Original Articles

Chromosome Assignment of *Cd36* Transgenes in Two Rat SHR Lines by FISH and Linkage Mapping of Transgenic Insert in the SHR-TG19 Line

(SHR / *Cd36* / transgenic lines / FISH / linkage mapping)

F. LIŠKA[1], G. LEVAN[2], K. HELOU[2], M. SLADKÁ[1], M. PRAVENEC[1, 3], V. ZÍDEK[3], V. LANDA[3, 4], V. KŘEN[1, 3]

[1]Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Prague, Czech Republic

[2]Department of Cell and Molecular Biology and Genetics, Lundberg Laboratory, Gothenburg University, Gothenburg, Sweden

[3]Institute of Physiology, Academy of Sciences, Czech Republic

[4]Institute of Molecular Genetics, Academy of Sciences, Czech Republic

Abstract. The chromosome position of the *Cd36* insert was determined by FISH in two rat transgenic lines (SHR/Ola-TgN(EF1a*Cd36*)10Ipcv (SHR-TG10) and SHR/Ola-TgN(EF1a*Cd36*)19Ipcv (SHR-TG19). The *Cd36* transgene construct labelled with digoxigenin-11-dNTP was used as a probe in the FISH analysis. In accord with the previous finding that the SHR-TG10 harbours 6–8 copies of the transgene, the signals from both metaphase and interphase nuclei of SHR-TG10 preparations were rather strong and the probe hybridized to both copies of chromosome 1 at band q55. The probe hybridization to SHR-TG19 metaphase preparations also showed homozygosity of the transgene with localization of both copies to chromosome 11 at band q11. The signals were distinct but much weaker compared to the SHR-TG10, which again is in accord with the fact that the SHR-TG19 line harbours only a single copy of the transgene. In order to look for a possible impact of the insertion site neighbourhood upon the transgene phenotypic effect, we performed linkage mapping of the transgene in the SHR-TG19 line. By linkage mapping, the placement of the transgene to the proximal part of RNO11 was confirmed, the critical interval being 4 cM between *D11Rat20* and *D11Rat21*, in good agreement with the RH map. Within the close neighbourhood of the inserted *Cd36* transgene, there are several genes known to be expressed in kidney, and so the influence of some regulatory sequences enhancing kidney expression of the *Cd36* transgene can be envisaged.

Originally, the Cd36 was found on platelets as an integral membrane glycoprotein (Okumura and Jamieson, 1976) and underlies the human Nak[a] blood group polymorphism (Yamamoto et al., 1990). Cd36 functions as a thrombospondin and collagene receptor, binds oxidized low density lipoproteins (LDL), phospholipids and aged or *Plasmodium falciparum*-infected red blood cells. Cd36 was found to be expressed by several different cell types and involved in diverse physiological and pathological events, such as coagulation, host defence, inflammation, angiogenesis, lipid metabolism, scavenging, etc. (Telen, 2000; Febbraio et al., 2001). The implication of Cd36 in many different biological processes defines it as a multiligand scavenger receptor (Silverstein and Febbraio, 2000).

The *Cd36* gene is located to rat chromosome 4 at the peak of quantitative trait locus (QTL) linkage to spontaneously hypertensive rat (SHR) defects in glucose and fatty acid metabolism, triglyceridemia and hypertension, and it was recently shown to be defective in the spontaneously hypertensive rat strain SHR/OlaIpcv (Aitman et al., 1999). In order to analyse the causal involvement of *Cd36* in these disorders, we have derived several SHR transgenic lines, which express the introduced wild-type allele of the *Cd36* gene on the SHR background carrying the *Cd36* deletion variant (Pravenec et al., 2001). A relatively low level of wild *Cd36* expression in SHR-*Cd36* transgenic lines was shown to ameliorate metabolic disturbances compared to the intact SHR strain. In one of these lines, the SHR-TG19, with increased expression of wild-type *Cd36* in kidneys and liver, the blood pressure was significantly reduced (Pravenec et al., 2000). In order to elucidate the

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Corresponding author: Vladimír Křen, Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, Albertov 4, Prague 2, 128 00, Czech Republic. Tel.: +420 (2) 2496 8147; fax: +420 (2) 2491 8666; e-mail: vkren@lf1.cuni.cz.

Abbreviations: FISH – fluorescence *in situ* hybridization, QTL – quantitative trait locus, RH map – radiation hybrid map, SHR – spontaneously hypertensive rat.

position of transgenes in the genome of SHR recipients, the chromosomal position of the *Cd36* insert was determined by fluorescence *in situ* hybridization (FISH) in two transgenic lines (SHR-TG10 and SHR-TG19) and, in addition, linkage mapping of the *Cd36* insert in the SHR-TG19 line was performed using a segregating backcross population.

Material and Methods

Animals

The production and characteristics of SHR/Ola-TgN(EF1a*Cd36*)10Ipcv (SHR-TG10) and SHR/Ola-TgN(EF1a*Cd36*)19Ipcv (SHR-TG19) transgenic lines were described earlier (Pravenec et al., 2001). BN.*Lx.Cd36*.1K is a triple congenic strain (Šeda et al., 2002) that besides the differential segment of RNO8 (*Lx*) of PD/Cub origin and RNO20 (RT1) of SHR/OlaIpcv origin carries a short SHR chromosomal segment of RNO4 with the deletion variant of *Cd36* (Šeda et al., 2002).

The backcross population was prepared by mating F_1(SHR-TG19 x BN.*Lx.Cd36*.1K) x BN. Lx.*Cd36*.1K. SHR-TG19 animals were homozygous transgene carriers and so F_1 hybrids were hemizygous.

FISH

FISH was performed essentially according to Pinkel et al. (1986) with minor modifications (Helou et al., 1998). In brief, the transgenic construct clone (1 μg) was labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) by nick translation (Life Technologies Inc., Gaithersburg, MD). The appropriate length (100 to 500 bp) of the nick-translated fragments was monitored by checking the nick translation products on a 1% agarose gel. Eight hundred ng of labelled DNA were mixed with 50 μg unlabelled rat c_0t1 DNA, which was included in order to suppress probe hybridization to repetitive sequences. The probe DNA mixture was ethanol precipitated and dissolved in hybridization buffer (50% formamide, 2 x SSC, 10% dextran sulphate). After denaturation, the probe mixture was applied to rat C-metaphase preparations from bone marrow, which were prepared as described (Sladká et al., 1992). Rat metaphase chromosome slides were previously denatured at 73°C for 2 min in 70% formamide, 2 x SSC. Hybridization was allowed to proceed in a moist chamber for 48 h at 37°C. Subsequently, the slides were washed for 15 min at 45°C in 55% formamide, 2 x SSC, and then in 2 x SSC (pH 7.0) at 43°C for 9 min. The labelled probe molecules were detected with FITC antidigoxigenin (Oncor, Inc., Gaithersburg, MD) and the slides were washed 3 times in PBS and 0.1% Nonidet NP-40. Finally, chromosome spreads were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) in an antifade solution (Vectashield®, Vector Laboratories, Inc., Burlingame, CA). The images were captured using the Leica DM RXA in combination with the Q-FISH software for microphotography.

Cd36 genotyping

Primers for PCR were designed in putative exons 5 and 7, respectively (forward RCD36FAT355F: 5'-AGT TCG CTA TTT AGC CAA GG-3' and reverse RCD36FAT675R: 5'-AGG ATA AAA CAC ACC AAC TGT-3'), to amplify a fragment of 328 bp from the intronless transgenic construct. Hemizygous transgene carriers are positive and non-carriers are negative. Amplification of an about 800 bp fragment from the *Cd36* gene on chromosome 4 served as an internal control (Fig. 1).

Linkage mapping

Seventy-five backcross BN.*Lx.Cd36*.1K x F_1(BN.Lx.*Cd36*.1K x SHR-TG19) animals were genotyped. Triple congenic strain BN.*Lx.Cd36*.1K was used for the derivation of the backcross progeny because multiple microsatellite polymorphisms with the SHR strain could be exploited for genotyping. Genomic DNA was isolated from tail biopsy by phenol-chloroform extraction and ethanol precipitation. Primers for rat microsatellite markers (*D11Mit1, D11Mit4, D11Rat20, D11Rat21, D11Rat28, D11Rat29, D11Rat40* and *D11Rat79*) were obtained from Research Genetics or synthesized by Gibco BRL according to published sequences (Rat Genome Database – http://rgd.mcw.edu/). PCR and PAGE analysis were performed according to Pravenec et al. (1996). Map Manager QTX version 12 (http://mcbio.med.buffalo.edu/mapmgr.html) was used for linkage evaluation.

Results and Discussion

1/ The assignment of Cd36 insert in the SHR-TG10 and SHR-TG19 transgenic lines by FISH

FISH was used to determine the chromosomal location of *Cd36* inserts in the SHR-TG10 and SHR-TG19 transgenic lines. The transgene construct was used as a probe in the FISH analysis. In the SHR-TG10 line, the probe hybridized to both copies of chromosome 1 at band q55 (Fig. 2a). The signals from both metaphase and interphase nuclei were rather strong, which was in good accord with our previous finding that the SHR-TG10 line harbours 6–8 copies of the transgene (Pravenec et al., 2001). The probe hybridization to the SHR-TG19 metaphase preparations showed homozygosity of the transgene and localization to both copies of chromosome 11 at band q11 (Fig. 2b). The signals were distinct but much weaker than in the SHR-TG10, which is in agreement with the fact that the SHR-TG19

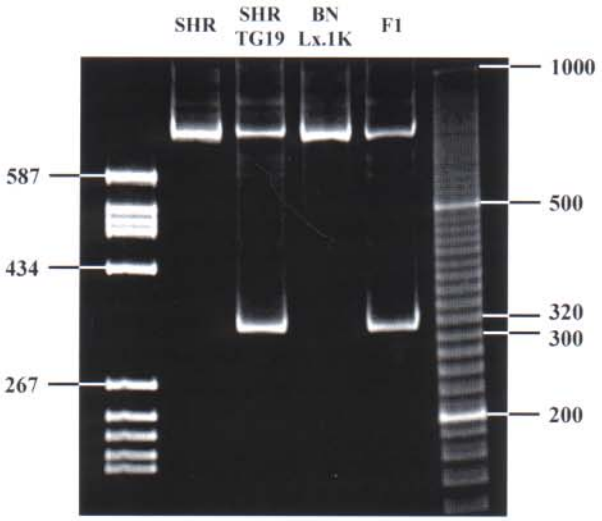

Fig. 1. PCR detection of the transgenic Cd36 insert.
Rat DNA was isolated by phenol-chloroform extraction from tail biopsy, and amplified with primers F: 5'-AGT TCG CTA TTT AGC CAA GG-3' and R: 5'-AGG ATA AAA CAC ACC AAC TGT-3', in exons 5 and 7, respectively, according to rat-human homology. In transgenic animals, an approximately 320 bp fragment was obtained, as expected due to lack of introns in the *Cd36* transgene. The larger fragment represents amplification of a deletion variant of the *Cd36* gene on RNO4. First lane: pBR322 DNA *Hae*III digest, 2nd lane: SHR/OlaIpcv, 3rd lane: SHR-TG19, 4th lane: BN.*Lx.Cd36*.1K/Cub, 5th lane: F_1(SHR.TG19xBN.*Lx.Cd36*.1K), the last lane: 20 bp low ladder.

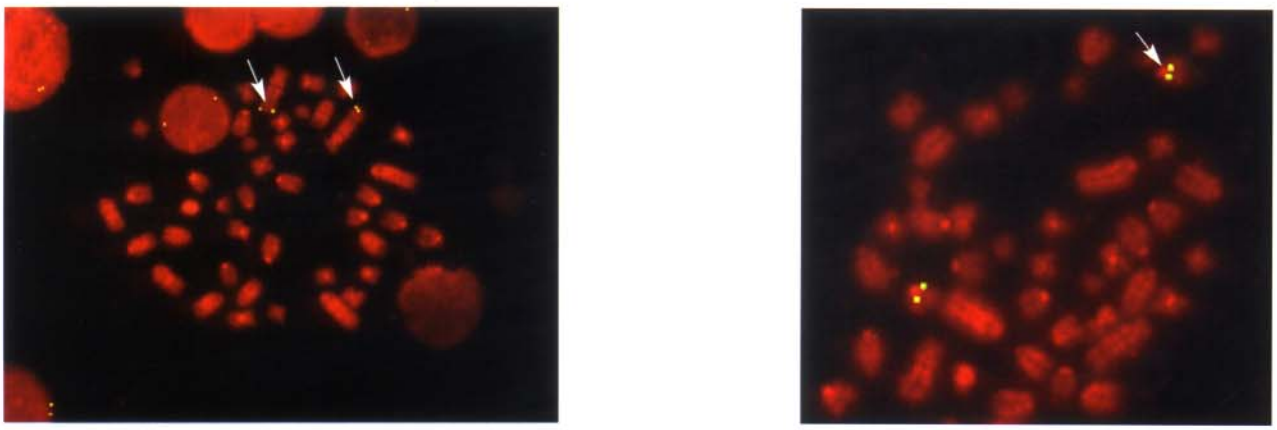

Fig. 2. FISH – TG10, TG19
Determination of the chromosomal insertion sites (arrows) of the Cd36 transgene by FISH. Hybridization signals can be seen on RNO1q55 in the SHR-TG10 transgenic lines (a) and on RNO11q11 in the SHR-TG19 transgenic lines (b).

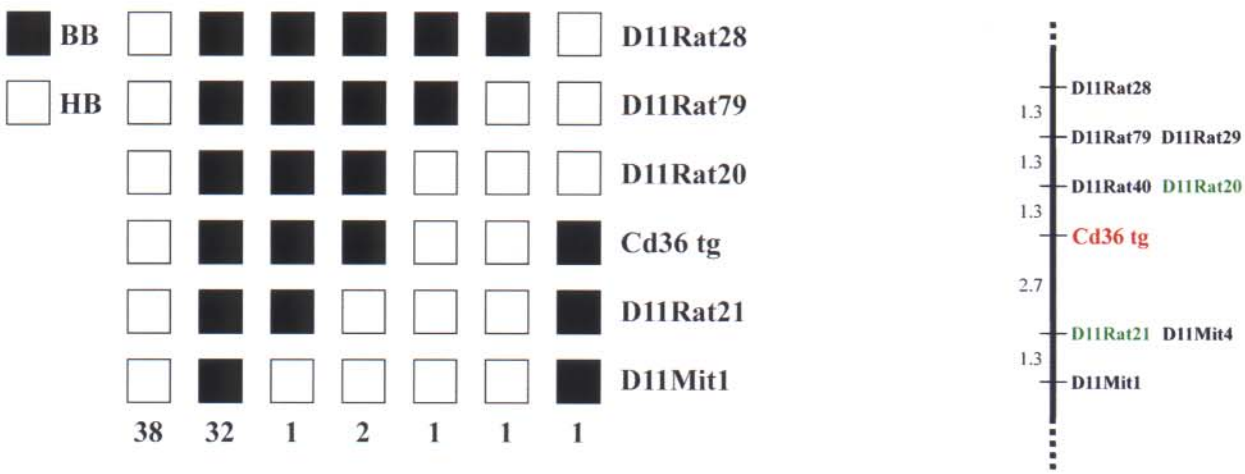

Fig. 3. Linkage mapping of the *Cd36* transgenic insertion site in the SHR-TG19 line.
A) Haplotype figure from BN.*Lx.Cd36*.1K x F_1(BN.*Lx.Cd36*.1K x SHR.TG19) backcross showing part of RNO11 with loci linked to the *Cd36* transgene. Black boxes represent the BN.*Lx.Cd36*.1K allele, whereas the white boxes are hetero- or hemizygous combinations of the BN.*Lx.Cd36*.1K allele with the SHR-TG19 allele. The number of animals for a given haplotype is indicated at the bottom of each column. B) Partial linkage map of an RNO11 segment containing the transgene, derived from the haplotype analysis shown in A). The numbers represent distances between linked loci in centimorgans (Kosambi map function).

Table 1. Genes and ESTs found between D11Rat20 and D11Rat21 by Virtual Comparative Mapping Tool (http://rgd.mcw.edu/VCMAP/.

cR	Name	Characteristics and expression		Mouse orthologue and its expression	
568.4	D11Rat21				
568.5	Rn.41680	2ESTs	ovary (AI547883), placenta, adult lung, brain, liver, kidney, heart, spleen, ovary, muscle, embryos 8, 12, and 18dpc		
568.6	Rn.1528	7ESTs	ovary, lung, pineal gland	Mm.170971	kidney, mammary gland, cerebellum, medulla oblongata, adrenal gland, diencephalon, spinal ganglion, aorta and vein, thymus, heart, lung
574	Rn.5790	Atp5j	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit F6 – ubiquitously expressed	Mm.353	ubiquitously expressed
576.2	Rn.19066	1EST	ovary (AI059097)		
578.9	Rn.16839	7ESTs	spleen, embryo 13dpc, kidney, brain, ovary, placenta, lung, liver, heart, muscle		
594.8	L11926	App	amyloid precursor protein (APP) gene, 5' end	Mm.15571	ubiquitously expressed
605.6	Rn.18313	4ESTs	placenta, embryo 8dpc	Mm.77895	caecum, colon, corpora quadrigemina, embryonic body between diaphragm region and neck, eyeball, forelimb, head, hypothalamus, medulla oblongata, skin, spinal cord, spinal ganglion, sympathetic ganglion
612.7	Rn.40214	5ESTs	spleen, heart, embryo 13dpc, kidney, ventricle 15dpc	Mm.31546	colon, embryo, infiltrating ductal carcinoma, kidney, lung, lymph, mammary gland, pooled lung tumours, spleen, uterus
617.4	D11Rat20				

line harbours only a single copy of the transgene. It is worth mentioning that reduction in the copy number often results in a marked increase in expression of the transgene and is accompanied by decreased chromatin compaction and decreased methylation at the transgene locus. Vice versa, the presence of multiple homologous copies of a transgene within a concatemeric array can have a repressive effect upon gene expression in mammalian systems (Garrick et al., 1998).

2/ Linkage mapping of the transgene in the SHR-TG19 line

The increased transgenic expression of the wild-type *Cd36* in the kidney of the SHR-TG19 was associated with a significant decrease in blood pressure compared to the SHR-TG10 and SHR strains (Pravenec et al., 2000). In the SHR-TG19 line, we decided to determine the precise position of the transgene on RNO11 in relation to surrounding genetic markers in order to

look for a possible impact of the insertion site neighbourhood upon the transgene expression and phenotypic effects.

By linkage mapping, we were able to confirm placement of the transgene to the proximal segment of RNO11, the critical interval being 4 cM between *D11Rat20* and *D11Rat21* (Fig. 3a, 3b). This result confirmed the FISH analysis and represents the first step towards detailed mapping and identification of the molecular basis of increased renal expression of the transgene. Markers *D11Rat20* and *D11Rat21* are placed in the rat radiation hybrid (RH) map at positions 617.4 and 568.4, respectively, with logarithm of odds (LOD) > 3.0. The distance between them is 49 cR_{3000} (http://rgd.mcw.edu/) and if an average of 12.5 cR_{3000}/1 cM is taken (Watanabe et al., 1999), our linkage results are in good agreement with the RH map. Moreover, the map bin defined by the RH positions allowed us to identify RH mapped ESTs and genes that could be considered to be candidate sequences possibly influencing the transgenic *Cd36* expression. The interval contains eight Unigene clusters and genes according to http://rgd.mcw.edu/tools/vcmap/vcmap.cgi?MapName=Rat+VirtualMap+5.0_HS&Chr=11&FirstPos=568.4&SecondPos=617.4). Several of the candidates, situated between *D10Rat20* and *D10Rat21*, are known to be expressed in kidney: *App* (amyloid β(A4) precursor protein, I11926), Rn.5790 (*Atp5j* – ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit F6), Rn.40214 (ESTs, homologous to Mm.31546 ESTs) and Rn.16839 (ESTs). However, the expression of these genes and ESTs is not restricted to kidney (Table 1).

Generally, the low copy number is probably associated with increased transgene expression in the SHR-TG19 compared to the SHR-TG10 line. However, it is possible that the strong transgene expression in kidney might be influenced by the genomic context of *Cd36* insertion in proximal RNO11. In turn, the increased renal expression of *Cd36* might determine the phenotypic differences between TG19 and TG10 transgenic lines. Several genes in the critical region are expressed in kidney; therefore, some regulatory sequences driving renal expression should exist there. Consequently, one might speculate that if the *Cd36* transgene were inserted in the vicinity of the above-mentioned regulatory sequences, they might enhance the expression of the *Cd36* transgene. Although the mechanism of the strong expression in kidney could be hypothesized, the exact explanation will need cloning and precise molecular characterization of the insertion site. The most straightforward approach to define the flanking DNA sequences seems to be sequence analysis of inverse PCR products (e.g. Wiliams et al., 2002).

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