Articles

Chromosomal Mapping of *HCaRG*, a Novel Hypertension-Related, Calcium-Regulated Gene

(chromosome / localization / gene / comparison / species)

N. SOLBAN[1], P. DUMAS[1], F. GOSSARD[1], Y. SUN[1], M. PRAVENEC[2,3], V. KŘEN[2,3], R. LEWANCZUK[4], P. HAMET[1], J. TREMBLAY[1]

[1]Centre Hospitalier de l'Université de Montréal, Montréal, Canada

[2]Institute of Physiology, Academy of Sciences, Prague, Czech Republic

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

[4]Department of Endocrinology, University of Alberta, Edmonton, Canada

Abstract. We recently identified a novel gene that is negatively regulated by extracellular calcium concentration with higher levels of transcripts in hypertensive animals (SHR). We named this gene *HCaRG* (Hypertension-related, Calcium-Regulated Gene). In this work we report the chromosomal localization of the *HCaRG* gene among different species. We identified a *Bgl*II RFLP between BN.*lx* and SHR rats. We then analysed the strain distribution pattern of this RFLP in 31 RIS, originating from BN.*lx* and SHR rats, and compared it to the segregation of 475 markers localized in the rat genetic map. *Hcarg* localizes to the rat chromosome 7 between the markers Mit3 and Mit4. This region is homologous to human chromosome 8q21-24. We identified three clones in Genbank that contain the sequence of *HCaRG*. It was therefore possible to narrow down the localization of human *HCaRG* to chromosome 8q24.3. Furthermore, a suggestive localization of mouse *Hcarg* based on conservation of linkage between human and mouse is on chromosome 15. We previously identified a putative calcium-binding motif (EF-Hand) and a nuclear receptor-binding domain (LxxLL) in the rat sequence of the HCaRG protein. Sequence comparison between five different species showed that these domains are highly conserved. Furthermore, a search of ESTs in Genbank for homologous sequences showed that HCaRG is expressed only in eukaryotes, particularly in mammals.

HCaRG, a novel hypertension-related calcium-regulated gene, was recently isolated in a screen of rat parathyroid cells cultured in low-calcium medium. Molecular cloning and analysis revealed that *HCaRG* encodes a nuclear protein of 224 amino acids (a.a) containing 4 overlapping putative 'leucine zipper' consensus motifs and an EF-hand calcium-binding-like motif. A nuclear receptor-binding motif was also identified in its sequence. HCaRG mRNA levels are negatively regulated by extracellular calcium levels and its basal levels are significantly higher in spontaneously hypertensive rats (SHR) when compared to normotensive animals (BN.*lx* or WKY) (Solban et al., 2000).

Human HCaRG is expressed preponderantly in the parathyroid gland, kidney, heart, stomach, jejunum, liver and adrenal glands. Comparison of HCaRG expression between foetal and adult organs revealed that HCaRG mRNA is less expressed in all foetal tissues compared, particularly in the heart, kidney and liver. Its levels are also decreased dramatically in several cancerous cell lines.

We also demonstrated that in renal ischaemia-reperfusion experiments HCaRG mRNA declined rapidly to its lowest levels at 3 h and 6 h of reperfusion. These values then increased steadily to higher than baseline at 48 h of reperfusion (Solban et al., 2000). Human embryonic

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Corresponding author: Johanne Tremblay, Laboratory of Cellular Biology of Hypertension, Centre Hospitalier de l'Université de Montréal (CHUM), Campus Hôtel-Dieu 3840 St. Urbain St., Montréal, Québec H2W 1T8, Canada. Tel.: (514) 890-8000 (ext. 12721); fax: (514) 412-7152; e-mail: johanne.tremblay@umontreal.ca.

Abbreviations: a.a – amino acid(s), BN – Brown-Norway, EST – expressed sequence tag, RFLP – restriction fragment length polymorphism, RIS – recombinant inbred strain, SDP – strain distribution pattern, SHR – spontaneously hypertensive rat.

kidney cells (HEK293) stably overexpressing HCaRG have a much lower proliferation rate than control cells. Taken together, these results suggest that HCaRG is potentially involved in the regulation of renal cell proliferation.

In this report, we describe the chromosomal localization of the *Hcarg* gene on rat chromosome 7 in a region of conserved synteny with human chromosome 8q21-24 that is associated with several bone diseases, including osteopetrosis and multiple exostosis and several human neoplasms. We identified three clones in Genbank containing the full-length *HCaRG* sequences. These clones were localized on human chromosome 8q24.3, confirming our initial prediction. A suggestive localization of mouse *Hcarg* based on the conservation of linkage between human and mouse is on chromosome 15. Sequence comparison of HCaRG between five different species shows a high degree of homology and the conservation of the identified motifs. Furthermore, a search of translated ESTs showed that *HCaRG* is a highly conserved gene found only in eukaryotes.

Material and Methods

Southern blot analysis

Genomic DNA was extracted from the liver of SHR and Brown-Norway (BN) rats as well as from 33 recombinant inbred strains (RIS) as described earlier (Hamet et al., 1992). Restriction digestions and preparation of Southern blots were performed following standard methods. The probe used corresponds to a 437-bp coding region of rat *HCaRG*, labelled using the random primers DNA labelling system (Life Technologies, Burlington, ON).

Restriction fragment length polymorphism (RFLP) analysis and chromosomal mapping

Southern blot analysis was performed with 10 μg genomic DNA of SHR and BN.*lx* rats after digestion with several restriction enzymes including *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Pst*I. Genomic DNA was extracted from 33 RIS and digested with *Bgl*II. The strain distribution pattern (SDP) of *HCaRG* RFLP in these RIS was correlated with those of 475 polymorphic markers mapped previously (Pravenec et al., 1996). The SDP of these markers are available from the Ratmap web site (http://www.ratmap.gen.gu.se). Linkage analysis was performed using the MapManager QT programme of Manly (version 3.0b21) (Manly, 1993). These RIS, originating from reciprocal crosses of normotensive BN.*lx* and hypertensive SHR, are described elsewhere (Pravenec et al., 1989). They are the only set of rat RIS available for the study of the genetics of hypertension and related traits. Human *HCaRG* chromosomal location was first determined by rat–human synteny, and confirmed with the identification of three clones submitted to Genbank (accession numbers AF124523, AF146367, and AF118808). A suggestive chromosomal localization of mouse *Hcarg* was determined by the conservation of linkage between human and mouse available through Genbank.

Protein comparison and taxonomy report

The human HCaRG protein was compared to all available ESTs in Genbank using the tBLASTn programme which translates all ESTs. The taxonomy report was simultaneously generated based on the information in the NCBI taxonomy database. Protein sequences of HCaRG from different organisms were aligned using the ClustalX1.81 programme.

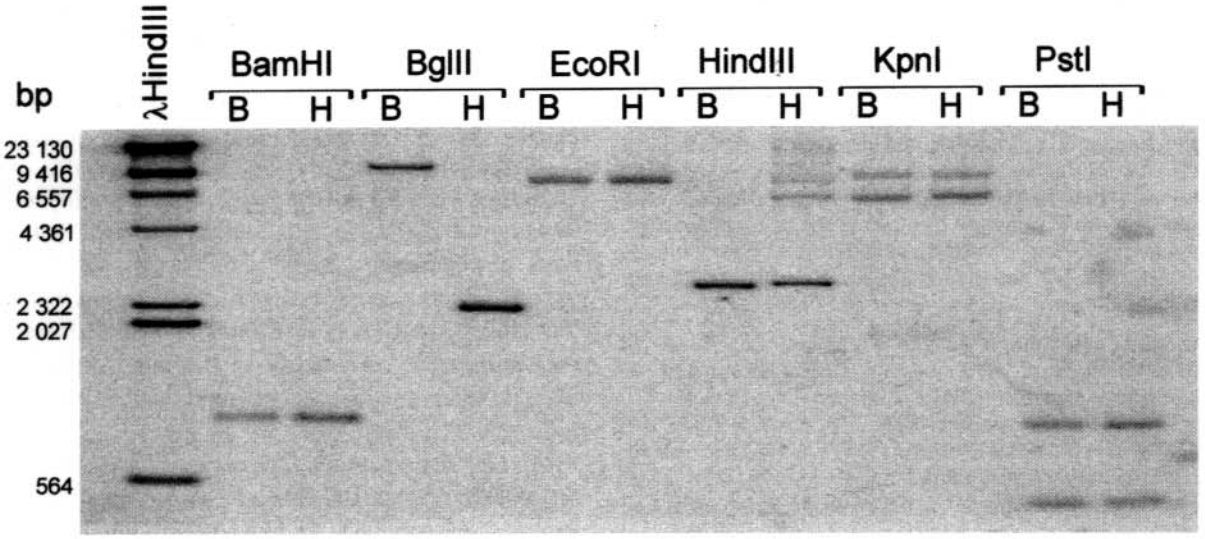

Fig. 1. Determination of a RFLP near the *Hcarg* gene. Genomic DNA from BN.*lx* (B) or SHR (H) rats was extracted and digested with different restriction enzymes. Digestion with *Bgl*II revealed a 12-kb band in BN.*lx* rats and a 2.2-kb band in SHR.

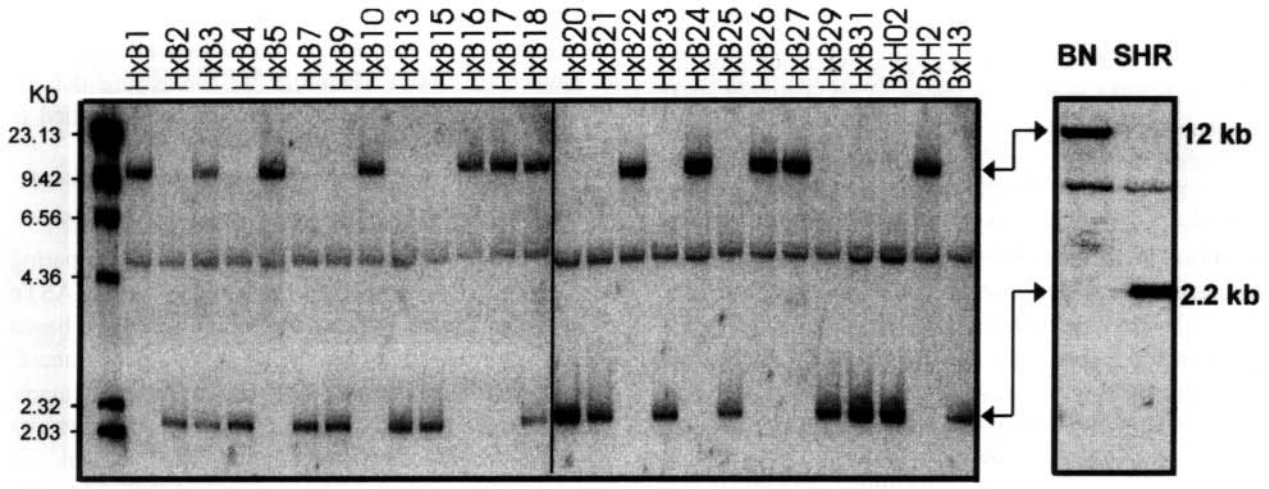

Fig. 2. Strain distribution pattern of RFLP. Strain distribution of *Hcarg* DNA polymorphism from progenitors SHR and BN.*lx* rats (right) as well as in a subset of 26 of their 33 RIS (left). Genomic DNA was extracted, digested with *Bgl*II and resolved on gel. The *Hcarg* radiolabelled probe revealed bands with B, 12 kb or H, 2.2 kb genotype from BN.*lx* and SHR, respectively (right). Strains heterozygous at the *Hcarg* locus were excluded from the SDP analysis.

Results

Chromosomal localization of HCaRG

From several restriction digestions of rat genomic DNA (Fig. 1), a *Bgl*II polymorphism was found near the *Hcarg* gene by the presence of a 12-kb genomic DNA band in BN.*lx* and 2.2 kb in SHR rats (Figs. 1 and 2 right). We used this polymorphism to determine the chromosomal localization of rat *Hcarg* in RIS derived from reciprocal crosses (BXH and HXB) between SHR and BN.*lx* progenitors (Fig. 2, left). The allelic distribution of the *Bgl*II polymorphism in 31 out of the 33 RIS homozygous for *HCaRG* was compared to the strain distribution of 475 markers in the same RIS (see Material and Methods). We found that the *Hcarg* locus co-segregated with D7Cebrp187s3/D7Cebr77s1 on chromosome 7 with recombination fraction of zero in these 31 RIS. Therefore, the *Hcarg* gene was assigned to a 4.4-cM long region between Mit3 and Mit4 of rat chromosome 7 (Fig. 3 left). A possible position of human homologous gene region, based on conserved linkage of rat chromosome 7, is chromosome 8q21-24. In a search of *Hcarg* homologous sequences in Genbank, homologies were found with three clones from chromosome 8 containing ZFP7. It was therefore possible to narrow down the localization of *HCaRG* to chromosome 8q24.3, confirming our initial prediction (Fig. 3 center). This region contains loci involved in several bone diseases, including osteopetrosis and multiple exostosis and several human neoplasms (McKusick et al., 1994; Knuutila et al., 1998). Comparison of human *HCaRG* to the available human genome sequence at NCBI permitted us to identify two other sequences homologous to *HCaRG* on chromosome 4 and on chromosome 6. These sequences are identical to each other, 641 a.a long (while complete human *HCaRG* is 672 a.a), and are 95% identical to human *HCaRG*. It is possible that these sequences code for a homologue of *HCaRG* or a member of the same family. However, it remains to be verified that the same sequence is found on two different chromosomes, since the human genome sequence is still in its draft form. A suggestive position of mouse *Hcarg* was determined based on the conservation of linkage between human and mouse. Mouse *Hcarg* localizes to chromosome 15 between 32 and 44 cM.

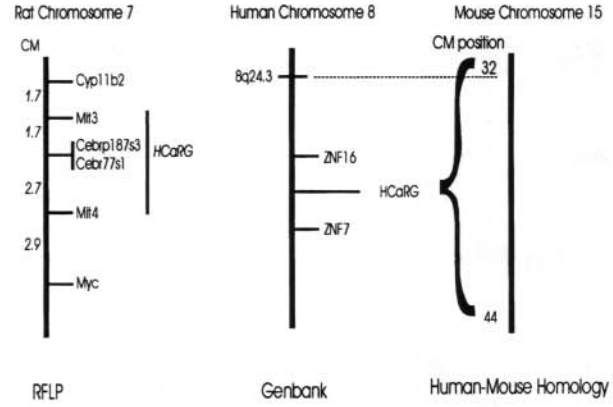

Fig. 3. Chromosomal localization of *HCaRG* in different species. A *Hcarg Bgl*II polymorphism was used as marker of genomic DNA from SHR or BN.*lx*. The allelic distribution of this polymorphism in RIS was compared to the strain distribution of 475 markers. *Hcarg* co-segregated with D7Cebrp187s3/D7Cebr77s1 of rat chromosome 7 in 31 out of 33 strains. cM represents the distance in centimorgans. In the middle, the position of the human homologous gene is depicted based on information obtained from Genbank. On the right side a suggestive position on mouse chromosome 15 is derived from the human–mouse homology database at NCBI.

Sequence comparison

In order to find similar sequences we compared the human HCaRG protein sequence to all available ESTs in Genbank using the tBLASTn programme. This programme dynamically translates all ESTs and compares the sequence to all possible open reading frames. We identified similar sequences in pig and cow. The pig sequence is 152 a.a and the cow sequence is 139 a.a while the complete sequence of HCaRG is 224 amino acids. Nucleotide comparison shows 79% identity between the human and the rat sequences with 93% identity between the rat and mouse HCaRG. Figure 4 shows the amino acid sequence alignment of rat, human, and mouse HCaRG cloned in our laboratory to the pig and cow sequence. At the amino acid level, 80% identity was found between the human and the rat and 95% between the rat and the mouse, 86% identity was calculated between the pig and the cow. The EF-hand domain is conserved in all five species with 8 out of the 10 most conserved a.a. The nuclear-receptor interaction domain is composed of the consensus sequence LxxLL (where x is any a.a) (Heery et al., 1997; Montminy, 1997). This motif is conserved in rat, mouse and human. However, in pig and cow an isoleucine replaces the last leucine (LxxLI). Some reports showed that the receptor interaction domain could be composed of isoleucine motifs: LxxII (Webb et al., 2000). An imperfect LxxLL sequence was recently identified in the chicken p160 coactivator molecule. This imperfect sequence, LxxIL, kept its capacity to interact with the nuclear receptor (Arai et al., 2001). Therefore, we can assume that the putative nuclear-receptor interaction domain identified in HCaRG is conserved in all five species.

Taxonomy report

The taxonomy report was generated by comparing human *HCaRG* to translated ESTs using the tBLASTn programme. Figure 5 shows the report generated based on the information in the NCBI taxonomy database. Most hits (identical sequences) are in eutheria (mammals) with the highest score in *Homo sapiens*. This was expected since we used the human HCaRG sequence to search ESTs, a score of 367 corresponds to an identity of 100%. This figure also shows hits in other organisms, but the score is below 100, and usually corresponds to a very short fragment being similar. No hits were observed in prokaryotes, using this search or with more precise searches (data not shown). *HCaRG* is therefore a gene mostly expressed in mammals.

Discussion

We identified a RFLP between BN.*lx* and SHR rats; this permitted us to localize rat *Hcarg* to chromosome 7. On the basis of conservation of synteny, we suggested the assignment of *HCaRG* on human chromosome

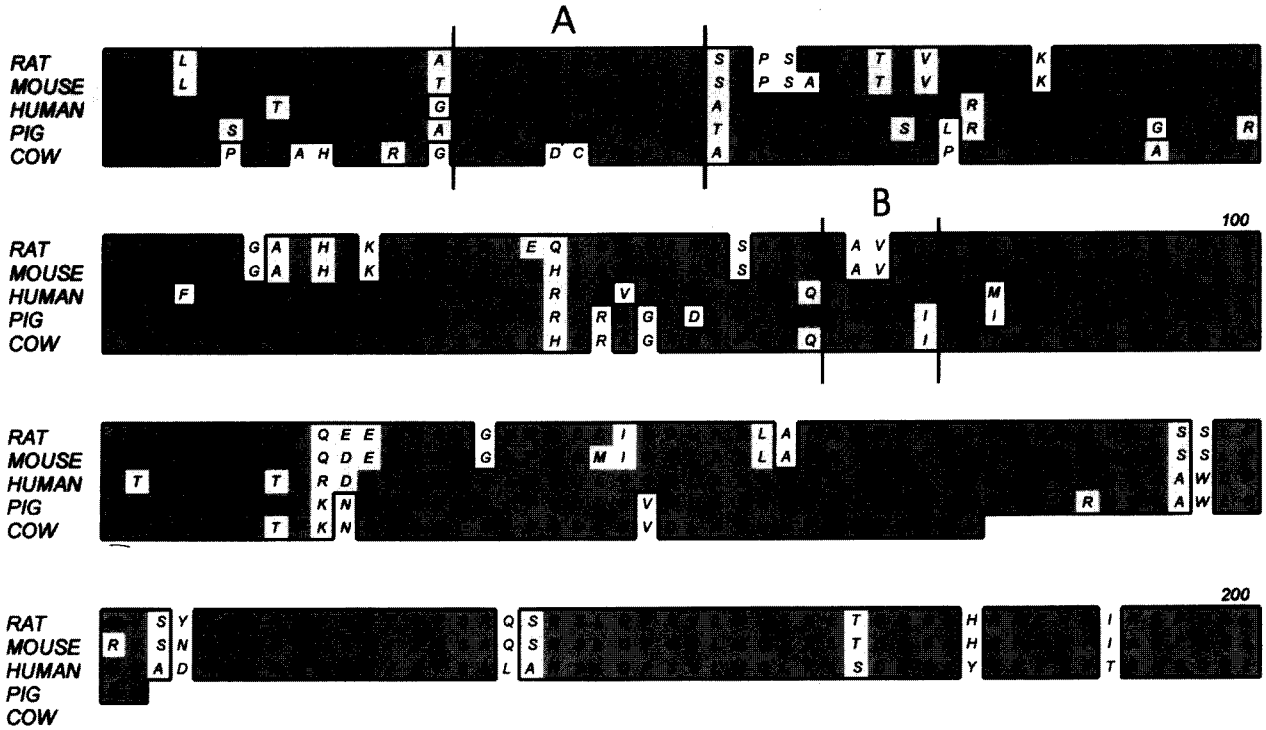

Fig. 4. Multiple species alignment. Similar sequences were found with the tBLASTn programme comparing the human HCaRG protein to translated ESTs. The sequences were then aligned using ClustalX1.81. Identities are shown in dark gray; similarities are in light gray. The putative EF-hand motif (A) and the nuclear receptor-binding domain (B) are conserved in all five species.

		Score
Eukaryota (eukaryotes)		
. . Coelomata (animals)		
. . . . . Euteleostomi (vertebrates)		
. . . . . Tetrapoda (vertebrates)		
. . . . . .Amniota (vertebrates)		
. . . . . Eutheria (mammals)		
. . . . . .Homo sapiens (human)----------	211 hits	367
. . . . . .Sus scrofa (pig)----------	10 hits	263
. . . . . .Mus musculus (house mouse)----------	81 hits	263
. . . . . .Bos taurus (cow)----------	36 hits	260
. . . . . .Rattus norvegicus (norway rat)----------	12 hits	250
. . . . . .Rattus sp. (rats)----------	8 hits	178
. . . . . Gallus gallus (chicken)----------	1 hit	37
. . . . . .Xenopus laevis (african clawed frog)----------	5 hits	97
. . . . . Danio rerio (zebrafish)----------	2 hits	38
. . . . . Bombyx mori (domestic silkworm)----------	2 hits	36
. . Glycine max (soybean)----------	1 hit	55

Fig. 5. Taxonomy report. The taxonomy report was generated by comparing the human HCaRG protein sequence to translated ESTs using the tBLASTn programme. The report shows the number of BLAST hits in different species. The highest numbers of hits are in eutheria (placental mammals) with the highest level of significance. HCaRG is not found in prokaryotes but only in eukaryotes, particularly in mammals.

8q21-24. In a recent search of *HCaRG* homologous sequences in Genbank, homologies were found with three clones from chromosome 8 containing the zinc-finger protein 7 (ZFP7). It was therefore possible to localize *HCaRG* on chromosome 8q24.3, confirming our initial prediction. Interestingly, this region contains loci involved in several bone diseases, including osteopetrosis, multiple exostosis, and early-onset osteoarthritis (McKusick et al., 1994). Osteopetrosis represents a group of genetically and clinically heterogenous disorders characterized by increased bone density and abnormalities of skeletal modelling. One form of osteopetrosis associated with renal tubular acidosis and characterized by deficiency of carbonic anhydrase II has been localized at position 8q22. Multiple exostosis, an abnormal dominant-inherited disease characterized by displacement of areas of the growth plate on bone metaphysis, has been localized at 8q24.11-8q24.13 (Mertens et al., 1994). Calcium pyrophosphate-deposition disease (CPDD), also called "chondrocalcinosis" or "pseudogout," is a disorder characterized by deposition of calcium-containing crystals in the joint tissue, which leads to arthritis-like symptoms. A locus for early-onset CPDD and severe degenerative osteoarthritis was found on chromosome 8q. The high level of expression of HCaRG in the parathyroid glands and its co-regulation with parathormone by extracellular calcium suggests a role for HCaRG in calcium metabolism (Solban et al., 2006).

Furthermore, this region is implicated in the 8;14 translocation associated with Burkitt's lymphoma (Adams et al., 1983), and is often abnormal in cancers. Chromosome 8 abnormalities can be detected in 15% of patients with acute myeloid leukaemia (AML), and recent work showed that the region 8q22 to 8qter might be of particular pathogenic importance (Batanian et al., 2001). Gains of the 8q region were also reported in prostate cancer (El Gedaily et al., 2001); among other regions, 8q21 and 8q24 were found to be amplified. Amplifications of *MYC* have also been found in a large fraction of hormone-refractory prostate cancer (Nupponen and Visakorpi, 2000). Gains and high-level amplification at 8q were more frequent in metastatic gastrointestinal stromal tumour (GIST) (El-Rifai et al., 2000). We observed that HCaRG levels were decreased in all cancerous cell lines studied, and also decreased in a glioblastoma, a partly differentiated renal cell carcinoma and a moderately differentiated hepatocellular tumour, when compared to the same amount of normal RNA of adjacent tissues excised from the same operational site (Solban et al., 2000). Chromosome 8 abnormalities in certain cancers could disrupt the *HCaRG* gene, leading to uncontrolled proliferation.

We previously evaluated *Hcarg* expression after unilateral renal ischaemia/reperfusion in uninephrectomized rats (Solban et al., 2000). This process of kidney injury and repair recapitulates many aspects of development. It involves de-differentiation and regeneration of epithelial cells, followed by differentiation (Molitoris et al., 1988; Bacallao and Fine; 1989; Wallin et al., 1992). We observed that Hcarg mRNA declined rapidly to its lowest levels at 3 h and 6 h of reperfusion, then increased steadily to higher than baseline at 48 h of reperfusion. In contrast, the levels of the proto-oncogene *c-myc*, which is correlated with hyperplastic response in mammalian cells (Nakajima et al., 1996), was rapidly increased following renal ischaemia and reperfusion. In the same study we showed that overexpression of HCaRG in a human embryonic kidney cell line (HEK293) inhibited proliferation, as demonstrated by cell counting and ^{3}H-thymidine incorporation. Furthermore, these cells exhibit characteristic features of differentiation: lower proliferation rate, higher protein content, increased production of ANP, and desmosomes (Devlin et al., in preparation). Taken together, this suggests that *Hcarg* contributes to kidney cell differentiation.

In conclusion, we have localized rat *Hcarg* on chromosome 7, and the human gene on chromosome 8q24.3. This region contains loci involved in several bone diseases and is associated with Burkitt's lymphoma. Amino acid comparison with translated ESTs showed that HCaRG is only expressed in eukaryotes, mostly in mammals. Two putative domains of HCaRG including an EF-hand domain and a nuclear receptor interaction domain are conserved among species.

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