



Structure and promoter analysis of murine *CAD* and *ICAD* genes

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Abstract

Caspase-activated DNase (CAD) degrades chromosomal DNA during apoptosis, whereas ICAD (inhibitor of CAD) inhibits the CAD's DNase by binding to it. Here, we describe the assignment of murine *CAD* and *ICAD* genes to the distal part of murine chromosome 4. Molecular cloning and structural analysis indicated that *CAD* and *ICAD* genes are comprised of 7 and 6 exons, respectively. Two different *ICAD* mRNAs coding for two forms of ICAD proteins (ICAD-S and ICAD-L) were found to be produced by alternative splicing of intron 5. The *CAD* and *ICAD* mRNAs were detected ubiquitously in various murine tissues. Analyses of the promoter activity with a series of deletion mutants of their 5' flanking regions indicated that a 190-bp 5' flanking region of the *CAD* gene was sufficient to promote the transcription. Whereas, a 120-bp flanking region of *ICAD* gene was required to promote its transcription. These regions do not show similarity between *CAD* and *ICAD* genes, suggesting that expression of *CAD* and *ICAD* genes is regulated by different mechanisms.

Keywords: apoptosis; CAD; ICAD; alternative splicing; expression; promoter

Abbreviations: CAD, caspase-activated DNase; ICAD, inhibitor of CAD; DFF, DNA fragmentation factor; RFLP, restriction fragment length polymorphism

Introduction

Apoptosis is a cell death process that removes useless, harmful, or senescent cells from the body.¹ This process is characterized morphologically by cell shrinkage, chromatin

condensation, and formation of apoptotic bodies,² and biochemically by degradation of chromosomal DNA into nucleosomal units.³ Most of the apoptotic stimuli activate caspases, which are cysteine proteases that cleave various substrates such as poly(ADP) ribose polymerase, lamin, and fodrin to promote the apoptotic process.^{4–7} We and others recently showed that a DNase (CAD, caspase-activated DNase; also called CPAN, caspase activated nuclease, as well as DFF-40, DNA fragmentation factor-40) is activated during apoptosis, and cleaves the chromosomal DNA.^{8–12} In the growing, non-apoptotic cells, CAD is complexed with its inhibitor (ICAD, inhibitor of CAD; also called DFF-45) and predominantly localized in the cytoplasm. When cells are induced to die by apoptotic stimuli such as Fas ligand or the kinase inhibitor staurosporine, caspases are activated. The activated caspases cleave ICAD at two positions, which releases CAD from ICAD.¹²

Murine and human CADs are comprised of 344 and 338 amino acids, respectively, with 76% identity.^{8–11} Murine ICAD exists in two forms – long and short (ICAD-L and ICAD-S) which consist of 331 and 265 amino acids, respectively.⁸ Murine ICAD-L is 76% identical to human DFF-45,¹³ suggesting that murine ICAD is the murine counterpart of human DFF-45. In addition, CAD and ICAD have a homologous domain of about 100 amino acids at the N-terminus.¹¹ A similar domain has also been found in several proteins of unknown function.¹⁴

In this report, we showed that the murine *CAD* and *ICAD* genes are localized close to one another on murine chromosome 4. The isolation and structural analysis of their chromosomal genes indicated that *CAD* and *ICAD* comprise about 11 and 16 kb DNA, and are split by 6 and 5 introns, respectively. A comparison of the *ICAD* chromosomal gene with its mRNAs indicated that the *ICAD-L* and *ICAD-S* mRNAs are generated by alternative splicing of intron 5. The *CAD* and *ICAD* genes are expressed ubiquitously in similar tissues. However, the 5' flanking sequences of *CAD* and *ICAD* genes necessary to promote the transcription were not well conserved between the two genes, suggesting that the expression of the *CAD* and *ICAD* genes is regulated differently.

Results

Assignment of *CAD* and *ICAD* genes on murine chromosome

The murine chromosomal location of *Icad* and *Cad* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J x *Mus spretus*)F₁ x C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2700 loci that are well distributed among all the autosomes as well as the X chromosome.¹⁵ C57BL/6J and *M. spretus* DNAs were

digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using murine cDNA probes. The 5.3 and 3.8 kb *SphI* *M. spretus* RFLPS (see Materials and Methods) were used to follow the segregation of the *Icad* locus and the 15.0 and 2.6 kb *BglII* fragments were used to follow the *Cad* locus in backcross mice. The mapping results indicated that the two genes are linked and are located in the distal region of murine chromosome 4 linked to *Htrld*, *Nppa* and *Gnb1*. Although 100 mice were analyzed for every marker and are shown in the segregation analysis (Figure 1), up to 178 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination

frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere- *Htrld*-5/119-*Nppa*-0/109-*Icad*-1/160-*Cad*-3/178-*Gnb1*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are *-Htrld*-4.2 ± 1.8- [*Nppa*, *Icad*]-0.6 ± 0.6-*Cad*-1.7 ± 1.0-*Gnb1*. No recombinants were detected between *Nppa* and *Icad* in 109 animals typed in common suggesting that the two loci are within 2.8 cM of each other (upper 95% confidence limit).

Chromosomal gene structure of the murine *CAD* and *ICAD* genes

To determine the exon-intron organization of *CAD* and *ICAD*, DNA fragments carrying the *CAD* and *ICAD* chromosomal genes were isolated from a murine genomic library. Restriction-enzyme mapping and PCR analyses of these DNA fragments indicated the clones Q9 and P1 contain the entire *CAD* and *ICAD* chromosomal genes, respectively. A comparison of the genomic sequences with that of the *CAD* cDNA indicated that the murine *CAD* chromosomal gene consists of about 11 kb, and is split by six introns (Figure 2A). The *ICAD* chromosomal gene was found to consist of about 16 kb, and to be split by five introns (Figure 2B). The nucleotide sequences of the coding region of chromosomal *CAD* was completely identical to the *CAD* cDNA sequence. The nucleotide sequence of the *ICAD* chromosomal gene carried two silent mutations at positions 249 (G – A) and 378 (T – C). All of the splice donor and acceptor sites conformed to the GT – AG rule for nucleotides immediately flanking exon borders.¹⁶ The N-terminal region of about 100 amino acids is homologous between *CAD* and *ICAD*.¹¹ This region was encoded by two exons in both *CAD* and *ICAD*.

There are two isoforms for murine *ICAD* mRNA (*ICAD-L* and *ICAD-S*), which code for proteins of 331 and 265 amino acids, respectively.⁸ *ICAD-S* is the same as *ICAD-L* truncated at the 261 amino acid position, and carries an extra 4 amino acids at the C-terminus. The 3' non-coding region of the *ICAD-S* mRNA diverges from that of *ICAD-L* mRNA. A comparison of the nucleotide sequence of the *ICAD* chromosomal gene with *ICAD-L* and *ICAD-S* cDNAs indicated that *ICAD-L* mRNA contains exons 1 – 6. In contrast, the *ICAD-S* mRNA contains only exon 1 – 5, and its sequence continues into intron 5 (Figure 2C).

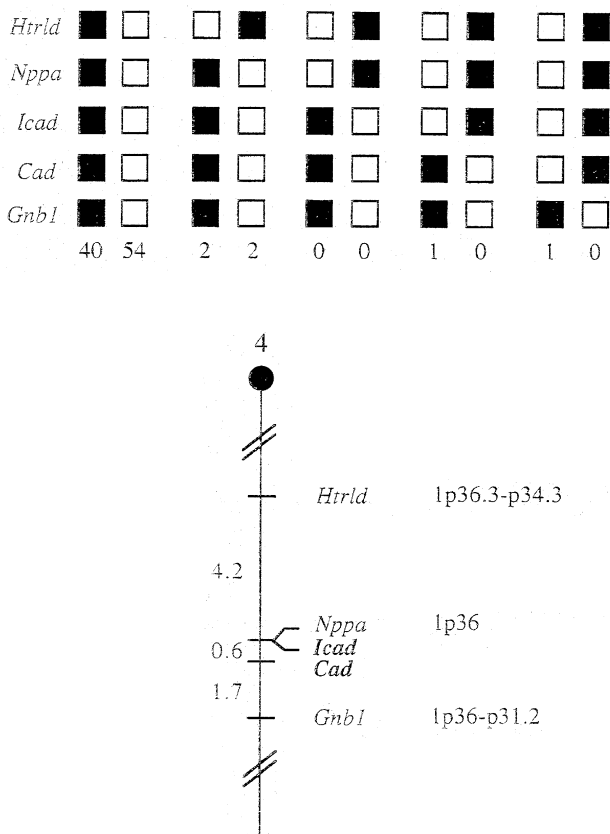


Figure 1 *Icad* and *Cad* map in the distal region of murine chromosome 4. *Icad* and *Cad* were placed on murine chromosome 4 by interspecific backcross analysis. The segregation patterns of *Icad* and *Cad* and flanking genes in 100 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 100 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J x *M. spretus*) F₁ parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 4 linkage map showing the location of *Icad* and *Cad* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD, USA)

Expression of *CAD* and *ICAD* mRNAs in murine tissues

The tissue distribution of the *CAD* and *ICAD* mRNAs was examined by Northern hybridization. As shown in Figure 3A, most tissues were found to express an mRNA of about 2.5 kb that hybridized with the *CAD* probe. Some tissues such as spleen, thymus, small intestine and lymph nodes expressed a high level of *CAD* mRNA. The skeletal muscle also showed a strong band hybridizing with *CAD* probe, but its size (about 1 kb) was significantly smaller than that found in other tissues. When Northern hybridization was carried out with the coding sequence of *ICAD* cDNA, two bands of 3.8 and 2.3 kb were

detected in various tissues, particularly in the spleen, thymus and prostate (Figure 3B). The upper 3.8 kb mRNA hybridized with the 3' non-coding regions of *ICAD-S* and *ICAD-L* cDNAs (data not shown). While, the lower 2.3 kb mRNA hybridized only with the 3' non-coding region of *ICAD-L* cDNA. The length of intron 5 is about 1.5 kb, which agrees with the difference in length between the upper and lower *ICAD*

mRNAs. These results suggest that the upper band of 3.8 kb is the mRNA for *ICAD-S*, and carries the unspliced intron 5. Whereas, the lower band of 2.3 kb represents the *ICAD-L* mRNA in which intron 5 is spliced out. The similar intensity of the upper and lower bands hybridizing with the *ICAD* probe (Figure 3b) indicates that *ICAD-S* and *ICAD-L* mRNAs are expressed at a similar level in most tissues.

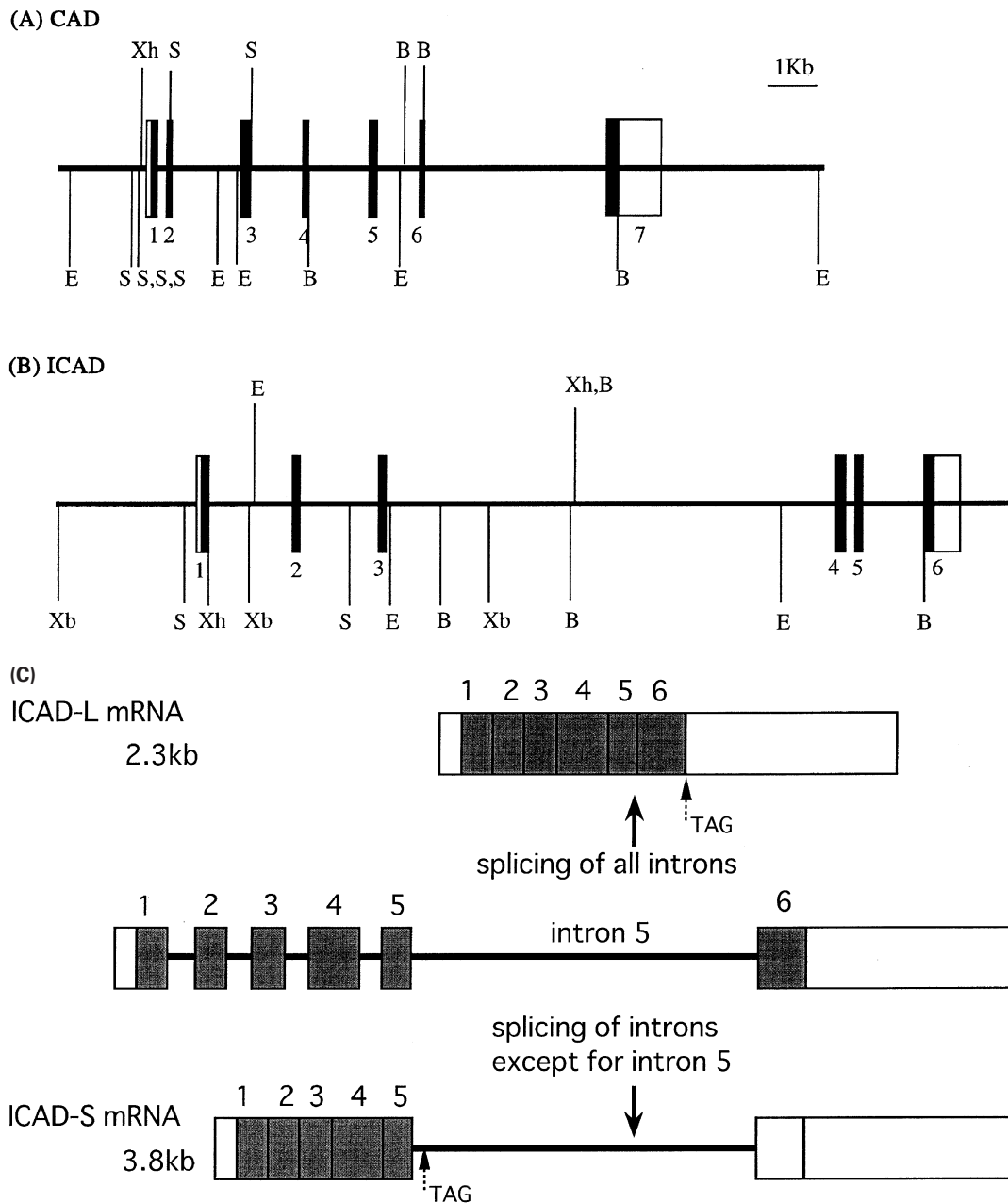


Figure 2 Chromosomal gene structures for the *CAD* and *ICAD* genes. (A and B) The organization of the *CAD* and *ICAD* chromosomal genes. The structures of *CAD* (A) and *ICAD* (B) are schematically shown. The boxes and lines between them represent exons and introns, respectively. The filled areas represent the coding regions, while the non-coding regions are indicated by open areas. The restriction enzyme sites for *Bam*HI (B), *Eco*RI (E), *Sma*I (S), and *Xho*I (Xh) on the both genes, and for *Xba*I (Xb) on the *ICAD* gene are shown. (C) Generation of two isoforms of *ICAD* mRNAs, *ICAD-L* and *ICAD-S*. A close-up view of the *ICAD* precursor RNA (middle), *ICAD-L* mRNA (top) and *ICAD-S* mRNA (bottom) are shown. Boxes represent exons, which are numbered above boxes. The shaded and open areas represent the coding and non-coding regions, respectively. The precursor RNA undergoes splicing of intron 5 to produce *ICAD-L* mRNA, while intron 5 remains unspliced to generate *ICAD-S* mRNA. The positions of the first termination codon are indicated by arrows

The promoter sequence of *ICAD* and *CAD* chromosomal genes

The 5' flanking sequences (660 and 730 bp, respectively) of the murine *CAD* and *ICAD* genes are shown in Figure 4A and B. To determine the transcription initiation sites for *CAD* and *ICAD* mRNAs, 5'-RACE procedure was carried out with mRNA from murine WR19L T cell lymphoma cells. As shown in Figure 4C, PCR with an anti-sense primer at 371 nucleotides downstream of the ATG initiation codon in the *CAD* cDNA produced a major band of 460 bp. The same procedure for *ICAD* mRNA with an antisense primer at 614 nucleotides downstream of the ATG codon gave a major band of 740 bp. The major PCR products for *CAD* and *ICAD* mRNAs were then subcloned and sequenced. Most of the PCR products for *CAD* mRNA started around the G at position +1, 43 nucleotides upstream from the initiation codon, while minor PCR products (less than 10%) started from the G at position -37 (Figure 4A). On the other hand, most of the PCR products for *ICAD* mRNA started from the A at position +1, 88 nucleotides upstream from the initiation codon (Figure 4B). These bases were therefore tentatively assigned as the transcription initiation sites for *CAD* and *ICAD* mRNAs. There is no 'TATA-like' box immediately upstream of the transcription initiation site in either the *CAD* or *ICAD* gene. A sequence of about 500 nucleotides up to position -600 in the *CAD* gene is extremely rich in G and C nucleotides (75% G/C). In contrast, the 5' promoter region of the *ICAD* gene was rich in A and T nucleotides (42% G/C). There was a B1-like repetitive sequence of about 340 bp at 320 nucleotides upstream of the transcription initiation site of the *ICAD* gene.

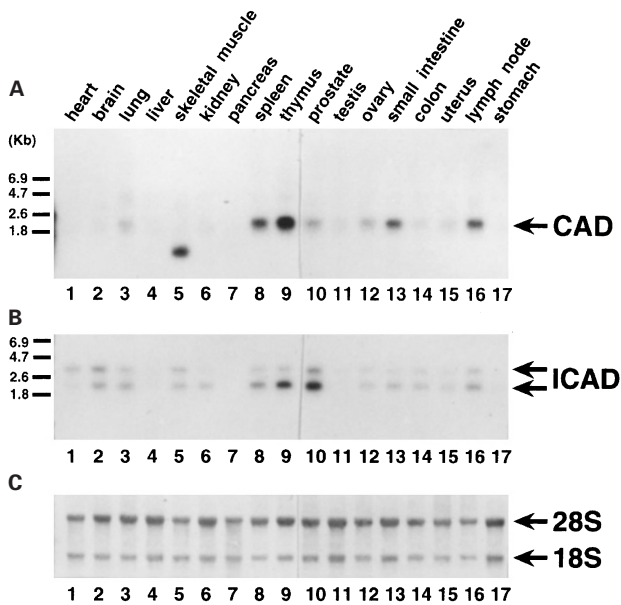


Figure 3 Expression of the *CAD* and *ICAD* mRNAs in murine tissues. Poly(A) RNA was prepared from the indicated tissues by using the Quick PrepTM micro mRNA preparation kit (Pharmacia). Two micrograms of RNA from each source was subjected to electrophoresis and analyzed by Northern hybridization as described previously. ²⁸³²P-labeled murine *CAD* cDNA (A) or *ICAD-L* cDNA (B) was used as the probe. The filter was stained with methylene blue, and is shown in (C). The positions of 28S and 18S rRNAs are indicated by arrows on the right

Promoter analysis of *CAD* and *ICAD* genes in human HeLa and Jurkat cells

To examine the promoter activity of the 5' flanking region of *CAD* and *ICAD* genes, a 544-bp fragment carrying the sequence of -590 to -46 of *CAD* gene or a 297-bp fragment carrying the sequence of -305 to -8 of *ICAD* gene was introduced into the upstream of the *luciferase* reporter gene (pBPA). As shown in Figure 5A, compared with the basic promoter in pBPA, the flanking region of *CAD* gene in pCADP stimulated transcription of the *luciferase* gene about 120 or 25 times in HeLa and Jurkat cells, respectively. When a longer fragment (4.7 kb) carrying the 5' upstream flanking region was used to drive the reporter gene, the luciferase activity in the transfected cells was comparable to that found with pCADP, suggesting the control element(s) responsible for the *CAD* gene transcription is within the 554 bp fragment in pCADP. To determine regulatory elements in this region, a series of deletion mutants in the 5' flanking region of the *CAD* gene was then joined to *luciferase* gene, and the promoter activity was assayed. As shown in Figure 5A, deletion of the sequence from -590 to -190 had no significant effect on the promoter activity. On the other hand, the deletion of the sequence from -189 to -133 caused a sixfold or threefold reduction of the promoter activity in HeLa and Jurkat cells, respectively. These results suggest that a transcriptional regulatory element(s) are on the region from -189 to -133 of the *CAD* gene.

The 297-bp DNA fragment of the 5' flanking region of *ICAD* gene in pICADP plasmid also stimulated transcription of the *luciferase* gene about 100 times and 30 times in HeLa and Jurkat cells, respectively (Figure 5B). Activation of transcription by a longer fragment containing the sequence up to 3.7 kb was similar to that obtained with the 297-bp fragment (data not shown), suggesting that the transcription of *ICAD* gene is controlled by the 297-bp fragment in the upstream of the transcription initiation site. Deletion of the sequence from -305 to -119 had little effect on the promoter activity. Further deletion up to -80 caused a significant reduction of the promoter activity in HeLa cells, although its effect in Jurkat cells were less significant. Whereas, when the sequence was further deleted up to -56, the promoter activity dropped in both HeLa and Jurkat cells.

Discussion

Murine and human *CAD* have an identity of 76% at the amino acid sequence level. We previously showed that there are intact and processed pseudo genes for human *CAD*.¹¹ The intact human *CAD* gene is localized on the short arm of chromosome 1 (1p36.3).¹¹ Here, we cloned a single intron-containing gene for murine *CAD*, and assigned it to the distal part of murine chromosome 4, which is syntenic to the short arm of human chromosome 1 (1p36). These results suggest that there is a single functional gene for *CAD* per murine haploid genome. Murine *ICAD* inhibits the *CAD*'s DNase activity, and has an identity of 76.1% with human DFF-45, one of the subunits of DNA fragmentation factor in apoptosis.¹³ We

cloned an intron-containing gene for murine *ICAD*, and showed that it is closely localized with the *CAD* gene on murine

chromosome 4. Human *DFF-45* was recently assigned to the region of 1p36.3,¹⁷ where the *CAD* gene is located.¹¹ These

(A) *CAD*

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-650                                -600  CADP :>
TGCTTTAGAGAATCTTAAACGCTTAAAAAGCACAAACAAACCCAGCGAACAACAAAACCCCAAAAC
                                SRY
                                -550
GACACGGATCGAAGTGTAGCCTACCCGGAGCCAGAGCTACAGCTTTTAGTCTCTGGGCAACTGCTCTGT
                                RFX1 Myb
                                -500                                -450
TCAGTAAACGCATGCAGACGAGGACAAGCGGGGCTCGAATCCGATTCCGGACACGAAGGCTGAGATGCGC
                                Ets
                                -400
TGCAGGAGGGCTGGAACAGGCACTGGCCGCGAGTCCCTCCAAGGCCAGGTGTCAGGAGGCTCCGCCCC
                                MyoD SP1
                                -350                                -300
CGGGTGCCTGCCGAAGACCGAGGCCACGAGGGTCACAGAGCGCCGCGAAACGACAGCGGGGGCCGCT
                                AP-4
GCGATGTCCGGCTCCGCTATCTCGAGGGGTCTCGGTCCAGCCGCCCAACCCCGGGCTCTGAGGAGG
                                GATA-1 SP1
                                -200                                -250
GCAGCAGAGGAGCAGCCGGGCTGCCTCTGCCAGGGCCCGGGCCGAGCTCCGCTCCCATAGCAACCAG
                                AP-4 HEN1 RFX1
                                -150                                -100
GCCGGCCCGCGCGGTACGCCTGCCGCTCCGCGCTGCGCATCCAGGCTCGAGGATTC
                                SP1 AP-2 CPT3 :> CPT4 :>
TGGCTGGGGAAGTCTCGAGTCCGACGTTTCCGGTTGCACCTACCTACGTAGAGAAAAGAGTTCCGGG
                                MZF-1 Ets
                                +1
CCGGCTTACGCACACAGTTCCGGGAGGAACGTTACAAGTCCAGTGCCTACCGTACCGTACCGCCCTG
                                <
CA ATG
    
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(B) *ICAD*

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                                -700
GCTTGTAATCATATCCTTTAAACTTCAAAGTCACAAAACCCAGCTCTGAAAACACACAAACACACACA
                                -650                                -600
CACACAATTTCTCTTTTAGTCTCTGGCTGTGTAGAACTCACTATGTAGACCAGGCTGCCCTGGAA
                                -550
CTCATAAAGATCCTTCTGCCTCCTGAGGATTAAGGCCCTGAGCCACACACCAGTTCAATGGCACTCT
                                -500                                -450
TAAAACATGAATATGCATCTGGAGAGATGGCCAGCAGTTAAGAGCACTGACTGCTCTTCAAAGGTCTT
                                -400
GAGTTCAAATCCCAGCAACCACATGATGGCTCACAAACCATCCGTAATGAGATCTGATGCCCTTCTTGGT
                                -350
GTGCTGAGGACAGCTACAGTGTACTTATGTATAAATAAACTTTAAAAAGGAAAAAATCATGAA
                                -300                                -250
ICADP :> TATATGGCTGGCAATGTAGTGTAGAACTATGCTTACCATACATGAGACCCCGGGTTTTCCATCATACAGT
                                c-Rel
                                -200
CTTTTAGAGAAGAAAAGGAGGGGAAAGTAAACAGGTTAGGGGAAAAGAAAACAACAGAAAAAAGAAATCT
                                MZF1 MZF1 SRY
                                -150                                -100
CCAATCTGGACCACCTTCCCTAACTGAATCCCCCGCCCTGGCTTTGAAACCAGAAACTACAATTTCCC
                                STAT MZF1 SP1 IPT2 :> Ikaros
                                -50
GTCGTGCCCTGGAAACCGCGTATTAGCCGCGACTAGAACTACATCTCCCGGGCTGCTTGGTCTCGCCT
                                c-Rel Myc Gfi-1
                                +1
AACTTGCTGAGAGAACTACATCTCCCGTCAGGCGGGGAGGAGGTTGTCAAAGCTTAGGGAAGCGGCT
                                <
CTGGCTACCTGGGATTTCCAGAGTCCGCAAGTCCCACTTTGGAG ATG
    
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(C)

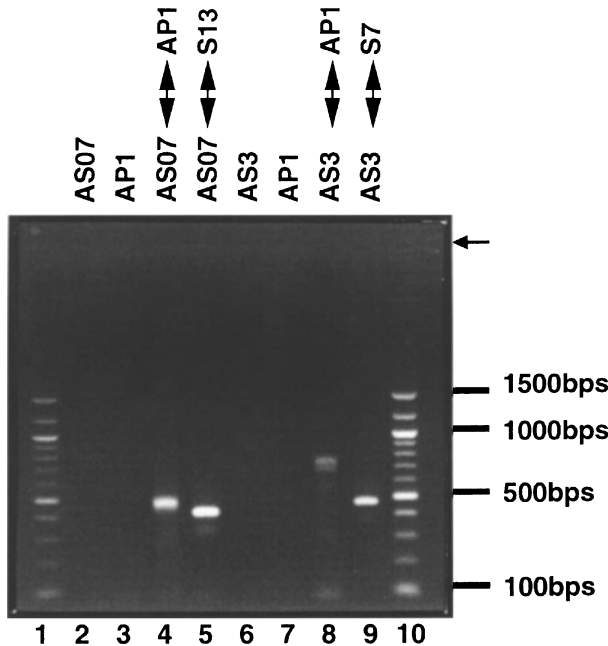


Figure 4 The 5' flanking region of the *CAD* and *ICAD* chromosomal genes. (A and B) Nucleotide sequence of the 5' flanking region of murine *CAD* (A) and *ICAD* (B) chromosomal genes. The nucleotide sequence is numbered from the major transcription initiation site. The major transcription initiation site is indicated by a bold arrow, while the minor initiation site is indicated by a thin arrow. The B1-like repetitive sequence in *ICAD* gene is doubly underlined. The binding sites for transcription factors were identified with MatInD and MatInspector programs,²⁹ and are underlined. (C) 5' RACE procedure for the *CAD* and *ICAD* mRNAs. Poly(A) RNA from murine WR19L cells were reverse-transcribed with an anti-sense primer specific for *CAD* (lanes 2–5) or *ICAD* (lanes 6–9), and subjected to the 5' RACE procedure as described in Materials and Methods. PCR was carried out with a specific anti-sense primer (AS07 for *CAD* and AS3 for *ICAD*) and a sense primer (AP1) in the adaptor (lanes 4 and 8). As controls, PCR was carried out with the anti-sense primer alone (lanes 2 and 6) or the sense adaptor primer alone (lanes 3 and 7). PCR was also carried out with the sense and anti-sense primers (AS07 and S13 for *CAD*, and AS3 and S7 for *ICAD*) that are specific to the *CAD* or *ICAD* mRNA, respectively (lanes 5 and 9). The DNA size marker (100 bp DNA ladder, New England Biolabs) was subjected to electrophoresis in parallel, and the size is shown on the right in bp. An arrow indicates the origin of electrophoresis

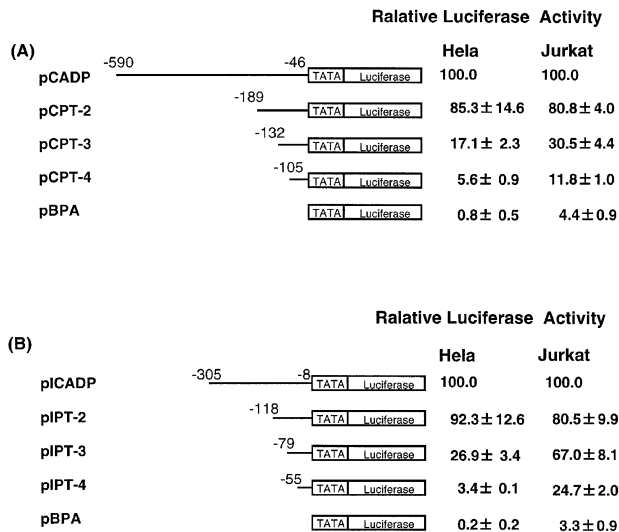


Figure 5 Promoter activities of 5' flanking region of *CAD* and *ICAD* genes. The promoter regions of *CAD* (A) and *ICAD* (B) genes connected to the *luciferase* gene are shown by horizontal bars. The numbers above each bar indicate positions from the major transcription initiation site. TATA and Luciferase indicate the TATA box, and firefly *luciferase* gene, respectively. BPA represents pBPA-TATALuc vector.²⁷ The promoter activity of each construct was determined in HeLa and Jurkat cells, and relative luciferase activities are expressed as a percentage of that of pCADP (A) or pICADP (B), respectively. Transfection was done four times, and the average values are shown with standard deviation

results suggest that human *DFF-45* is the human ortholog of *ICAD*, and that there is a single chromosomal gene for *ICAD* per murine haploid genome. We have compared our interspecific map of chromosome 4 with a composite murine linkage map that reports the map location of many uncloned murine mutations (provided from Murine Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME, USA). *Icad* and *Cad* were mapped in a region of the composite map that lacks murine mutations with a phenotype that might be expected for an alteration in these loci (data now shown).

ICAD, which is more abundant than *CAD*, can bind to *CAD*.^{8,12} In growing cells, *CAD* exists as a complex with *ICAD*,^{10,13,18} suggesting that *CAD* and *ICAD* function as subunits. The N-terminal domain of about 100 amino acids is homologous between *CAD* and *ICAD*. However, no homology is apparent between the other parts of these molecules.¹¹ Nonetheless, *CAD* and *ICAD* are localized in the same chromosomal region in human and mouse. Since functional *CAD* cannot be synthesized without *ICAD*,^{8,18} *CAD* and *ICAD* may have evolved as a set of genes. To further examine this possibility, it would be necessary to determine the detailed arrangement of the *CAD* and *ICAD* genes on human and murine chromosomes, as well as on the chromosomes of other organisms. The N-terminal homologous domain of *CAD* and *ICAD* is coded by two exons in both genes, and introns in this domain are inserted at similar positions in the *CAD* and *ICAD* genes. However, intron 1 is inserted after the third letter of a codon in the *CAD* gene, while it is inserted after the first letter of a codon in the *ICAD* gene, suggesting that this domain may

not have derived from a common ancestral gene. Rather, the sequence encoding the N-terminal portions of *CAD* and *ICAD* may have converged from different ancestral genes to a similar sequence to acquire a similar function. Although the function of the N-terminal domain of *CAD* and *ICAD* has not yet been elucidated, it is possible that it works as a homophilic interacting domain as found for the death and CARD domains.^{19–21}

Structural analysis of the murine *ICAD* gene indicated that two different mRNAs for *ICAD* (*ICAD-L* and *ICAD-S*) are generated by alternative splicing of intron 5. *ICAD-L* and *ICAD-S* mRNAs were expressed at a similar level in various murine tissues. Equal amounts of *ICAD-S* and *ICAD-L* proteins were also detected by Western blotting in human and murine cell lines,^{18,22} suggesting that the human *ICAD* gene also produces *ICAD-S* and *ICAD-L* mRNAs by alternative splicing. We have recently found that *ICAD-L* but not *ICAD-S* has an ability to work as a chaperone to enhance the correct folding of the *CAD* protein during *CAD* synthesis, although both *ICAD-L* and *ICAD-S* proteins efficiently inhibit the *CAD*' DNase activity.¹⁸ Whether *ICAD-S* has a specific function or not, remains to be determined.

Murine *CAD* and *ICAD* mRNAs were expressed rather ubiquitously in various tissues, although its expression level in the lymphocytic tissues such as the thymus, spleen and lymph nodes was relatively high. Yet, the characteristics of the 5' flanking promoter region were significantly different between the *CAD* and *ICAD* genes. The promoter region of the murine *CAD* gene was very G and C rich as found in many ubiquitous house-keeping genes. On the other hand, the G and C content in the promoter region of murine *ICAD* was much lower than for *CAD*. The promoter analysis of the *CAD* and *ICAD* genes indicated that the sequence up to -189 of the *CAD* gene is sufficient to stimulate the transcription in HeLa and Jurkat cells. Several cis-regulatory elements for binding of various transcription factors such as RFX-1, SP-1, AP-2, MZF-1 and Ets are in this region. The *ICAD* gene expression required the sequence between -118 and -8 of the *ICAD* gene, in which binding sites for Ikaros, c-Rel, Myc and Gfi-1 can be found. Except for MZF-1 and Ikaros, these transcription factors are rather ubiquitously expressed in various tissues, as found for *CAD* and *ICAD* genes. Although it will be necessary to determine which transcription factors are responsible for the expression of the respective genes, these results suggest that expression of *CAD* and *ICAD* genes are regulated by different mechanisms.

Materials and Methods

Isolation of murine chromosomal genes for *CAD* and *ICAD*

4×10^5 plaques of λ FIXII carrying a genomic DNA fragment from 129/SV mice (Stratagene, La Jolla, CA, USA) were screened under high stringency conditions using a DNA fragment carrying murine *CAD* or *ICAD-L* cDNA⁸ as probes. Recombinant λ DNAs that were positive with

the probe were subcloned into pBluescript II, characterized by restriction enzyme mapping and PCR (polymerase chain reaction) using primers in exons. The DNA appropriate DNA fragments were then characterized by DNA sequencing analysis.

Hybridization and DNA sequencing

Colony, plaque, and Northern hybridizations were carried out as described previously.²³ The probe DNA was prepared by PCR using the appropriate plasmid DNA as a template, and labeled with α -³²P-dCTP (New England Nuclear, Wilmington, DE, USA) using a random primer labeling kit (Boehringer Mannheim, Germany). DNA sequencing was carried out using a DNA sequencer (model 373A, Applied Biosystems, Foster City, CA, USA) and a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems).

5' RACE

The 5'-RACE was carried out using a kit from Clontech Laboratories (Palo Alto, CA, USA) according to the protocol provided. In brief, the first-strand cDNA was synthesized using a primer (5'-CTGGCTCA-CATGATGTAGGAGATC-3') at the nucleotide position of 373–396 for *CAD*, and a primer (5'-GATAAACTCAGCTCTGG-3') at the nucleotide position of 751–767 for *ICAD*. The *CAD*- and *ICAD*-specific cDNAs were then amplified using a primer (ASO7; 5'-GGCCAG-CAGCTTTTGCCTCAG-3') at the nucleotide position of 352–372, and a primer (AS3; 5'-TCCACTTCTCATTGCAGGCC-3') at the nucleotide position of 264–283, respectively. The PCR products were cloned in pGEM^R-T Easy Vector (Promega, Madison, WI, USA), and their DNA sequences were determined.

Interspecific murine backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J \times *M. spretus*) F₁ females and C57BL/6J males as described.¹⁵ A total of 205 N₂ mice were used to map the *Icad* and *Cad* loci (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described.²⁴ All blots were prepared with Hybond-N⁺ nylon membrane (Amersham, Arlington Heights, IL, USA). The probe for *Icad* was an about 1.1 kb fragment of murine cDNA and the probe for *Cad* was an about 1.6 kb fragment of murine cDNA. The probes were labeled with α -³²P-dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of 0.8–1.0 \times SSCP, 0.1% SDS, 65°C. Fragments of 21.0 and 3.6 kb were detected in *Sph*I digested C57BL/6J (B) DNA and a fragments of 5.3, 3.8 and 3.6 kb were detected in *Sph*I digested *M. spretus* (S) DNA using the *Icad* probe. *Bgl*II fragments of 11.0, 1.8, and 1.5 (B) and 15.0 and 2.6 kb (S) were detected with *Cad* probe. The presence or absence of the *M. spretus*-specific fragments, which co-segregated for each probe, was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Icad* and *Cad* including *Htrld*, *Nppa* and *Gnb1* has been reported previously.^{25,26} Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Transfection and luciferase assay

A series of fragments containing various length of the 5' flanking region of murine *CAD* and *ICAD* genes were prepared by PCR. After

confirming its authenticity by DNA sequencing, they were joined to the *luciferase* gene in pBPA-TATALuc vector, which contains a TATA box derived from murine *G-CSF* gene upstream of the firefly *luciferase* gene.²⁷ Human HeLa and Jurkat cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS, Gibco BRL) or RPMI1640 medium containing 10% FCS, respectively. HeLa cells (5×10^3 cells) were transfected with 500 ng of the reporter plasmid by lipofection using spherical cationic polyamidoamine polymers (SuperFect Transfection Reagent, QIAGEN). As an internal control of the transfection, 100 ng of pBOS-Rluc plasmid carrying the sea pansy *luciferase* gene (Promega) in pEF-BOS vector was included in each transfection. After culturing cells at 37°C for 24 h, the luciferase activity in the cell lysates was measured using Dual Promoter Luciferase Assay Kit from Promega. The firefly luciferase activity of each sample was normalized to the sea pansy luciferase activity. Jurkat cells (4×10^5 cells) were transfected with 1 μ g of the reporter plasmid and 200 ng of the pBOS-Rluc plasmid by the lipofection method using DIMREA-C Reagent from Gibco BRL. Cells were cultured at 37°C for 30 h, then the luciferase activity in the cell lysates was measured as described above.

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