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Chromosomal Localization of the Human Genes, *CPP32, Mch2, Mch3,* and *Ich-1,* Involved in Cellular Apoptosis

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Members of the ICE/CED-3 protease family appear to play an essential role in programmed cell death process. In this paper the chromosomal localization of the human genes *CPP32, Mch2, Mch3* and *Ich-1* is reported, obtained by Radiation Hybrid Mapping. *CPP32* was assigned to chromosome 4q33–q35.1, *Mch2* to chromosome 4q25–q26, *Mch3* to chromosome 10q25.1–q25.2 and *Ich-1* to chromosome 7q35. *Ich-1* was found to map very close to the marker WI-9353. The possible overlapping of the two independent locus assignements is considered. The genomic distribution of these genes is discussed, with particular reference to the co-location with some human genetic diseases all characterized by autosomal dominant inheritance and by similar malformative features.

Apoptosis is a selective, controlled and genetically programmed cell death process that occurs to replace redundant or unnecessary cells, in tissue morphogenesis and remodeling, and in the normal turnover of cells, as a specific type of terminal cell differentiation, but it is also triggered in response to pathogenic invasion to halt the spread to neighboring cells.

Apoptotic suicide has many advantages over other forms of cell death, such as necrosis, owing principally to the membrane integrity that is maintained through the entire process, without the occurrence of inflammation. The distinctive nature of this highly ordered process is also suggested by other features, such as chromatin condensation, fragmentation and margination, internucleosomal DNA cleavage, cytoskeletal disruption, cell shrinkage, membrane blebbing and budding to produce membrane-bound apoptotic bodies that are subsequently engulfed by neighboring cells or professional macrophages (1).

There is a substantial evidence that inappropriate apoptosis may contibute to the pathology of several human diseases. These can be divided into disorders of excessive apoptosis (such as neurodegenerative diseases or ischemic damage) and those were insufficient apoptosis occurs (such as autoimmune syndromes, cancers and sustained pathogenic infections) (2) (3) (4) (5) (6) (7).

Disease association, genetic analysis and *in vitro* reconstitution of apoptotic events have helped to define a biochemical pathway that accounts for many of the key events that occur in dying cells *in vivo.* At the heart of this process, proteases related to mammalian interleukin-1β converting enzyme (ICE) and to nematode CED-3 appear to play an essential role. CED-3 was initially identified by genetic analysis of the nematode *C. elegans* as the product of one of the two genes (the other being *ced-4*) that were absolutely required for programmed cell death to occur (8). *Ced*-3 was found to encode a cysteine protease that was highly related to mammalian ICE, an enzyme responsible for the proteolytic maturation of pro-interleukin-1β to the biologically active inflammatory cytokine (9) (10) (11). Although this initially implicated ICE itself in mammalian apoptosis, it now seem clear that other ICE-homologues are more

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likely candidates as functional counterparts of CED-3 in higher organisms. Molecular cloning has identified several human homologues of ICE and CED-3 including ICErel-II, ICErel-III, ICH-1 (equivalent to murine Nedd-2), CPP32 (apopain, Yama), Mch2 and Mch3 (12) (13) (14) (15) (16) (17).

Members of the ICE/CED-3 protease family cluster into two subfamilies. Those related to ICE (ICErel-II and ICErel-III) can be structurally distinguished from those related to CED-3 (ICH-1/Nedd2, CPP32/apopain, Mch2 and Mch3).

At least some of these proteases have been directly implicated in mammalian apoptosis, particularly those belonging to the CED-3 subfamily (18).

Here we report on the fine mapping of the human cell death genes *CPP32* (isoform α and β), *Mch2* (isoform α and β), *Mch3* (isoform α) and *Ich-1* (*Ich-1L* and *Ich-1S*) obtained by radiation hybrids (RH mapping). The genomic distribution of these genes is also discussed.

MATERIALS AND METHODS

Primer designing. Oligonucleotides for PCR were selected from the *CPP32α*, *Yama/CPP32β*, *Mch2α*, *Mch2β*, *Mch3α*, *Ich-1S* and *Ich-1L* cDNA sequences (14) (15) (16) (17) (13) present in the GenBank database by OLIGO software (Version 4.0) (19).

We have considered the 3′ portion of the sequences because this region is virtually free of introns (20), therefore primers designed from such part of the gene will amplify fragments in the human genomic DNA whose lenght can be predicted from the cDNA sequence. Secondly, since the 3′ untranslated regions are poorly conserved, there is a good probability that the designed primers will selectively amplify a distinct human fragment even within a rodent background when somatic hybrids are used. Finally, related members of a gene family can be very similar in the coding region but normally they are considerably different in the less conserved 3′ untranslated part.

We have aligned the partial sequences (about 250-300 nucleotides) of these genes together with other members of the ICE / CED-3 family by CLUSTAL W software (Version 1.4) (21).

We have excluded as target sequences for the primer selection the regions more conserved between the members of the family or very closed (about 20 bp) to the polyA tract, where signals of polyadenylation can be present.

The selected primers were:

CPP32α FOR: 5′ AGTATGACATTTCACGGGAGATTT 3′

CPP32α REV: 5′ ACAAAATTGTCACATAGAAACACA 3′Expected PCR product size: 153 bp

CPP32β FOR: 5′ AACAGAATTTGAGTCCTTTTCCTT 3′

CPP32β REV: 5′ TCACTTGGCATACAAACTAAAAAA 3′ Expected PCR product size: 148 bp

*Yama** FOR: 5′ TATTCTTGGCGAAATTCAAAGGAT 3′

*Yama** REV: 5′ AAAGTAGCGTCAAAGGAAAAGGAC 3′ Expected PCR product size: 158 bp

Mch2α FOR: 5′ ATAAATATCGTTAGGGTGAAGCAT 3′

Mch2α REV: 5′ CCCTGCAGTTTATTAAAAAATAAT 3′ Expected PCR product size: 200 bp

Mch2β FOR: 5′ CAAAATCCTCAGGAAATTAGATAA 3′

Mch2β REV: 5′ TGACAAAATACAAGTTAAATCAGC 3′ Expected PCR product size: 109 bp

Mch3α FOR: 5′ TGCAAAATCTGTTATAGCTTTAAA 3′

Mch3α REV: 5′ ACTATTAGGCAGAAAAAAACAGTC 3′ Expected PCR product size: 195 bp

Ich-1L - FOR: 5′ GTGCAAGGAAATGTCTGAATACTG 3′

Ich-1L - REV: 5′ ATCCTGAAGATCAAAGGCTCTATC 3′ Expected PCR product size: 154 bp

Ich-1S - FOR: 5′ CTGAAGAAGCAAACATGACTAGAG 3′

Ich-1S - REV: 5′ GGCAAAGATCAATAAGGTACAAAT 3′ Expected PCR product size: 178 bp

* This primer pair is alternative to the previous pair, but equivalent.

TABLE 1
Retention Scores for the Genes on the Genebridge4 Radiation Hybrid Panel

Gene	Retention profile
CPP32α	00000001000000000100011010000100001001100100010000000010000000100010000001000000001010000000 1
*Yama/	20000201200000000100011010002100001001120101010020002210000000100210020001000000001010000200 1
CPP32β	00000001000000000100011010000100001001100101010000002010000020100010000001000000001010000000 1
Mch2α	10000011010000000000001010100100111000010010010100001120001000001000100000010000000010100001010
Mch2β	10000011010000000000001010100100111000010010020100001100001000001000100000010000000010102001010
Mch3α	11001000100120000210001000211001201000000110110010010001000100000100000100002210111020100000 0
Ich-1S	00000001100000100010001001001100000001110100001001101010001000000100000001000000011010000010 0
Ich-1L	00000001200000000010001002001100000001110100001001101010001000000100000001000020110110000010 0

Note. A hybrid scored 1 indicates presence of the sequence in that hybrid. A hybrid scored 0 indicates absence of the sequence. Unknown/uncertain data are indicated with 2's. The hybrids in the data vector are arranged according to the official Genebridge4 order.

* Retention profiles obtained with use of two distinct primer pairs.

Radiation hybrid mapping. The radiation hybrid mapping was performed by the Genebridge 4 whole-genome Radiation Hybrid Panel (Research Genetics, Huntsville, Al., U.S.A.) consisting of 93 genomic DNAs from the same number of human-on-hamster somatic cell lines, plus the two control DNAs (HFL donor and A23 recipient) (22).

Twenty-five ng of genomic DNA were used for amplification in 10 μl of PCR buffer (16.6 mM (NH4)2SO4; 67 mM Tris-HCl ph 8.3; 0.01 % Tween-20; 1.5 mM MgCl2) containing 800 nM of each of the forward and reverse primers, 0.2 units of DNA polymerase (RTB polymerase; Bioline, Italy) and 25 μM of each of the four dNTPs. The mix was covered with 5 μl of mineral oil.

Cycling conditions were: 1 min and 15 sec at 94 °C, followed by 35 cycles of 15 sec at 94 °C, 25 sec at 58 °C (62.5 °C for *CPP32β* and *Ich-1L*), 30 sec at 72 °C, and a final extension step for 1 min and 30 sec at 72 °C (PTC-225 Peltier Thermal Cycler, MJ Research).

The PCR reactions were mixed with 5 μl of loading buffer (30 % glycerol; 0.25 % Orange G; 25 mM EDTA) and separated on 2.5 % agarose gel in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) containing ethidium bromide. The gel was electrophoresed in the same buffer at 100 Volts for 45 min.

The screening results were processed by the RHMAPPER software program (23) at the Whitehead Institute/MIT Center for Genome Research (Cambridge, Mass., U.S.A.).

RESULTS AND DISCUSSION

Polymerase chain reaction (PCR) primers specific for the 3′ untranslated region of the human *CPP32 α*, *Yama/CPP32β*, *Mch2α*, *Mch2β*, *Mch3α*, *Ich-1L* and *Ich-1S* cDNAs were designed and used to PCR amplify the human / hamster somatic cell hybrid DNAs (Genebridge 4 whole-genome Radiation Hybrid Panel). The expected size for all the PCR products was confirmed and the retention pattern data for every gene processed by RHMAPPER software program.

The retention scores obtained for the genes on the Genebridge 4 Panel are shown in table 1. Multipoint analysis placed both the *CPP32α* and *Yama/CPP32β* sequences on chromosome 4q33-q35.1, 1.6 cR(3000) from D4S2865 (WI-4886) (that maps at 188.585 Mb from the p-telomere) and 3.4 cR from D4S2827 (WI-3160) (at 200.913 Mb), with a lod score $>$17.

Both *Mch2α* and *Mch2β* sequences were assigned to chromosome 4q25-q26, 3.5 cR from D4S3178 (WI-9414) (at 117.284 Mb) and 1.5 cR from D4S1651 (CHLC.GATA4C04.520) (at 121.01 Mb), with a lod score $>$17.

Mch3α sequence was mapped on chromosome 10q25.1-q25.2, 0.1 cR from D10S2189 (WI-9448) (at 109.205 Mb) and 3.5 cR from D10S562 (at 112.932 Mb), with a lod score $>$ 15.

Both *Ich-1L* and *Ich-1S* sequences were placed on chromosome 7q35, 1.4 cR from WI-9353 (at 157.220 Mb) and 3.5 cR from D7S676 (at 158.330 Mb) with a lod score $>$ 15.

The cytological localization was deduced from LDB (14) informations about the framework markers flanking the genes. The placement maps are shown in fig. 1, 2 and 3.

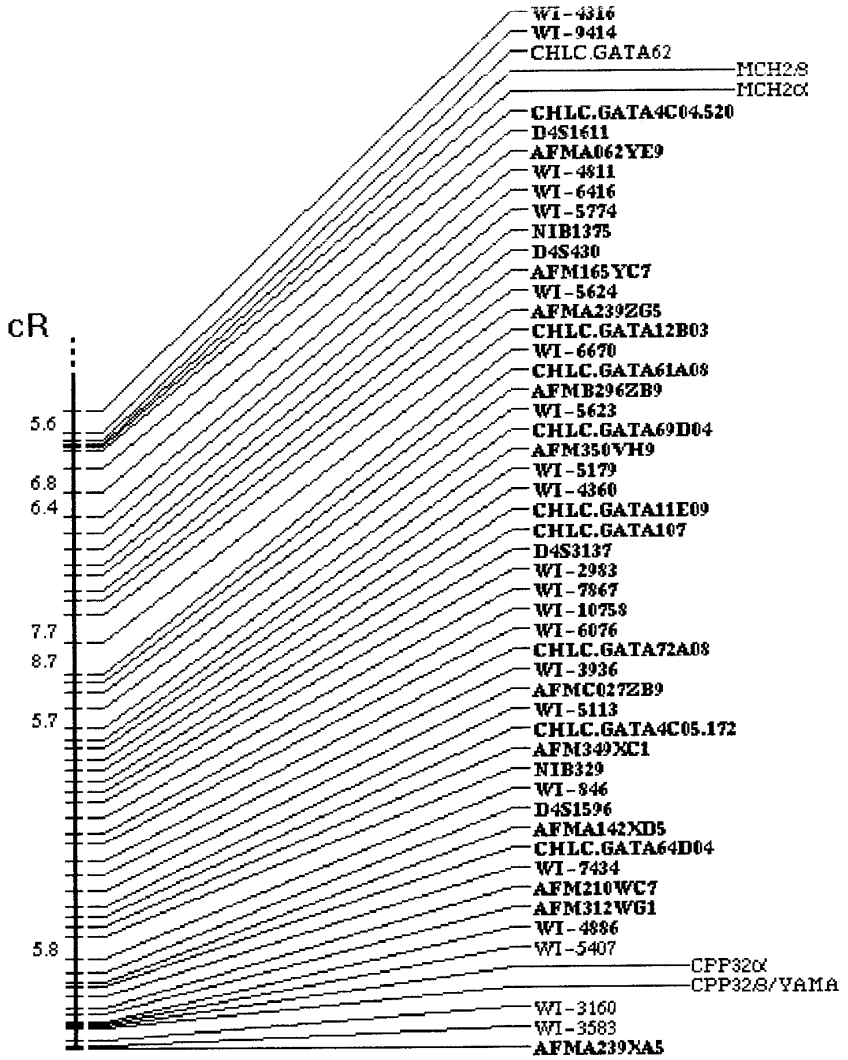

FIG. 1. Portion of chromosome 4 radiation hybrid framework map with *Mch2* and *CPP32* genes placed on the q25−q26 and q33−q35.1 regions, respectively. Note: 1 cR(3000) = 1 centiRay(3000rad) = 1 % frequency of breakage between two markers at 3000 rad ≈ 300 Kb (25).

The four members of the CED-3 subfamily, although functionally related, appear to map to distinct chromosomes or to different regions on the same chromosome.

On the other hand, as expected, sequences derived from alternative *CPP32*, *Mch2* and *Ich-1* cDNA isoforms show identical position on the framework map. This is in agreement with the hypothesis that these six mRNA species may represent alternatively spliced isoforms from three parental *CPP32*, *Mch2* and *Ich-1* messenger RNAs. (14) (16) (13)

With regard to the chromosomal localization of *Ich-1S* and *Ich-1L* sequences, by using both primer pairs (one specific for apoptosis positive regulator ICH-1S, the other specific for the negative regulator ICH-1L) two identical retention patterns were obtained. Multipoint analysis placed the sequences on the framework map with a slight position difference (1.4 cR, corresponding to about 400 Kb), compared to the marker WI-9353. This marker was obtained at

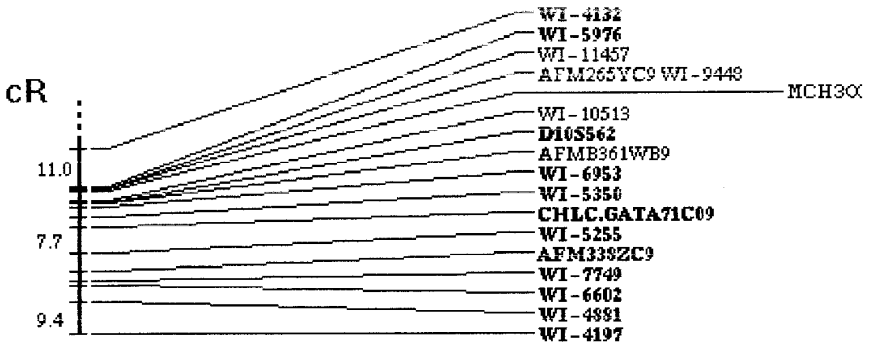

FIG. 2. Portion of chromosome 10 radiation hybrid framework map with *Mch3* gene placed on the q25.1−q25.2 region.

the Whitehead Institute by using primers from *Ich-1S* Genbank sequence, but it was never published as the location of *Ich-1S* and it is still not reported as such in Genbank. The distance between the location of WI-9353 and that of *Ich-1* obtained in the present study is too short to prove that there two different genes are involved, since the resolution bounds of the panel are of about 1000 Kb. Therefore it is possible that our primers and the WI-9353 primers identified the same *Ich-1S* gene.

The essential role that these proteases play in mammalian apoptotic cell death has been already well substantiated by a great deal of protease inhibitor studies (18). Analysis of tissue distribution demonstrated a wide expression of *CPP32, Mch2, Mch3* and *Ich-1* transcripts in several embryonic and adult human cell lines (14) (16) (17) (13).

Inappropriate apoptosis might produce a pathological expression . Therefore, *CPP32, Mch2, Mch3* and *Ich-1* might be candidate genes for human disorders due to excessive or insufficient apoptosis. A homozygote nonsense mutation for one of these genes is expected to produce a generalized adverse effect, possibly leading to embryonic or fetal death, whereas heterozygosity might be tolerated (although the mutation would produce dominant effects). The inspection of the list of human genetic disorders mapped to chromosomal bands 4q25-q26, 4q33-q35.1, 10q25.1-q25.2 and 7q35 revealed the presence of some candidate genetic diseases: Rieger syndrome (4q25-q26), William syndrome (4q33-q35), Crouzon craniofacial dysostosis (10q25-q26) and holoprosencephaly type 3 (7q34-q36). Rieger syndrome (OMIM 180500) is a dominantly inherited disorder characterized by hypodontia, malformation of the eye, anal stenosis and renal malformation. The charac-

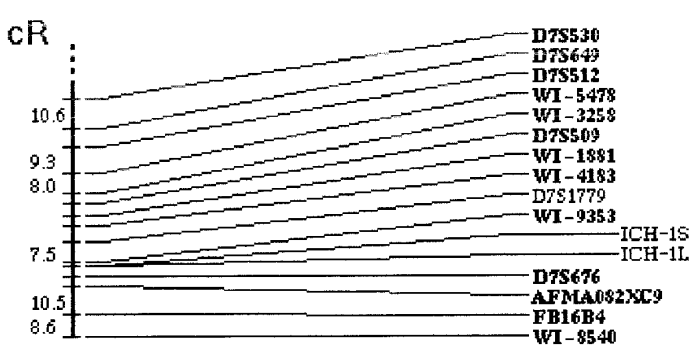

FIG. 3. Portion of chromosome 7 radiation hybrid framework map with *Ich-1* gene placed on the q35 band.

teristic facies consists of broad nasal root and maxillary hyoplasia with protruding lower lip. Wiiliams syndrome (OMIM 194050) is an autosomal dominant disorder which includes arterial stenosis, elfin face, mental and statural deficiency, characteristic dental malformation, infantile hypercalcemia and eye malformations. Crouzon craniofacial dysostosis (OMIM 123500) is characterized by cranial synostosis, eye malformations, parrot-beaked nose, short upper lip, hypoplastic maxilla and a relative mandibular prognatism. Holoprosencephaly (OMIM 142945) includes cyclopia or other milder ocular malformations, a nose-like structure above the eye, midface hypoplasia, microcephaly, mental retardation and cleft lip and palate.

These diseases are all inherited as autosomal dominant trait and all show eye, nose and mouth anomalies. Thesese similarities and the fact that members of the CED-3 subfamily map in the same chromosomal subregions suggest that these genes might be involved. Since in the proteolytic cascade each activated enzyme may influence the cleavage of a specific subset of target substrates, the differences in the pathological phenotypes could be easily explained. On the other hand, these diseases show genetic heterogeneity, variable penetrance and expressiveness and often they occur associated with specific chromosomal deletions . Since the size of deletions is variable and so it is for the clinical presentation of the syndromes, the role of one or more adjacent genes in determining the pathological phenotypes is possible, but presently hard to be defined. In spite of that, given the undeniable role of apoptosis in tissue morphogenesis and remodeling, screening for mutations in *CPP32*, *Mch2*, *Mch3* and *Ich-1* genes in these genetic disorders might be worth to be considered.

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