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## Structural organization, expression and chromosomal mapping of the mouse cystatin-C-encoding gene (*Cst3*)

(Recombinant genomic DNA; gene mapping; exon; intron; promoter sequence; proteinase inhibitor; multigene family)

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### SUMMARY

Cystatin C (CstC) is a potent cysteine-proteinase inhibitor. The structure of the mouse CstC-encoding gene (*Cst3*) was examined by sequencing a 6.1-kb genomic DNA containing the entire gene, as well as 0.9 kb of 5' flanking and 1.7 kb of its 3' flanking region. The sequence revealed that the overall organization of the gene is very similar to those of the genes encoding human CstC and other type-2 Cst, with two introns at positions identical to those in the human gene. The promoter area does not contain typical TATA or CAAT boxes. Two copies of a Sp1-binding motif, GGGCGG, are present in the 5' flanking region within 300 bp upstream from the initiation codon. A hexa-nucleotide, TGTTCT, which is a core sequence of the androgen-responsive element (ARE), is found in the promoter region. This region also contains a 21-nucleotide sequence, 5'-AGACTAGCAGCTGACTGAAGC, which contains two potential binding sites for the transcription factor, AP-1. The mouse *Cst3* mRNA was detected in all of thirteen tissues examined by Northern blot analysis. *Cst3* was mapped in the mouse to a position on distal chromosome 2.

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### INTRODUCTION

The cystatins (Cst) are a group of potent cysteine-proteinase inhibitors. There are at least five distinct types in the Cst superfamily, each type consisting of several

proteins (Rawlings and Barrett, 1990; Devos et al., 1993). CstC belongs to the family of type-2 Cst and consists of 120 aa, with two intrachain disulfide bonds (Barrett et al., 1986).

Although the proteinase-inhibiting function of the CstC has been thoroughly investigated, less is known about its broader biological role. Recent reports indicate that CstC may play a role in cancer progression (Sloane, 1990), bone resorption (Lerner and Grubb, 1992), modulation of neutrophil chemotactic activity and inflammation (Leung-Tack et al., 1990a,b), and resistance to viral infection (Collins and Grubb, 1991). Furthermore, a point mutation in the *CST3* gene, resulting in a Leu→Gln substitution, is the primary cause of autosomal dominant hereditary disorder, hereditary CstC amyloid angiopathy (HCCAA) (Grubb et al., 1984). As young adults, carriers

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Abbreviations: aa, amino acid(s); ARE, androgen-responsive element(s); bp, base pair(s); Chr, chromosome; Cst, cystatin(s); *Cst3*, gene (DNA) encoding CstC; *Cst1*, gene (DNA) encoding CstSN; *Cst2*, gene (DNA) encoding CstSA; *Cst4*, gene (DNA) encoding CstD; HCCAA, hereditary CstC amyloid angiopathy; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); RFLP, restriction-fragment length polymorphism; W, A or T.

of this mutation suffer from repeated and massive brain hemorrhages due to deposition of the mutant protein in the walls of the cerebral arteries.

The gene structures for several type-2 *Cst* have been determined. The human *CST1* and *CST2* genes (Saitoh et al., 1987), and the human *CST3* (Abrahamson et al., 1990) and *CST4* (Freije et al., 1991) genes show very similar structural organization with respect to the number and position of the introns. Structural analysis of the genes for these proteins will be necessary to understand the function and evolution of the members in the cystatin multigene superfamily. In this paper, we report the structural organization and expression of the mouse *Cst3* gene, compare its regulatory elements with that of other *Cst* genes and map the *Cst3* gene in the mouse.

## EXPERIMENTAL AND DISCUSSION

### (a) Isolation of the mouse *Cst3* gene

Using two primers *mcy3*: (5'-ATG GCC AGC CCG CTG CGC TCC TTG-3') and *mcy4*: (5'-GGC ATT TTT GCA GCT GAA TTT TGT CAG-3'), a 420-bp DNA fragment within the coding region was generated from mouse *Cst3* cDNA, labeled with <sup>32</sup>P by random primer extension, and used as a probe to screen a  $\lambda$ FixII genomic DNA library from 129/Sv mice (purchased from Stratagene, La Jolla, CA, USA). Among several hybridizing clones, one clone,  $\lambda$ cyg13, was chosen for characterization. Southern blot and PCR analysis indicated the presence of the mouse *Cst3* gene in a 15-kb DNA insert. Southern blot analysis of the genomic DNA did not show any evidence for the presence of a *Cst3* pseudogene. A 6.1-kb genomic DNA fragment containing the entire gene, as well as 0.9 kb of 5' flanking and 1.7 kb of 3' flanking region, was subjected to nt sequencing.

### (b) Structural analysis of the mouse *Cst3* gene

The 6125-nt sequence covering the entire mouse *Cst3* gene is shown in Fig. 1. Comparison of the mouse *Cst3* gene sequence with that of the corresponding cDNA (Solem et al., 1990) revealed that the gene contains two intron sequences located between the nt triplets encoding aa 55–56 and 93–94 of the proposed mature polypeptide chain, exactly as in the human *CST3* gene. The presence of two introns, at homologous positions, has also been reported in the other type-2 *Cst* genes fully characterized to date: the human *CST1*, *CST2* and *CST4* genes. The intron-exon junctions in the mouse *Cst3* gene are all close matches to the consensus sequences for the donor and acceptor splice sites of introns (Mount, 1982).

Some differences were observed between the exons of the genomic DNA sequence and the published cDNA.

The five differences are summarized in Table I. Two of these positions, 994 and 3421, are in the coding region. The nt<sup>994</sup> in exon 1 results in a GCC codon (coding for Ala) towards the C-terminal part of the leader sequence. However, a GGC codon (coding for Gly) was reported at this position in the published cDNA. This GCC codon found in the genomic DNA exists in the corresponding site of the rat and human *Cst3* cDNAs. The nt<sup>3421</sup> in exon 2 forms a TTG codon for Leu (TTT coding for Phe in cDNA). The differences between genomic and cDNA sequence may be due to an error during cDNA synthesis or due to polymorphism between the mouse strains 129/Sv and BALB/c.

### (c) 5'-flanking sequence of the mouse *Cst3* gene

The sequence of the 0.9-kb segment flanking exon 1 of the mouse *Cst3* gene at the 5' end, did not reveal a typical TATA or CAAT box in the suggested promoter area. However, a TATA-box-like TAAA sequence is present at 78–82 nt upstream from the start codon. A similar slightly atypical TATA-box is found at the homologous position in the human *CST3* gene (ATAAAA), the human *CST4* gene (ATAAAT), the human *CST1* and *CST2* genes (ATAAAA). The TATA-box is preceded by a Sp1-binding GC-box sequence (Pugh and Tjian, 1990) with the core consensus sequence, GGGCGG, ending 23 nt upstream from the AT-sequence. A corresponding sequence in the human *CST3* gene is located slightly closer to the TATA-box (distance 16 nt). In the human *CST4* gene, a GC-box is also found in the immediate 5'-flanking region (upstream distance from the AT-sequence 41 nt). By contrast, in the human *CST1* and *CST2* genes, a segment similar to the CAAT consensus is found instead of the GC box.

A core sequence of the *ARE*, TGTTCT, is found 22 nt downstream from the TAAA sequence. This hexanucleotide is located in a partial palindromic setting. Some naturally occurring sequences and synthetic constructs containing this core sequence in a partial palindromic structure were shown to be inducible with androgens (Ham et al., 1988). This *ARE* is not found in the promoters of any other type-2 *Cst* genes published. However, it is present in *Cst*-related protein-encoding genes whose expression is regulated by androgen in the ventral prostate and lachrymal gland (Chamberlain et al., 1983).

An exact match of nine nt with the pituitary transcription factor (Pit-1) recognition element is centered around nt –795 from the start codon, but is probably of low significance for the expression of the gene because multiple recognition elements have been shown to be needed for markedly increased expression of the rat prolactin gene by Pit-1 (Ingraham et al., 1988). The motif

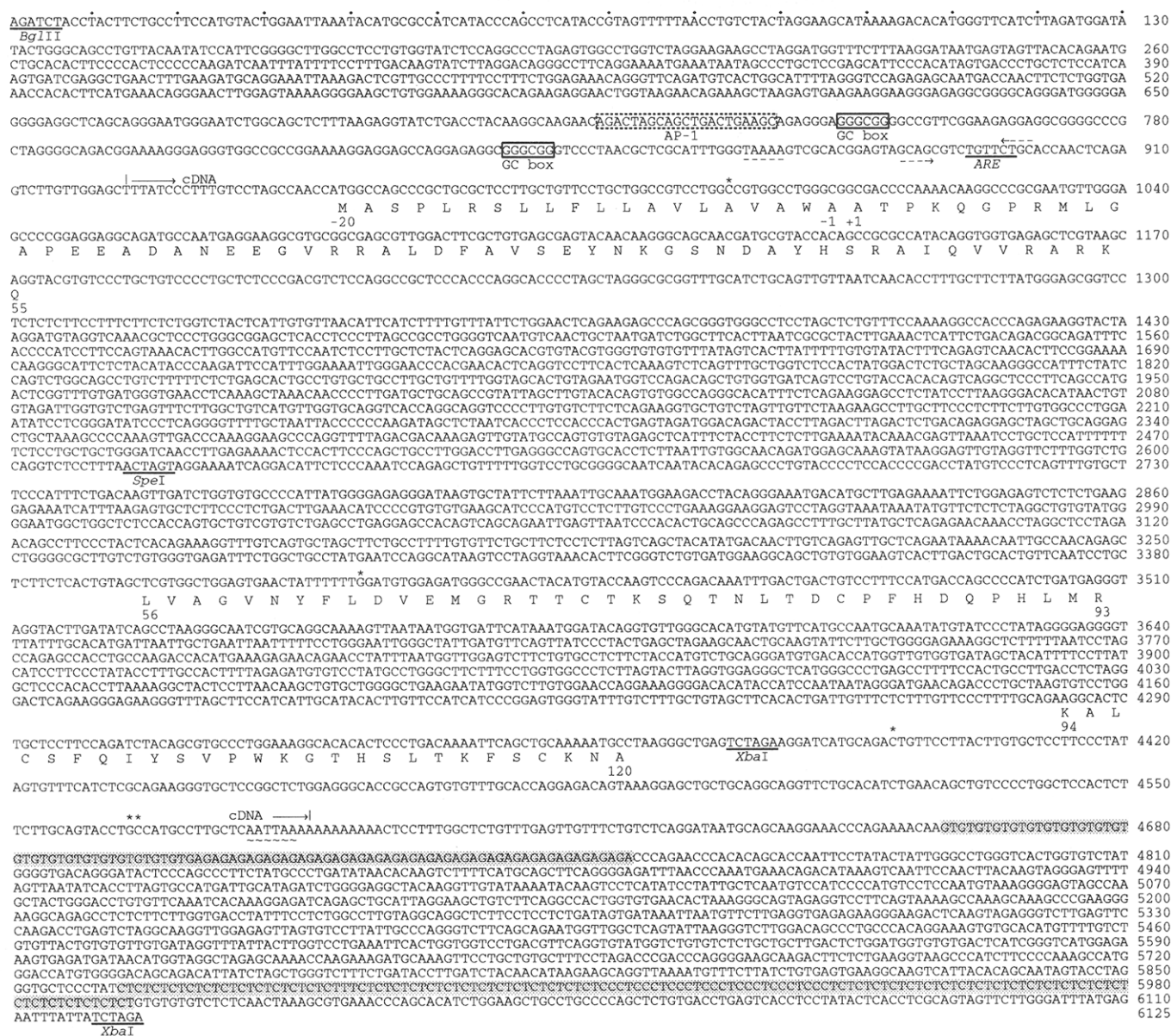


Fig. 1. Nucleotide sequence of the mouse *Cst3* structural gene and flanking regions. The complete sequence of a 6125-nt *BglIII-XbaI* fragment is shown above. The deduced aa sequence is shown below the coding part of the exons. Numbering of aa is based on the human CstC sequence, with negative numbers for residues in the putative signal peptide. A potential binding site for the transcription factor AP-1 is shown by the dashed box. The core sequences of the two binding sites for transcription factor Sp1 (GC box) are shown by solid boxes. The potential *ARE* in a partial palindromic setting is marked. The TATA-box-like TAAA sequence is shown (dashed underline). The boundaries of the published cDNA are also indicated. Those nt that differ from the published cDNA are indicated by asterisks. The poly(A)-addition signal sequence is shown by a wavy underline. Two regions of dinucleotide repeat sequences are indicated by shaded blocks. The nt sequence reported in this paper has been submitted to the GenBank data base under accession No. U10098.

recognized by the leader binding protein (LBP-1), 5'-WCTGG-3' or its inverse, that is present in several copies in the HIV-1 promoter and contribute to its basal function (Jones et al., 1988), is strikingly abundant in the 5'-flanking region of the mouse *Cst3* gene. Another five LBP-1 motifs are found within a 300-bp segment in the first part of the first intron.

Transcription factor AP-1-binding sites that bind the Jun-Fos protooncogene complexes contain the consensus sequence 5'-TGACTCAGC. The mouse *Cst3* promoter

contains two AP-1-like binding sequences within the sequence 5'-AGACTAGCAGCTGACTGAAGC, immediately upstream from the first Sp1-binding site. This 21-mer sequence contains direct repeats of two adjacent potential AP-1-binding sites, each slightly deviating from the consensus sequence. It has been shown that two adjacent AP-1-like binding sites act synergistically to confer inducibility beyond that observed for a single AP-1 consensus sequence (Friling et al., 1992). The presence of the two AP-1-like binding sites in the promoter indicates that

TABLE 1

Differences between the mouse *Cst3* gene sequence and that of the published cDNA (Solem et al., 1990)

Position <sup>a</sup>	Genomic DNA <sup>b</sup>	cDNA <sup>c</sup>	aa <sup>d</sup> (genomic/cDNA)
994	C (11)	G	Ala/Gly
3421	G (5)	T	Leu/Phe
4393	C (5)	T	-
4564-5	GC (3)	AT	-

<sup>a</sup> The nt positions refer to Fig. 1.

<sup>b</sup> The nt refers to the genomic DNA sequence. Sequence was determined on both strands (number of independent sequencing runs in parentheses).

<sup>c</sup> The nt refers to the cDNA sequence.

<sup>d</sup> The aa encoded by nt in columns Genomic DNA and cDNA.

transcription factor AP-1 may play a role in the *Cst3* gene expression. There is evidence that induction of gene expression by TGF- $\beta$  is mediated by transcription factor AP-1. Autoregulation of TGF- $\beta$  expression is mediated by the binding of AP-1 to a loose consensus binding site, TGAGACA, in the TGF- $\beta$  promoter (Kim et al., 1990). A strong positive regulation of the *Cst3* gene by TGF- $\beta$  in serum free mouse embryo cells has also been reported (Solem et al., 1990). The presence of AP-1-like binding sites in the mouse *Cst3* promoter suggests that cystatin C induction by TGF- $\beta$  may be mediated by the AP-1 complex.

The 5'-flanking region of the human *CST3* gene has a notably high G+C content, with >70% G+C in the 400 bp sequence upstream from the start codon. The G+C-rich region also includes the coding part of exon 1 and the 5' part of the first intron, which together represents a 900-bp segment with a G+C content of 73%, and contains CpG/GpC dinucleotides in a ratio close to unity (Abrahamson et al., 1990). The immediate 5'-flanking region of the mouse *Cst3* gene does not have such a strikingly high G+C content, but is more similar to the human *CST1*, *CST2* and *CST4* genes in having a GC content of approx. 60%. However, the CpG/GpC ratio is 1/1.3 in the 300 bp region upstream from the start codon (as compared to 1/4.1 over the entire 6.1-kb sequenced region), differing markedly from ratios of 1/6, 1/9 and 1/16 for the human *CST4*, *CST1* and *CST2* genes, respectively. Thus, the mouse *Cst3* gene 5'-flanking region is not a typical housekeeping gene promoter having extremely high G+C content. Rather it is similar to these promoters and the human *CST3* promoter because it displays several Sp1-binding sites and contains a high number of CpG dinucleotides. This may indicate a low degree of methylation due to constant transcription of the gene (Bird, 1986). Proposed promoter regions of several type-2 *Cst* are compared in Fig. 2.

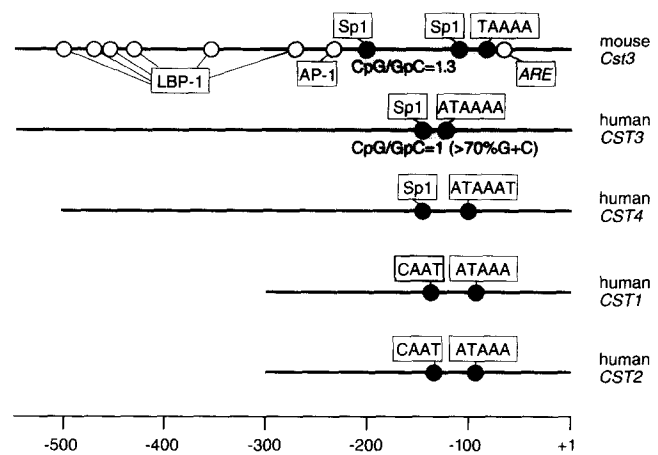


Fig. 2. Schematic structures of the proposed promoter regions of genes encoding type-2 *Cst* genes. The immediate 5'-flanking region of the mouse *Cst3* gene is compared to the sequenced parts of the corresponding regions in the genes for human *CST3*, *CST4*, *CST1* and *CST2*. TATA box-like sequences are also shown. +1 denotes the start codon. LBP-1 motifs are depicted only for the mouse *Cst3* gene. CpG/GpC ratios indicated are calculated from the shaded regions. Data for the human genes are from the literature (Saitoh et al., 1987; Abrahamson et al., 1990; Freije et al., 1991).

#### (d) 3'-Flanking sequence of the mouse *Cst3* gene

Sequence determination of the mouse *Cst3* gene 1.7-kb 3'-flanking segment revealed no alternative polyadenylation signals in addition to the one present in the corresponding cDNA, 213 bp downstream from the stop codon. Analysis of short tandem repeats within the entire gene sequence revealed the presence of (GT)<sub>21</sub> and (GA)<sub>26</sub> in the region immediately 3'-flanking the polyadenylation signal and three stretches of perfect CT repeats, (CT)<sub>13</sub>, (CT)<sub>14</sub> and (CT)<sub>25</sub>, 1300 bp further downstream.

#### (e) Genetic mapping of the mouse *Cst3* gene

Analysis of two multilocus crosses was used to define the Chr location for the mouse *Cst3* gene: (NFS/N or C58/J  $\times$  *M. m. musculus*)  $\times$  *M. m. musculus* (Kozak et al., 1990) and (NFS/N  $\times$  *M. spretus*)  $\times$  *M. spretus* or C58/J (Adamson et al., 1991). DNAs extracted from parental mice and progeny of the crosses were typed by Southern blotting analysis for RFLPs of *Cst3* using the mouse *Cst3* cDNA as probe. *Sst*I digestion produced fragments of 8.6 and 6.0 kb in *M. spretus* and 6.0 and 2.8 kb in NFS/N and C58/J mice, and *Bam*HI fragments of 22.0 and 18.8 kb were detected in *M. m. musculus* and NFS/N, respectively.

Inheritance of the parental fragments was followed in both crosses and compared with inheritance of almost 650 markers previously typed and mapped to all 19 autosomes and the X chromosome. As shown in Fig. 3, *Cst3* showed linkage to markers on distal Chr 2 and could be positioned proximal to *Psp* (encoding parotid secretory protein) and *Emv15* (ecotropic murine virus 15)

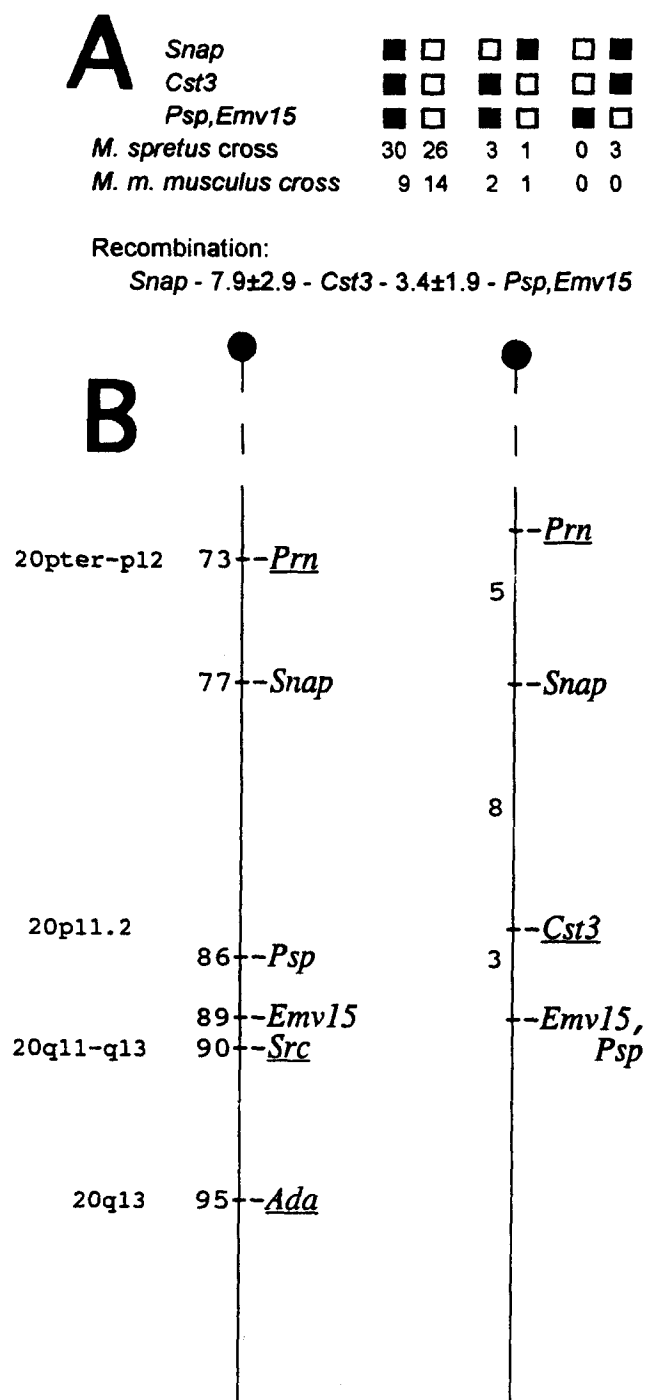


Fig. 3. Genetic mapping of the mouse *Cst3* gene. (A) Segregation of *Cst3* with markers on Chr 2. Blackened squares represent heterozygous mice, open squares are homozygous mice. The numbers under each column represent the number of progeny in each of the two backcrosses with the indicated genotype. Recombination frequencies are given below the figure. The numbers given between adjacent loci represent percent recombination  $\pm$  the standard error calculated according to Green (1981). (B) Chr 2 linkage maps. The map on the right was generated from the two crosses described here and indicates the position of *Cst3* relative to the other markers typed in this cross. Distances between adjacent markers (in centiMorgans) are indicated to the immediate left of the map. The map on the left is an abbreviated version of the composite genetic map (Siracusa and Abbott, 1993). Numbers to the left of the map are centiMorgan distances from the centromere. Human map locations for homologs of the underlined mouse genes are indicated to the far left of this map.

and distal to *Snap* (encoding synaptosomal associated protein), markers which were typed in these crosses as previously described (Joseph et al., 1990; Grimaldi et al., 1992). It has recently been shown that the human homolog of this gene maps to 20p11 (Schnittger et al., 1993). This is consistent with our results, since the distal end of mouse Chr 2 contains a substantial region of linkage conservation with human Chr 20 (Siracusa and Abbot, 1993; Fig. 3b). The human *CST3* is part of a cluster which includes up to eight members of the *CST* gene family (Schnittger et al., 1993) further suggesting that the mouse homologs of these genes are likely to map to the same site on Chr 2.

#### (f) Expression of the mouse *Cst3* gene

We examined the expression of the mouse *Cst3* gene in 13 different tissues by Northern blot analysis using the mouse *Cst3* cDNA probe. As expected, *Cst3* mRNA was detected in all tissues examined, including stomach, brain, intestines, liver, muscle, spleen, heart, kidney, lung, pancreas, testis, uterus and ovary (data not shown). The pattern of the mouse *Cst3* gene expression is similar to that of its human counterpart. Both species show expression of the gene in all tissues examined with high level of *Cst3* messenger RNA in brain and testis, and lowest level in pancreas. This overall similarity between the two species indicates that mouse may be suitable for generating an animal model for the human genetic disease HCCA.

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