Structure and chromosomal assignment of the human lectin-like oxidized low-density-lipoprotein receptor-1 (*LOX-1*) gene

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We have reported the cDNA cloning of a modified low-density-lipoprotein (LDL) receptor, designated lectin-like oxidized LDL receptor-1 (LOX-1), which is postulated to be involved in endothelial dysfunction and the pathogenesis of atherosclerosis. Here, we determined the organization of the human *LOX-1* gene, including the 5′-regulatory region. The 5′-regulatory region contained several potential *cis*-regulatory elements, such as GATA-2 binding element, *c-ets*-1 binding element, 12-*O*-tetradecanoylphorbol 13-acetate-responsive element and shear-stress-responsive elements, which may mediate the endothelium-specific and inducible expression of LOX-1. The major transcription-initiation site was found to be located 29 nucleotides downstream of the TATA box and 61 nucleotides upstream from the translation-initiation codon. The minor initiation site was found to be 5 bp downstream from the major site. Most of the promoter activity of the *LOX-1* gene was ascribed to the region (−150 to −90) containing the GC and CAAT boxes. The coding sequence was divided into 6 exons by 5 introns. The first 3 exons corresponded to the different functional domains of the protein (cytoplasmic, transmembrane and neck domains), and the residual 3 exons encoded the carbohydrate-recognition domain similar to the case of other C-type lectin genes. The *LOX-1* gene was a single-copy gene and assigned to the p12.3–p13.2 region of chromosome 12. Since the locus for a familial hypertension has been mapped to the overlapping region, *LOX-1* might be the gene responsible for the hypertension.

Key words: chromosomal assignment, genomic organization.

INTRODUCTION

Functional changes of endothelial cells are implicated in the earliest stage of the pathogenesis of atherosclerosis [1]. These changes include the impairment of the release of nitric oxide, and the enhanced expression of chemoattractant molecules and adhesion molecules for leucocytes, and of growth factors for smooth-muscle cells in endothelial cells. Accumulating evidence suggests that oxidized low-density lipoprotein (OxLDL) plays a critical role in the changes in endothelial function [2,3].

Although several kinds of scavenger receptor expressed in macrophages have been identified, the endothelial OxLDL receptor has not been molecularly identified [4–10]. Recently, we cloned cDNA for the major endothelial OxLDL receptor, lectin-like OxLDL receptor-1 (LOX-1), from cultured bovine endothelial cells [11]. LOX-1 is a type-II membrane protein which belongs structurally to the C-type lectin family. LOX-1 was expressed not only in cultured endothelial cells and vascular-rich organs, but also in intact endothelium *in vivo* and atheromatous intima. To date, cDNAs for bovine, human, rat and mouse LOX-1 have been cloned [11–13]. Unlike human and bovine LOX-1, mouse and rat LOX-1 had a triple-repeat structure of the neck domain that connects the transmembrane domain and the carbohydrate-recognition domain (CRD). LOX-1 binds negatively charged phospholipids and apoptotic/aged cells as well as OxLDL [14].

The genetic study of the *LOX-1* gene may provide further information concerning the physiological and pathological roles of LOX-1. Here we describe the structural organization of the *LOX-1* gene and its choromosomal location.

EXPERIMENTAL PROCEDURES

PCR

LA *Taq* (2.5 units; Takara) was used for PCR to perform accurate amplification of DNA in 50 μl of LA PCR buffer (Takara) with 2.5 mM $MgCl_2$/0.4 mM dNTPs/0.2 μM primer pairs. At least two clones were sequenced to confirm the accuracy of the sequences of the PCR products.

Cloning of the 5′-promoter region of the human *LOX-1* gene

For the numbering of the cDNA and promoter sequence for *LOX-1*, the first transcription-initiation site was assigned 1. We amplified the 5′-promoter region of the *LOX-1* gene using the Human promoter finder DNA walking kit (Clontech). The tagged human genomic library was subjected to PCR: 98 °C, 20 s; 68 °C, 15 min, for 30 cycles using a primer for the tagged sequence of the library, A-1 (5′-GTAATACGACTCACTATAGGGC-3′), and 5′-TTCCAAATTCAAGCTAAGAATGAGAGAGTGAAGCA-3′, which is complementary to *LOX-1* cDNA (bp 27–61). The reaction mixture from the first PCR was subjected to a second PCR using A-2 (5′-ACTATAGGGCACGCGTGGT-3′), a primer for the tagged sequence nested to A-1,

Abbreviations used: OxLDL, oxidized low-density lipoprotein; LOX-1, lectin-like OxLDL receptor; TRE, 12-*O*-tetradecanoylphorbol 13-acetate-responsive element; CRD, carbohydrate-recognition domain; 5′-RACE, 5′-rapid amplification of cDNA ends; FISH, fluorescence *in situ* hybridization; SRE, sterol-regulatory element; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SSRE, shear-stress responsive element.

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and 5′-GAGTGAAGCAGTCACGAACTTCAACAAACTA-AAAT-3′, which is complementary to *LOX-1* cDNA (1–36), under the same conditions as the first PCR. The specifically amplified product, with a length of 2500 bp, was gel-purified, subcloned in pGEM-T vector (Promega), and the sequence analysed by the dideoxy chain-temination method [15]. The product covered the 5′-promoter region of the *LOX-1* gene (−2463 to +30).

Isolation of genomic clones

A human genomic DNA library constructed in EMBL3SP6/T7 (Clontech) was screened with *LOX-1* cDNA (1–946) labelled with the random primer method using [α-^{32}P]dCTP. Six positive clones were obtained. The inserts of clones H, J and S were analysed extensively. Clone H covered the *LOX-1* gene from the 5′-flanking region to intron 1; clone J was from intron 3 to the 3′ end; and clone S was from intron 2 to intron 3.

The genomic DNA fragments containing intron 1 and intron 2 were obtained by nested PCR using the human genomic DNA library as a template. PCR for genomic DNA around intron 1 was performed with the following primer pairs; lox1 and lox6 for the first PCR and lox2 and lox5 for the second. For intron 2, primer pairs lox3 and lox8 were used for the first PCR, and lox4 and lox7 were used for the second PCR. Then the exon–intron boundaries were determined by sequencing the amplified DNA fragments and the inserts of clone S and J. The sequences of the PCR primers were as follows: lox1, 5′-CAGTCTCTCATTCT-TAGCTTGAATTTGGAA-3′; lox2, 5′-ATGACTTTTGATG-ACCTAAAGATCCAGACT-3′; lox3, 5′-GTCCTTTGCCT-GGGATTAGTAGTGACCATT-3′; lox4, 5′-TAGTGACCA-TTATGGTGCTGGGCATGCAAT-3′; lox5, 5′-AATGGTCA-CTACTAATCCCAGGCAAAGGAC-3′; lox6, 5′-ATTGCAT-GCCCAGCACCATAATGGTCACTA-3′; lox7, 5′-CTTGT-GTTAGGAGGTCAGACACCTGGGATA-3′; and lox8, 5′-TCTGGTGAGTTAGGTTTGCTTGCTCTTGTG-3′.

Primer-extension analysis

An oligonucleotide (5′-TGGATCTTTAGGTCATCAAAAG-3′) complementary to *LOX-1* cDNA (65–86) labelled with Infrared Dye 41 (LI-COR) was incubated with RNA samples [2 μg of poly(A)$^+$ RNA from human placenta or *Escherichia coli* tRNA] at 80 °C for 15 min in 20 μl of 0.8 M NaCl/80 mM Pipes (pH 6.3)/2 mM EDTA/50 % formamide. The reverse-transcription reactions proceeded for 1 h at 42 °C in 10 μl of 75 mM KCl/3 mM $MgCl_2$/50 mM Tris/HCl (pH 8.3)/1 mM each of the dNTPs/10 units of RNase inhibitor (Promega)/10 μM dithiothreitol/200 units of Molony murine leukaemia virus reverse transcriptase. The primer-extension reactions were separated by electrophoresis, running alongside the DNA-sequence reactions of the genomic DNA around the transcription-initiation site that was sequenced using the same primer.

5′-Rapid amplification of cDNA ends (5′-RACE)

5′-RACE was performed with 5′-RACE-ready cDNA from human placenta (Clontech), anchor primer (5′-CTGGTT-CGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′) and *LOX-1*-specific primer (5′-CATCCAGAATGGAAAACTGG-AATAGG-3′), which is complementary to *LOX-1* cDNA (647–672). Thermal cycles (30 rounds) were then carried out (94 °C, 45 s; 60 °C, 45 s; 72 °C, 2 min). The amplified cDNA fragments were separated in 0.8 % agarose gel, purified from gel and subcloned in pGEM-T vector. The sequence of the insert was analysed as above.

Luciferase reporter vector construction

The DNA fragment of the 5′-promoter region was amplified by PCR using the 5′-promoter-region DNA (−2463 to +30) for the template, the upstream primers tagged with the *Sac*I restriction site (see below) and the downstream primer tagged with the *Bgl*II restriction site (5′-CCAGATCTGAGTGAAGCAGTCACGA-ACTTCAA-3′). The amplified fragments were digested with *Sac*I and *Bgl*II and subcloned between the *Sac*I and *Bgl*II sites of the pGL3basic vector. The constructs were named according to the region covered by the inserts, e.g. p(−310/+30), p(−230/+30) etc. The sequences of the upstream primers were as follows: p(−310/+30), 5′-CCGAGCTCGGCACATTTTTTACAAAT-GTAGTG-3′; p(−230/+30), 5′-CCGAGCTCTCCTCTGAT-GCTCATGAAAAATAG-3′; p(−189/+30), 5′-CCGAGCT-CTACTTAGCGAAATATCCTGAAACA-3′; p(−166/+30), 5′-CCGAGCTCACCTTCAGAATCACCACTTTCTCC-3′; p(−150/+30), 5′-CCGAGCTCCTTTCTCCACCTGCAATA-CACATA-3′; p(−90/+30), 5′-CCGAGCTCAGCAAAGCC-TCTCCTTCCTCCTAC-3′; p(−50/+30), 5′-CCGAGCTCA-CTTCTGCAGAAGCTCACATATTT-3′; and p(−20/+30), 5′-CCGAGCTCCTTCTATTAGATAACAGTAGCTAT-3′.

Measurement of luciferase activity and β-galactosidase activity

HeLa cells were seeded on 6-well plates to give 40–60 % confluency at the transfection. The appropriate reporter vector (0.5 μg) was co-transfected with pCMV·Sport-βGal vector (Gibco; 0.5 μg) using the CalPhos Maximizer transfection kit (Clontech) according to the manufacturer's instructions. Luciferase and β-galactosidase activities were measured 48 h after transfection with the Luciferase assay system and the β-galactosidase assay system (Promega), according to the manufacturer's instructions. The relative values of luciferase activity were determined by normalizing with β-galactosidase activity for transfection efficiency. Results are expressed as the means ± S.E.M. Statistical analysis was performed using analysis of variance and Student's *t*-test.

Southern-blot analysis

Human genomic DNA (4 μg) prepared from human placental tissue was digested with restriction endonucleases, *Eco*RI, *Hin*d III and *Bam*HI, separated on a 0.7 % agarose gel, and blotted to a membrane (Gene Screen Plus, DuPont). The membrane was hybridized at 65 °C, using a 630 bp cDNA fragment (485–1114) as a probe, in a hybridization buffer containing 1 M NaCl, 1 % SDS and 250 μg/ml denatured salmon sperm DNA. The membrane was then washed three times at 50 °C, for 15 min in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS and then autoradiographed with an imaging plate and analysed using the BAS 2000 system (Fujifilm).

Isolation of *LOX-1* genomic DNA clones from the chromosome-12-specific library

Approximately 10^6 clones from a human chromosome-12 genomic library in Charon40 (American Type Culture Collection) were screened using human *LOX-1* cDNA as a probe under the high-stringency hybridization conditions as used for Southern-blot analysis.

DNA sequencing

The nucleotide sequence of the *LOX-1* genomic clones was determined by the dideoxy chain-termination method using

Figure 1 Nucleotide sequence of the promoter region of human *LOX-1*

The transcription-initiation site is assigned +1. GATA-2, potential GATA-2 binding sites; c-ets-1, potential *c-ets-1* binding site.

Sequenase (U.S. Biochemicals) in combination with appropriate oligonucleotide primers. Nucleotide sequences were analysed using Genetyx Gene Analysis System software (Genetyx).

Fluorescence *in situ* hybridization (FISH)

The R-banded human metaphase chromosome slides were prepared from peripheral blood by standard methods [16]. The probe labelled with biotin-16-dUTP was a mixture of *LOX-1* genomic DNA that, in total, covered the regions from the 5′-flanking region to exon 3 and from exon 4 to exon 6. FISH was carried out according to Kagiyama et al. [17] with slight modifications. Hybridization signals were detected with a DETEK 1-f signal-generating system (Enzo Diagnotics) for four plasmid DNA probes, and chromosomes were counterstained with 4,6-diamino-2-phenylindole dihydrochloride for identification. Fluorescent signals were analysed on a Zeiss Axiophoto fluorescence microscope system.

RESULTS AND DISCUSSION

Transcriptional enhancers in the 5′-flanking region of the *LOX-1* gene

A genomic DNA fragment of the 5′-flanking region of the *LOX-1* gene about 2500 bp long was obtained. The identity of the fragment was confirmed by the presence of the same sequence obtained from a phage clone covering the overlapping region (clone H).

As shown in Figure 1, we determined the sequence of the 5′-flanking region, and searched for transcriptional-enhancer elements in a database, TRNASFAC, with a threshold of 0.88 [18]. Although only a few details are known about the factors that modulate LOX-1 expression, a number of potential transcription-factor-binding sites were found in the sequence.

The GATA-2-binding sites were present at −180 and −1676 bp and are known to mediate endothelium-specific expression of endothelin-1 [19]. The binding motif for *c-ets-1* was found at −2274 bp. *c-ets-1* has been shown to be expressed in endothelial cells during angiogenesis and tumour vascularization, and to be responsible for endothelium-specific expression of a vascular endothelial growth-factor receptor, *flt* [20,21]. These sites may mediate endothelial expression of LOX-1.

12-*O*-Tetradecanoylphorbol 13-acetate-responsive elements (TREs) were found at −60, −984 and −1714 bp. TRE is the binding site for transcription factor activating protein-1, and is responsible for the induction of transcription mediated by protein

kinase C [22,23]. As phorbol ester induces LOX-1 expression in bovine aortic endothelial cells (N. Kume, unpublished work), TREs found in the present study may mediate the induction by phorbol ester.

Shear-stress responsive element (SSRE, GAGACC) was reported to mediate the transcriptional induction of the platelet-derived growth-factor B-chain gene in response to haemodynamic factors [24,25]. SSRE is present in shear-inducible genes expressed in endothelial cells, e.g. endothelial nitric oxide synthase, monocyte chemoattractant protein-1, transforming growth factor-β and intercellular cell-adhesion molecule 1, and is also present in *LOX-1* (-1447 and -1011 bp) [26]. Indeed, the expression of LOX-1 is induced by shear-stress in cultured endothelial cells (T. Murase, unpublished work). The SSRE found in the present study may be involved in this process. The expression of LOX-1 is also induced by tumour necrosis factor-α (N. Kume, unpublished work). Since SSRE was also reported to act as the nuclear factor κB-binding site, the elements may also be involved in the nuclear factor κB-mediated induction of LOX-1 expression [27,28].

Sterol-regulatory elements (SREs) are known to mediate the regulation of gene expression by cholesterol, e.g. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, HMG-CoA reductase and LDL receptor [29–31]. Under the regulation by this system, cholesterol is endogenously synthesized from acetyl-CoA and acetoacetyl-CoA and exogenously taken up from LDL, when needed. SREs were not found within the 5′-upstream region of LOX-1 obtained in the present study. This is consistent with the finding that the expression of LOX-1 is not restricted by the overload of LDL (T. Aoyama, unpublished work), although SRE might be located outside the analysed region. This finding supports the role of LOX-1 in the unregulated accumulation of LDL in atheroma.

LOX-1 gene promoter

TATA and CAAT boxes were found in the proximal part of the 5′-flanking region of the *LOX-1* gene. The TATA box (TATTTAAA) was located at -29 bp and the CAAT box (CCAAT) at -99. The locations were consistent with those of other genes. The presence of these elements suggested that *LOX-1* is not a house-keeping gene but an inducible regulated gene.

To clarify the complete structure of the *LOX-1* gene, the 5′ end of the corresponding mRNA was determined by primer-extension analysis using human placenta as the source of LOX-1 mRNA. As shown in Figure 2, a major transcription-initiation site was found to be an adenosine nucleotide corresponding to a site 61 bp upstream from the first nucleotide of the methionine initiation codon. A second prominent band that corresponds to the adjacent adenosine was also detected. This minor site was located at a position $+5$ bp relative to the major transcription-initiation site. The transcription-initiation sites were mapped to two adenosine residues 61 and 55 bp upstream from the translation-initiation site.

To confirm the results of the primer-extension analysis, the transcriptional start site of the *LOX-1* gene was also examined by 5′-RACE analysis. 5′-RACE amplified DNA fragments about 730 bp long from cDNA of the placenta. The fragments were subcloned in a plasmid vector and subjected to sequencing. DNA sequencing revealed that one fragment started with an adenosine nucleotide, which was 55 bp upstream from the methionine initiation codon, and the other started with another adenosine nucleotide 61 bp upstream. These results are consistent with those obtained using primer extension. Both techniques placed the two initiation sites of *LOX-1* transcription 29 and 34 bp downstream of a consensus TATA box sequence.

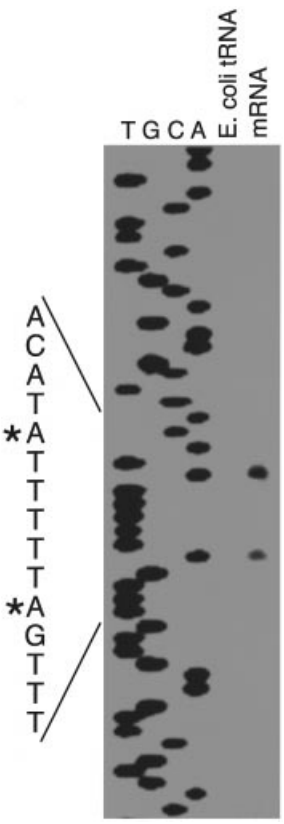

Figure 2 Identification of the transcription-initiation sites of human *LOX-1* by primer-extension analysis

Primer-extension analysis was performed on placental poly(A)$^+$ RNA using an end-labelled primer, which is complementary to the 5′ end of the LOX-1 mRNA. mRNA from human placenta (mRNA in Figure) and, for control, *E. coli* tRNA were analysed using a synthetic oligonucleotide complementary to *LOX-1* cDNA (65–86) as a primer (5′-TGGATCTTTAGGTCATCAAAAG-3′). Sequence reactions of *LOX-1* genomic DNA using the same primer were run in parallel (A,G,C,T) on a 4% sequencing gel. Asterisks indicate the transcription-initiation sites.

Promoter activity

To identify DNA elements important for basal expression of LOX-1, we constructed a series of 5′ deletions through the promoter region (Figure 3). These constructs were co-transfected into HeLa cells with pCMV·Sport-βGal to normalize luciferase activities with the differences in transfection efficiency. Among them, the longest construct of the *LOX-1* genomic fragment, p($-310/+30$), spanning bp -310 to $+30$, showed the highest activity. Serial deletions from -310 to -150 bp caused no significant change in the promoter activity. Deletion of the sequences between bp -150 and -90 significantly reduced *LOX-1* promoter activity to 24% of p($-310/+30$) ($P < 0.05$), suggesting the presence of positive regulatory elements in this region. The region contained GC and CAAT boxes, which probably mediate the positive regulatory roles. Further deletion from -90 to -50 and from -50 to -20, the area containing TATA box, resulted in a further decrease in activity to less than 3% of p($-310/+30$) ($P < 0.05$).

Analyses of the 5′-flanking region of the *LOX-1* gene have identified a proximal fragment of 180 bp (-150 to $+30$) that is

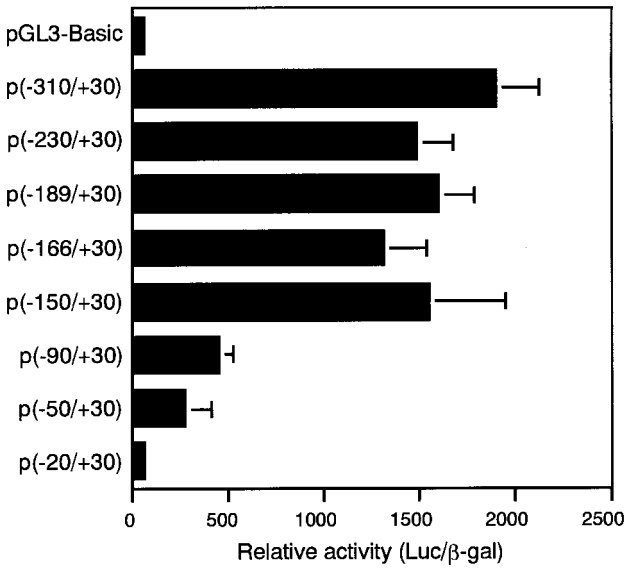

Figure 3 Analysis of the promoter function of the *LOX-1* 5′-flanking region

A panel of constructs for the *LOX-1* promoter coupled to the reporter gene, luciferase (Luc), was transfected into HeLa cells. All constructs were co-transfected with pCMVβgalSPORT to correct the transfection efficiency. The luciferase activity conferred by each construct was expressed as activity relative to that raised by the promoter-less control plasmid pGL3-Basic. Values are expressed as the means $\pm$ S.E.M. of three independent experiments. Each plasmid name indicates the beginning and the end of the region of *LOX-1* inserted into the vector.

sufficient to regulate basal promoter activity. This proximal promoter region contains a canonical TATA box, a CAAT box and a GC box that may be a target for Sp1, a ubiquitous and constitutive transcription factor that binds to the promoters of many genes [32].

Exon–intron organization of human *LOX-1*

As shown in Figure 4, the *LOX-1* gene was found to span more than 7 kb. *LOX-1* consisted of 6 exons interrupted by 5 introns. The exons ranged in size from 102 to 1722 bp. Exons 1–5 ranged from 102 to 246 bp, whereas the last exon, exon 6, was relatively very long, being 1722 bp. Excluding exon 6, the average exon length was 148 bp, which is consistent with the reported average exon size of 137 bp [33]. The positions of the exon–intron boundaries could be determined by comparison with the cDNA sequence. Splice-acceptor and -donor sequences (Table 1) completely agreed with the GT–AG rule [34] and conformed to the consensus proposed by Mount [35]. No differences between the cDNA and genomic sequences were observed. In the coding region, one splice junction (20%) occurred between amino acid codons (type 0), three (60%) occurred after the first nucleotide of a codon (type 1), and one (20%) occurred after the second nucleotide of a codon (type 2). The frequency of type-1 junctions was more than the reported average of that in the vertebrate genes (type 0, 41%; type 1, 36%; and type 2, 23% [36]).

The *LOX-1* gene structure suggests a relationship between the exon–intron architecture and protein structure. Exon 1 encodes the 5′-untranslated region and cytoplasmic domain, exon 2 encodes the remainder of the cytoplasmic domain and the transmembrane domain, exon 3 encodes the neck domain and exons 4–6 encode the lectin domain, as in a number of other C-type lectins, and the 3′-untranslated region [37] (Figure 4).

At the 3′ end of the *LOX-1* gene, exon 6 contained the consensus polyadenylation signal, AATAAA. Poly(A)$^+$ was found to be attached to LOX-1 mRNA 23 bp downstream from the AATAAA motif [38]. Further downstream from this region,

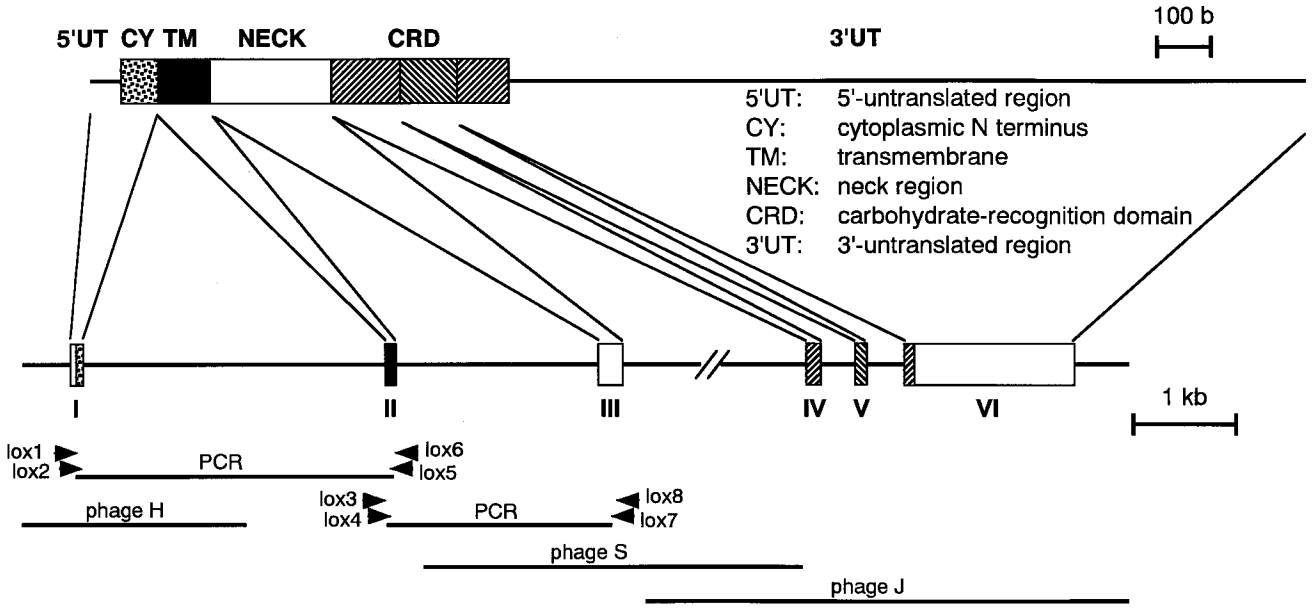

Figure 4 Structural organization of human *LOX-1* gene and mRNA

The upper scheme indicates the domain structure of the LOX-1 protein encoded by LOX-1 mRNA. The translated region is indicated by boxes: dotted box, cytoplasmic region; black box, transmembrane domain; open box, neck region; striped box, CRD domain. The lower scheme indicates the exon–intron organization of the human *LOX-1* gene. Exons are indicated by boxes numbered I–VI; introns, and 5′- and 3′-flanking sequences are indicated by lines. The cloning strategy in the present study is also shown below. Arrow heads indicate PCR primers. The cloned DNA fragments are indicated by lines.

Table 1 Exon–intron boundaries of human *LOX-1*

Sequences of exon–intron boundaries are shown. Sequences of exons are in upper-case letters, and those of introns are in lower-case letters. Amino acids whose codons are interrupted by introns are also shown.

		Sequence at exon–intron junction			
		5′-Splice donor		3′-Splice acceptor	Amino acid interrupted
1	136	GCT AAA G gtactag	(intron 1)	gctaaag GT CTT CAG	Gly
2	102	ATG CAA T gtaagtg	(intron 2)	gattcag TA TCC CA	Leu
3	246	TGT TCA G gtattgg	(intron 3)	ttcccag CT CCT TG	Ala
4	140	T GAT CTG gtgagtg	(intron 4)	cccacag GAC TTC A	Leu–Asp
5	116	CC CAC TT gtaagtt	(intron 5)	cctgcag A TTT AGA	Leu
6	1722	TTGTGTAC			

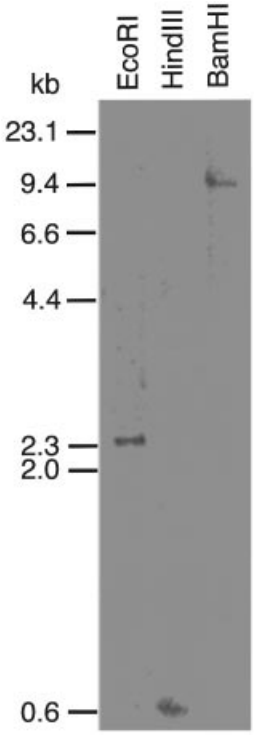

Figure 5 Southern-blot analysis of total genomic DNA

Human genomic DNA (15 μg) was completely digested with *Eco*RI, *Hin*dIII and *Bam*HI, separated by electrophoresis in 0.8% agarose gel, and transferred to nylon membrane. Then the membrane was hybridized with a ^{32}P-labelled *LOX-1* cDNA (485–1114).

the *LOX-1* gene had a striking representation of the trinucleotides TGT in conjunction with oligo-T stretches (a G/T cluster), which is frequently seen in genes in the region 18 bp downstream from the AATAAA motif. Therefore, this region was regarded as the 3′ end of the *LOX-1* gene.

C-type animal lectins, which have a Ca^{2+}-dependent CRD, have been classified into seven groups. The classification is based on the overall architecture of the protein, the position of the CRD relative to other domains, or the degree of similarity among their CRD [39]. Briefly, these groups are: I, proteoglycans; II, type-II membrane proteins including hepatic lectins, the Kupper cell receptor and the CD23 lymphocyte-activation antigen; III, collectins, which include the mannose-binding proteins, the pulmonary surfactant apoprotein and conglutinin; IV, L-, P- and E-selectins; V, a second group of type-II membrane proteins including NKR-P1, Ly49 and CD69; VI, the mannose receptor family; and VII, the free CRD.

In amino acid sequence, LOX-1 resembles group-V proteins. The organization of the *LOX-1* gene was also similar to that of genes of group-V proteins. The positions of the introns in the CRD of LOX-1 corresponded exactly to those in mouse NKR-P1 and mouse Ly49. The first intron, which separates the cytoplasmic tail from the transmembrane domain, was in a similar position to that found in NKR-P1, CD69 and Ly49 genes. *LOX-1* had the exon coding the neck domain located between the transmembrane domain and CRD, present in these families. This additional exon encodes an α-helical coil that serves as a stalk for the CRD. These properties suggest that the *LOX-1* gene is derived by gene duplication from a common ancestor of group-V proteins.

Southern-blot analysis of the *LOX-1* gene

Other group-V lectins, such as mouse and rat NKR-P1 and mouse Ly49, were reported to be produced by alternative splicing from a family of multiple genes [40]. To determine whether LOX-1 is a product of a single gene, Southern-blot analysis of the total genomic DNA was carried out (Figure 5). Each lane containing the digest with *Eco*RI, *Hin*dIII and *Bam*HI showed a single band, probed with a 630 bp fragment (485–1114) of *LOX-1* cDNA. These findings suggest that LOX-1 is encoded by a single-copy gene.

Chromosomal assignment

Dib et al. have reported the mapping of 5264 microsatellites containing $(CA)_n$ dinucleotide repeats [41]. One of them was identical to the $(CA)_n$ repeat present in the 3′-untranslated region of human *LOX-1* cDNA. It was reported to be located on chromosome 12. To confirm the chromosomal localization, we screened the human chromosome-12 genomic DNA library. Using *LOX-1* cDNA as a probe, eight clones giving positive hybridization signals under high-stringency conditions were obtained. Sequencing the inserts of the clones identified the sequence of *LOX-1* cDNA fragments in all eight clones.

We then performed FISH on normal metaphases, using *LOX-1* genomic DNA fragments as probes. Approximately 100 early metaphases were analysed, 90% of which displayed twin signals on chromosome 12 at the distal portion of 12p12.3–p13.2. About 10% of the metaphases showed a single signal at the same

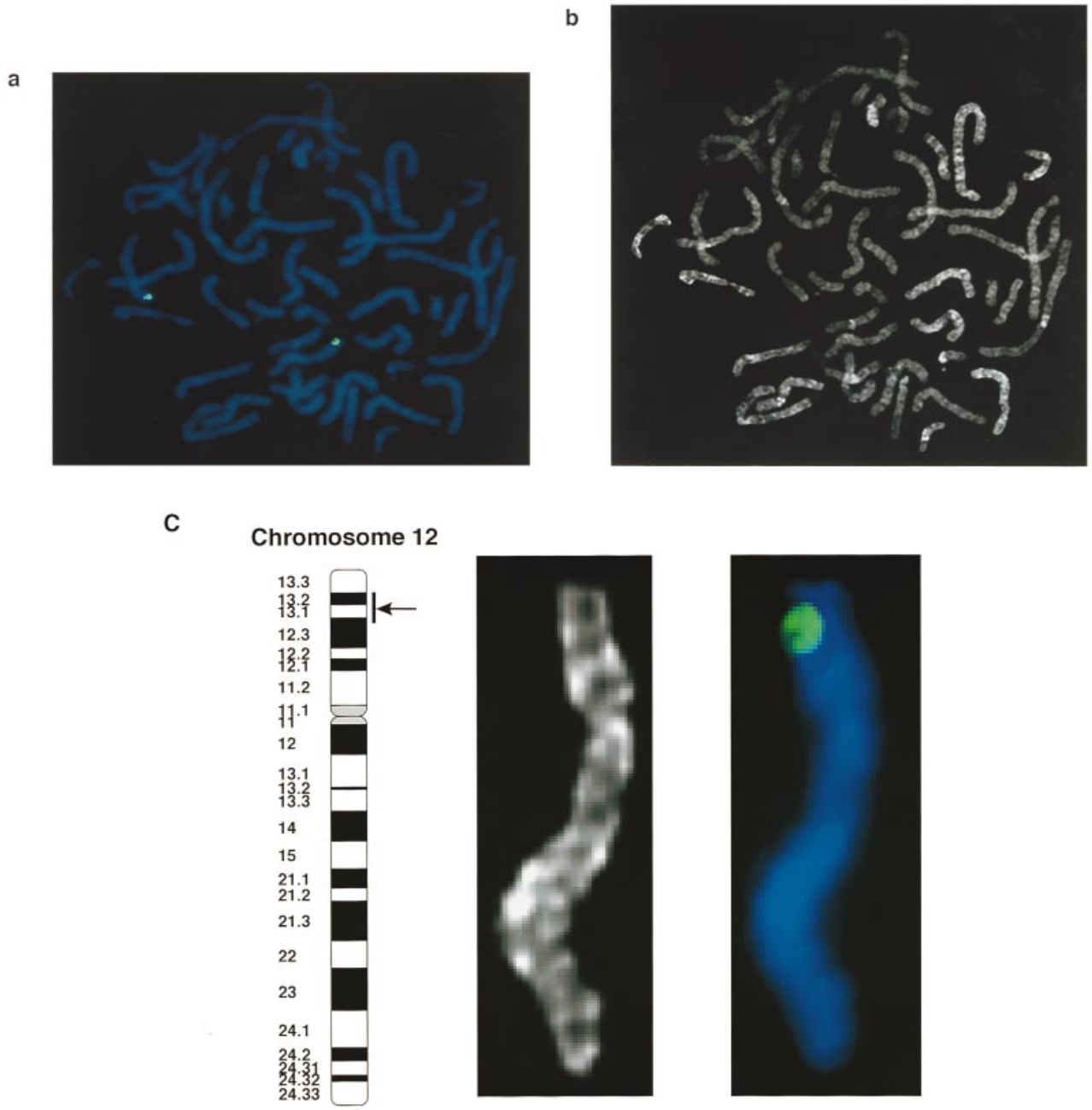

Figure 6 Location of human *LOX-1* on chromosome 12p12.3–p13.2

(**a**) Human metaphase chromosome following FISH with four plasmid DNA probes. Twin signals of biotin-labelled (green) DNA probe for the *LOX-1* gene were detected at band 12p12.3–p13.2 on both chromatids of chromosome 12. (**b**) Converted R(G)-banded chromosomes were viewed using a UV filter. The microphotograph was reproduced in black and white by computer-based image processing. (**c**) Ideogram of human chromosome 12 with the map position of human *LOX-1*. The other panels show chromosome 12 with a hybridization signal in its chromatid and converted R-banded chromosome.

position on chromosome 12 (Figure 6). As a result, the *LOX-1* gene was assigned to band 12p12.3–p13.2 on the short arm of human chromosome 12. Interestingly, the deletion of chromosomal region 12p12.3 was reported to be associated with the development of a familial hypertension [42]. In addition, we have found the enhanced expression of LOX-1 in hypertensive rats [12]. Thus, our results suggest that changes in the expression level of LOX-1 might affect the development of hypertension, and that *LOX-1* might be the gene responsible for a type of familial hypertension. If *LOX-1* is not the responsible gene, the polymorphism of the microsatellites found in the *LOX-1* gene would be a help to identify the hypertension gene.

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