for QTL mapping. They are highly polymorphic and are randomly dispersed throughout the genome.[60,61] Microsatellites consist of tandemly repeated nucleotides and are easily typable by PCR.[62-64] The AFLP technique which leads to the detection of AFLP markers is based on the combined used of restriction enzymes and selective PCR primers.[65] Multiple polymorphism are simultaneously visualized without the need of prior information on genomic sequences. These polymorphisms mostly consist of presence/absence polymorphisms (dominant/recessive markers).

If a QTL is located near a genetic marker, the chance of recombination in F_1 gametocytes is small. So, in the majority of F_2 animals, the parental QTL and marker allele will stay together on the chromosome. Evidence for a QTL is based on the LOD score, indicating the log10 of the odds favoring linkage.[59] Due to genomic conservation among species it is possible to compare the genetic maps of different species. Candidate genes may be detected by comparing the chromosomal segments that contain a QTL with the homologous segments of a species in which more genes have been mapped. As previously indicated, for the genetic analysis of genes and QTLs, we have used F_2 progenies of inbred strains and recombinant inbred (RI) strains.

In a previous study, rat inbred strains were tested for their response to a cholesterol-rich diet.[66] The strains BN.*lx*/Cub and LEW/OlaHsd were reported to be hyperresponders with respect to serum cholesterol level, whereas the BC/CpbU and SHR/OlaIpcv were reported to be hyporesponders. The F_2 population used for the studies described in this thesis is based on a cross between the BC and LEW inbred strains. The RI strains are derived from the BN and SHR inbred strains. RI strains are produced by crossing of two inbred strains followed by 20 or more generations of brother x sister mating, starting with F_2 animals. RI strains represent an array of genotypes with a random assortment of unlinked genes that have become fixed after several rounds of segregation and reassortment during several generations of brother-sister mating. It is an advantage of the RI strains that a given random combination of alleles can be tested repeatedly. This is particularly important if traits are to be analyzed which require the sacrifice of the animal for assay. The data

obtained are cumulative, i.e. results obtained at different times and in different laboratories on different individuals of the RI strain set can be combined and analyzed together. Since the genetic traits are stable (genetically fixed) only a few individuals from each RI strain need to be typed. A limitation of RI strain analysis might be that in a given set of RI strains the number of allele combinations is restricted, and depends on the progenitor strains used.

Besides these four inbred strains and their related crosses, we have also used the radiation hybrid (RH) panel of Research Genetics (Huntsville, USA). The RH panel was used for the localization of genes which were not polymorphic between the four rat inbred strains and could, therefore, not be localized in the genetic map of the RI strains or in the genetic map of the F_2 progeny.

Scope of the thesis

This thesis aims to contribute to the value of the rat as an animal model for studying the genetic background of differences in serum and liver cholesterol levels and susceptibility for dietary cholesterol. In chapters 2 and 3 the two inbred strains BN and SHR, and recombinant inbred (RI) strains derived from these two progenitors, are used for genetic screening. In chapter 2, the genetic map of the RI strains is expanded with AFLP (Amplified Fragment Length Polymorphism) markers, whereas in chapter 3, the *Fabp6* gene is sequenced for both the BN and SHR inbred strains. The gene is localized in the radiation hybrid map. Also the localization of a *Fabp6* pseudogene, as found in SHR has been performed in the backcross progeny of the rat inbred strains BN and SHRSP.
Chapter 4 describes the development of the genetic map based on the F_2 progeny from the cross between the BC and LEW inbred strains.
In chapter 5-7, these 192 F_2-animals were screened for QTLs and for candidate genes involved in the regulation of cholesterol levels or related levels. Several QTLs and candidate genes are mapped by using the F_2 progeny and some candidate genes have been mapped by using the radiation hybrid panel. The thesis is concluded with a general discussion.

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Chapter II

Genetic map of AFLP markers in the rat (*Rattus norvegicus*) derived from the HXB/Ipcv and BXH/Cub sets of recombinant inbred strains

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Biochemical Genetics, accepted

Abstract

The amplified fragment length polymorphism (AFLP) technique has been used to enhance marker density in a large set of recombinant inbred strains (HxB and BxH) derived from a spontaneously hypertensive rat (SHR/OlaIpcv) and a Brown-Norway (BN.*lx*/Cub) inbred strain. Thirteen different primer combinations were tested and a total of 191 polymorphic bands were detected. From these polymorphic bands 89 AFLP markers could be assigned to specific chromosomes. Several of these AFLP markers were mapped to regions with low marker density, thus filling up gaps in the existing genetic map of these recombinant inbred strains. These results substantiate the value of the AFLP technology in increasing marker density in genetic maps.

Introduction

Recombinant inbred (RI) strains constitute a useful tool in gene mapping and analysis of complex traits.[1-6] In the rat, six different sets of RI strains have been reported.[7-9] The HXB and BXH strains, which represent the largest set of rat RI strains, are derived from a spontaneously hypertensive rat (SHR/OlaIpcv) (H) and a Brown-Norway strain (BN.*lx*/Cub) (B).[10-12] The linkage map, constructed with this set of RI strains, includes 482 markers.[13] The usefulness of this set of RI strains in genetic studies of complex traits like hypertension and accompanying metabolic disorders can be improved by the addition of more markers to the map.[8] Although the linkage map of the rat consists predominantly of microsatellite markers, also other kinds of markers have been used to develop the genetic map of the rat.[14-16] The amplified fragment length polymorphism (AFLP) fingerprinting technique has high potential to become another valuable genome mapping tool.[17] The technique rapidly generates hundreds of highly replicable markers from DNA of any organism. Previously we have experimented with the AFLP technology in the rat[18-20] using the restriction enzyme combination *Sse*I/*Mse*I. In the current study we have used *Eco*RI/*Taq*I as enzyme combination in order to increase the number of polymorphic markers in the HXB and BXH sets of recombinant inbred strains.

Animals, Materials and Methods

Origin of recombinant inbred strains.

The RI strains were derived from spontaneously hypertensive rats (SHR/OlaIpcv) (H) and Brown Norway rats (BN.*lx*/Cub) (B). The BN.*lx* is a BN congenic strain that carries a segment of chromosome 8 from the PD strain with the polydactyly-luxate syndrome.[10] As a consequence, the RI strains exhibit polymorphisms on chromosome 8 that may originate from the PD strain. A total of 36 RI strains were originally derived from crosses between female SHR and male BN.*lx* rats (HXB/Ipcv; n=26; at the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague) or female BN.*lx* rats and male SHR (BXH/Cub; n=10; at the Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Prague). Of these 36 original strains, DNA was available for genetic analysis. Currently, 31 surviving strains are still available (21 HXB/Ipcv and 10 BXH/Cub strains) and most of these have reached more than 45 generations of brother-sister inbreeding. All these strains were beyond F20 when the DNA was isolated from a piece of the tail using a standard procedure.[21]

AFLP protocol.

The AFLP procedure has been developed by KeyGene N.V. (Wageningen, The Netherlands), which has filed property rights on this technology.[22] (patent #0534858). The main steps of the AFLP procedure have been described by Vos et al.[23] Present AFLP analysis was performed according to their protocol, but instead of the *Sse*I/*Mse*I restriction enzyme set we used the *Eco*RI/*Taq*I set.[24] All oligonucleotides used as primers or adapters (Table 1) were synthesized by Pharmacia Biotech Benelux (Roosendaal, The Netherlands). Briefly, total genomic DNA was digested with *Eco*RI and *Taq*I and adapters were ligated. PCR primers overlap the adapters and restriction sites and are provided with a specific nucleotide extension at their 3' ends, which enables further selection of the sequences to be amplified. The resulting template-DNA was pre-amplified (non-radioactive PCR) with primers carrying one selective nucleotide (Adenine for both the *Eco*RI and the *Taq*I primer). The final amplification (radioactive PCR; γ^{33}P-labelled primers) was carried out using primer combinations with two extra selective nucleotides. The products were separated on polyacrylamide gels and visualized by exposure to X-ray films. Amplification products were scored visually as present or absent. Phenotypes were scored independently by two individuals.

Nomenclature of AFLP markers.

AFLP markers were designated by the primer combination code (i.e. the combination of primers that were used to amplify the fragment: A, C, E, G, H, I, J, P, Q, T, U, W, or Y; see table 2) and a ranked number (Table 3). Polymorphic AFLP bands were ranked according to their mobility in a sequencing gel. The size of the fragments in the gel was estimated relative to a molecular weight marker of known length (10-base DNA ladder; Allele Sizing Set, Boehringer Mannheim GmbH, Mannheim, Germany). In order to conform rat gene and locus symbols with those of other mammalian species, we decided to indicate these anonymous polymorphic DNA bands with symbols according to the suggestions of the International Rat Genetic Nomenclature Committee[26] e.g. marker P4 (= primer combination E43/T32,size = 428 bp) located on chromosome 1 is given the symbol *D1Utr1*.

Data analysis and map construction.

Possible segregation distortion of AFLP marker inheritance from the expected 1:1 Mendelian ratio was analysed using a χ^2 goodness-of-fit test with 1 d.f. ($P>0.05$). AFLP markers were assigned to chromosomes by linkage analysis with an existing map for these strains.[12,13] Strain distribution patterns of the markers of the existing map have been published elsewhere.[27] For this purpose the strain distribution patterns of the genetic markers were entered into the Map Manager computer program of Manly[28] (version 2.5b3. Details of this mapping method have been described previously.[12]

Linkage groups were merged where they were known to be separate segments of a chromosome. Output from Map Manager was converted to linkage maps using the graphics program MapChart.[29]

Results

Polymorphism rate.

This survey revealed that for each primer combination the number of bands that could be scored on gels ranged from 78 to 112, with a mean of 91 and an average of 14.7 polymorphic bands (Table 2). Only AFLP-bands within 50-460 bp were scored, because bands > 460 bp have low intensity and poor reducibility. For the different primer combinations, no significant correlation between the total number of bands and the number of polymorphic bands was found (Pearson's r = -0.0093, n = 13, p = 0.976). Out of a total of 1178 AFLP bands 191 (16.2%) were polymorphic between SHR/Ola and BN.*lx*/Cub. Eighteen of these proved to be allelic sets of bands (thus representing nine fragment length polymorphisms). The remaining 173 bands were presence/absence type polymorphisms. In total 100 of these bands were entered into Map Manager. The other bands were excluded due to segregation distortion, or because the intensity of the band was too low. A typical fingerprint is given in Fig. 1.

Linkage map.

Eighty-nine out of 100 AFLP markers could be mapped onto the existing RI linkage map. Table 3 shows the characteristics of the mapped AFLP markers. The position of these markers on the genetic maps is indicated in Fig. 2. About 58.4% and 41.6% of the mapped markers were specific to BN.*lx*/Cub and to SHR/OlaIpcv, respectively. Each autosome contained at least one new AFLP marker. There was a significant correlation between number of mapped AFLP markers per chromosome and the physical size (Table 3)(Pearson's r = 0.7684, n = 20, $p<0.001$) or genetic size (Fig. 2)
(Pearson's r = 0.7117, n = 20, $p<0.001$) of the chromosome.

AFLP band T16 (*D19Utr7*), which is present in the BN.*lx*/Cub strain, seems to contain a dinucleotide repeat. This assumption is based on observation of the repetitive pattern with fading of bands with two nucleotides between each band (Fig. 1).

Table 1. AFLP adapters and primers used in this study

Adapters and primers	selective bases	Primer code	Adapter and primer sequences or primer combination code
EcoRI –adapter:			5'-CTCGTAGACTGCGTACC-3' (top strand)
			3'-CATCTGACGCATGGTTAA-5' (bottom strand)
EcoRI –primers:	+ 1 (A)	E01	5'-GACTGCGTACCAATTCA-3'
	+ 3 (AAG)	E33	5'-GACTGCGTACCAATTCAAG-3'
	+ 3 (ACA)	E35	5'-GACTGCGTACCAATTCACA-3'
	+ 3 (ACT)	E38	5'-GACTGCGTACCAATTCACT-3'
	+3 (ATA)	E43	5'-GACTGCGTACCAATTCATA-3'
TaqI-primers :	+ 1 (A)	T01	3'-AAGCCAGTCCTGAGTAG-5'
	+ 3 (AAC)	T32	3'-CAAAGCCAGTCCTGAGTAG-5'
	+ 3 (AAG)	T33	3'-GAAAGCCAGTCCTGAGTAG-5'
	+ 3 (AGA)	T39	3'-AGAAGCCAGTCCTGAGTAG-5'
	+ 3 (AGT)	T42	3'-TGAAGCCAGTCCTGAGTAG-5'
	+3 (ATC)	T44	3'-CTAAGCCAGTCCTGAGTAG-5'
	+ 3 (ATG)	T45	3'-GTAAGCCAGTCCTGAGTAG-5'
TaqI-adapter :			5'-CGGTCAGGACTCAT-3' (top strand)
			3'-CAGTCCTGAGTAGCAG-5' (bottom strand)

Table 2. Comparison of 13 primer combinations for their capacity to generate AFLP marker polymorhisms in the two rat inbred strains SHR and BN.

Primer Combination*	Number of visible bands	Number of polymorphic bands	Number of bands entered into Map Manager	Number of mapped AFLP markers
E33/T33 (A)	94	19	10	10
E33/T44 (C)	81	11	6	6
E35/T32 (E)	82	16	6	5
E35/T39 (G)	94	12	5	4
E35/T42 (H)	93	18	11	10
E35/T44 (I)	78	10	8	4
E38/T33 (J)	87	13	5	5
E43/T32 (P)	81	16	7	5
E43/T33 (Q)	98	12	9	9
E33/T32 (T)	106	21	12	12
E43/T45 (U)	87	16	6	6
E33/T42 (W)	112	8	6	4
E43/T42 (Y)	85	19	9	9

* The code of the primer combination is given in parentheses.

Table 3. Characteristics of the mapped AFLP markers.

Alias[@]	Marker name	Product size (bp)	Present BN	/Absent SHR	Alias[@]	Marker name	Product size (bp)	Present BN	/Absent SHR
Chromosome 1 (301 Mb; 141 cM)[#]					*Chromosome 7* (153 Mb; 79 cM)				
P4	D1Utr1	428	+	-	H2	D7Utr5	110	+	-
P5	Allele P4[*]	418	-	+	*Chromosome 8* (142 Mb; 84 cM)				
W5	D1Utr2	188	-	+	Y15	D8Utr1	175	-	+
A15	D1Utr3	153	-	+	Y16	D8Utr2	173	+	-
A10	D1Utr4	195	-	+	E10	D8Utr3	160	+	-
A11	D1Utr5	188	+	-	H3	D8Utr4	124	-	+
T7	D1Utr6	381	-	+	H10	D8Utr5	351	+	-
T19	D1Utr7	124	+	-	*Chromosome 9* (135 Mb; 79 cM)				
U6	D1Utr8	272	-	+	P9	D9Utr1	56	+	-
U7	Allele U6[*]	269	+	-	C5	D9Utr2	458	+	-
E9	D1Utr9	178	+	-	A13	D9Utr3	165	-	+
Chromosome 2 (268 Mb; 111 cM)					A14	Allele A13[*]	157	+	-
Y9	D2Utr1	387	-	+	*Chromosome 10* (128 Mb; 93 cM)				
Q13	D2Utr2	177	+	-	Q18	D10Utr1	107	-	+
T8	D2Utr3	359	-	+	T10	D10Utr2	328	-	+
U9	D2Utr4	224	-	+	T12	D10Utr3	296	+	-
U10	Allele U9[*]	221	+	-	T20	D10Utr4	57	-	+

U4	D2Utr5 P4*	459	+	-
A6	D2Utr6	339	+	-
A9	D2Utr7	239	+	-
G6	D2Utr8	82	+	-
E4	D2Utr9	299	+	-
E5	Allele E4*	297	-	+
E11	D2Utr10	149	+	-
H4	D2Utr11	161	-	+
P7	D2Utr12	236	+	-
Chromosome 3 (203 Mb; 94 cM)				
C10	D3Utr1	291	+	-
Y18	D3Utr2	145	+	-
C12	D3Utr3	117	+	-
T9	D3Utr4	357	-	+
T15	D3Utr5	182	+	-
U1	D3Utr6	125	+	-
H6	D3Utr7	205	-	+
I6	D3Utr8	373	+	-
J5	D3Utr9	234	+	-
Chromosome 4 (198 Mb; 104 cM)				
Y19	D4Utr2 P4*	142	+	-
H7	D10Utr5	220	+	-
Chromosome 11 (110 Mb; 49 cM)				
I3	D11Utr1	155	+	-
Chromosome 12 (76 Mb; 43 cM)				
T13	D12Utr1	194	+	-
T14	Allele T13*	189	-	+
Chromosome 13 (118 Mb; 46 cM)				
P8	D13Utr5	215	+	-
Y13	D13Utr6	271	-	+
Q14	D13Utr7	172	-	+
Q7	D13Utr8	373	-	+
Chromosome 14 (116 Mb; 69 cM)				
Q12	D14Utr2	183	-	+
Q8	D14Utr3	347	+	-
A5	D14Utr4	355	-	+
P6	D14Utr5	308	+	-
Q15	D14Utr6	146	-	+
A12	D14Utr7	173	+	-
U8	D14Utr8	261	+	-
Chromosome 15 (111 Mb; 69 cM)				
C6	D15Utr2	360	-	+

T11	D4Utr3	304	+	-	Q9	D15Utr3	324	+	-	
W4	D4Utr4	234	-	+	E7	D15Utr4	208	-	+	
Chromosome 5 (181 Mb; 98 cM)					E8	Allele E7*	206	+	-	
A4	D5Utr1	442	+	-	*Chromosome 16* (101 Mb; 46 cM)					
C8	D5Utr2	315	+	-	T2	D16Utr1	460	-	+	
H5	D5Utr3	193	+	-	*Chromosome 17* (100 Mb; 50 cM)					
H13	D5Utr4	378	+	-	J1	D17Utr1	287	-	+	
I1	D5Utr5	90	+	-	*Chromosome 18* (95 Mb; 52 cM)					
Chromosome 6 (158 Mb; 73 cM)					G3	D18Utr2	191	+	-	
Y12	D6Utr1	288	+	-	H12	D18Utr3	367	-	+	
Y14	D6Utr2	204	+	-	*Chromosome 19* (77 Mb; 44 cM)					
G7	D6Utr3	58	-	+	J4	D19Utr2	141	+	-	
A7	D6Utr4	324	-	+	W2	D19Utr3	295	+	-	
H8	D6Utr5	253	-	+	C11	D19Utr4	209	-	+	
I5	D6Utr6	252	+	-	Y10	D19Utr5	344	+	-	
Chromosome 7 (153 Mb; 79 cM)					Y11	Allele Y10*	332	-	+	
J2	D7Utr1	185	-	+	Q11	D19Utr6	264	-	+	
W3	D7Utr2	242	+	-	T16	D19Utr7	154	+	-	
U5	D7Utr3	289	-	+	*Chromosome 20 (67* Mb; 37 cM)					
G1	D7Utr4	408	-	+	J3	D20Utr4	176	+	-	
G2	Allele G1*	406	+	-						

@ The alias consists of the primer combination code (see Table 1) and the ranking number.
* These length polymorphisms were characterized by obvious shifts in the AFLP fingerprint.
# The physical and genetic size of the chromosome is given in parentheses and has been taken from Scheetz *et al*.[25]

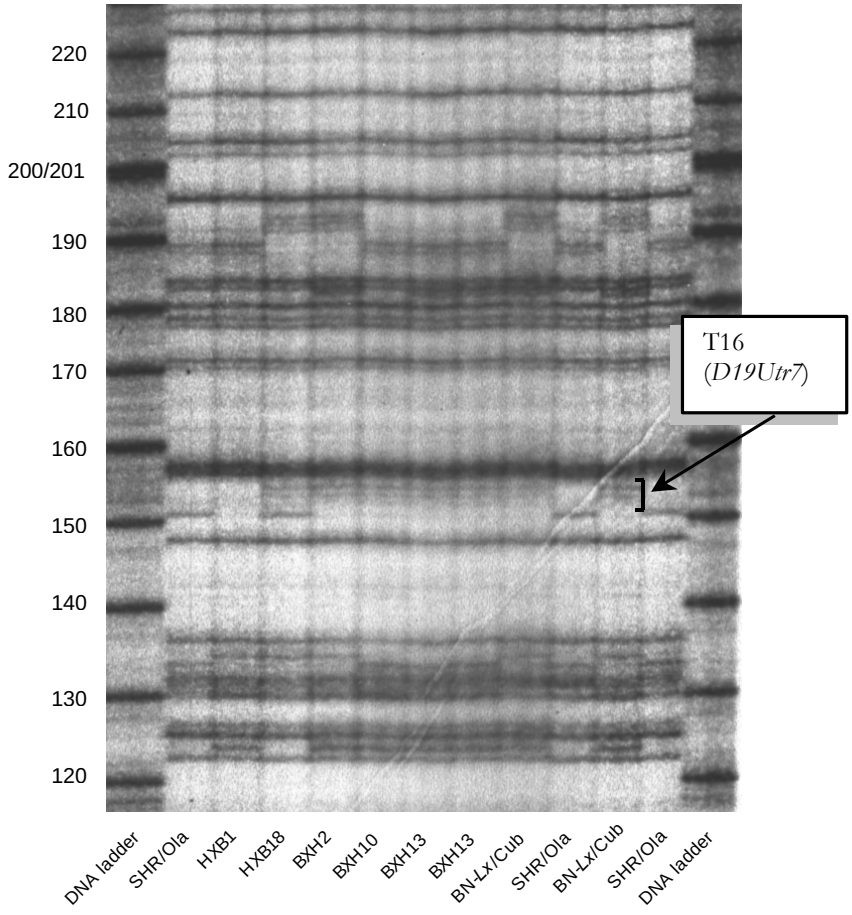


Fig. 1. Section of autoradiograph showing AFLP fingerprint of genomic DNA of the BN.*lx*/Cub, SHR/OlaIpcv, two HXB/Ipcv and three BXH/Cub strains. Primer combination T (see Table 2) was used for AFLP. The shadow bands, which are indicated by an arrow result from the apparent strand slipping during PCR amplification (dinucleotide repeat).

Discussion

We have previously experimented with the AFLP technology in the rat.[18-20] In those studies the restriction enzyme combination *Sse*I/*Mse*I was used and four primer combinations were tested. We contributed 18 AFLP markers to the linkage map of the rat: seven AFLP markers were assigned to specific chromosomes by analysis of a (BNxACI)F_1xACI backcross progeny.[19] Another 11 AFLP markers were mapped by using the panel of the HXB/BXH RI strains.[19] In crop plants the restriction enzyme combination *Eco*RI/*Mse*I has been recommended for producing AFLP markers.[30] However, we now know that the use of this restriction enzyme combination in vertebrate DNA results in an excessive number of fragments in the restriction fragment library. To reduce the number of fragments efficiently, primer extensions of three nucleotides will often not be sufficient. Therefore, in mammals and birds the use of *Taq*I instead of *Mse*I has been suggested.[24] This restriction enzyme combination, when combined with selective amplification using primers containing three nucleotide extensions, was expected to result in satisfactory AFLP patterns for vertebrate DNA. Furthermore, it was reasoned that this restriction enzyme combination produces more polymorphic AFLP patterns in mammals and birds because the *Taq*I site contains a CG dinucleotide within its sequence, which is a site of frequent polymorphisms in vertebrate DNA.[31] This prompted us to use *Eco*RI and *Taq*I as restriction enzymes for fingerprinting rat DNA. The number of mapped AFLP markers per primer combination now ranges from 4 to 12 (Table 2), whereas the number of mapped AFLP markers per primer combination using the enzyme combination *Sse*I/*Mse*I and the same panel of HXB/BXH RI strains ranged from 2 to 5.[19] Thus, the *Eco*RI/*Taq*I enzyme combination seems to be more suitable for DNA fingerprinting in rats.

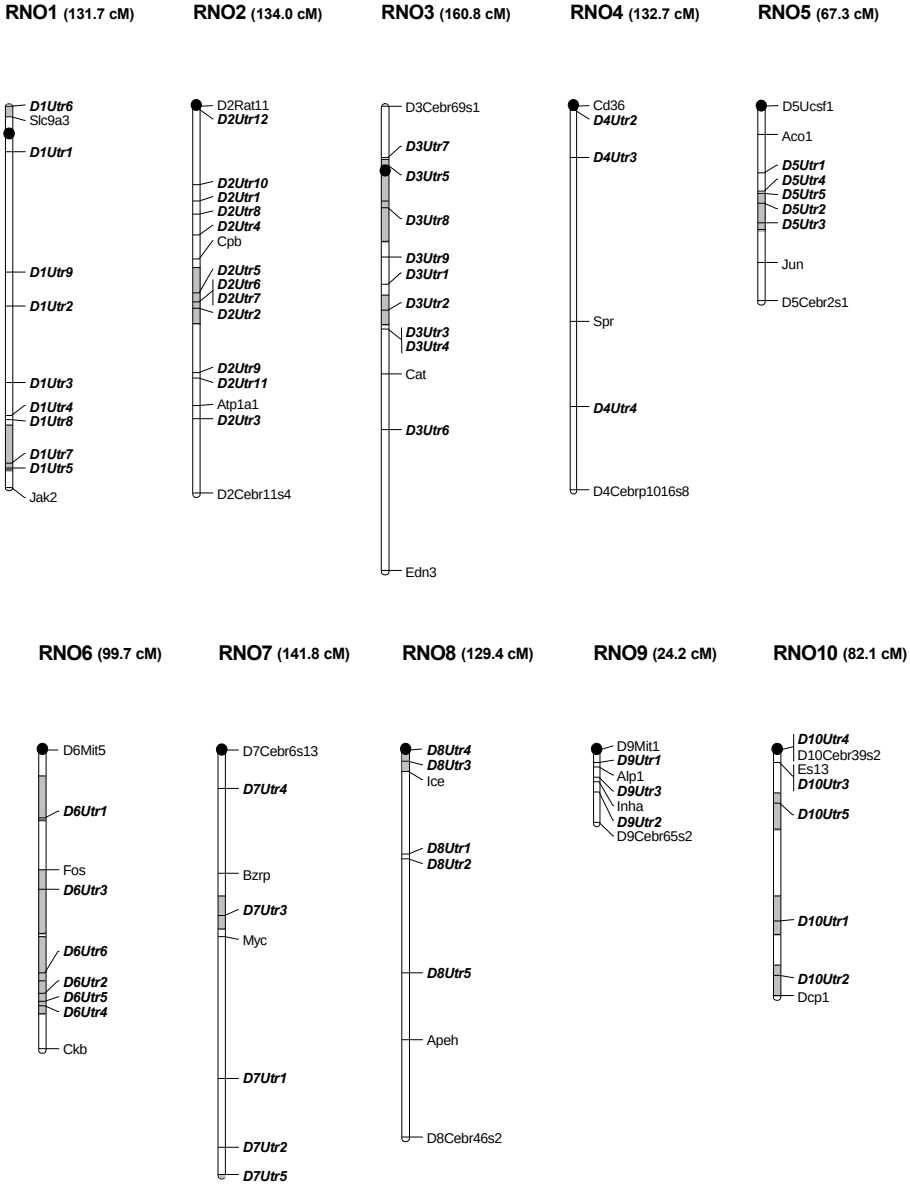
In plants the distributions of AFLP markers across the genome is not random and strong clustering of AFLP markers around the centromere has been described (e.g. Alonso-Blanco et al.[32] and references cited herein). In those studies the restriction enzyme combination *Eco*RI/*Mse*I was used. Inspection of Fig. 2 for both the centromeric position and the genetic location of AFLP markers shows that in the rat, at least when using *Eco*RI/*Taq*I as restriction enzymes, there is no evidence of clustering of AFLP markers around the centromere. Whether the absence of clustering is due to the restriction enzyme combination used or because of characteristics specific for rat (or vertebrate) DNA, remains to be investigated.

Fig. 2 demonstrates that several gaps (>10 cM) in the genetic map of the RI strains could be filled up with AFLP markers (on chromosomes 1, 2, 3, 5, 6, 7, 10, 12, 15, 19 and 20). Other AFLP markers could be mapped in regions outside the segments covered by previously mapped markers (on chromosomes 1, 7, 8 and 10).

The AFLP markers proved to be helpful in more precisely allocating QTLs onto the chromosome. For example, the *D19Rat52 - Hmox* region of chromosome 19 contains a QTL influencing basal serum HDL_2 cholesterol level[3] and has now been filled up with two AFLP markers (*D19Utr2* and *D19Utr5*). Also, Kren and co-workers[11] described a QTL on the telomeric part of chromosome 7 (in the vicinity of *D7Mit17*) that is controlling limb morphogenesis. In this region the AFLP marker *D7Utr5* could be placed. Furthermore, Panczak et al.[35] found on the telomeric part of chromosome 13 (nearby the *D13Mit4* marker) a QTL involved in graft-versus-host reaction. In the vicinity of this marker we could localize *D13Utr7*. Thus, for these traits AFLP markers might help in fine mapping the responsible QTLs.

Eleven AFLP markers could not be assigned to the map. This might indicate that these are located on the telomeric regions of the chromosomes. With increasing density of the linkage map these markers will eventually also be placed on the linkage map.

The AFLP technique enables the rapid increase of genetic markers on the rat genome map. One restriction enzyme combination like *Eco*RI and *Taq*I can be used with 4096 different primer pairs (+3 extensions on both primers). The total rat genome is estimated to be 3001 Mb and 1503 cM.[25] Assuming 7.7 (useful) AFLP markers per primerset (see Table 2; 100 AFLP markers entered in Map Manager, thirteen different primer sets tested), this would give 10 AFLP markers/Mb or 21 AFLP markers/cM. Thus the AFLP technique is a powerful tool to generate abundant markers in a short time period. Especially, in recombinant inbred strains where co-dominant scoring is not necessary, and thus no specific software is needed, these markers can effectively been used for extension of the genetic map.


RNO1 (131.7 cM)
D1Utr6
Slc9a3
D1Utr1
D1Utr9
D1Utr2
D1Utr3
D1Utr4
D1Utr8
D1Utr7
D1Utr5
Jak2
RNO2 (134.0 cM)
D2Rat11
D2Utr12
D2Utr10
D2Utr1
D2Utr8
D2Utr4
Cpb
D2Utr5
D2Utr6
D2Utr7
D2Utr2
D2Utr9
D2Utr11
Atp1a1
D2Utr3
D2Cebr11s4
RNO3 (160.8 cM)
D3Cebr69s1
D3Utr7
D3Utr5
D3Utr8
D3Utr9
D3Utr1
D3Utr2
D3Utr3
D3Utr4
Cat
D3Utr6
Edn3
RNO4 (132.7 cM)
Cd36
D4Utr2
D4Utr3
Spr
D4Utr4
D4Cebrp1016s8
RNO5 (67.3 cM)
D5Ucsf1
Aco1
D5Utr1
D5Utr4
D5Utr5
D5Utr2
D5Utr3
Jun
D5Cebr2s1
RNO6 (99.7 cM)
D6Mit5
D6Utr1
Fos
D6Utr3
D6Utr6
D6Utr2
D6Utr5
D6Utr4
Ckb
RNO7 (141.8 cM)
D7Cebr6s13
D7Utr4
Bzrp
D7Utr3
Myc
D7Utr1
D7Utr2
D7Utr5
RNO8 (129.4 cM)
D8Utr4
D8Utr3
Ice
D8Utr1
D8Utr2
D8Utr5
Apeh
D8Cebr46s2
RNO9 (24.2 cM)
D9Mit1
D9Utr1
Alp1
D9Utr3
Inha
D9Utr2
D9Cebr65s2
RNO10 (82.1 cM)
D10Utr4
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Es13
D10Utr3
D10Utr5
D10Utr1
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Dcp1

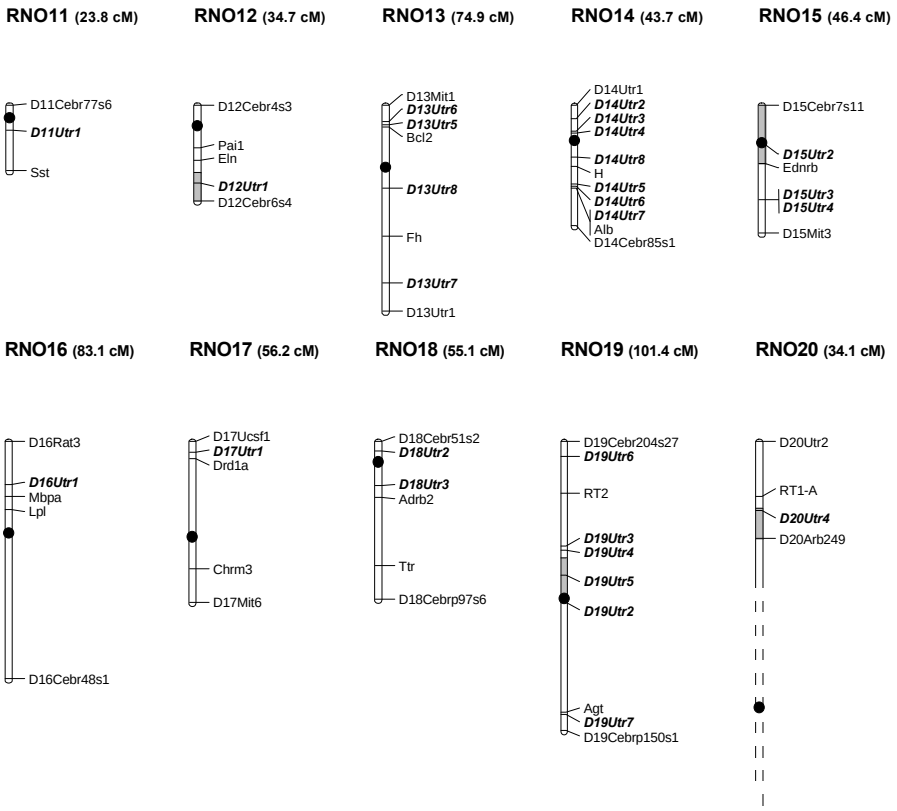

Fig. 2. Linkage map of AFLP markers on rat chromosomes. AFLP markers are given in bold/italics characters. For reference, a few landmark markers (out of the 482 previously mapped genetic markers) are also indicated. The centromeric positions is shown as a closed circle. The position of the centromere is based on the chromosome reports presented at the "Impact of Rat Genome Mapping on Biomedical Research- October 4 to 6, 1998 in Hannover-Germany[33]and on Szpirer et al.[34] The genetic length (in cM, based on the results from the HXB/Ipcv and BXH/Cub strains) of each chromosome is given in parentheses in the heading. Solid bars indicate regions of the chromosome where gaps of >10 cM could be filled with AFLP markers. The 95% confidence levels of the distance between an AFLP marker and the flanking markers is not included in this figure, but will be supplied upon request by the corresponding author.

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Chapter III

Localization of *Fabp6* and identification of a *Fabp6* pseudogene in the rat (*Rattus norvegicus*)

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Abstract

Fabp6 codes for ileal lipid binding protein, one of the fatty acid binding proteins (FABPs). The expression of the gene is regulated by bile acids in the ileum. A rat specific primer combination for *Fabp6* was used for linkage analysis in a radiation hybrid (RH) panel. Rat *Fabp6* was found to be located on rat chromosome 10, closely to the marker D10Got43. This location is supportive for the previously expressed view that *Fabp6* is a candidate gene for the regulation of rat liver cholesterol levels. The present study also revealed the existence of a *Fabp6* pseudogene. This pseudogene occurs in four related rat inbred strains (SHR; SHRSP; WKY; and OKA), but not in 62 other commonly used rat inbred strains. By analyzing the progeny of (SHRSPxBN)xBN backcross, the *Fabp6* pseudogene could be localized on rat chromosome 15.

Introduction

In the rat marked strain-specific differences exist in the susceptibility for dietary cholesterol. In previous studies we have found a quantitative trait locus (QTL) for postdietary liver cholesterol concentration on chromosome 10 (RNO10)[1]. Based on homology with mouse and human, *Fabp6* was suggested as a candidate gene. *Fabp6* is a member of a family of genes coding for fatty acid-binding proteins. These proteins constitute a rather conserved group of cytosolic low molecular mass proteins (14-16 kDa). They are tissue-specific and are able to bind long-chain fatty acids and their CoA derivates[2,3].

Fabp6 codes for the ileal lipid binding protein. This protein occurs in ileum, ovary and adrenal gland and, unlike the other members of the family, has a low binding affinity for long-chain fatty acids, but a high affinity for bile acids[4,5]. Recently it has been shown in humans that bile acids regulate the expression of the gene[6,7]. FABP6 might function as a cytosolic receptor for bile acids[8]. The chromosomal location and the nucleotide sequence of the gene is known in mouse and man[4,9].

In rat, *Fabp6* has not been mapped yet, but the mRNA sequence of the gene is known (NM_017098, NCBI GenBank). In mouse and human the gene contains four exons. Based on sequence homology we have selected primers for the amplification of rat *Fabp6* (exon2, intron2, exon3, intron3 and exon4). These primers were used for studying the chromosomal location of *Fabp6* in rat.

Animals, Materials & Methods

Animals, hybrids, DNA.

DNA from the SHR/OlaIpcv (SHR) and the BN.*lx*/Cub (BN) was used to determine the sequence of the *Fabp6* gene[10,11]. A whole genome rat/hamster (T55) Radiation Hybrid (RH) panel was purchased from Research Genetics (Huntsville, Ala) for the localization of *Fabp6*. This panel has been constructed by fusing irradiated cells from a Sprague Dawley fibroblast line (RatFR) with a recipient hamster line (A23). RatFR donor cells were irradiated with 3000 rad prior to fusion with A23 recipient cells. Genomic DNA of 66 commonly used rat inbred strains (see Table 3) was screened for presence of the *Fabp6*-ps (pseudogene). For the localization of the pseudogene a (BN x SHRSP) x BN backcross was used. The DNA from 366 backcross animals was screened.

PCR protocol and sequencing.

Primers for amplification of rat *Fabp6* sequences were designed, based on homology with mouse *Fabp6*. Primers complementary to the sequence at the start of

mouse exon2, exon3 and exon4 and the end of exon2 and exon3 were used in order to amplify rat exon2, intron2, exon3, intron3 and part of exon4 (Table 1). In the 10 µl PCR reactions, 100 ng genomic rat DNA, 0.2 mM dNTPs, 1 mM primer and 1 U *Taq* (HT Biotechnology LTD, Cambridge, UK) were used. Standard PCR included an initial 5 min. denaturation step performed at 94°C followed by 30 cycles of amplification beginning with a 1 min. denaturation at 94°C, a 2 min. annealing at 55°C, and a 2 min. extension at 72°C. The reaction was terminated with a final 5 min. step at 55°C followed by a 10-min extension at 72°C. The PCR-products were separated and visualized on an 1% agarose gel. The PCR products were isolated from gel and sequenced by DNA Sequencing Core (Leiden, The Netherlands) using the Perkin-Elmer Big-Dye Terminator cycle sequencing kit.

Radiation hybrid mapping, chromosomal mapping.

For localization of *Fabp6* on the RH map, 25 ng DNA of the hybrids was used in 10 µl PCR reactions, amplifying the *Fabp6* gene. Two primers (5'-TGTCAGTCTGTAGGTCTTGA-3')FW and FA6RN4, specific for the intron between exon3 and exon4 were used. The forward primer was labeled with [γ-^{32}P]ATP (Amersham, UK). The reaction was performed as described above. The PCR products were separated in a polyacryl-amide gel and visualized autoradiographically. The obtained results were sent to the RH mapping server of the Otsuka Gen Research Institute (http://www.otsuka. genome.ad.jp/ratmap). For the localization of the *Fabp6* pseudogene, the primers FA6RN2 and FA6RN3 (Table 1) were used. The PCR products were visualized on a 3% agarose gel and were scored as present and absent. For chromosomal mapping the program MapMaker/EXP Ver. 3.0 from the Whitehead Institute was used.

Results

The primers described in Table 1 were used in different combinations for amplification of genomic DNA of the rat inbred strains BN.*lx*/Cub (BN) and SHR/OlaIpcv (SHR). In Table 2 the primer combinations that have been used are shown together with the sizes of PCR products that are common in BN and SHR. After sequencing these products, we found the nucleotide sequence to be identical in BN and SHR. Figure 1 illustrates the sequence as obtained for this rat *Fabp6* gene. After alignment of this sequence with the sequence of rat *Fabp6* mRNA and genomic mouse *Fabp6*, the exons and introns could be distinguished (GenBank acc. num.: 8393345). The number and size of the exons in the rat (Fig. 2.) are similar to those of mouse and human[13]. The sequences of the exons of the BN and SHR are completely identical to the rat mRNA sequence (GenBank acc. num.: 8393345) whereas a 98% homology is found for mouse *Fabp6*[4]. In contrast, the two introns differ considerably between mouse and rat.

Table 1. Primers designed for amplification of rat *Fabp6* (based on data taken from references Crossman *et al.*[4] and Gong *et al.*[12])

Rat primer name	Primer sequence (5'-3')	Location in mouse
FA6RN1	AACTTCAAGATCATCACAGAGG	Start Exon 2 (FW)
FA6RN2	CCCAGTCTTACTCTGGGGGC	End Exon 2 (FW and REV)
FA6RN3	TTCCCCAACTATCACCAGACTTC	Start Exon 3 (FW and REV)
FA6RN4	ATCTCCACCATCGGGGATGTGA	Start Exon 4 (FW)

Table 2. Product size (bp) found in BN and SHR progenitor inbred strains when using different primer combinations.

	Primer (REV)					
	FA6RN2		FA6RN3		FA6RN4	
Primer (FW)	BN	SHR	BN	SHR	BN	SHR
FA6RN1	72	72	1125	1125/*206*[*]		*258*[*]
FA6RN2			1073	1073/154[*]		*206*[*]
FA6RN3					950	950/75[*]

[*]The figures in italics correspond to the product sizes found for the *Fabp6* pseudogene

As the *Fabp6* sequences of the BN and SHR progenitor inbred strains were completely identical, linkage analysis could not be performed with the use of the recombinant inbred (RI) strains. Therefore, the location of rat *Fabp6* was studied by using a radiation hybrid (RH) panel (Research Genetics, Huntsville, USA). A primer, specific for rat *Fabp6* was selected from the intron sequence between exon3 and exon4 (Fig. 1, bp 1538-1559). The PCR products of this primer and FA6RN4 were scored and the results were sent to the RH mapping server at the Otsuka Gen Research Institute. *Fabp6* was placed on the RH map in the vicinity of D10Got43 (LOD: 16.47, 308.5 cR).

Besides the PCR products found both in BN and SHR, additional, small size, products were obtained when genomic DNA of the SHR was amplified (Table 2). No extra PCR product was detected with either the BN or SHR when the specific rat intron primer combination was used. We have sequenced the 258 bp product of FA6RN1 and FA6RN4 (Fig.3). This sequence turned out to be completely identical to the sequence of exon2, exon3 and first part of exon4 of mouse *Fabp6* and the sequences of exon2, exon3 and first part of exon4 as established in BN and SHR inbred strains (Fig. 1 and Fig. 2). This PCR product contains the sequence of the *Fabp6* gene without the introns, thus indicating the existence of a *Fabp6* pseudogene (*Fabp6*-ps), present in SHR, but not in BN.

In order to test the strain distribution pattern of *Fabp6*-ps, 66 commonly used rat inbred strains were screened for presence or absence of the pseudogene. Four out of these 66 strains (SHR; SHRSP; WKY; and OKA) were found to possess the pseudogene (Table 3). Linkage analysis of the Fabp6-ps was performed in a (BNxSHRSP) x BN backcross panel. From this backcross DNA of 366 animals was used for screening. Significant linkage (placement at log-likelihood threshold 7) was found on RNO15 in the vicinity of the maker D15Mit2 (Fig. 4).

Table 3. Rat inbred strains (n = 66) screened for the presence (+) or absence (-) of the *Fabp6* pseudogene (*Fabp6*-ps). (For specifications of the rat inbred strains, see Bender et al.[14]).

Fabp6-ps (+)	*Fabp6*-ps (-)
SHR; SHRSP; WKY; OKA	A2; ACI; AGUS; ALC; AS; AO; AUG; AVN; BBWB; BC; BDII; BDIV; BDIX; BDE; BDVII; BDX; BH; BN; BN.*lx*; BP; BS; BUF; CAP; CHOC; COP; DA; DZB; E3; F344; GC; Hooded; LE; LEH; LEW; LH; LOU; MHS; MNS; MW; NAR; NEDH; OM; PAR; PD; PVG; R; RHA; RNU; SD; SDH; SDL; SPRD; SPRD-CU3; SR; SS; U; WAG; WF; WOK.1A; WOK.1W; AMORAT; ARISTORAT

AACTTCAAGATCATCACAGAGGTCCAGCAGGATGGAGAGAACTTCACCTGGTCCCAGTCTTACTCTG
GGGGCAACATCATGAGCAACATGTTCACCATTGGCAAAGAATGTGAAATGCAGACCATGGGGGGCA
AGAAGTTCAAG **144**
GTGAGAGACCCCTGGCCGTCCTTCCAGCCAGAAGTCAGCTTCTCTACTTCAGCTATGACCCCTCTG
CTCCTCAGGTTGACCTTCAGCATAAAAAGCTTTCTTTCTTTCCTTCTTTCTTCCTTCCTTCCTTTCT
TCCTAATTGGTTATTTTATTTATTTACATTTCAAATGCTGTCCTCTTCCCATGCAAACCTTTCTTACT
TGGAAATTTAATGTTTCTGCTAGGTAATGGCTTCCTTCAGCAGGAAACACAACACCAAGATAGATAC
AAAGAGCTGGTATGCTGCTGGGTGTGGCGGTGCATATTAATCCCAGGACTTGGGGGACAGAGTTC
CAGACCAATCTAGTCTACATAAGACAGCCAGGAATACACAGAGAAATACTGTCTCAAGGAATGAAA
GAAAAAAAAGAAAGAAAAAAAAGGAAAAAAAAGCTGAGCATTTGCTGAACGTTTAACATAATTCACC
TCANACACTTACTTACTGAATAGTCTACCTACCTACCTGCCTAGCTGTCTACATACCTATCATCTCT
TAATCTCTCATCTCTCTCTCCTATCTGTCCACGTATCTATCATTGAGGGTGGTGTGGTATAGGGG
TTGNGTGGTNTGGGGGTAGTGTTANAGGCATACACTGTCATGAGCAGCTTTAAAAAAAAAATAAGA
GTGCTGGGAATCTGAACTCCAGTNTTTATCCTCTGAGNNATCTTCCCCCCACCCCTCCCTTCCCCT
TGGTTTCGAGACCCTTGGGCAGGGCAGGCAATCATTTTGTCAACGGAGGCCAGCCCTACAGGTGT
TATTTTTAAAATCGACCTAAGCGATGCCAGAAAGGAATCATTAGGCCGTGACTAGAGGCTTCATATT
CAATGCTCCCTCTC TCTCGTAGGCAACCGGAAGATGGAGGGGGCAAGGGGNGTAG **1063**
GCAACCGTGAAGATGGAGGGTGGCAAGGTGGTGGCAGACTTCCCCAACTATCACCAGACTTCGGA
GGTCGTGGGTGACAAGTTGGTGGAG **1153**
GTGAGTGTGCAAAGGTTCCTACTATTTCTATTTTTTTTTTTTTTTANTTTTTTTTCGGAGCTGGGGA
CCGAACCCAGGACCTTGCGCTTGCTAGGCAAGCGCTCTACCACTGAGCTAAATCCCCCAACCCCCC
TACTATTTCTATTTTAAAGGGGCGCCTGCTGAGGCTCAGGGACAAGCCCGTGGCTTGCCTCAGGTCA
CACAGCTAGTACACCATGGAGGCAAGACCAGCTGACTTCTGGAGGATGTTCTCAGCCTCTCCTAGAG
GATATAGGGCATAAGGAAAAGAACCAAAGTTAGTGTCTTCTGCTCTGGGTGAAAACTGGCCCGTTGG
AAGATTTGATGAGAGCACCTTTTGATTTGATTTGAATACTTTTCCGTGGTGAAATGTCAGTCTGTAGG
TCTTGATTTGCAAGCAATGTTCCCCAGCCTGAGGTGTGCAGGGAGAGCTGAGGGCAGGAGACATGG
AGCAGCAGGTTTGTGTTCACAAGACTTAATCTTCAAACAATGAGCCAACCCTCAGTTTGCCCCTCTGT
ATAATGGGAACAACTGGGGCTCTTGGGAATTAGAGATGGAATAGTATGAAAAGGGGCCTGGCATGG
AGTAGTCAAAAGGAGCAGTCATCACATAGAGGCTAACAGGTCTGCAGCCCATCACTCTTGCCCCTGC
TCAGCCTTTTACCCCATTTTTCTGCCGCGTGACTCCCGTAACTGACAGGGAATCTAAGGCCCAGAAA
GCAGTGTGCTTGCCTTGCATCCACCCAGGATTAGCAATGGTAGCTGGCCTTCCCTTCAGCTGTGCCC
CTCGATCATGTCAGGGCCACCGCCTGAGGGAATACTCTGACTCCCTCCCTCTGATCTGGTTTCTTCT
TTCAG **2028**
ATCTCCACCATCGGGGATGTGACC **2052**

Fig 1. The sequence of *Fabp6* as found in BN.*lx*/Cub and SHR/OlaIpcv (Genbank acc. nrs. : AY049763 and AY049762, respectively).

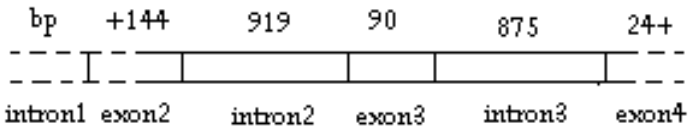

Fig. 2. Primary structure of the rat *Fabp6* gene.

Exon2:
AACTTCAAGATCATCACAGAGGTCCAGCAGGATGGAGAGAACTTCACCTGGTCCCAGTCTTACTCTGGG
GGCAACATCATGAGCAACATGTTCACCATTGGCAAAGAATGTGAAATGCAGACCATGGGGGGCAAGAAG
TTCAAG

Exon3:
GCAACCGTGAAGATGGAGGGTGGCAAGGTGGTGGCAGACTTCCCCAACTATCACCAGACTTCGGAGGT
CGTGGGTGACAAGTTGGTGGAG

Exon4:
ATCTCCACCATCGGGGATGTGACC.

Fig. 3. The sequence of *Fabp6*-ps as found in SHR/OlaIpcv (GenBank acc. nr.: AY049764).

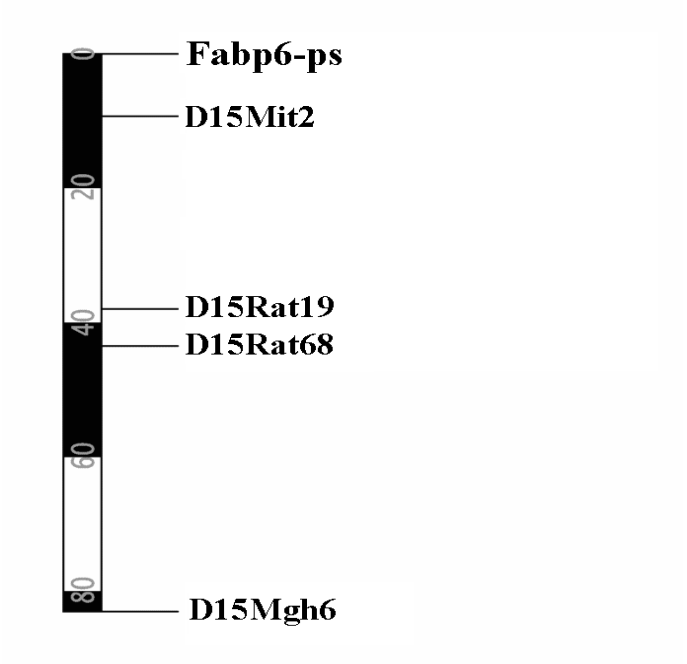

Fig. 4. The genetic map of RNO15 based on (BNxSHRSP) x BN backcross (n = 366) with the *Fapb6*-ps included.

Discussion

The fatty acid-binding proteins (FABPs) belong to a conserved group of cytosolic low mass proteins. The nine family members have 20-70 % identity in their amino acid sequence in human and mouse. All members have unique tissue-specific expression patterns. It is suggested that the proteins have specialized functions in different tissues[3]. The human *FABP6* (fatty acid binding protein 6 or ileal lipid binding protein) gene encodes a protein that appears to be the cytosolic receptor for bile acids that have undergone sodium-dependent active transport into the enterocyte[8]. In a previous study we analyzed liver cholesterol concentration in rat recombinant inbred (RI) strains derived from the progenitors BN.*lx*/Cub and the SHR/OlaIpcv. A quantitative trait locus (QTL) was found on chromosome 10 in the vicinity of D10Mit4[1]. *Fabp6* was suggested as a candidate gene, but the location of this gene in the rat still needed to be established.

In the search for rat *Fabp6* polymorphism, in the present study exon2 through exon4 were amplified and sequenced but the sequence in rat inbred strains BN and SHR were found to be identical. After alignment of the sequence with the *Fabp6* sequence of the mouse and the mRNA sequence of the rat[4,12], the exons could be distinguished from the introns. The sequences of the three exons (144 bp, 90 bp and 24 bp) were identical to the sequence as reported by Gong et al. [12]. But when compared to the mouse, four bp in exon2 and two bp in exon3 were found to be different. In the mouse the introns are 995 bp and 1295 bp, whereas in rats these are 919 bp and 875 bp, respectively.

Linkage analysis revealed *Fabp6* to be located on chromosome 10, close to D10Got43. In the high density integrated genetic linkage map, as published by Steen et al. [15], this marker is linked to D10Rat41 (279 cR; 28,4 cM) which is closely linked to the QTL marker D10Mit4[1]. Recently, another gene involved in the hepatic control of lipid metabolism, sterol regulatory element binding transcription factor 1 (*Srebf1*), has been mapped on RNO10[16]. But *Fabp6* maps more closely to the QTL than *Srebf1*.

We have found evidence for the existence of a *Fabp6* pseudogene (*Fabp6*-ps) on rat chromosome 15 in the vicinity of the marker D15Mit2. Prinsen and co-workers[17] have reported the occurence of a *Fabp3* pseudogene in humans. Pseudogenes may arise from gene duplication and increased crossing-over between homologous chromosomal regions or by reverse transcription from mRNA. The absence of introns and the location of *Fabp6*-ps on a different chromosome than *Fabp6* seem to indicate that the origin of *Fabp6*-ps is through reverse transcription ('processed gene') rather than through gene duplication and crossing-overs. It is unlikely that *Fabp6*-ps is coding for a physiologically active product. Most nucleotide sequences that consist of exons without introns are inactive. Also the strain distribution pattern

is not supportive for an essential role in the animal's physiology. The pseudogene is found to occur in only four out of 66 rat inbred strains. These four strains (SHR; SHRSP; WKY and OKA) all originate from the same outbred Wistar colony at the Kyoto School of Medicine, Japan[18]. The SHR and the SHRSP develop spontaneous hypertension but the WKY strain is frequently used as the normotensive control strain for the SHR. But, though there is no evidence for a present function, pseudogenes may play a major role in the evolution of species since these DNA sequences can readily serve as reservoirs to be changed into genes with new functions .

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Chapter IV

A rat linkage map based on BC x LEW intercross

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Folia Biologica Praha, 2002, 48:120-123

Abstract

A genetic linkage map consisting of 258 polymorphic loci has been constructed on the basis of an F_2 intercross between the BC/CpbU and LEW/OlaHsd inbred rat strains. When compared to previously published maps a discrepancy was found for rat chromosome 7. The map spans a sex-averaged genetic length of 1790 cM and has an average marker spacing of 7.7 cM. It was estimated that this genetic map is linked to about 90% of the DNA in the rat genome. Because LEW/OlaHsd and BC/CpbU strains differ for dietary cholesterol susceptibility and hepatic copper content, the map is considered to be a valuable tool for studying the genetic background of these complex traits.

Introduction

The laboratory rat provides several models for human diseases and is increasingly being used for genetic studies. Our group has focused on the genetic analysis of rat strain differences in dietary cholesterol susceptibility[1] and hepatic copper content[2]. Previously, we have searched for the genetic components associated with these phenotypes using a set of recombinant rat inbred strains derived from SHR/OlaIpcv and BN-*Lx*/Cub progenitor strains. These strains differ for the two parameters mentioned above[3,4,5]. The BC/CpbU (BC) and LEW/OlaHsd (LEW) strains also differ for dietary cholesterol susceptibility[6] and liver copper content[2]. This prompted us to produce an F_2-intercross derived from the BC and LEW rat inbred strains. Before quantitative trait loci (QTLs), controlling these phenotypes, can be identified, a genetic map based on genotype data from the cross between BC and LEW must be developed. Here we report this genetic map. It is based 192 F_2-intercross progeny and 258 DNA markers (mainly microsatellites) covering all 20 autosomes and the X chromosome.

Materials and Methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals and housing.

All animals were kept under SPF conditions and a 12 hours per day light-regimen (7.00 h - 19.00 h). The other laboratory conditions, temperature and humidity, were also controlled. The LEW/OlaHsd (obtained from Harlan, UK) and BC/CpbU (obtained from the Central Laboratory Animal Institute of the Utrecht University, The Netherlands) strains, which differ for dietary cholesterol susceptibility[6] and liver copper content[2], were used as progenitor strains. The F_1-generation was derived by reciprocal matings of LEW and BC animals. One [BC female x LEW male] F_1 male was mated with seven F_1 females to produce the F_2-progeny (92 males and 100 females; 2-3 litters/F_1 female). Three F_2 females were found to possess the LEW genotype on the X-chromosome, probably due to non-disjunction of the sex-chromosomes in the F_1 male. These three animals were excluded from the construction of the linkage map of the X-chromosome.

Genome scan.

DNA of each F_2 animal and the (grand-)parents was extracted from the spleen using a standard procedure[7]. A total of 256 microsatellite (SSLP) markers, one RFLP (for the *Lcat* gene, coding for lecithin cholesterol acyl transferase[3]) and a 17-bp repeat (see below), polymorphic between the BC and LEW strain, were used for screening. These markers were dispersed throughout the rat genome. Primers flanking the rat microsatellite markers (defined as D*x*Wox*y* (n = 2), D*x*Arb*y* (n = 6), D*x*Mco*y* (n = 1), D*x*Mit*y* (n = 3), D*x*Mgh*y* (n = 2) and D*x*Rat*y* (n = 242)) were obtained from Research Genetics Inc. (MapPairs™, Huntsville, USA). The primers flanking the 17-bp repeat (coding for mevalonate pyrophosphate decarboxylase), were based on the paper of Kato *et al.*[8] and were synthesized by Amersham Biosciences (Buckinghamshire, UK). When polymorphisms between BC and LEW differed less than 10 basepairs, the forward primer was 5'-end labelled with [γ-^{32}P]dATP (Amersham, 10 µCi/µl). Twenty ng genomic DNA was used in the PCR mixture. After the PCR reaction, the products were heated for 5 minutes at 95°C and then separated on a standard 5% (w/v) polyacrylamide gel[9]. If the polymorphism of the microsatellites between BC and LEW differed more than 10 bp, 100 ng genomic DNA was used for the PCR reaction. The non-radioactive PCR products were separated on a 3% (w/v) agarose gel and visualized by ethidium bromide staining (0.5 µg/ml PCR products). The PCR reaction was performed under standard conditions.

All genotype scoring was done independently by two persons. After one turn of genotype scoring, all differences between the independent reads were checked. All indications of a double recombination event were re-scored and, if necessary, re-typed. In the LEW inbred strains heterozygosity was found for 4% of the markers whereas no heterozygosity was found in the BC parental strain.

Map construction.

The segregation ratio for the individual markers was checked by means of the Chi-squared goodness-of-fit test. None of these markers had a significant segregation distortion. The genetic map distance for the markers was computed with the computer package JoinMap™, version 3.0[10]. For the establishment of linkage groups we used a critical minimal LOD score of 3.0. For calculation of map distances and estimating most likely gene orders, we used a critical LOD score of 0.05. Recombination frequencies were converted to map distances in centiMorgans with the Kosambi function. Output from JoinMap™ was converted to figures using the graphics program MapChart[11].
Linkage groups were merged where they were known to be separate segments of a chromosome. Output from Map Manager was converted to linkage maps using the graphics program MapChart.[29]

Results and Discussion

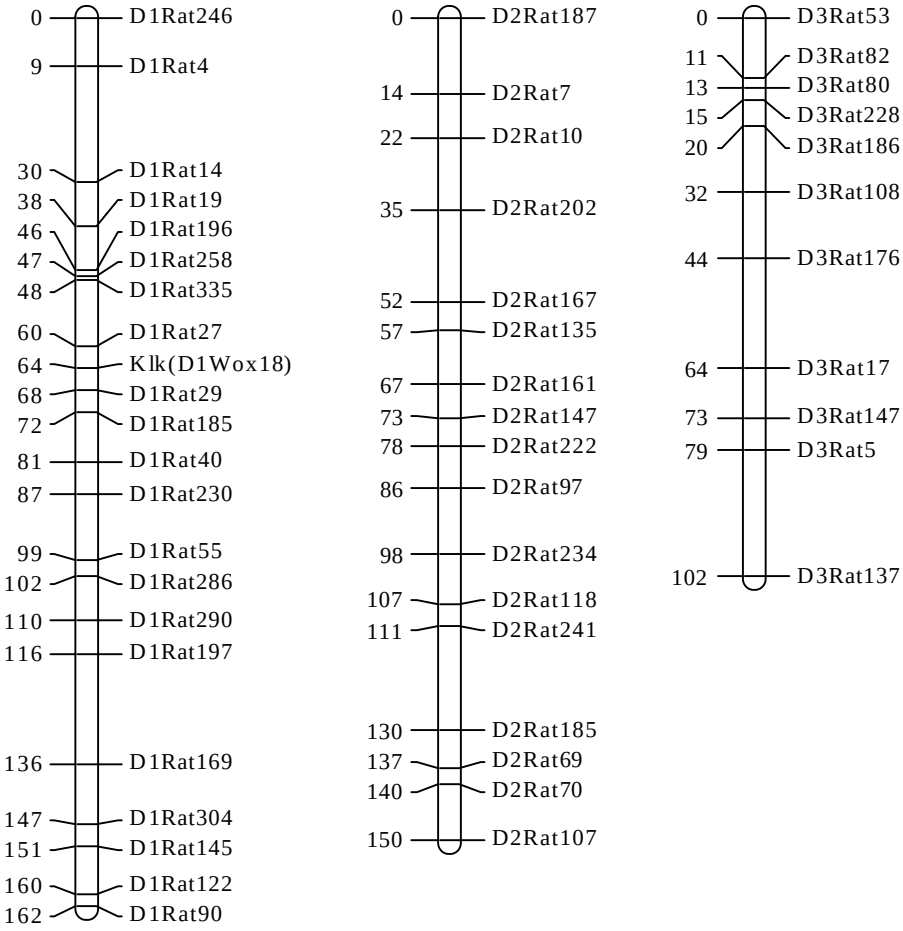
Table 1 and Fig. 1 show the marker distribution on the rat chromosomes. The generated map spans a total length of 1790 cM with an average spacing of consecutive markers of 7.7 cM. The largest gap is 26.1 cM (on the X chromosome). The total length of the rat genome might be estimated according to method 3 of Chakravarti *et al.*[12]. Using this method we estimated the genetic length of the rat genome to be 1993 cM. Thus the present genetic map covers about 90% of the rat genome.

The order of the markers in our genetic map was generally consistent with other published rat maps. However, when compared with the high-density map of MIT based on the (SHRSPxBN)F_2 intercross[13] the position of D7Rat111 and D7Rat94 is reversed. This discrepancy might be caused by the fact that we have used a relatively large intercross (192 rats) when compared with the intercross of MIT (46 rats).

In the mouse, within-strain microsatellite polymorphism of up to 30% of these markers has been found in C3H, but not in DBA, C57BL or BALB/c inbred strains[14]. The 4% polymorphic microsatellites as found in the LEW inbred strain but not in the BC strain might be due to a similar strain specific difference in microsatellite polymorphism. Since for each of the polymorphic microsatellite markers neither of the two LEW alleles were identical to the BC allele, this polymorphism in the parental strain did not hamper the construction of the genetic map.

In summary, we believe that our map, together with other maps facilitates the genetic characterization of the rat and contributes to the rat as a model organism for human disease studies.

Fig. 1. A genetic linakge map of 258 loci of the rat. The names of the DNA markers are listed on the right, and distances (cM, Kosambi mapping function) are listed on the left.


RNO1
0 D1Rat246
9 D1Rat4
30 D1Rat14
38 D1Rat19
46 D1Rat196
47 D1Rat258
48 D1Rat335
60 D1Rat27
64 Klk(D1Wox18)
68 D1Rat29
72 D1Rat185
81 D1Rat40
87 D1Rat230
99 D1Rat55
102 D1Rat286
110 D1Rat290
116 D1Rat197
136 D1Rat169
147 D1Rat304
151 D1Rat145
160 D1Rat122
162 D1Rat90
RNO2
0 D2Rat187
14 D2Rat7
22 D2Rat10
35 D2Rat202
52 D2Rat167
57 D2Rat135
67 D2Rat161
73 D2Rat147
78 D2Rat222
86 D2Rat97
98 D2Rat234
107 D2Rat118
111 D2Rat241
130 D2Rat185
137 D2Rat69
140 D2Rat70
150 D2Rat107
RNO3
0 D3Rat53
11 D3Rat82
13 D3Rat80
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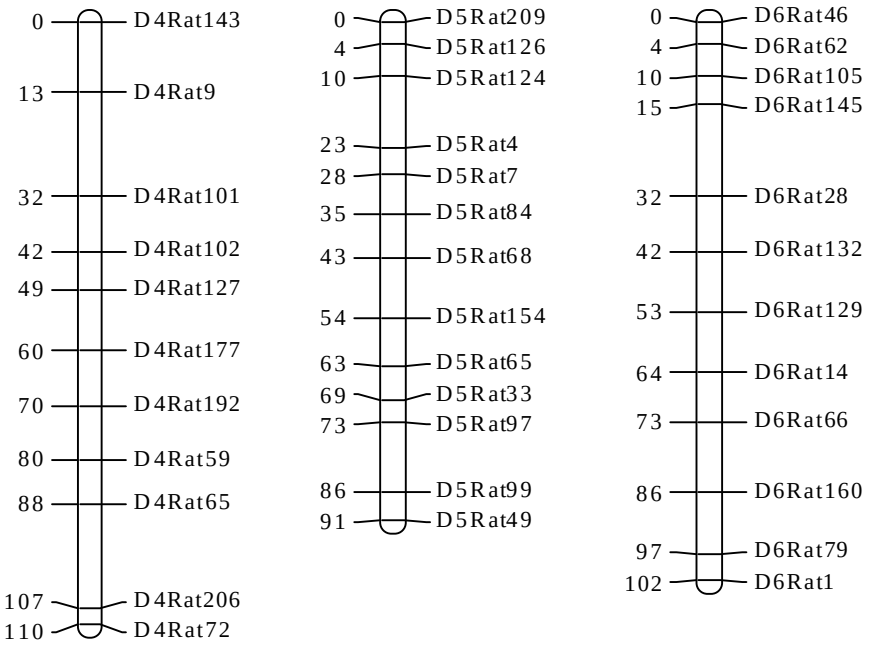
64 D3Rat17
73 D3Rat147
79 D3Rat5
102 D3Rat137


RNO4
0 D4Rat143
13 D4Rat9
32 D4Rat101
42 D4Rat102
49 D4Rat127
60 D4Rat177
70 D4Rat192
80 D4Rat59
88 D4Rat65
107 D4Rat206
110 D4Rat72
RNO5
0 D5Rat209
4 D5Rat126
10 D5Rat124
23 D5Rat4
28 D5Rat7
35 D5Rat84
43 D5Rat68
54 D5Rat154
63 D5Rat65
69 D5Rat33
73 D5Rat97
86 D5Rat99
91 D5Rat49
RNO6
0 D6Rat46
4 D6Rat62
10 D6Rat105
15 D6Rat145
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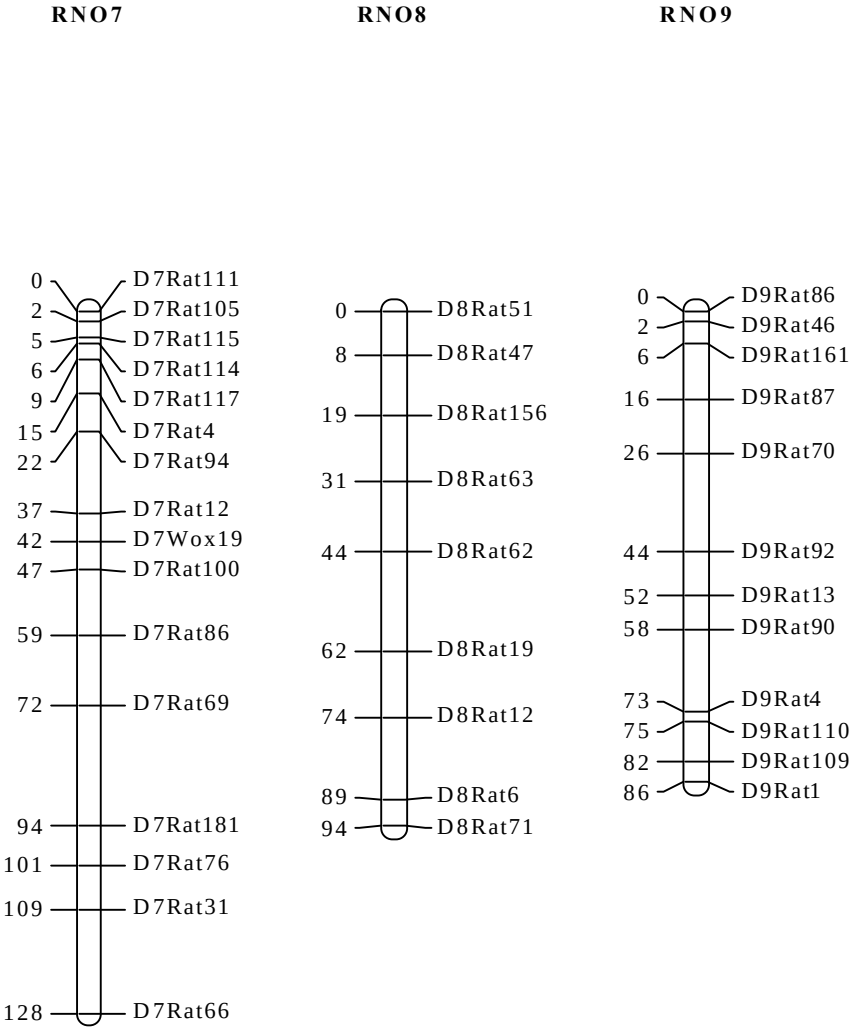
73 D6Rat66
86 D6Rat160
97 D6Rat79
102 D6Rat1


RNO7
RNO8
RNO9
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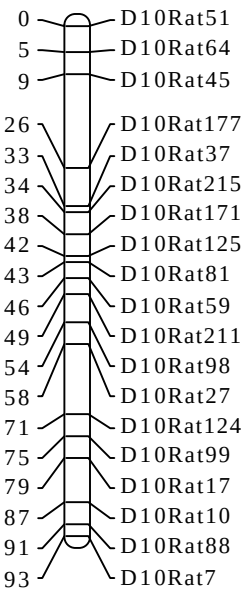
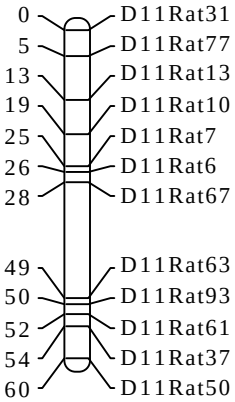
2 D7Rat105
5 D7Rat115
6 D7Rat114
9 D7Rat117
15 D7Rat4
22 D7Rat94
37 D7Rat12
42 D7Wox19
47 D7Rat100
59 D7Rat86
72 D7Rat69
94 D7Rat181
101 D7Rat76
109 D7Rat31
128 D7Rat66
0 D8Rat51
8 D8Rat47
19 D8Rat156
31 D8Rat63
44 D8Rat62
62 D8Rat19
74 D8Rat12
89 D8Rat6
94 D8Rat71
0 D9Rat86
2 D9Rat46
6 D9Rat161
16 D9Rat87
26 D9Rat70
44 D9Rat92
52 D9Rat13
58 D9Rat90
73 D9Rat4
75 D9Rat110
82 D9Rat109
86 D9Rat1

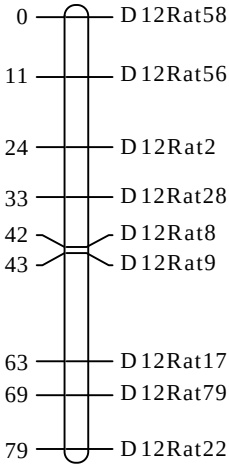
RNO10

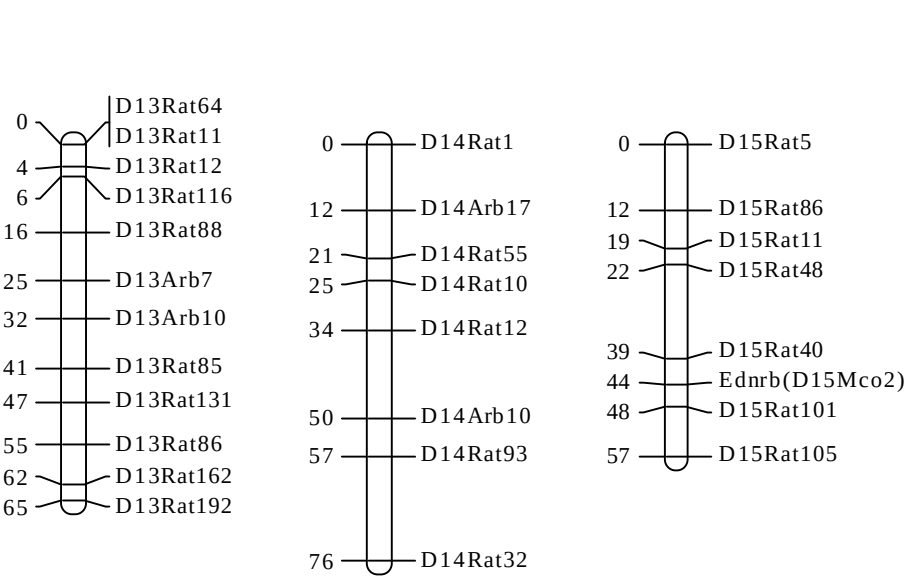


RNO11

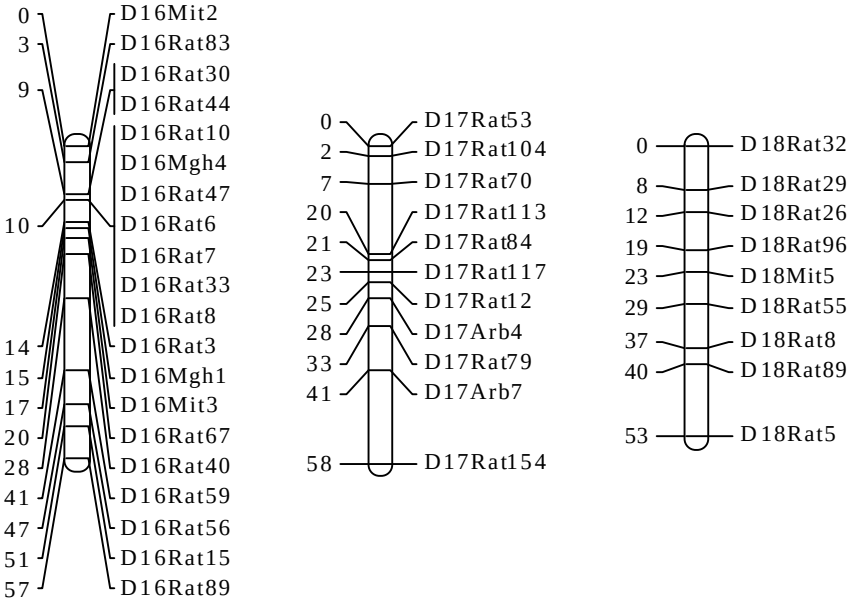


RNO12




RNO13
RNO14
RNO15
0
4
6
16
25
32
41
47
55
62
65
D13Rat64
D13Rat11
D13Rat12
D13Rat116
D13Rat88
D13Arb7
D13Arb10
D13Rat85
D13Rat131
D13Rat86
D13Rat162
D13Rat192
0
12
21
25
34
50
57
76
D14Rat1
D14Arb17
D14Rat55
D14Rat10
D14Rat12
D14Arb10
D14Rat93
D14Rat32
0
12
19
22
39
44
48
57
D15Rat5
D15Rat86
D15Rat11
D15Rat48
D15Rat40
Ednrb(D15Mco2)
D15Rat101
D15Rat105

RNO16 **RNO17** **RNO18**



RNO19 **RNO20** **RNOX**

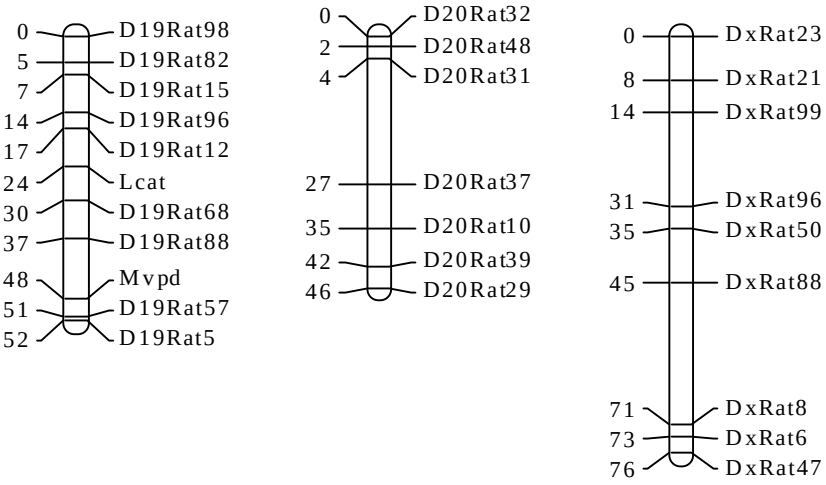


Table 1. Distribution of the polymorphic DNA markers by chromosome

		Marker distribution			
	Number of markers	Maximal distance between adjacent markers (cM)	% chromosome within 5 cM of markers	% chromosome within 10 cM of markers	% chromosome within 20 cM of markers
RNO					
1	22	21	38	76	90
2	17	20	25	56	100
3	11	23	20	50	90
4	11	19	10	50	100
5	13	13	25	75	100
6	12	16	18	45	100
7	16	22	33	67	93
8	9	18	13	25	100
9	12	19	36	73	100
10	19	18	78	89	100
11	12	20	45	91	91
12	9	20	13	63	88
13	12	10	36	100	100
14	8	19	14	57	100
15	8	17	29	71	100
16	18	13	71	88	100
17	11	18	60	80	100
18	9	13	38	88	100
19	11	11	50	90	100
20	7	23	50	83	83
X	9	26	28	75	88

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phenotypes map to chromosomes that contain genes regulationg blood pressure in the spontaneously hypertensive rat. *J Clin Invest*. 98:856-862.

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Chapter V

Quantitative trait loci influencing blood and liver cholesterol concentration in rats

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submitted

Abstract

The LEW/OlaHsd and BC/CpbU rat inbred strains differ markedly in blood and hepatic cholesterol levels, both before and after a cholesterol-rich diet. To define loci controlling these traits and related phenotypes, an F_2 population derived from these strains was genetically analyzed. For each of the 192 F_2 animals phenotypes were determined and genomic DNA was screened for polymorphic microsatellite markers. Significant quantitative trait loci (QTLs) were detected for basal serum cholesterol level on chromosome 1 (*D1Rat335-D1Rat27*; total population, LOD: 9.6; females, LOD: 10.3) and chromosome 7 (*D7Rat69*; males, LOD: 4.1), for postdietary serum cholesterol level on chromosome 2 (*D2Rat69*; total population, LOD: 4.4) and chromosome 16 (*D16Rat6-D16Rat44*; total population, LOD: 3.3), for postdietary serum phospholipid level on chromosome 11 (*D11Rat10*; total population, LOD: 4.1; females, LOD: 3.6), and for postdietary serum aldosterone level on chromosome 1 (*D1Rat14*; females, LOD: 3.7) and chromosome 18 (*D18Rat55-D18Rat8*; females, LOD: 2.9). In addition, QTLs with borderline significance were found on chromosomes 3, 5-11, 15 and 18.

Introduction

Atherosclerosis is a complex disorder in which genetic as well as environmental factors play a role. A high serum cholesterol concentration is one of the risk factors in the development of this disease. Circulating cholesterol levels do not only reflect dietary habits; epidemiological studies revealed consistent higher than average serum cholesterol levels only in particular individuals after a high dietary cholesterol intake.[1] Individual differences in serum cholesterol concentration exist also after a diet with low fat and/or cholesterol content. Similar variability in serum cholesterol levels can also be observed in laboratory animals like mice[2], rabbits[3] or rats[4] in response to control diets as well as high fat and high cholesterol diets.[5-8] Differences, observed between inbred strains of these species indicate that both the basal serum cholesterol concentration and the rate of increase in serum cholesterol levels after a cholesterol-rich diet are under genetic control.

In order to genetically analyse these phenotypes in laboratory rats, we performed a total genome scan of an F_2 population derived from the BC/CpbU and LEW/OlaHsd rat inbred strains. Both cholesterol (basal and postdietary serum level and postdietary liver concentration) and cholesterol-related traits (postdietary serum phospholipid, aldosterone and corticosterone levels) were measured in the F_2 animals. The cholesterol-related traits were included into this study because in the F_2 population these parameters were significantly associated with postdietary serum cholesterol level. The results of this QTL-analysis and possible candidates genes located in the vicinity of some QTLs will be discussed.

Methods

Animals and housing.

All animals were kept under SPF conditions with 12 hours per day light (7.00 h - 19.00 h) and controlled temperature and humidity regimen. Six males and six females of the inbred strains BC/CpbU (BC) (obtained from the Central Laboratory Animal Institute of the Utrecht University, The Netherlands) and LEW/OlaHsd (LEW) (obtained from Harlan, UK) were tested for cholesterol response, as described in the experimental protocol (see below). From each gender and strain the two out of six most extreme (serum cholesterol) responding animals were used for reciprocal matings. The F_1-hybrids were intercrossed producing an F_2 progeny.

Experimental protocol.

After weaning, at the age of three weeks, the animals were fed a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands) till the age of seven weeks. The chemical composition of this commercial diet has previously been described.[7] Then, at the age of seven weeks, the commercial diet was supplemented with 5.0% (w/w) olive oil (Reddy, Van de Moortele NV, Oudenbosch, The Netherlands) and 2.0% (w/w) cholesterol (USP, Solvay/Pharmaceuticals BV, Weesp, The Netherlands) during four weeks. This pelleted test-diet had been stored in the freezer until use. The animals had free access to food and water before and during the test-period. At the start and at the end of the test-period, after a 16 hour fast, blood samples were taken by orbital puncture under diethyl-ether anaesthesia (between 8.00 and 10.00 h). Serum was collected after centrifugation and stored at -20°C. Following the last blood sampling, the rats were anaesthetised with diethyl-ether, exsanguinated via aorta puncture and the livers and spleens were removed. The tissues were frozen immediately at -70°C.

Chemical analyses.

In a total of 192 F_2-animals (92 males and 100 females) basal serum cholesterol levels, postdietary liver cholesterol concentrations, and serum phospholipid, cholesterol, aldosterone and corticosterone levels were determined. Lipids were extracted from liver homogenates according to a modification of the method of Abell *et al.*[9] Liver cholesterol, serum cholesterol and serum phospholipids were measured enzymatically using the appropriate kits supplied by Boehringer Mannheim GmbH (Mannheim, Germany). Serum aldosterone and corticosterone concentrations were determined by radioimmunoassays as previously described.[10]

Genome scan.

DNA was isolated from the spleen using a standard procedure.[11] A total of 256 microsatellite (SSLP) markers, polymorphic between the BC and LEW strain, were used for screening of the F_2-progeny. These markers were randomly dispersed throughout the rat genome. Primers flanking the microsatellites were obtained from Research Genetics Inc. (MapPairs™, Huntsville, USA). When a microsatellite in BC and LEW differed less than 10 basepairs, the forward primer was 5'-end labelled with [γ^{32}P]dATP. Twenty nanogram genomic DNA was used for PCR according to manufacturers protocol. Products were separated in standard sequencing gels.[12] When the microsatellites differed more than 10 bp between BC and LEW, 100 ng genomic DNA was used in the PCR reactions. These non-radioactive PCR products were separated in standard agarose gels.

Map construction.

Segregation ratio of the genotypes of individual markers was checked by means of the Chi-squared goodness-of-fit test. None of the markers showed significant segregation distortion. The genetic map distance for the markers was computed with JoinMap™, version 3.0.[13] For the establishment of linkage groups, a critical minimal LOD score of 3.0 was used. For calculation of map distances and estimating most likely gene orders, a critical LOD score of 0.05 was used. Recombination frequencies were converted to map distances in centiMorgans using the Kosambi function. Output from JoinMap was converted to figures using the graphics program MapChart[14].

Statistical analysis.

Both for the parental strains and for the F_2-intercross rats, all statistical analyses of the measured phenotypes were carried out according to Petrie and Watson[15] using a SPSS PC+ computer program.[16] Two-side probabilities were estimated throughout.

a. Parental strains.

The phenotypic characteristics of the BC and LEW rats were checked for normality using the Kolmogorov-Smirnov one-sample test. All results within groups were found to be normally distributed. Student's one sample *t* test for paired data was used to evaluate changes with time within groups. The significance of the differences between groups was calculated by a two-way analysis of variance (ANOVA) with strain and gender as main factors. Homogeneity of the variances was tested by Bartlett's test. For some phenotypes the variances had to be equalized by transformation (i.e. logarithmic) of the data.[15] After transformation the variances were similar and the transformed within-group data still were normally distributed. For the serum and liver parameters the two-way ANOVA was performed with body weight as covariate, because there were significant differences in body weight between the two strains. If the ANOVA showed significant effects the group means were further compared with the unpaired Student's *t* test. These tests were performed with pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested using a *F* test. To take into account the greater probability of a type I error due to multiple comparisons, the level of significance for the Student's *t* tests was pre-set at $P<0.05$/[times a group is used for a comparison] instead of $P<0.05$, according to Bonferroni's adaptation.[15] In all other cases, the probability of a type I error <0.05 was taken as the criterion of significance.

b. F_2-animals.

Within each gender, all traits were normally distributed (Kolmogorov-Smirnov one-sample test). Gender appeared to have a significant influence on the phenotypes (unpaired Student's *t* test, $p<0.05$), except on final body weight and baseline serum cholesterol level. For the genetic analysis of the gender-influenced traits in the combined male and female population, these phenotypes were first normalized in each gender. The measured levels were subtracted by the mean established for that gender and then divided by the standard deviation of that gender. Within the combined male and female population the (transformed) variables were normally distributed according to the Kolmogorov-Smirnov one-sample test.

QTL analysis.

The location of the QTLs affecting the measured (transformed) quantitative traits were determined using the interval-mapping module of the MapQTL computer package (version 4.0).[17] QTL analysis was also performed by MQM-mapping[17] on the MapQTL computer program. QTL-likelihood plots were produced by using the markers that flank the LOD score peak of identified QTLs in the interval-mapping method, as cofactors for QTL mapping on the MapQTL computer program.

For each trait and chromosome the LOD score thresholds were calculated by permutation analysis[18] in order to achieve the genome-wide significance levels of 5%. Based on the paper of Lander and Kruglyak[19] an association was assumed suggestive when the LOD score was between 57.6% and 100% of the LOD score threshold for significance. If a DNA marker and the trait of interest are segregating independently, the values of the trait will be equally distributed among the homozygote and heterozygote genotypes. All data within genotype groups were found to be normally distributed by using the Kolmogorov-Smirnov one-sample test. For each group (females and males, females or males), co-segregation of phenotypes with alleles at marker loci was evaluated by comparing the values between different genotypes via one-way ANOVA with or without body weight as co-variate. For serum and liver parameters body weight was used as co-variate, since in F_2 rats there were weak, but significant, associations between these parameters and body weight. In the ANOVA tests, homogeneity of variances was tested (Bartlett's test). When necessary, the variances were equalized by logarithmic or logistic transformation of the data.[15] After transformation the within-group data were still normally distributed.

Results

Parental strains (Table 1)

At the beginning of the test period all rats were of the same age, but body weight of LEW rats, when compared to BC rats, was higher. Males and females of the BC strain had equal body weights. In contrast, initial body weight of LEW males was higher when compared with LEW females. As would be expected body weight increased, in an identical fashion, in the two inbred strains during the course of the experiment. At the end of the test period LEW rats had a higher body weight than BC rats and males were significantly heavier than females.

Pre-experimental serum cholesterol levels (baseline values) of the BC rats were significantly higher than those of LEW rats. Baseline serum cholesterol levels were similar in males and females of the BC strain. In LEW females these levels were significantly higher when compared with LEW males. Since there were significant differences in initial body weight, group means of baseline serum cholesterol levels were also compared with an ANOVA test with initial body weight as covariate. Now, the gender effect on baseline serum cholesterol concentration disappeared, but there tended to be a strain effect.

The high-fat, high cholesterol diet produced in LEW, but not in BC rats (in fact in BC males there was a significant decrease), an increase in serum total cholesterol level. This increase was more pronounced in female when compared with male LEW rats. Gender and strain significantly affect final serum cholesterol concentration. However, after correction for body weight the sex-effect was borderline significance, but the strain-effect was still highly significant. Group mean postdietary serum phospholipid levels of the LEW strain were higher than in the BC strain; this strain effect reached the level of statistical significance only in the females. After correction for body weight there was a significant strain-effect.

Although serum cholesterol levels in males of the BC strain responded with a decrease to a high fat, high cholesterol diet, the postdietary liver cholesterol concentration was significantly higher in male BC rats when compared with male LEW rats. Females when compared with males of the LEW, but not of the BC strain, had significantly elevated hepatic cholesterol levels. In the two-way ANOVA with final body weight as covariate these effects did not reach the level of statistical significance. Group mean circulating adrenal steroids levels were higher in BC rats when compared with LEW rats. However, after correction for body weight this strain effect was only significant for serum aldosterone concentration.

Genetic mapping of quantitative traits (Tables 2 and 3, Figs. 1-5)

The results of the QTL analysis using the MapQTL software are summarized in Table 2. For initial body weight male or female-specific QTLs were mapped on chromosomes 5 and 7 (Fig. 1A), respectively. QTLs for males *plus* females were found for initial body weight on chromosomes 15 (Fig. 1B) and 18, and for final body weight (females; males *plus* females) on chromosome 17. Kovacs *et al.*[20] and Klöting *et al.*[21] also found QTLs influencing body weight on rat chromosomes 5 and 18.

The genome-wide scan revealed regions with significant linkage of the cholesterol and related phenotypes to loci on chromosomes 1, 2, 7, 11, 16 and 18 (Figs. 2-5 and Table 2). QTLs with borderline significance were found on rat chromosomes 3, 5-11, 15 and 18 (Table 2). Table 2 also illustrates the existence of sex-specific and sex-independent QTLs.

Table 3 shows the phenotypes of each of the three genotypes (LL, LB and BB) segregating at selected loci on chromosomes that were significantly associated with a trait. The allele frequencies were not statistically different ($p>0.05$) from the expected ratio, 1(LL):2(LB):1(BB) with one exception: *D1Rat335* in females. However, the closely linked marker *D1Rat27* showed no segregation deviation in females, and the ANOVA results of both *D1Rat27* and *D1Rat335* ($p<0.001$) point to a QTL also in females; the LOD score for baseline serum cholesterol level being somewhat higher with *D1Rat335* as a marker. Therefore, taken together, the segregation distortion at *D1Rat335* is not a serious problem for the quantitative trait linkage analysis.

Table 1. Some phenotypic characteristics of the BC and LEW rats[a]

	Strain							*Post-hoc* comparison			
	BC		LEW		Anova[b]			males vs. females		BC vs. LEW	
Measure	**males**	**females**	**males**	**females**	S	G	S x G	BC	LEW	**males**	**females**
Body weight (g)											
Day 0	109±13	106±5	217±13	160±8	**<0.001**	**<0.001**	**<0.001**	0.530	**<0.001**	**<0.001**	**<0.001**
Day 28	223±17	177±4	341±35	219±12	**<0.001**[e]	**<0.001**[e]	0.950[e]	**0.001**	**<0.001**	**<0.001**	**<0.001**
0 vs. 28[d]	**<0.001**	**<0.001**	**<0.001**	**<0.001**							
Serum cholesterol level (mmol/l)											
Baseline	3.5±0.3	3.5±0.2	2.6±0.1	2.9±0.1	0.075	0.769	0.496	0.907	**0.002**	**<0.001**	**<0.001**
Postdietary	2.4±0.4	3.8±0.6	4.5±1.5	15.6±6.1	**<0.001**[e]	0.055[e]	0.597[e]	**0.001**	**0.006**	**0.015**	**0.005**
B vs. P[d]	**0.004**	0.154	0.018	**0.004**							
Serum phospholipid level (mmol/l)											
Postdietary	1.5±0.2	1.6±0.2	1.7±0.5	2.6±0.6	**0.018**	0.100	0.476	0.342	**0.011**	0.492	**0.008**

Table 1. continued

Liver cholesterol concentration (ìmol/g wet weight)											
Postdietary	173±27	164±21	1222±10	193±36	0.0814	0.322	0.177	0.621	**0.010**	**0.011**	0.225
Postdietary serum adrenal steroid level (nmol/l)											
Aldosterone	1.4±0.4	1.4±0.5	0.8±0.3	0.6±0.3	**0.011**	0.464	0.890	0.859	0.431	0.035	**0.023**
Corticosterone	184±219	718±422	46±41	204±219	0.487[e]	0.358[e]	0.649[e]	**0.020**	0.173	0.188	0.036

[a] Values are means ± SD for 6 (serum parameters and body weight) or 4 (liver cholesterol concentration) animals per group

[b] P-values in two-way ANOVA with main factors *strain* and *gender*. S, effect of strain; G, effect of gender; SxG, interaction. Significant effects (P<0.05) are indicated in bold characters. For statistical analysis of serum and liver parameters body weight was used as co-variate.

[c] P-values in *unpaired* Student's *t* test. Significant differences (P<0.0167) for serum cholesterol and bodyweight; P<0.025 for the other parameters) are indicated in bold characters.

[d] P-values in *paired* Student's *t* test. Significant differences (P<0.0167) are indicated in bold characters.

[e] ANOVA after logarithmic transformation of the data.

Table 2. Summary of the QTLs.

Phenotypic trait	Population	Peak LOD[2]	Chrom	Meth.[1]	Location
Initial body weight	males	2.2	5	No	D5Rat68-D5Rat154
	females	**3.4**	7	No	D7Rat94-D7Rat12
	males and females	**3.6**	15	No	D15Rat5-D15Rat86
	females	2.1	15	No	D15Rat5-D15Rat86
	males and females	3.0	18	No	D18Rat26
Final body weight	males and females	3.2	17	No	17Rat104
	females	2.5	17	No	D17Rat70
Baseline serum cholesterol	males and females	**9.6**	1	No	D1Rat335-D1Rat27
	females	**10.3**	1	No	D1Rat335-D1Rat27
	females	3.0	5	No	D5Rat4
	males	**4.1**	7	Yes	D7Rat69
	males and females	3.0	11	Yes	D11Rat50
	males	2.5	15	Yes	D15Rat40-D15Mco2
Postdietary serum cholesterol	males and females	**4.4**	2	No	D2Rat69
	males	2.2	3	Yes	D3Rat53-D3Rat82
	females	3.6	5	Yes	D5Rat209
	males and females	2.1	15	Yes	D15Rat105
	males and females	**3.3**	16	No	D16Rat6-D16Rat44
Postdietary serum phospholipids	males and females	3.5	3	Yes	D3Rat53
	males	2.9	3	Yes	D3Rat53
	males and females	2.5	9	Yes	D9Rat109
	males	2.8	9	Yes	D9Rat109
	males and females	**4.1**	11	No	D11Rat10
	females	**3.6**	11	No	D11Rat10
	males	2.7	18	Yes	D18Rat5
Postdietary liver cholesterol	males and females	2.2	6	Yes	D6Rat66
	females	2.3	10	No	D10Rat211
Postdietary serum aldosterone	females	**3.7**	1	Yes	D1Rat14
	females	2.4	7	Yes	D7Rat4-D7Rat94
	females	**2.9**	18	Yes	D18Rat5-D18Rat8
Postdietary serum corticosterone	females	3.9	6	Yes	D6Rat160-D6Rat79
	males	2.9	8	Yes	D8Rat51
	females	2.3	18	Yes	D18Mit5-D18Rat55

1. QTLs were investigated using the MapQTL software on data collected from 192 (LEWxBC) F_2-intercross rats. The interval mapping module with ("*Yes*") or without ('*No*') co-factors was used.
2. Data are shown only when significant of suggestive correlations were found. Significant results are indicated in bold characters.

Discussion

In the present study, genome wide scanning of 192 F_2 animals derived from the rat inbred strains BC and LEW, for associations between marker genotypes and quantitative traits related to dietary cholesterol susceptibility resulted in the localization of quite a few QTLs with borderline significance (on rat chromosomes 3, 5-11, 15 and 18; Table 2) and seven significant QTLs (on rat chromosomes 1, 2, 7, 11, 16 and 18; Tables 2 and 3, Figs. 2-5). The body weight QTLs did not localize to regions were the QTLs for cholesterol and related phenotypes were found (Table 2; Fig. 1A versus Fig. 2B). Thus, it could be excluded that the QTLs for the cholesterol and related phenotypes are QTLs controlling interstrain differences in body weight.

Kovács et al.[22] reported significant linkage of the marker *D1Mit14* with basal serum cholesterol levels in a study of a backcross derived from SHR/Mol and BB/OK rats. Genetic analyses of OLETF x (OLETF x Fischer 344) rats revealed statistically significant linkage between *D1Rat306* and basal serum cholesterol levels.[23] These QTL regions are located at the telomeric part of the q-arm of rat chromosome 1, and do not co-localize with the QTL region *D1Rat335-D1Rat27* as found in this study (Fig. 2A). The markers *D1Rat306* and *D1Mit14* are located at approximately 133 cM in the Rat Genome Database map, whereas *D1Rat27* is located at 45 cM.

Interestingly, the region around *D1Rat27* (Fig. 2a) contains the *Apoe* gene.[24] Apolipoprotein E (APOE) plays a pivotal role in the catabolism of triglyceride-rich lipoproteins by serving as a ligand for lipoprotein receptors. The *Apoe* gene might be a positional candidate for this QTL. In humans, allelic variation of the *APOE* gene has been associated with differences in serum total cholesterol levels.[25]

For mice several genetic analyses have been published that revealed QTLs influencing hepatic or plasma cholesterol concentration after a high-cholesterol diet.[2,26,27] However, to the best of our knowledge this is the first report dealing with rats in which a genome-wide search successfully identified multiple chromosomal regions linked to circulating cholesterol levels *after a high-fat high-cholesterol diet* (Tables 2 and 3, Fig. 3). This study also supports our previous findings of a QTL on rat chromosome 2 influencing postdietary IDL cholesterol levels.[28] Bottger *et al.*[29] localized the gene (*Lpl*) coding for lipoprotein lipase to rat chromosome 16. In the radiation hybrid map *Lpl* is located near the marker *D16Rat6*.[24] In this study the latter marker is located in the vicinity of the QTL controlling serum cholesterol levels on rat chromosome 16. LPL plays a major role in lipoprotein metabolism[30] and in humans LPL mutations have been

associated with atherosclerosis and dyslipidemia.[31] Thus *Lpl* could be the gene on rat chromosome 16 that is controlling postdietary circulating cholesterol levels.

Up until now two QTLs controlling serum phospholipid levels have been described in the rat. Both Bottger *et al.*[29] and Kovács *et al.*[32] found a QTL for basal serum phospholipid levels on rat chromosome 4. Postdietary HDL_2 phospholipids were associated with the same region of rat chromosome 4[32] or with rat chromosome 20.[29] In the present study, we could not confirm the aforementioned associations. However, we now found a QTL for postdietary serum phospholipid levels on rat chromosome 11 (Tables 2 and 3, Fig. 4). As to the gene involved we can only speculate. This segment of rat chromosome 11 is homologous with mouse chromosome 16 and human chromosome 3q, where the gene for CTP:phosphocholine cytidylyltransferase (*Pcyt1a*) is located. This gene is involved in hepatic phospholipid metabolism.[31] Based on homology, one might speculate that this gene is responsible for the strain difference in serum phospholipid level in high-fat, high-cholesterol fed rats.

The reciprocal relationship between cholesterol metabolism and adrenal steroids hormone activity is well established. Therefore, we anticipated that QTL affecting serum cholesterol concentration might also be associated with variations in serum corticosterone. However, no significant QTL for corticosterone were identified. Interestingly, QTLs for aldosterone, were revealed on regions of chromosomes 1 and 18 where QTL for high blood pressure have previously been identified [34,35]. The genes responsible for these aldosterone variations are not known but may, indirectly relate to the regulation of electrolyte metabolism [34,36].

In summary, the present study indicates that rat chromosomes 1-3, 5-11, 15, 16, and 18 each contain at least one QTL involved in blood and/or hepatic lipid concentrations (or related phenotypes). Since the QTL mapping data were obtained with a relatively small number of animals, further experiments, including the development of congenic strains or knockout strains after gene cloning, are necessary to precisely map the QTLs and to confirm the role of the suggested candidate genes.

Table 3. Co-segregation-analysis results in F_2 progeny of LEW and BC rats[a]

Marker	Population	Phenotypic trait	*P* Chi-squared test	Genotype[b]			LOD score[c]	*P* (one-way ANOVA[d]
				LL	LB	BB		
Chromosome 1								
D1Rat14	Females	Aldosterone (4)	0..458	0.9±0.6 (29)	0.6±0.3 (43)	0.6±0.3 (24)	3.7	0.015[e]
D1Rat335	Males + females	Cholesterol (0)	0.219	2.9±0.4 (53)	3.2±0.4 (84)	3.4±0.5 (55)	8.4	<0.001
	Females	Cholesterol (0)	0.034	2.8±0.4 (32)	3.2±0.4 (37)	3.5±0.5 (31)	8.7	<0.001
D1Rat27	Males + females	Cholesterol (0)	0.769	2.9±0.4 (50)	3.2±0.4 (90)	3.4 ±0.4 (50)	8.2	<0.001
	Females	Cholesterol (0)	0.409	2.8±0.3 (29)	3.2±0.5 (43)	3.5±0.5 (27)	8.3	<0.001
Chromosome 2								
D2Rat69	Males + females	Cholesterol (4)	0.185	5.4±2.1 (41)	4.7±1.9 (91)	3.9±1.5 (58)	4.4	<0.001[e]
Chromosome 7								
D7Rat94	Females	Body weight (0)	0.189	129±12 (21)	132±11 (44)	145±21 (32)	3.4	0.002[e]
D7Rat12	Females	Body weight (0)	0.583	130±13 (22)	134±13 (48)	144±21 (29)	2.1	0.014[e]
D7Rat69	Males	Cholesterol (0)	0.549	2.9±0.4 (26)	3.2 ± 0.4 (40)	3.2±0.4 (24)	4.1	<0.001

Table 3. Continue

Chromosome 11								
D11Rat1	Males + females	Phospholipids (4)	0.064	1.7±0.4 (39)	1.5±0.3 (91)	1.5±0.3 (61)	4.1	0.001
	Females	Phospholipids (4)	0.773	1.7±0.5 (27)	1.4±0.4 (50)	1.3±0.3 (22)	3.6	<0.001
Chromosome 15								
D15Rat5	Males + females	Body weight (0)	0.809	145±25 (44)	152±25 (99)	161±27 (48)	3.3	0.008
D15Rat68	Males + females	Body weight (0)	0.930	146±25 (46)	152±26 (92)	162±26 (49)	2.9	0.007
Chromosome 16								
D16Rat6	Males + females	Cholesterol (4)	0.118	5.2±2.0 (60)	4.5±1.8 (85)	4.0±1.7 (47)	3.3	0.007
D16Rat44	Males + females	Cholesterol (4)	0.067	5.2 ±2.0 (61)	4.5±1.8 (84)	4.0±1.7 (44)	3.3	0.006
Chromosome 18								
D18Rat55	Females	Aldosterone (4)	0.2439	0.9±0.6 (18)	0.6±0.3 (56)	0.7±0.4 (23)	2.3	0.034[e]
D18Rat8	Females	aldosterone (4)	0.212	0.9±0.5 (17)	0.6±0.4 (48)	0.7±0.3 (29)	1.4	0.050[e]

(0) = initial or baseline (4) = postdietary

[a] Values are means±SD; muber of rat is given in parentheses. Body weight is in g; serum cholesterol and phospholipid level is in mmol/l; aldosterone level is in nmol/l;. Some DNA samples failed to give a conclusive genotype, hence te number of rats typed varied slightly between each lccus.

[b] L = LEW allele and B = BC allele [c] LOD scpres reported are at the markers indicated. In some instances the LOD score between markers is higher (see Figs. 1-5)

[d] One-way ANOVA with main factor genotype. For statistical analysis of serum parameters body weight was used as co-variate

[e] P-value after logarithmic transformation of the data .

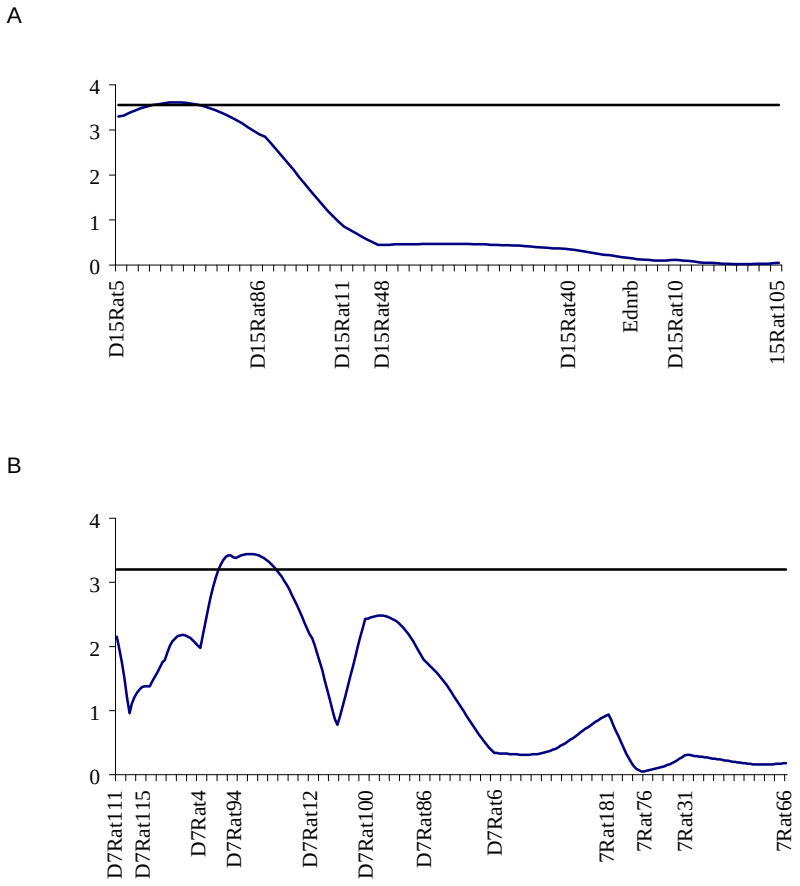

Fig. 1. The LOD score plot for initial body weight in the (LEW x BC) F_2-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage.
(A) Rat chromosome 7 (RNO7), *females*.
(B) Rat chromosome 15 (RNO15), *males plus females*.

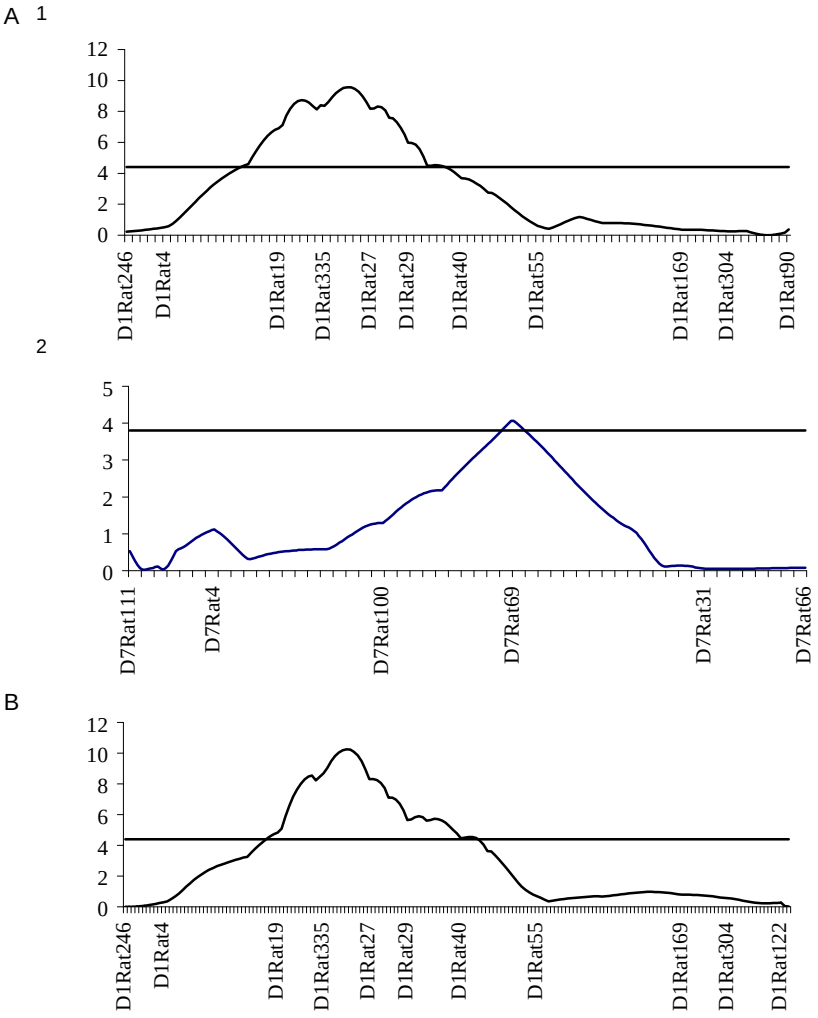

Fig. 2. The LOD score plot for baseline serum cholesterol level in the (LEW x BC)F_2-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage. (A) RNO1; 1 = *males plus females*, 2 = *females*. (B) RNO7, *males*.

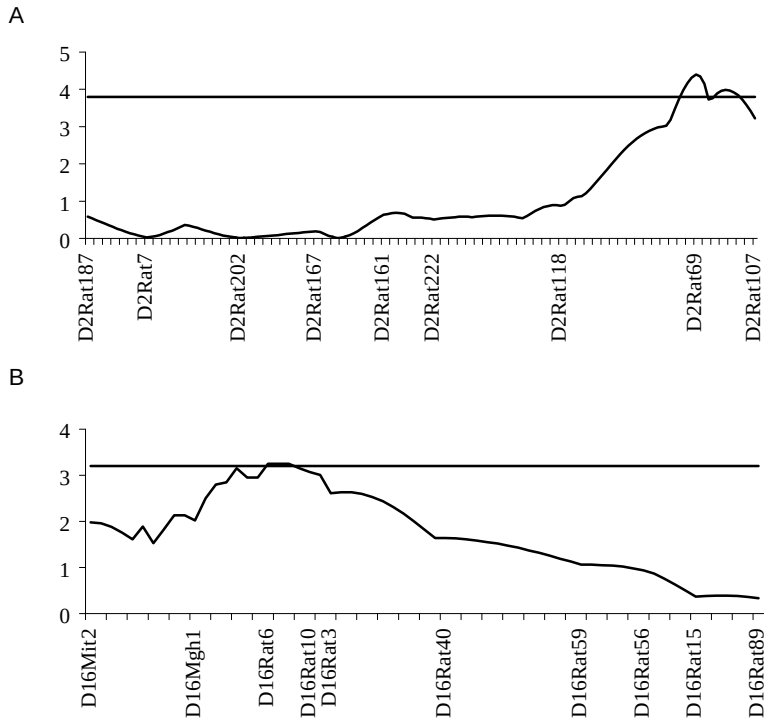

Fig. 3. The LOD score plot for postdietary serum cholesterol level in the (LEW x BC)F_2-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage. (A) RNO2, *males plus females*. (B) RNO16, *males plus females*

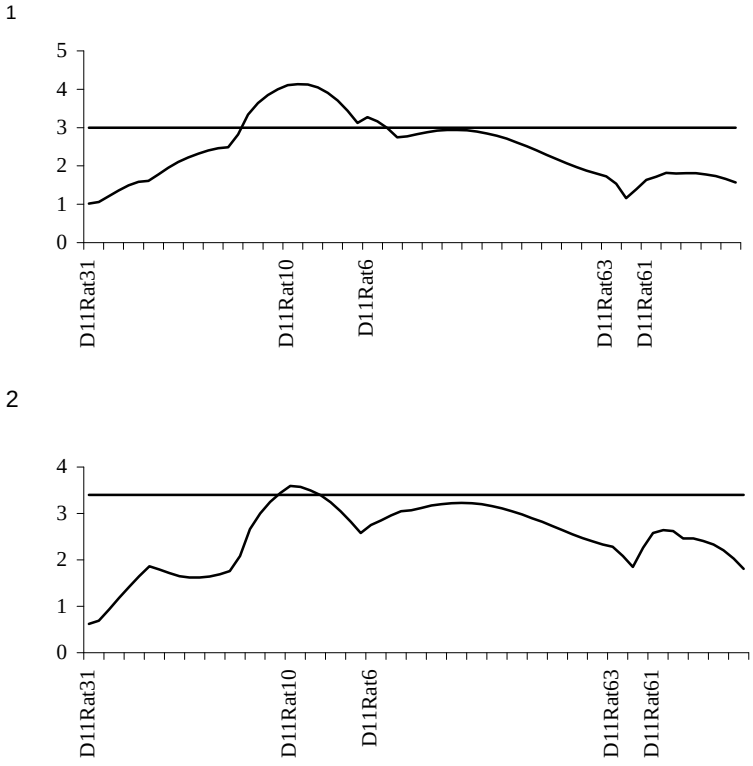

Fig. 4. The LOD score plot for postdietary serum phospholipid level in the (LEW x BC)F_2-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage.
RNO11; 1 = *males plus females*, 2 = *females*.

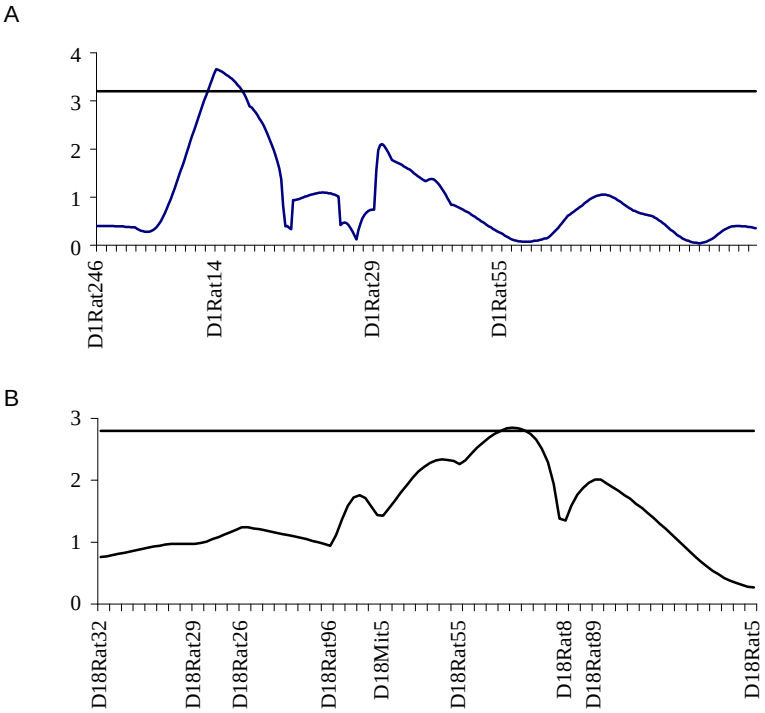

Fig. 5. The LOD score plot for postdietary serum aldosterone level in the (LEW x BC)F_2-intercross. The thick horizontal line represent the threshold value of the LOD score considered as significant for linkage. (A) RNO1, *females*. (B) RNO18, *females*.

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Chapter VI

Chromosomal localization of genes involved in biosynthesis, metabolism or transport of cholesterol in the rat

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Cytogentics and Genome Research, accepted

Abstract

Several genes involved in biosynthesis, transport or metabolism of cholesterol have been localized on rat chromosomes by using a radiation hybrid (RH) panel. The genes, coding for squalene epoxidase (*Sqle*), mevalonate kinase (*Mvk*), and farnesyl diphosphate farnesyl transferase 1 (*Fdft1*) which are involved in the cholesterol biosynthesis, have been mapped on chromosome 7, 12, and 15, respectively. The genes coding for phospholipid transfer protein (*Pltp*), sterol carrier protein-2 (*Scp2*), ATP binding cassette reporter A7 (*Abca7*), scavenger receptor class B, type 1 (*Cd36l1*), steroidogenic acute regulatory protein (*Star*), and lecithin:cholesterol acyl transferase (*Lcat*), which are involved in the transfer and/or metabolism of cholesterol, have been mapped on chromosome 3, 5, 7, 12, 16, and 19, respectively.
Each of the genes *Scp2*, *Sqle* and *Fdft1* maps close to a QTL for serum total cholesterol in rat, suggesting that these three genes might represent candidate genes for the previously mapped QTLs.

Introduction

The rat is a frequently used model in studies on the etiology and therapy of hypercholesterolemia. Impaired cholesterol metabolism is one of the predisposing factors in the process of this disease[1-3]. In a -previous study, involving the F_2 progeny of a cross between two rat inbred strains differing in their response to dietary cholesterol, we have found several quantitative trait loci (QTLs) that are associated with cholesterol and other lipid levels in blood and liver[4].
For the present study we have selected primers to amplify part of the genes known to be involved in the biosynthesis, transport and metabolism of cholesterol and have used a radiation hybrid (RH) panel in order to localize these genes on rat chromosomes. Comparing the results of this study with the QTL mapping data may reveal possible candidate genes for these QTLs.

Material & Methods

Radiation Hybrid (RH) mapping.

A whole genome rat/hamster (T55) RH panel of 106 hybrids was purchased from Research Genetics (Hunstville, USA). This panel has been constructed by fusing irradiated cells from a Sprague Dawley fibroblast line (RatFR) with a recipient hamster line (A23). RatFR donor cells were irradiated with 3000 rad prior to fusion with A23 recipient cells.

For localization of the genes on the RH map, 25 ng DNA of the hybrids was used in 10 µl PCR reaction. The reactions were performed as described by Research Genetics Inc. (Hunstville, USA). Primers were designed based on mouse or rat sequences (Table 1). For the localization of *Lcat,* the primers described by Bottger *et al.*[5] were used. The PCR products were separated on a 3% (w/v) agarose gel and visualized with ethidium bromide staining. Screening results were submitted to the RH mapping server of Rat Genome Database (RGD) (http://rgd.mcw.edu) and Otsuka Gen Research Institute (http://www.otsuka.genome.ad.jp/ratmap).

Sequencing

Introns of the genes *Sqle, Fdft1* and *Pltp* were amplified in order to differentiate between rat and hamster patterns. The amplified introns of these genes were sequenced (DNA Sequencing Core, Leiden, The Netherlands) using the Perkin-Elmer Big-Dye terminator cycle sequencing kit.

Table 1. The primers used to amplify genes involved in the biosynthesis, transport and metabolism of cholesterol.

Genes	GenBank Accession number	Forward primer (5'-3')	Reverse primer (5'-3') [a]
Mvk	NM_031063	GACAGCAGGCTGACACAGTAGGCC	ACAGGTACACACGGGGATAAAGCA
Sqle[b]	NM_017136	CGGAGCTTGTTCTGGTCGATCCCA	GAAGGAAGCTTGCTGGCATGGTCCGC
Fdft1[c]	NM_019238	CCACGATCTCCCTGGAGTTT	GGCGAGAAAGGCCGATTCCC
Pltp[d]	NM_011125	GGAACCTTCAGGAGGATGTATAAC	CGGAATTCCATATCCAGGTTGCCG
Scp-2	M34728	GTTTTCCCGAAGCTGCCAGC	CAAAGAAGCTACGTGGGTGG
Abca7	AF213395	ATTTCTTTGGAACAGCTTGCTGTC	AGAGTCAGTGTGTGGCCAGCCCCGAA
Cd36l1	AB002151	GCTACTGCTGCTCTGGCCACTGT	GATGAGCGAGGGCACCATGAGAA
Star	NM_031558	GGTCCTGAAGAAGATTGGAA	ATGGTCTTTGGCAGCCACCC
Lcat	NM_017024	TGATGGCTTCATCTCTCTCG	AGGGGGAAGTTGTGGTTATG

a The primers have been chosen based on homology between human, mouse and rat and are selected out of the mRNA sequence. The primers are located in the exons and sometimes overlap an intron.

b The intron, amplified as a part of the gene, has been sequenced in the rat inbred strain BC/CpbU, (GenBank acc. nr: AF434745).

c The intron, amplified as a part of the gene, has been sequenced in four rat inbred strains: BC/CpbU, BN.*lx*/Cub, LEW/OlaHsd and SHR/OlaIpcv (GenBank acc. nr: AF434741-AF434744).

d The intron, amplified as a part of the gene, has been sequenced in the rat inbred strain BN.*lx*/Cub, (GenBank acc. nr: AF4347405).

Results & Discussion

In previous studies, several quantitative trait loci (QTLs) for cholesterol related parameters have been localized on the genetic map of the rat[4-7]. In this study we have used a rat/hamster radiation hybrid (RH) panel for mapping a number of genes that code for proteins known to be involved in the biosynthesis, transport and/or metabolism of cholesterol. Figure 1 presents a schematic overview of the steps in the cholesterol biosynthesis that are catalyzed by enzymes encoded by genes involved in this study.

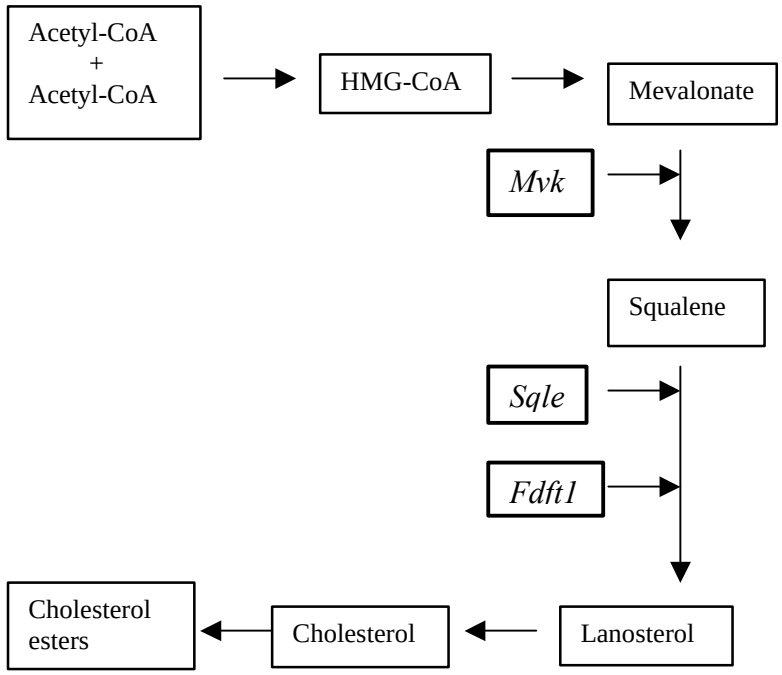


Fig 1. Schematic pathway of cholesterol biosynthesis.
(Abbrevations: *Mvk* : Mevalonate kinase; *Sqle*: Squalene epoxidase; *Fdft1*: farnesyl diphosphate farnesyl transferase1)

The *Mvk* gene codes for mevalonate kinase, a key-enzyme in cholesterol biosynthesis. *Mvk* is involved in the conversion of mevalonate to squalene[8] and is one of only two enzymes in the pathway in which a defect has been reported in a clinical disease[9]. With primers designed on the basis of the published rat sequence[10] (Table 1) the RH panel was screened and the gene could be located on rat chromosome 12 (RNO12), in the vicinity of the marker D12Wox1 (LOD 11.5, 550.8 cR; Fig. 2). The position is in accordance with the homology of rat chromosome 12 with mouse chromosome 5 and human chromosome 12

The *Sqle* gene codes for squalene epoxidase (Fig. 1), which catalyzes the first oxygenation step in cholesterol biosynthesis. The gene product is suggested to be one of the rate-limiting enzymes in this pathway as it has a low specific activity when compared to squalene synthase (*Fdft1*) [11]. *Sqle* was found to be localized on RNO7 close to the marker D7Rat67 (LOD 16.2; 558 cR; Fig. 2). In humans this gene has been assigned to a segment of chromosome 8 that is homologous to a region of RNO7 and MMU15. Kato *et al.*[6] have found a QTL for total serum cholesterol levels on RNO7, in the vicinity of the marker D7Mit10, which is about 10 cM apart from D7Rat67. When estimating the 95% confidence interval for each QTL (as determined by calculating the genetic distance based on the drop of 1.0 LOD unit from the peak), it seems that the interval of the QTL as reported by Kato *et al.*[6] and the position of *Sqle* are overlapping. Thus, the *Sqle* gene might be considered as a candidate gene for this QTL.

Fdft1 (farnesyl diphosphate farnesyl transferase 1, or squalene synthase) catalyzes the first reaction of the isoprenoid metabolic pathway, a route specifically committed to sterol biosynthesis[12]. The gene has been mapped on RNO15 in the vicinity of the marker D15Rat86 (LOD: 13.1, 200 cR; Fig. 2). *Fdft1* has previously been mapped to human chromosome 8 and mouse chromosome 14. The regions to which the gene has been mapped in these species are homologous to the region of rat chromosome 15 where *Fdft1* is located. A QTL for serum cholesterol levels has been found by Kato and co-workers[6] near D15Rat64. As the position of *Fdft1* and the position of the QTL is only 6 cM apart, we assume that *Fdft1* is the candidate gene for this QTL.

In addition to the three genes involved in the cholesterol biosynthesis, another six genes involved in the transport and/or metabolism of cholesterol have been mapped by using the RH panel.
The gene coding for phospholipid transfer protein (*Pltp*) was found to be located on RNO3, close to the marker D3Rat5 (LOD: 8.4, 928 cR; Fig. 2). *Pltp* has found to be located on human chromosome 20 and mouse chromosome 2. The human and mouse chromosomal regions where *Pltp* is situated is homologous wit rat chromosome 3. The protein plays a role in the regulation of high density lipoprotein (HDL) metabolism[13]. The two major functions are phospholipid transfer and HDL conversion[14].

The gene *Scp2* is coding for sterol carrier protein2. This protein regulates the availability of cholesterol for various cellular processes[15]. *Scp2* overexpression enhances the rate of cholesterol cycling, thus reducing the availability of cholesterol for cholesterol ester synthesis, and alters the activity of the cellular cholesterol pool involved in regulating apolipoproteinA-I-mediated high-density lipoprotein cholesterol secretion[16,17]. A discrepancy was found in mapping the *Scp2* gene. In the RH map of the RGD *Scp2* maps in the vicinity of the marker D5Rat216 (101.3 cR) whereas and in the RH map of the Otsuka GEN Research Institute the gene was localized near D5Rat94 (LOD: 5.5, 1027 cR). In the mouse *Scp2* is located on chromosome 4 (52 cM). Based on homology between mouse chromosome 4, human chromosome 1 and rat, the most likely position of this gene in the rat should be on chromosome 5 in the vicinity of marker D5Rat33 (899 cR; Fig. 2). Therefore we conclude that the position determined through the RH server of the Otsuka GEN Research Institute is the correct position of this gene. Previously, *Scp*2 has been suggested as a candidate gene for a QTL controlling serum total cholesterol levels after a cholesterol-rich diet on rat chromosome 2[4], but the present localization excludes this proposition. Instead the male-specific QTL described by Klöting *et al*. [7] is located on chromosome 5 (D5Mgh14-D5Mgh15) close to the *Scp2* gene. Therefore we assume that this gene rather represents this latter QTL.

The gene *Abca7* (ATP-binding cassette reporter A7) was mapped on RNO7 (D7Rat72, LOD: 11.6, 0 cR; Fig. 2). *Abca7* is a member of the *ABC1* family, which plays an important role in cholesterol metabolism[18]. Mutations may result in familial HDL deficiency, whereas the complete absence of *ABC1* results in Tangier disease[19]. In humans this gene is located on HSA19p13.3, which is homologous to segments of MMU10 and RNO7.

The gene *Cd36l1*, coding for scavenger receptor class B type 1, previously known as *SR-BI*, was found to be located on RNO12 in the vicinity of D12Rat36 (LOD 12.0, 431.3 cR; Fig. 2). In mouse and humans *Cd36l1* is located on chromosome 5 and chromosome 12, respectively.

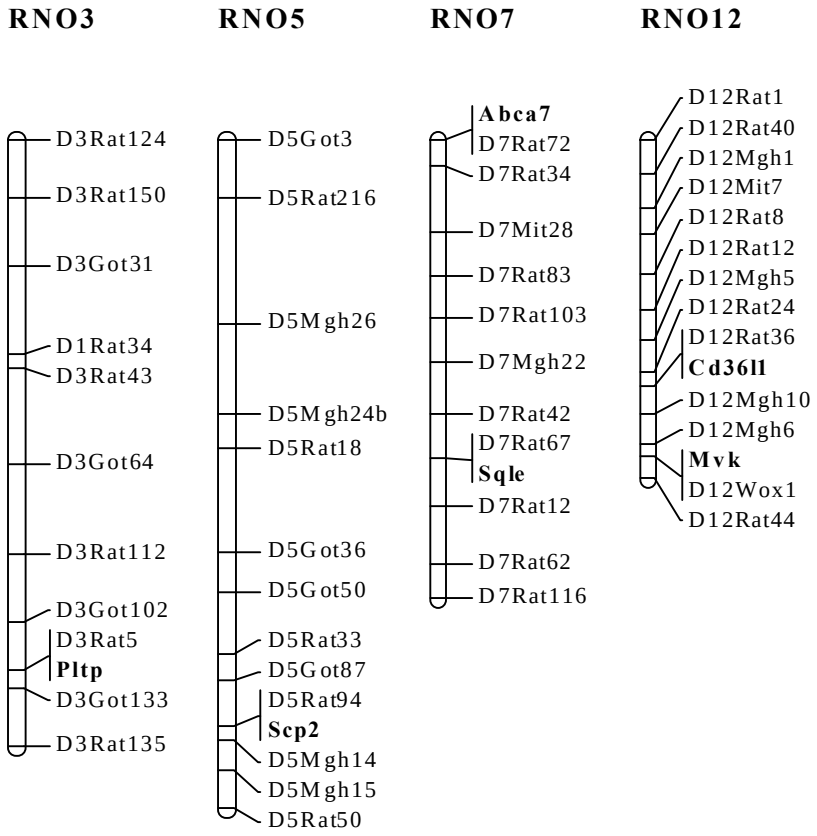
Therefore based on homology this gene should be located in the vicinity of the gene *Mvk*. The protein produced by this gene is a high-density lipoprotein receptor which is involved in the absorption of dietary cholesterol[20] and in the selective uptake of cholesteryl esters from high-density lipoprotein by various organs, including liver and steroidogenic tissues[21]. It also facilitates non-lipoprotein cholesterol uptake as well as free-cholesterol efflux from cultured cells[22]. The present assignment of *Cd36l1* confirms the finding of Johnson *et al.*[23].

The gene that codes for the steroidogenic acute regulatory protein (*Star*) could be mapped on RNO16 linked to D16Rat34 (LOD 9.9, 753.7 cR; Fig. 2). The position found for *Star* in rats is in accordance with the anticipated homology with mouse (chromosome 8) and human (chromosome 8). *Star* is a mitochondrial protein that is involved in the acute production of steroids in the adrenal glands and gonads in response to corticotropin (ACTH) and luteinizing hormone (LH) respectively. It regulates the transfer of cholesterol from the outer to the inner mitochondrial membrane where it is converted into pregnenolone[24].

Finally, the gene that codes for lecithin:cholesterol acyltransferase (*Lcat*) was mapped on RNO19 near the marker D19Rat22 (LOD: 10.1, 403.3 cR; Fig. 2). This position is in agreement with the position of *Lcat* as found in the genetic linkage map of the rat by Bottger *et al.* [5]. The enzyme is synthesized in the liver of mammals and it is responsible for the esterification of intravascular cholesterol[25]. *Lcat* is activated by apoAI, a component of HDL. The QTL controlling HDL cholesterol, which has been found on RNO19[5], is not in the vicinity of the *Lcat* gene. In humans *LCAT* is located on chromosome 16q and in mice on chromosome 8.

In conclusion, we have localized three genes involved in the biosynthesis and six genes involved in the transport and/or metabolism of cholesterol. Based on the chromosomal location of these genes and the position of previously mapped QTLs we may assume that three of the presently mapped genes (*Scp2*, *Sqle*, and *Fdft1*) each represent a candidate gene for previously mapped QTLs.

Fig. 2. The radiation hybrid map of the chromosomes that contain one or more of the genes (in bold) localized in this study.


RNO3
D3Rat124
D3Rat150
D3Got31
D1Rat34
D3Rat43
D3Got64
D3Rat112
D3Got102
D3Rat5
Pltp
D3Got133
D3Rat135
RNO5
D5Got3
D5Rat216
D5Mgh26
D5Mgh24b
D5Rat18
D5Got36
D5Got50
D5Rat33
D5Got87
D5Rat94
Scp2
D5Mgh14
D5Mgh15
D5Rat50
RNO7
Abca7
D7Rat72
D7Rat34
D7Mit28
D7Rat83
D7Rat103
D7Mgh22
D7Rat42
D7Rat67
Sqle
D7Rat12
D7Rat62
D7Rat116
RNO12
D12Rat1
D12Rat40
D12Mgh1
D12Mit7
D12Rat8
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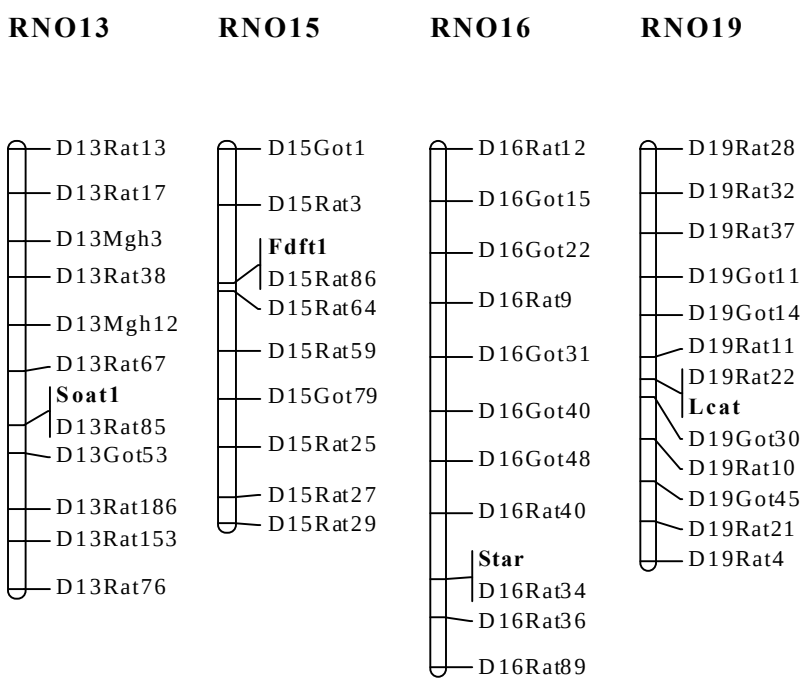
D12Mgh6
Mvk
D12Wox1
D12Rat44


RNO13
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D13Rat17
D13Mgh3
D13Rat38
D13Mgh12
D13Rat67
Soat1
D13Rat85
D13Got53
D13Rat186
D13Rat153
D13Rat76
RNO15
D15Got1
D15Rat3
Fdft1
D15Rat86
D15Rat64
D15Rat59
D15Got79
D15Rat25
D15Rat27
D15Rat29
RNO16
D16Rat12
D16Got15
D16Got22
D16Rat9
D16Got31
D16Got40
D16Got48
D16Rat40
Star
D16Rat34
D16Rat36
D16Rat89
RNO19
D19Rat28
D19Rat32
D19Rat37
D19Got11
D19Got14
D19Rat11
D19Rat22
Lcat
D19Got30
D19Rat10
D19Got45
D19Rat21
D19Rat4

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Chapter VII

Sequencing and Chromosomal Assignment of the Rat Endothelial-derived Lipase Gene (*Lipg*)

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DNA Sequence, 2001, 12:285-287

Abstract

Part of the nucleotide sequence of the *Lipg* gene in the rat was established using primers based on the mRNA sequence described in the mouse. The rat intron sequence served as a template for designing primers for the specific amplification of rat *Lipg*.
A rat-hamster radiation hybrid (RH) panel was used for chromosomal assignment of the rat *Lipg* gene. The *Lipg* gene was found to be located on rat chromosome 18 in the vicinity of the marker D18Mit11; a region reported to be homologous with both human and mouse chromosome 18.

In man three lipase genes are known to be involved in HDL cholesterol metabolism: lipoprotein lipase[1], hepatic lipase[2], and endothelial-derived lipase[3]. In the rat the gene for lipoprotein lipase (*Lpl)* is known to be located on chromosome 16[4,5] and the gene for hepatic lipase (*Hl*) on chromosome 8[6], but the gene for endothelial derived lipase (*Lipg*) has not yet been mapped. It has been suggested that endothelial-derived lipase plays a role in regulating lipid metabolism in the vessel wall[7] and in vascular disease[7,8]. In humans the *LIPG* gene has been assigned to chromosome 18 (OMIM™). In the mouse the gene has not been mapped yet, but the mRNA sequence of mouse *Lipg* has been established (GenBank: NM_010720). This mouse sequence was used for designing primers for the amplification of rat *Lipg* sequences. These primer sequences are: FW(mouse position bp 851-874), 5'-CGATGCAGACTTTGTGGATGTCCT-3' and REV(mouse position bp 1065-1090), 5'-GACTCTCTGGTGA ATCAGGACAAGCC-3'. Since in our previous study we analyzed a set of recombinant inbred strains derived from the progenitor strains BN.*lx*/Cub and SHR/OlaIpcv[4], the resulting amplification products of these two inbred strains were sequenced and screened for polymorphism (fig. 1). The 1165 bp long sequences were found to be completely identical in both strains. The outer regions of the sequence (bp 1-161 and bp 1087-1165) were identical to mouse mRNA sequence bp 851-1090. Therefore, we assume that in the rat these sequences are also exons and that the sequence in between is an intron. As expected for an intron, this middle sequence starts with the nucleotides GT and ends with AG. As no polymorphism was detected between the *Lipg* sequences of the BN and SHR rat inbred strains, the localization of the *Lipg* gene could not be studied by linkage analysis using the recombinant inbred strains. Therefore, a rat/hamster RH panel (Research Genetics, Hunstville, USA) was used for the chromosomal localization of the rat *Lipg* gene. A primer-combination, specific for amplifying a part of the intron of rat *Lipg*, was designed: (IntronFW, 5'-TCCTGTGGGATCCAGATCACTC TCAGT-3'; bp 823-850, fig.1 and IntronREV, 5'-CATCCCCTG TGTCCTCTGTCCTGTGTT-3'; bp 1053-1079, fig. 1), and the RH panel was screened for amplification of the 256 bp *Lipg* sequence. The results were analyzed (http://www.otsuka.genome.ad.jp/menu/RH.html), and *Lipg* was found to be located on rat chromosome 18 in the vicinity of the marker D18Mit11 (LOD 6.77) at 57.3 cR. This position supports the homology between segments of HSA18 and RNO18[9]. Also a large segment of mouse chromosome 18 is homologous with RNO18[10]. Interestingly, Mehrabian et al. [11] described a quantitative trait locus (QTL) that affects serum HDL cholesterol levels after a high fat diet on mouse chromosome 18. *Lipg* might be the candidate gene for this QTL in the mouse.

CGATGCAGACTTTGTGGATGTCCTGCATACCTACACGCTGTCCTTTGGCTTGAGCATTGGGATTCGGAT
GCCCGTGGGTCACATTGACATCTATCCCAATGGTGGTGACTTCCAGCCCGGCTGCGGATTCAACGATGT
CATGGGATCTTTCGCATATGGAA
GTGAGTTCGTACGCATCTGTCTCGTGTTTGATCCGCTCTGTCCATCGTGTGAGTCAGCCCAAGCTGTGA
GTGGAGCTAGCGGAGTTCAATTGATCACTCCTGTCTCTTTCCACAGTGGTGAGGCACATCGCTTGCCTG
TTAACAGCATTCACCCGCTTCCAAATCACAGTCGATACTGATTAATTTTCTTTTTTTGCTTGAGACAGGG
TTTTACTATGTAGCCCTGGCTGGCCTGGAAATTGATATGTAGACCAGGCTGGATCCAAACTTACAGAGA
TTTGCCTCCCACTGCCTTCTGAGTTAAAGATATGTGCTACCATGCCCAGCATTTATGTCTAGTATTTCTA
TTCAGACAGCTGTTAGAGCTCAGTGACTCTACCACATGGTTGCCCTTTGACACTCGATTTTCCTCCTCCT
CTTCTTCCCCTTCCCCCCTCTCCTCATCCCCCTCCTTTCTGTTTTAATGTTTTGTTTTGTTTTGTTTTTTT
GAGACAGGGTCTCTATGTAGTCTCTGTTGTACTGGAGCTTGCTATGTAGAACAGGCTGGCTTCGAACTC
GCAGAGGCTTTCCTGTCTTCCACCTTGGAGTGTTGGGAATAAAGGCACGTGCCACCGCTCCTGGTGGAC
AGCCTCCTGTTGATGTCCATCCCTTCAAAATACGGGTCCTGTGGGATCCAGATCACTCTCAGTGTGTGA
ACTCTCTTCTCTCTATTTCTTGGAGTCAATGCTGTGGCCTTCTCTCATGGAGTGGACAGTTACATGGTTG
GAGTGGTCTTCAATCCCTCTGCCTCTTACTAGGAGCTTGGAATACCATTGAGCCTGTGCTAAGAGTCAC
AGACCGCTGAGTGTGTGTGTATTGATTACTGAGCGGTGGAATCACCCAGAAATGGCTCATCCCCTGTGT
CCTCTGTCCTGTGTTTCCTCAG
CAATCTCAGAGATGGTGAAATGCAAGCACAAGCGAGCCGTACACCTCTTTGTCGACTCTCTGGTGAATC
AGGACAAGCC

Fig. 1. The sequence of the PCR product amplified with the primers for the *Lipg* gene as found in both the BN.*Lx*/Cub and the SHR/OlaIpcv rat (exon sequences are underlined).
(GenBank acc. Nrs. AY027561 (BN) and AY027562 (SHR).

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Chapter VIII

General Discussion

Introduction

The laboratory rat has been extensively characterized for biochemical and physiological mechanisms involved in the pathogenesis of human disorders. Therefore, this species has become a major animal model in biomedical research. The availability of inbred strains reproducing the main features of monogenic or polygenic human diseases, is important for the identification of genetic defects relevant to these disorders. In rats, the quantitative trait loci (QTL) approach has been commonly used for the genetic mapping of loci involved in a wide range of complex diseases like dyslipidemia, hypertension, diabetes mellitus, obesity, autoimmunity, and abnormal behaviour.[1-9] Research for identifying genes controlling blood and liver cholesterol concentrations after a cholesterol-rich diet has been performed by Bottger *et al*. [10,11] The research as described in this thesis is a continuation of these latter studies and contributes to a better understanding of the rat as an animal model for dietary cholesterol induced diseases. In this thesis four rat inbred strains, BC/CbpU (BC), BN.*lx*/Cub (BN), LEW/OlaHsd (LEW) and SHR/OlaIpcv (SHR), have been used. The strains were selected on the basis of their response to a cholesterol-rich diet. [12] The BN and SHR have been used for the amplification of genes and the recombinant inbred (RI) strains, which are derived from the progenitors BN and SHR, have been used for the construction of an AFLP linkage map. The BC and LEW have been used to create an F_2 progeny. These animals have been used for the localization of quantitative trait loci (QTLs) controlling serum and liver cholesterol and related phenotypes. In addition, genes which could be candidate genes for some of the QTLs have been identified and assigned to chromosomes.

Genetic map of AFLP markers as derived from the HXB/Ipcv and BXH/Cub sets of recombinant inbred strains.

Although the genetic map of the rat is well developed, there are still areas with a relatively low marker density. To obtain more markers in these areas the AFLP (Amplified Fragment Length Polymorphism) technique can be used. Hundreds of markers can be generated in a relatively short time period. Moreover, the AFLP technique provides an affective tool for the study of species relationships and for the construction of phylogenetic trees in animals. [13] Besides these advantages, there is also a disadvantage of the technique. The AFLP method generates dominant rather than co-dominant markers. This is not a problem when using RI strains, but in case of a backcross or an F_2 intercross this might cause a problem, unless specialized software (AFLP Quantar) that can distinguish the homozygote "band absent", heterozygote "band present" and homozygote "band present" from each other, is available. Thus, by using a phosphor imaging system and quantification of band

intensities, the AFLP fragments are equal to co-dominant markers. This has been demonstrated by Ajmore-Marsan *et al*. [14] for cattle.
The genetic map as based on linkage studies in the RI strains, the genetic map as published on the internet by the Rat Genome Database (RGD; http://rgd.mcw.edu) and the genetic map as based on the F_2 progeny of the BC x LEW (chapter 4) have been compared for the genetic length of the chromosomes(Table 1). This comparison clearly demonstrates that the length of a chromosome is dependent on the segregating population that is used. This is not surprising, because this is one of the reasons why for QTL analysis in a certain cross, the genetic map of that cross has to be established. The linkage map as obtained with RI strains is much smaller than the genetic map based on the (BC X LEW) F_2 progeny and that of the RGD. The genetic map based on the RI strains shows a deviation in size because the map is based on only 30 strains and approximately 600 markers. It is well known that for a linkage map derived by evaluating small numbers of meiotic events, the confidence intervals for the map distances between individual loci are relatively large and thus the total length of such a genetic map is less reliable. The genetic of the RGD is also based on a small number of animals ($n = 45$[15]), but in this map 3824 markers have been used to establish the map. For establishing the genetic map of the F_2, 192 animals and 258 markers have been used. Since the number of meiotic events has more impact than the number of markers, we feel that the map obtained with (BC x LEW) F_2-intercross is highly reliable. A detailed comparison based on the order of the markers on the chromosomes, is only possible for the F_2 map and the RGD map, because in these two maps the same markers have been used.

Localization of Fabp6 and identification of a Fabp6 pseudogene in the rat.

The QTL analysis for cholesterol related traits in the RI strains has revealed a QTL controlling liver cholesterol on rat chromosome 10. Based on homology, the gene coding for the fatty acid-binding protein 6 (*Fabp6*) has been suggested as a candidate gene for this QTL.[10] However at that time the location of this gene was not yet known in the rat. We have now found that *Fabp6* is localized on rat chromosome 10. This localization is in accordance with the homology of rat chromosome 10 with mouse chromosome 11 and human chromosome 5. The position of *Fabp6* is extrapolated to other linkage maps of the rat. In the genetic map of the RI strains, we anticipated that *Fabp6* maps in the vicinity of the QTL controlling liver cholesterol concentration (Fig. 1). However, Pravenec and co-workers[16] suggested the sterol regulatory element binding factor 1 (*Srebf1*) as a candidate gene for this liver cholesterol QTL. This QTL has been confirmed in the F_2 progeny of the BC x LEW (see chapter 5), albeit that in this intercross the position of the QTL is closer to the *Srebf1* gene (Fig. 1).

In the SHR, we found a *Fabp6* exon sequence without introns, and have assumed that this sequence represents a pseudogene. However, the promoter region of this

gene has not yet been investigated. More research is needed in order to further characterize the suggested pseudogene.

Table 1. Comparison of the lengths of the individual linkage maps.

RNO	Map length (cM) RI strains	Map length (cM) RGD	Map length (cM) F_2 BCXLEW
1	131.7	149.3	162.3
2	134	112.5	149.9
3	160.8	94.2	101.7
4	132.7	102.2	110.5
5	67.3	105.7	91.5
6	99.7	85.2	101.6
7	141.8	88.6	128.3
8	129.4	85.3	93.5
9	24.2	79.5	85.7
10	82.1	95.4	92.5
11	23.8	41.6	59.5
12	34.7	54.0	78.7
13	74.9	44.2	65.3
14	43.7	71.2	75.6
15	46.4	66.5	57.0
16	83.1	45.5	56.9
17	56.2	47.6	58.3
18	55.1	52.4	53.1
19	101.4	48.1	51.6
20	34.1	48.2	46.5
X	16.9	44.6	76.1
total	1674.0	1561.8	1796.1

BC x LEW intercross.

In the genetic map of the F_2 progeny the average spacing between markers was found to be 7.7 cM. This makes the map very useful for the localization of QTLs (see chapter 5). As already mentioned in chapter 4, some of the parental animals of the LEW strain were heterozygous for some of the marker loci. This heterozygosity has mainly been found on rat chromosome 10. Although genetic contamination cannot be excluded it might be that some of the microsatellites are less stable in the LEW strain than in other inbred strains. Yuan and co-workers[17] also found allelic variation for several microsatellites within inbred mice strains. In the strains CBA/J and C3H/HeN, microsatellites have been screened for within-strain polymorphisms on

10 chromosomes and 33 polymorphism have been found. Fifteen out of these 33 polymorphisms were located on chromosome 7 and nine polymorphisms were found on chromosome 11. Mouse chromosome 11 is homologous to rat chromosome 10.

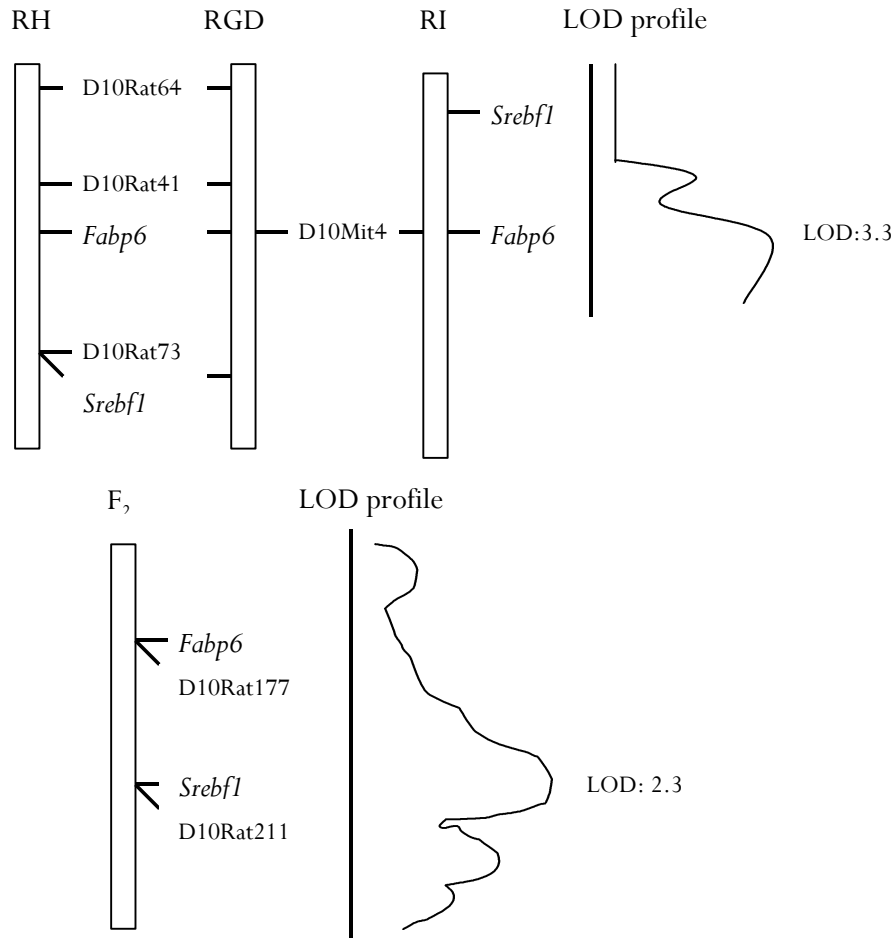

Fig. 1. The position of Fabp6 as found in the RH map is extrapolated to the genetic linkage map of the RI strains and the (BC x LEW) F_2 intercross. (RH = radiation hybrid map; RGD = the genetic map of rat genome database; RI = the genetic map of the RI strains; F_2 = the genetic map of the F_2 progeny of the BC x LEW)

However, no polymorphicity was found on rat chromosome 1, which is, at least in part, homologous to mouse chromosome 7. Yuan and co-workers[17] showed that the frequency of microsatellite polymorphisms is strain specific.

Quantitative trait loci influencing blood and liver cholesterol concentration in rats.

The genetic map of the F_2 progeny (see chapter 4) has been used for QTL analysis of the following traits: serum cholesterol levels before and after a cholesterol-rich diet, serum phospholipids levels, liver cholesterol concentrations and circulating steroid hormone levels after a cholesterol rich diet, and body weight. In total nine significant and nineteen suggestive QTLs have been found.
QTLs involving cholesterol and related phenotypes have also been reported by other authors. They found QTLs on chromosomes 1, 2, 4, 5, 7, 10, 15, 18, and 20.[1,10,11,18-22] Four of the previously reported QTLs have been confirmed in this study: on chromosome 2 (postdietary serum cholesterol levels), 5 (basaline serum cholesterol levels), 10 (liver cholesterol levels), and 15 (baseline serum cholesterol levels). There is increasing evidence that transcription factors play an important role in the regulation in cholesterol.[23] Thus, in different strains different QTLs can be expected to be found. In the mouse, also many QTLs involved in cholesterol metabolism have been localized.[24-31] The chromosomal position of the QTLs as described in chapter 4, have been compared with the chromosomal location of QTLs found in the mouse. Homologous location of the QTL for basal serum cholesterol levels on rat chromosome 1, has been confirmed by two studies in the mouse. Gu and co-workers[29] and Prucell-Huynh and colleagues[30] both found a QTL on mouse chromosome 7 in the vicinity the apoE gene. For serum total cholesterol levels, a QTL on rat chromosome 2 was found. On the homologous region of mouse chromosome 3 also a QTL for serum cholesterol levels was identified. [28] Furthermore on mouse chromosome 18 a QTL for phospholipids levels has been found. [32] Mouse chromosome 18 is homologous to rat chromosome 18 where we have found a suggestive QTL for this trait. These findings seem to indicate that these traits have been conserved in the evolutionary differentiation of these species and thus may play a major role in cholesterol metabolism.

Chromosomal localization of genes involved in biosynthesis, metabolism or transport of cholesterol in the rat.

In chapter 5 we have described the localization of several genes involved in cholesterol metabolism. Three genes involved in the biosynthesis of cholesterol have been localized. The gene coding for mevalonate kinase (*Mvk*) has not found to be associated with cholesterol biosynthesis in the rat, but in the mouse Welch *et al*. [24] have found an association of this gene with plasma cholesterol and in humans, Berger

and co-workers[33] found a inborn error of cholesterol biosynthesis caused by a deficiency of mevalonate kinase.
The gene squalene epoxidase (*Sqle*) has been assigned to human chromosome 8.[34] According to the virtual comparative (VC) mapping server of the RGD, *Sqle* is expected to be located at approximately 555.3 cR. This position is supportive for the assumption that *Sqle* is a candidate gene for the QTL controlling total serum cholesterol levels found on chromosome 7. [1] It is of interest to note that mouse chromosome 15 which is in part homologous to rat chromosome 7, possesses several QTLs that control cholesterol traits. [29,30]
The gene involved in the last step of cholesterol biosynthesis, farnesyl diphosphate farnesyl transferase 1 (*Fdft1*) has been mapped on rat chromosome 15, in a segment where Kato and colleagues[1] have found a QTL for serum cholesterol levels. Based on the VC map, the *Fdft1* gene was expected to be located at 240 cR. This position is approximately 40 cR distant from the position as found in the present study. In the mouse this gene was mapped in a study for the chromosomal organization of candidate genes involved in the cholesterol homeostasis, but no direct association was found with cholesterol synthesis. [24]

Another six genes involved in cholesterol metabolism and cholesterol transport have been mapped in chapter 6. Three of these genes are further discussed here. *Cd36l1*, the gene coding for scavenger receptor class B, type 1, has been mapped to rat chromosome 12. This gene was already mapped in the rat with the FISH technique, but a more precise position remained to be established. On the homologous region in the mouse (chromosome 5), three associations were found, one for serum unesterified cholesterol levels, one for serum total cholesterol levels and one for serum HDL cholesterol levels.[29] These authors suggested that SR-BI could be the major gene involved in this QTL. Welch and co-workers[24] found also associations on mouse chromosome 5, but they suggested that *Mvk* would be the major gene involved in these associations. As stated before, no association has been found between cholesterol and rat chromosome 12.
The gene coding for steroidogenic acute regulatory protein (*StAR*) was found to be located on rat chromosome 16. StAR protein controls the rate-limiting step in steroidogenesis: the transport of cholesterol from the outer to the inner mitochondrial membrane. [35] The presence of SREBP (sterol regulatory element binding proteins) response elements in both human and rat StAR promoters and the ability of SREBPs to regulate basal StAR gene promoter activity indicates that StAR is a important enzyme in cholesterol metabolism[35], but until now no direct association has been found between cholesterol and *StAR*.
The gene coding for lecithin:cholesterol acyl transferase (*Lcat*) has previously been mapped by Bottger *et al*. [11] In this study we confirmed the position in the RH map. In mouse, *Lcat* has been associated with basal cholesterol levels. [25,29]

In this thesis several QTLs and genes involved in cholesterol metabolism have been localized in the rat. The results of the QTLs remain preliminary until confirmed e.g. in congenic strains. Also, the precise role of the genes needs to be studied in more detail e.g. in knock-out rats. But, despite these limitations, we might conclude that the present study contributes to a better understanding of the rat as a model in cholesterol related research.

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Summary

Epidemiological studies in man and experimental studies in animals have shown that serum and liver cholesterol levels are influenced by both environmental and genetic factors. Research that aims to dissect which genetic factors are involved in the differences of blood and hepatic cholesterol levels requires standardization of both environmental circumstances and the genetic background. Therefore, genetically defined laboratory animals are indispensable for these studies. In this thesis the four rat inbred strains, BC/CpbU (BC), BN.*lx*/Cub (BN), LEW/OlaHsd (LEW) and SHR/OlaIpcv (SHR), selected for their response to a cholesterol-rich diet, have been used. BN and LEW are hyperresponders whereas BC and SHR are hyporesponders with respect to serum cholesterol levels. The strains differ also for liver cholesterol accumulation.

In chapters 2 and 3 the BN, SHR and the recombinant inbred (RI) strains, derived from the progenitors BN and SHR, have been used. In chapter 2, the genetic linkage map that is based on these RI strains has been extended by using amplified fragment length polymorphism (AFLP) markers. Thirteen primer combinations have been tested and 89 AFLP markers could be assigned to chromosomes.

In previous studies a QTL (quantitative trait loci) for liver cholesterol concentration after a cholesterol-rich diet was found on rat chromosome 10. Based on homology with mouse and man, *Fabp6* (fatty acid binding protein 6) has been suggested as a candidate gene for this QTL. In chapter 3, DNA from the BN and SHR have been used to amplify the *Fabp6* gene and the PCR products obtained from these animals have been sequenced. As no polymorphism could be detected between the BN and the SHR, the location of *Fabp6* has been determined by screening a 96 radiation hybrid (RH) panel with rat specific primers, which were based on the intron sequences. The chromosomal location was, as expected, on rat chromosome 10, in the vicinity of a QTL controlling liver cholesterol metabolism in the RI strains.

Amplification of the *Fabp6* gene also revealed a pseudogene (*Fabp6*-ps) in the SHR strain. Screening 66 commonly used rat inbred strains, revealed that three other stains (SHRSP, OKA and WKY) also possess *Fabp6*-ps. All four of these strain originate from the outbred Wistar colony maintained at the Kyoto School of Medicine in Japan. By analysing a (SHRSPxBN)xBN backcross, the pseudogene was found to be located on rat chromosome 15.

In chapters 4 and 5, the F_2 progeny of a cross between BC and LEW has been used. In chapter 4, the construction of a genetic linkage map, consisting of 258 polymorphic DNA markers, is presented. The genetic length of this map is about 1790 cM and the average marker space is 7.7 cM. In chapter 5, the F_2 progeny has been screened for their basal serum cholesterol levels (before a cholesterol-rich diet), and for the post-dietary (i.e. after a cholesterol-rich diet) levels of serum

cholesterol, liver cholesterol, serum phospholipids and circulating steroid hormones. These parameters were used for QTL analysis. For basal serum cholesterol levels, a QTL has been found on chromosome 1 in the region where the apolipoprotein E (*ApoE*) gene is located. Also, a QTL for this parameter was found on chromosome 7. For post-dietary serum cholesterol levels, two significant QTLs have been found, one on chromosome 2 and one on chromosome 16. Based on its position, *Lpl* (lipoprotein lipase) could be a candidate gene for the QTL on chromosome 16. For liver cholesterol concentrations, two suggestive associations have been found on chromosome 6 and 10. For serum phospholipids level, a QTL has been found on chromosome 11 with *Pcyt1a* (CTP:phosphocholine cytidylyltransferase) as a possible candidate gene. For postdietary aldosterone levels, two significant QTLs have been found on chromosome 1 and 18.

Chapters 6 and 7 deal with the chromosomal localization of genes that are involved in the biosynthesis, metabolism and transport of cholesterol by using the RH panel. In chapter 6, three genes involved in the biosynthesis, have been located on chromosome 7, 12, and 15, respectively. Six genes, all involved in cholesterol metabolism and transport, have been located on chromosome 3, 5, 7, 12, 16 and 19, respectively. The genes *Scp2* (sterol carrier protein-2; chromosome 5), *Sqle* (squalene epoxidase; chromosome 7), *Fdft1* (farnesyl diphosphate farnesyl transferase; chromosome 15) map close to a QTL for total serum cholesterol. In chapter 7, a recently discovered lipase gene, *Lipg* (codes for endothelial derived lipase) has been mapped in the rat RH map to chromosome 18.

In conclusion, this thesis describes the work that has been performed for increasing the marker density of the genetic map of the rat and for the localization of QTLs and genes involved in the cholesterol metabolism in this species. This contributes to the value of the rat as an animal model in studies towards the role of cholesterol in the pathogenesis of atherosclerosis an other cholesterol related diseases.

Samenvatting

Epidemiologische studies bij mensen en experimentele studies bij dieren hebben aangetoond dat het cholesterolgehalte in het bloed en de lever wordt beïnvloed door zowel genetische als omgevingsfactoren. Onderzoek naar de gevoeligheid voor voedingscholesterol is daarom moeilijk uitvoerbaar bij de mens. Om de genetische factoren, die bij de cholesterol stofwisseling een rol spelen te analyseren, moeten zowel de milieufactoren als de genetische achtergrond gestandaardiseerd zijn. Door gebruik te maken van inteeltstammen kunnen genetische verschillen en de daarbij betrokken genen sneller gedetecteerd worden.

In dit onderzoek is gebruik gemaakt van vier rat inteeltstammen, namelijk de BC/CpbU (BC), BN.*lx*/Cub (BN), LEW/OlaHsd (LEW) en SHR/OlaIpcv (SHR). De vier stammen zijn geselecteerd op basis van hun reactie op een cholesterolrijke voeding. BN en LEW reageren voor wat betreft de serum cholesterol spiegel sterk op een cholesterolrijke voeding en worden daarom hyperresponders genoemd, terwijl bij BC en SHR slechts een geringe stijging van het serum cholesterol plaatsvindt. Zij worden daarom hyporesponders genoemd. De 4 stammen verschillen ook met betrekking tot lever cholesterol accumulatie.

In de hoofdstukken 2 en 3 worden de BN, SHR en de hiervan afgeleide recombinant inteelt (RI) stammen gebruikt. RI stammen worden gevormd met de F_2 nakomelingen van een kruising tussen twee inteeltstammen; uitgaande van deze F_2 dieren vinden er vervolgens tenminste 20 generaties opeenvolgende broer-zuster paringen plaats. In hoofdstuk 2 wordt het onderzoek beschreven dat gericht is op de uitbreiding van de genetische kaart van de RI stammen met AFLP (amplified fragment length polymorphism) merkers. Met dertien verschillende primer-combinaties konden 89 AFLP merkers toegewezen worden aan chromosomen.

In voorafgaande studies is er een QTL (quantitative trait locus) voor lever cholesterol niveaus gevonden op rat chromosoom 10. Op basis van homologie is *Fabp6* (fatty acid binding protein 6) als een kandidaat-gen voor dit QTL gesuggereerd. In Hoofdstuk 3 wordt het DNA van de BN en SHR gebruikt om het *Fabp6* gen te amplificeren en te sequencen. Aangezien er geen verschil in nucleotiden volgorden gevonden werd, kon het gen niet geplaatst worden in de genetische kaart van de RI stammen. Daarom werden rat specifieke primers ontworpen (op basis van de intron sequenties) en met behulp van deze primers werd een radiation hybrid (RH) panel gescreend. Hiermee kon het gen geplaatst worden op rat chromosoom 10, in de buurt van het eerder gevonden QTL. Tijdens de amplificatie van het *Fabp6* gen, is er bij de SHR stam een DNA segment gevonden met alleen de exon-sequenties van dit gen. Screening van 66 andere rat inteeltstammen wees uit dat naast de SHR nog 3 andere stammen (OKA; SHRSP en WKY) ook dit pseudogen (*Fabp6*–ps) bezitten. Deze vier stammen komen allen uit dezelfde Wistar outbred

kolonie (Kyoto School of Medicine, Japan). Door analyse van een (SHRSPxBN)xBN terugkruising, kon het pseudogen gelokaliseerd worden op rat chromosoom 15.

In de hoofstukken 4 en 5 wordt de F_2 generatie van een kruising tussen BC en LEW gebruikt voor genetische analyse. In hoofdstuk 4 wordt het onderzoek beschreven dat geleid heeft tot het samenstellen van een genetische kaart, bestaande uit 258 DNA merkers (waarvan het overgrote deel bestond uit SSLP, simple sequence length polymorphism, merkers). De (genetische) lengte van deze kaart bedraagt ongeveer 1790 cM and de gemiddelde afstand tussen de merkers is 7.7 cM. De QTL-analyse met als parameters, zoals serumcholesterol niveau voor een cholesterolrijke voeding, serum cholesterol niveau, serum fosfolipiden spiegel, lever cholesterol concentratie en de spiegel van serum steroid hormonen allen na een cholesterolrijke voeding, gemeten in de F_2 dieren, wordt beschreven in hoofdstuk 5.

Voor de basaal serum cholesterol spiegel werd een associatie gevonden tussen de merkers D1Rat27 en D1Rat335, op chromosoom 1 in de buurt van het gen apolipoproteïne E, en op chromosoom 7. Voor serum cholesterol concentratie na een cholesterolrijke voeding zijn associaties gevonden op chromosoom 2 en 16. Op basis van de positie van het gen *Lpl* (coderend voor lipoproteïne lipase), zou *Lpl* een kandidaat gen kunnen zijn voor de QTL op chromosoom 16. Voor lever cholesterol concentraties werden suggestieve associaties gevonden met chromosoom 6 en chromosoom 10. Ook werd een significante associatie gevonden tussen de serum fosfolipiden concentratie en chromosoom 11, met *Pcyt1a* (CTP:fosfocholine cytidylyltransferase) als een kandidaat gen.
Tevens werden twee significante associaties voor aldosteron gevonden, een op chromosoom 1 en een op chromosoom 18.

In de hoofdstukken 6 en 7 wordt het onderzoek beschreven dat gericht is op de lokalisatie van genen welke betrokken zijn bij de biosynthese, transport en metabolisme van cholesterol.

In hoofdstuk 6 worden drie genen gelokaliseerd, welke betrokken zijn bij de biosynthese, op respectievelijk chromosoom 7, 12 en 15. Zes genen welke betrokken zijn bij het transport en metabolisme van cholesterol zijn geplaatst op respectievelijk chromosoom 3, 5, 7, 12, 16 en 19. Elk van de genen, die coderen voor sterol carrier proteïne-2 (chrom. 5), squalene epoxidase (chrom. 7) en farnesyl difosfaat farnesyl transferase (chrom. 15) zijn gelokaliseerd in de buurt van een QTL voor serum cholesterol spiegel. In hoofdstuk 7 wordt een pas ontdekt gen *Lipg*, dat codeert voor endothelial-derived lipase gelokaliseerd op chromosoom 18.

Concluderend kan gesteld worden dat het in dit proefschrift beschreven onderzoek geleid heeft tot de lokalisatie van een aantal genen welke betrokken zijn bij de cholesterol stofwisseling van de rat. Hiermee wordt een bijdrage geleverd aan de betekenis van de rat als diermodel voor het onderzoek naar de rol van cholesterol bij het ontstaan van hart- en vaatziekten.

Dankwoord

Zo dan nu misschien wel de belangrijkste pagina van het proefschrift, beter gezegd de meest gelezen pagina. Ik kan wel beginnen met de standaard zin van een proefschrift maak en schrijf je niet alleen, maar ik neem aan dat iedereen dit ondertussen wel weet of heeft ondervonden.
Allereerst wil ik natuurlijk mijn promoter, Bert, bedanken. Ik had me geen betere promoter kunnen wensen. Er was altijd interesse voor mijn onderzoek en je had altijd tijd als ik iets wilde vragen of overleggen. Hartelijk dank voor alles!
Hein, mijn co-promoter, jij ook bedankt voor alles. Het zal wel wennen voor jou zijn, maar dit is de enige pagina, met mijn cv, waar jij geen "suggesties" voor heb kunnen doen, hoewel deze suggesties altijd zeer leerzaam zijn geweest. Dankzij jouw kritische manier van het verbeteren van mijn manuscripten, is het een heel mooi proefschrift geworden, naar mijn mening. Misschien krijg je nu weer wat meer tijd voor het onderzoek.
Nu 2 andere steunpilaren in mijn onderzoek die ik moet bedanken, mijn paranimfen: Ria en Gert, jullie stonden altijd klaar voor mij en boden zowel raad als daad.
Ria, zonder jou hulp en kennis had ik het nooit gered. We hebben ook een gezellig tripje gehad naar Berlijn, waar je me ook nog wat culturele kennis heb bijgebracht. Hartelijke dank voor alles!!
Gert, hoewel je nu niet meer werkzaam bent bij onze afdeling, kan ik in geval van problemen je gelukkig nog altijd via e-mail bereiken (De Gert hulplijn). Ik ben erg blij dat je het zo naar je zin heb op je nieuwe werk, maar moet ook zeggen dat ik jouw gezelschap het laatste jaar gemist heb. Jij ook bedankt voor alles.
Giet, jou wil ik bedanken voor de analyses die jij in het begin van mijn onderzoek voor mij heb gedaan. Nog een paar maandjes werken en dan mag je van je pensioen gaan genieten.
Natuurlijk hebben alle medewerkers van Proefdierkunde ervoor gezorgd dat ik 4 jaar lang met plezier naar mijn werk ben gegaan, maar er zijn er een paar die ik specifiek wil noemen.
Inez, Petra, Marlies, Margot, Tonneke en Stephan, jullie bedankt voor alle gezellige klets-, horoscoop- en crypto-uurtjes.

Behalve de mensen op de werkvloer zijn er ook nog mensen uit mijn privé-leven, die vaak belangstellend vroegen hoe het ging. Bedankt voor jullie interesse, al is en was het vaak moeilijk voor te stellen wat ik precies deed, hopelijk brengt dit proefschrift hier iets meer duidelijkheid in.
Ome Ger, bedankt voor het onderwerpen van de omslag, het is een echt kunstwerk geworden.
Truus en Hotze, bedankt voor alles.
Pap, Mam, Karin en Mark, bedankt voor alle steun en interesse.

En nu als laatste, de belangrijkste persoon in mijn leven. Robert, jij was soms net de praatpaal en steunpilaar die ik af en toe nodig had. Dit hoofdstuk is afgesloten en het is tijd om aan een nieuw hoofdstuk te gaan beginnen, net zoals wij dit jaar samen gedaan hebben. Bedankt voor alles en een dikke kus!!

Curriculum Vitae

De schrijfster van dit proefschrift werd op 8 augustus 1973 geboren te Roemond. In 1990 werd het HAVO diploma behaald en in 1992 in VWO-diploma aan het Bisschoppelijk College Schöndeln te Roermond. In september dat jaar werd begonnen met de studie Biologie aan de Universiteit Utrecht. Na het afronden van een 9 maanden durende stage bij de afdeling Moleculaire Microbiologie, onder begeleiding van dr. T. Prinz en dr. J. Thomassen en een stage van 6 maanden bij de afdeling Pathologie van het academisch ziekenhuis Maastricht onder begeleiding van Freek Bot, werd in 1997 het doctoraal examen behaald. Van november 1997 tot mei 1998 was zij werkzaam als analiste bij de afdeling Pathologie van het academisch ziekenhuis Maastricht. In september 1998 werd begonnen met het AIO-schap bij de Hoofdafdeling Proefdierkunde, Faculteit Diergeneeskunde van de Universiteit Utrecht, onder begeleiding van Prof. Dr. L.F.M. van Zutphen en dr. H.A. van Lith. Het onderzoek beschreven in dit proefschrift is uitgevoerd bij deze afdeling.
Vanaf 1 augustus is zij werkzaam als Postdoc bij de afdeling Antropogenetica van het St. Radboud ziekenhuis te Nijmegen.

LIST OF PUBLICATIONS

* A. Bonné, M. den Bieman, G. Gillissen, V. Kren, D. Krenová, V. Bílá, V. Zídek, V. Kostka, A. Musilová, M. Pravenec, L.F.M. van Zutphen, H. A. van Lith. Genetic map of AFLP markers in the rat (*Rattus norvegicus*) derived from the HXB/Ipcv and BXH/Cub sets of recombinant inbred strains. (*Biochemical Genetics,* accepted)
* A. Bonné, C. Gösele, M. den Bieman, G. Gillissen, T. Keitler, H.A. van Lith, and L.F.M. van Zutphen. Localization of Fabp6 and identification of a fabp6 pseudogene in the rat (*Rattus norvegicus*). (sumitted)
* A.C.M. Bonné, M.G. den Bieman, G.F. Gillissen, B.F.M. van Zutphen, H.A. van Lith. 2002. A rat linkage map based on BC x LEW intercross. *Folia Biologica Praha,* 48:120-123.
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* A.C.M. Bonné, M.G. den Bieman, G.F. Gillissen, H.A. van Lith, B.F.M. van Zutphen. Chromosomal localization of genes involved in biosynthesis, metabolism or transport of cholesterol in the rat (*Cytogenetics and Genome Research, in press*)
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* Bonné, A.C.M., den Bieman, M., Gillissen, G.F., Kren, V., Krenova, D., Lankhorst, Æ., van Zutphen, L.F.M., Zidek, V., Pravenec, M., van Lith, H.A. 1999. Fine Mapping of a QTL for basal serum HDL_2 cholesterol level on chromosome 19 of the rat. Animal Research and Welfare: A partnership. Proceedings of the international joint meeting, XII ICLAS General Assembly & Conference, 7th FELASA symposium, Palma de Mallorca, Spain.
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