The Mouse Zic Gene Family

HOMOLOGUES OF THE DROSOPHILA PAIR-RULE GENE odd-paired*

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The mouse Zic gene, which encodes a zinc finger protein, is expressed in the developing or matured central nervous system in a highly restricted manner. We identified two novel Zic-related genes (Zic2, Zic3) through genomic and cDNA cloning. Both genes are highly similar to Zic(1), especially in their zinc finger motif. A comparison of genomic organization among the three Zic genes showed that they share common exon-intron boundaries and belong to the same gene family. Zic1, Zic2, and Zic3 were determined to mouse chromosome 9, 14, and X using an interspecific backcross panel. Northern blotting and ribonuclease protection showed that Zic2 and Zic3 are expressed in a restricted manner in the cerebellum at the adult stage. However, the temporal profile of the mRNA expression in the developing cerebella differ in the three Zic genes. Furthermore, we found that the Drosophila pair-rule gene, odd-paired is highly homologous to the Zic gene family. The similarity was not only the zinc finger motif, but also the exon-intron boundary was the same as those of mouse Zic gene family. These findings suggest that the Zic gene family and Drosophila odd-paired are derived from a common ancestral gene.

A molecular analysis of the early development of Drosophila elucidated the basic molecular mechanisms that establish the regional specification of the developing organism (Ingham, 1988). A parallel attempt has been made to understand developmental mechanisms of the mammalian central nervous system (CNS). The Drosophila genes involved in the generation of anterior-posterior differences have vertebrate homologues that appear to have developmental roles in the CNS. Several gene families that control the regionalization of the mammalian CNS have been isolated (Rubenstein et al., 1994). Often, structurally related genes that belong to the same gene family control the coordinated process of CNS development.

The Zic protein (Aruga et al., 1994) is a zinc finger protein with a molecular mass of 48 kilodaltons. The expression of Zic is restricted to the dorsal region of the neural tube at a specific embryonic stage, whereas in the adult, it is restricted to the cerebellar granule cells and neurons of a few other nuclei (Zic, zinc finger protein of the cerebellum). We speculate that the Zic gene is involved in regional specification and cell lineage determination based upon its expression profile.

In this study, we searched structurally Zic-related genes and identified novel genes (Zic2 and Zic3) that were highly homologous to the Zic gene. Their predicted amino acid sequences and genomic organization suggest that they belong to the same class of gene family. The chromosomal locations of Zic1, Zic2, and Zic3 were determined by means of an interspecific backcross panel. The expression was largely restricted to the cerebellum in adults.

Furthermore, we found homology between the Zic gene family and odd-paired, a Drosophila pair-rule segmentation gene (Benedyk et al., 1994). odd-paired is a gene required for the timely activation of engrailed and wingless in Drosophila embryos.

MATERIALS AND METHODS

Isolation of Genomic and cDNA Clones—Genomic clones of Zic1 were isolated from a BALB/c mouse genomic library (L1016, distributed by a Japanese Cancer Resource Bank) using the mouse Zic cDNA fragment (Aruga et al., 1994). Low stringency hybridization proceeded using a 129SV mouse genomic library (a gift from Dr. K. Yamamura, Kumamoto University) using a 32P-labeled cDNA fragment from PG-STzic-ZF (Aruga et al., 1994). We characterized the clones with the same restriction fragments according to genomic Southern blot analysis (data not shown). The hybridization conditions were 5 x SSPE, 1% SDS, 10% dextran sulfate, 200 μg/ml sonicated salmon testis DNA, 2 x 106 cpm/ml radioactive probes incubated at 50 °C for 18 h. Membranes were sequentially washed in 0.1 x SSC, 0.1% SDS at 42, 56, and 65 °C. The second intron of Drosophila odd-paired was isolated by means of the polymerase chain reaction (PCR) using the primer pair: 5’-CAGCTCCACACGGGAAA-3’ and 5’-TCCGTGTCGTGGCCTGTT-3’ based on the sequence of the second and third exons (Benedyk et al., 1994), respectively. The genomic DNA prepared from Drosophila melanogaster, Canton S, was used as a template.

The cDNA clones were isolated from cDNA libraries generated from mouse cerebella (Furuichi et al., 1989) or mice embryos (12 days postcoitum).2 Screening and sequencing analyses proceeded as described (Aruga et al., 1994). The nucleotide sequence data reported in this paper appears in the DDBJ, EMBL, and GenBank nucleotide sequence database with the following accession numbers, D70848 and D70849.

* This work was supported by grants from the Science and Technology Agency of Japan (supported by Dr. K. Yamamura, Kumamoto University) using a 32P-labeled cDNA fragment from PG-STzic-ZF (Aruga et al., 1994). We characterized the clones with the same restriction fragments according to genomic Southern blot analysis (data not shown). The hybridization conditions were 5 x SSPE, 1% SDS, 10% dextran sulfate, 200 μg/ml sonicated salmon testis DNA, 2 x 106 cpm/ml radioactive probes incubated at 50 °C for 18 h. Membranes were sequentially washed in 0.1 x SSC, 0.1% SDS at 42, 56, and 65 °C. The second intron of Drosophila odd-paired was isolated by means of the polymerase chain reaction (PCR) using the primer pair: 5’-CAGCTCCACACGGGAAA-3’ and 5’-TCCGTGTCGTGGCCTGTT-3’ based on the sequence of the second and third exons (Benedyk et al., 1994), respectively. The genomic DNA prepared from Drosophila melanogaster, Canton S, was used as a template.

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Chromosome Mapping Using Interspecific Backcross Panel—The three Zic genes were mapped in a panel of an interspecific backcross of (C57BL/6Rs (B) × Mus spretus (S))F1 × M. spretus (BSS) that was characterized for the segregation of more than 500 loci including centromeric heterochromatin (Hayashizaki et al., 1994). Variations in Zic genes were identified between the progenitor strains using PCR and sequencing gel or single strand conformation polymorphism analysis (Mashiyama et al., 1990). For these analyses, 50 ng of genomic DNA from the backcross panel was amplified with three sets of primers (which amplify part of intron 2 of Zic1 gene), 3′-GGTG-ACAA-5′ and 5′-GACAA-3′ (which amplify 3124 to 3283 bp), or 58°C (which amplify 3283 to 3452 bp). The PCR products were denatured at 80°C for 5 min in a formamide dye mixture and resolved by electrophoresis on 5% acrylamide, 8 M urea sequencing gel (for initiation. In C, the first and second methionine are both candidates for translation initiation.

RESULTS AND DISCUSSION

Identification of the Zic-related Genes in Mouse Genome—To test whether or not there are genes structurally related to Zic in the mouse genome, we performed a series of Southern blots using various regions of Zic cDNA (Aruga et al., 1994) (data not shown). Several bands were detected with probes containing the segments coding the zinc finger motif. When a mouse genomic library was screened with this probe at low stringency, we obtained several positive clones. These positive clones were classified into five types of Zic-related genes including Zic itself (hereafter, Zic is referred to as Zic1). Of these, we examined the expression in various organs of mice at various developmental stages and confirmed the expression of two genes. The cDNAs of two novel genes designated as Zic2 and Zic3 (Fig. 1) were isolated from the cDNA library prepared from mouse cerebella or embryos, and the nucleotides were sequenced.

Zic2 and Zic3 proteins had a calculated molecular mass of 55.3 and 50.6 kilodaltons. A comparison of the predicted amino acid sequences of the Zic1, Zic2, and Zic3 (Figs. 1 and 2) revealed that there are several similar regions in the three genes. The conserved regions are located in a linear fashion. The most extensive homology was found in the zinc finger domain (97% between Zic1 and Zic2, 91% between Zic1 and Zic3 and 91% Zic2 and Zic3 over 158 amino acids). We found that Zic1 bound to DNA probes containing the 5′-GACAA-
CAAAC-3' sequence recognized by GLI protein (Aruga et al., 1994). The high similarity in the zinc finger domain suggests that Zic2 and Zic3 bind similar sequences. There was also similarity in the three regions including the amino and carboxyl termini (Figs. 1 and 2). The overall homologies between the proteins are 64% (between Zic1 and Zic2), 64% (between Zic1 and Zic3), and 59% (between Zic2 and Zic3). Except for the zinc finger domain, the amino acid compositions were similar to that of the Zic1 protein. There were some polyalanine and polyhistidine sequences. The carboxyl-terminal flanking the zinc finger motif is enriched with serine/threonine residues. Zic2 is relatively large because of the insertion of extra sequences in the serine-rich region (Aruga et al., 1994).

In addition to the Zic2 and Zic3, we isolated Zic1 genomic clones. All three genes were composed of three exons, containing the zinc finger domain. The third exon contains large 3'-untranslated regions. The nucleotide sequences of the two introns adjoining the splice junctions are consistent with the recognized consensus sequence GT/AG (Fig. 2D). The exon-intron boundaries are conserved in homologous sites in the zinc finger motif. Thus, it seems likely that these three genes are derived from a common ancestral gene.

To determine the chromosomal locations of the mouse Zic genes, we analyzed the segregation of the gene by means of a BSS-Ros interspecific backcross. Differences in the length of the PCR products or in single strand conformation polymorphism analysis were used to segregate the alleles from C57BL/6Ros or M. spretus. The segregation of these differences was determined in progeny of this cross (Fig. 3, A and B). A comparison of the segregation of Zic1, -2, and -3 with characterized loci (Hayashizaki et al., 1994) revealed a dose linkage between Zic1 and D9Ncvs13 (3/56 recombinants), D9Ncvs15 (2/56 recombinants), Zic2 and D14Ncvs12, D14Ncvs59 (both 0/55 recombinants), Zic3 and DXNcvs1, Hprt (both 1/55 recombinants) (Fig. 3, A and B). The gene orders shown in Fig. 3C were indicated from the minimization of recombinations. Thus, the Zic1, -2, and -3 genes are located in mouse chromosomes 9, 14, and X, respectively.

In the regions where the Zic1 and Zic3 genes were mapped, there are some mutations that result in abnormal behavior. ducky (Snell, 1955) which causes abnormal behavior and histologically severe dysgenesis of hindbrain and spinal cord, as well as myelin deficiency, is located near the position of Zic1 and trembly which causes tremors and seizures in hemizygous males (Taylor et al., 1978) is 1 centimorgan proximal to the Hprt gene, at the position of Zic3 gene. We do not, however, have any concrete data linking these genes.

We found by means of Southern blotting that Zic1 has at least one homologous gene in various species including fish, amphibians, reptiles, and avians (Aruga et al., 1994). Correspondingly, we isolated Zic-related cDNAs from various species. It is likely that the Zic-opa gene family is phylogenically conserved.

The Zic Genes Are Highly Homologous to Drosophila odd-paired—The Zic1 gene was originally identified and character-
Fig. 3. Interspecific backcross analyses of assignment of the chromosomal locus of mouse Zic genes. A, DNA isolated from backcross progeny of (C57BL/6 × M. spreptus) female and M. spreptus male (Hayashizaki et al., 1994) were analyzed by electrophoresis of the PCR products and denaturing gels (Zic1) or denaturing gels (Zic2 and Zic3). While mapping the Zic3 gene, the C57BL/6 homozygous type (B/Y) appeared (data not shown). B, distribution of the haplotypes in the backcross progeny. B represents heterozygous, S homozygous. The markers are described by Hayashizaki et al., (1994). C, a schematic drawing of the position of the Zic1, Zic2, and Zic3 genes in each chromosome. The numbers in the left of the bars indicate the distance from the centromere of each chromosome.

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Fig. 4. The spatiotemporal distribution of Zic2 and Zic3 mRNA. In each panel, Zic2 and Zic3 mRNA were analyzed by Northern blotting and by the ribonuclease protection assay, respectively, except for B, A, tissue distribution in adult mice. B, Northern blots of the three Zic genes. In each panel, 20 μg of the total RNA from the cerebellum was separated by electrophoresis. The hybridized membranes were exposed to the films for 12 h (Zic1), 2 days (Zic2), and 14 days (Zic3) with an intensifying screen. C, expression in the developing brain. RNAs were extracted from the forebrain, midbrain, and hindbrain of developing mice. The upper band in the Zic2 panel seemed to be an artifact arising from the presence of the ribosomal RNA because the band disappeared when the poly(A)⁺ RNA were used (data not shown). D, temporal change in postnatal developing cerebellum. E, densitometric scanning of D and Zic1. The positions of introns in the three Zic genes and that of opa are derived from a common ancestral gene.

We compared the genomic organization of the three Zic genes and that of opa. The positions of introns in the three Zic genes were conserved in the opa gene (Fig. 2D). The genomic organization of opa, which is composed of three exons with a relatively long first intron (Benedyk et al., 1994), was also similar to that of Zic gene family. These similarities in the genomic organization suggest that the mouse Zic genes and Drosophila opa are derived from a common ancestral gene.

The homology between the Zic-opa and GLI-1 (Orenic and GLI-1) gene family is significantly high in their zinc finger domain. However, we consider that the Zic-opa gene family belongs to another subclass of zinc finger proteins for the following reasons. The exon-intron organization of the Zic-opa gene family is different from that of GLI1(-cil)-tra1) gene family (Ruppert et al., 1988;
The similarity in the non-zinc finger region in GL1, GL12, GL13, and CIp is not conserved in Zic1, Zic2, Zic3, and Opa, and vice versa.

Drosophila opa is a pair-rule gene, which is essential for the parasegmental subdivision of the Drosophila embryo; opa mRNA and protein are found throughout all segment primordia. It appeared that Opa does not act in a spatially restricted manner. However, the expression of Zic1 is a highly restricted in the adult mouse CNS, although the mRNA was found in a broad domain, namely, a large area of the alar plate of entire neural tube and the surrounding mesenchyme at specific embryonic stages (Aruga et al., 1994). There is apparent similarity in their expression during development.

Zic2 and Zic3 Are Expressed in the Cerebellum—We showed that Zic1 is expressed in the adult mouse cerebellum in a highly restricted manner (Aruga et al., 1994). To examine the expression of Zic2 and Zic3 in the CNS, we performed Northern blots and ribonuclease protection analyses. In the various organs from adult mice, Zic2 mRNA was detected only in the cerebellum as a single band of 3.5 kilobases (Fig. 4A). Zic3 mRNA was detected in the cerebellum and faintly present in the olfactory bulb according to the ribonuclease protection assay. There seemed to be less Zic3 mRNA than that of Zic1 and Zic2 as more time was required to visualize the Zic3 mRNA (3.5 kilobases) by autoradiography (Fig. 4B) after Northern blotting. The tissue distribution of Zic1 mRNA (Aruga et al., 1994) is similar to that of Zic3, whereas Zic2 is unique in that it was detected only in the cerebellum even after a longer exposure (data not shown).

We examined the mRNA levels in younger mice because Zic1 is expressed in the developing neural tissue at days postcoitus. The developmental profile in the whole brain (fore-, mid-, hindbrain) showed that throughout the embryonic days 12 to 18, the mRNA levels seemed to be relatively constant. The high level of mRNA in embryonic day 12 observed using the Zic1 probe (Aruga et al., 1994) was not found with Zic2 or Zic3. In the postnatal developing cerebellum (Fig. 4D), the profile differed among the three genes. The Zic3 gene peaked soonest at 5 days after birth, whereas Zic2 peaked at 11 days. Zic1 mRNA peaked in 5 to 7 days. These periods correspond to the stage when the precursors of granule cells are differentiating into the mature neurons. However, whether or not Zic2 and Zic3 are expressed in the granule cells as seen in Zic1 must be determined before their role in cerebellar development can be investigated.

Our preliminary data indicated that another gene (Zic4) is also expressed in the cerebellum. Combining with the highly restricted expression of the Zic1 gene in the cerebellar granule cells (Aruga et al., 1994), the Zic gene family is unique in that all of the members of the family are expressed in the adult cerebellum in a highly restricted manner.

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