The Retinoblastoma Interacting Zinc Finger Gene RIZ Produces a PR Domain-lacking Product through an Internal Promoter*

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The PR domain is a newly recognized protein motif that characterizes a subfamily of Krüppel-like zinc finger genes. Members of the PR domain family have been shown to play important roles in cell differentiation and malignant transformation. The RIZ gene is the founding member of this family; it was isolated because its gene products can bind to the retinoblastoma tumor suppressor protein, Here, we have studied the RIZ gene structure and expression. By immunoprecipitation and immunoblot analysis we identified two different RIZ protein products of 280 and 250 kDa, designated RIZ1 and RIZ2, respectively. The 280-kDa RIZ1 product comigrated with the RIZ cDNA-derived polypeptide. The 250-kDa RIZ2 product lacked the NH2-terminal PR domain of RIZ1; it comigrated with a truncated RIZ1 polypeptide that was isolated from an internal ATG codon. Both the full-length and the truncated RIZ1 polypeptide were located in the nucleus as shown by transfection and immunofluorescence analysis. We identified the RIZ2 transcripts and showed that they were produced by an internal promoter located at the 5′ boundary of coding exon 5. RNase protection analysis revealed similar ratios of RIZ1 and RIZ2 transcripts in most adult rat tissues except in testis, where RIZ1 was more abundant than RIZ2. These observations were strikingly similar to those described for the MDS1-EVI1 cancer gene, which also normally gives rise to a PR domain-lacking product, EVI1, because of an internal promoter.

The Krüppel-like family of zinc finger genes is estimated to consist of hundreds of human genes (1). A number of them are involved in human diseases especially cancer, including BCL6, PLZF, EVI1, and WT1, to name a few (2–5). This family is characterized by the Cys2-His2 zinc fingers and can be divided further into different subsets based on other structural features, which include the seven-amino acid H/C link between adjacent fingers (6) and several conserved NH2-terminal modules (7–11).

The PR domain is a newly recognized NH2-terminal module. It was first noted during our characterization of the RIZ gene and was named for the homologous 100-amino acid region shared between RIZ and the PRDI-BF1/BLIMP1 transcription repressor that promotes B lymphocyte maturation (12–15).

The RIZ gene was isolated because its gene products can bind to the retinoblastoma protein, a tumor suppressor known to function in gene expression through physical interaction with transcription factors (16, 17). The predicted rat and human RIZ proteins are of 1,706 and 1,719 amino acids, respectively, and are highly homologous (84% amino acid identity). Besides the zinc fingers, several other interesting motifs are also found in RIZ, including a region of homology to E1A, a GTPase motif, and an SH3 motif. Partial or variant human cDNA clones of RIZ have since been isolated using different strategies. One of these is termed GATA-3-binding protein G3B, which was isolated by functional screening using GATA-3 transcription factor protein as probe (18). Another is termed MTB-ZT, which was isolated through binding to the DNA element GTCATGAC responsible for the induction of human heme-oxygenase-1 gene during 12-O-tetradecanoylphorbol-13-acetate-induced differentiation of myelomonocytic cell lines (19). Together, these findings suggest that RIZ may function as a DNA-binding transcription factor.

The RIZ gene has been mapped to human chromosome band 1p36 near the marker D1S228 and the syntenic region in mouse chromosome 4 (19–21). Deletion and alteration of the 1p36 region are commonly found in a variety of human cancers, including neuroblastoma, hepatoma, and breast cancer. Recently, we showed that a known cancer gene, MDS1-EVI1, shares PR domain homology with RIZ (22). We also mapped the human PRDI-BF1/BLIMP1 gene to D6S447 on chromosome band 6q21-q22, a region commonly altered in B cell non-Hodgkin lymphoma and melanoma (23). These observations suggest that PR domain genes may play an important role in human cancer.

To help understand the function of RIZ, we have here further characterized its gene products. Previously, we have identified a 250-kDa RIZ protein in rat and human cells (13). We now show that this 250-kDa protein lacks the PR domain; we identified the full-length RIZ protein to be of 280 kDa. We show that transcripts for the 250-kDa protein were generated by an internal promoter. The finding revealed striking similarity between RIZ and the MDS1-EVI1 cancer gene.

Experimental Procedures

Cell Culture and Transfections—Rat B50 cells, human HT1080 fibrosarcoma cells, Y79 retinoblastoma cells, and SAOS2 osteosarcoma cells were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium plus 10% calf serum. For transfections of these cells, a calcium phosphate precipitation procedure was used (24). Cells were processed 36 h after withdrawal of DNA for immunoprecipitation, immunofluorescence, or chloramphenicol acetyltransferase (CAT)1 analysis. For normalization of transfection efficiencies in 3T3 cells, a β-galactosidase expression construct (pCMV-LacZ) was included in the cotransfections. CAT activity and the level of β-galactosidase expression were deter-

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1 The abbreviations used are: CAT, chloramphenicol acetyltransferase; DAPI, 4′,6-diamidino-2-phenylindole hydrochloride; PCR, polymerase chain reaction; kb, kilobase(s).
mined as described (25). Cell line HCR1, which stably expressed the cDNA-derived rat RIZ protein, was generated by G418 selection of pCMV-RIZ-transfected human HT1080 cells.

**Immunoprecipitation, Immunoblot, and Immunofluorescence—**
Monoclonal antibody 2D7, mouse serum KGSE, and rabbit serum KG7, 1.1S have been described previously (13). The monoclonal antibody P4E1 was generated by injecting mice with the glutathione S-transferase-PR domain (rat) fusion protein KGPR.

Immunoprecipitation and immunoblot were performed as described previously (13). Briefly, cells were lysed in ELB lysis buffer (250 mm NaCl, 0.1% Nonidet P-40, 50 mm Tris-HCl, pH 7.0). Cell extracts were incubated with RIZ serum (anti-KG7.1S) for 1 h followed by 1 h of incubation with protein A-Sepharose at 4 °C. Proteins bound to protein A-Sepharose were washed four times with ELB and were analyzed on 5% SDS-polyacrylamide gel. Immunoblotted was performed on Immobilon P filters (Millipore) using RIZ monoclonal antibodies P4E1 or 2D7 or mouse serum KGSE and alkaline phosphatase-conjugated goat anti-mouse IgG.

Immunofluorescence staining was performed on transfection SAOS2 cells seeded on a glass coverslip. After transfection, cells were washed in phosphate-buffered saline and fixed in methanol for 2 min on ice. Cells were then incubated with 2D7 antibody for 1 h in phosphate-buffered saline plus 4% milk. After washing 3 times with phosphate-buffered saline, the cells were further incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Cells were then washed with phosphate-buffered saline, stained for DNA with 0.5 μg/ml of DAPI, mounted, and examined using an epifluorescence microscope equipped with filters allowing discrimination between the fluorescein isothiocyanate (RIZ) and DAPI (all cells). 

cDNA and Genomic DNA Cloning—**The 5’ region of human RIZ cDNA was cloned by rapid amplification of cDNA ends using the Marathon ready cDNA from human fetal brain (Clontech) according to the manufacturer’s recommended procedures. The primer RP198 from nucleotide 823 to 852 was used: 5’-CCGAGATCAATTCCTGTTGTTCTTCTGTAAGATACA-3’. The 3’ region was cloned and characterized using IMAGE consortium cDNA clones 40662 and 163778 whose Expressed Sequence Tag sequence (GenBank accession nos. R56425 and H14131) overlapped with the previously published RIZ cDNA coding sequence (26).

The chromosome 1 cosmId library from the Reference Library Data base at the Max Plank Institute in Berlin (27) was screened using a human RIZ cDNA probe according to procedures recommended by the supplier. Partial rat RIZ gene was isolated from a rat λ genomic library (Clontech) and from a rat tesi cDNA library (Stratagene) that contained incompletely spliced cDNA. Partial mouse RIZ gene was isolated from 129SV1 J genomic library (Stratagene). The DNA sequence was determined using Sequenase (U. S. Biochemical Corp.) and oligonucleotide primers.

**Plasmid Constructs—**To generate the plasmid pKGPR for production of a glutathione S-transferase-PR domain fusion protein, a HindIII-StuI fragment (amino acids 57–214) of rat RIZ cDNA was cloned into the NcoI site of pSK-GEX vector. The fusion was confirmed to be in-frame by DNA sequencing analysis.

We have previously generated an EE epitope-tagged rat RIZ cDNA expression plasmid pCMV-RIZ (13). To express a more authentic RIZ protein lacking the EE tag, the tagged RIZ cDNA was first moved from pCMV-RIZ to the vector pCDNA3 by the HindIII-StuI cloning site to generate pRIZEE. A PCR fragment of 0.7 kb starting from the first Met residue was synthesized using the antisense primer RP9: 5’ to generate pcRIZEE. A PCR fragment of 0.7 kb starting from the first Met to the end of exon 7 and was cloned into the NcoI site from the first Met to the end of exon 7 and was cloned into the NcoI site of pKG-GEX vector. The fusion was confirmed to be in-frame and self-ligated.

**For the antisense rat RIZ RNA probe, the plasmid pSK12.1S was generated, which contained a 5’ end fragment of 0.7 kb of rat cDNA cloned into pBluescript. Antisense RNA was produced by T3 RNA polymerase.**

For promoter analysis, a 3.5-kb genomic DNA fragment containing intron 5 and exon 6 was first cloned into pBluescript to generate pSK5K. A PCR fragment was amplified from pSK5X with primer RP186 (5’-CCGCAAGCTTTGGGCAAGCTGTTTGCCATC-3’) and primer RP174 (5’-TCTTACCTTCTTCCGGCTCTTC-3’). The PCR fragment was cloned into the HindIII and XhoI sites in pBLCAT3 vector (28) to generate pCAT186. To construct exon 6 deletion mutant pCAT186 (residues 1–320) was cloned into pBluescript. Antisense RNA was prepared using RNAzol according to the manufacturer’s recommended procedures (Tel-Test, Inc.). 20–30 μg of total RNA was used for protection analysis as described (25). Antisense human RNA probe was generated by T3 RNA polymerase and [32P]UTP using Apol or BI restriction plasmid pCR96–102. Antisense rat RNA probe was generated by T3 RNA polymerase using AccI-restricted plasmid pSK12.1S. An equal amount of yeast RNA was used as negative control. The protected products were analyzed on DNA sequencing gel.

For primer extension analysis of human mRNA, the antisense oligomer RP171 was synthesized, which corresponds to amino acid residues 179–168: CTTGGATTCTTCCCTTTGTCCGCTTGG. The primer was end labeled with γ-32P-ATP and polynucleotide kinase. Extension reaction was performed on 30 μg of BAL1 RNA using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) according to standard procedures (25). Yeast tRNA was used as a negative control. Extension products were analyzed on DNA sequencing gel followed by autoradiography.

**RESULTS**

**Two Different Protein Products, RIZ1 and RIZ2—**To identify cellular RIZ protein products, rat brain tumor B50 cell extracts were immunoprecipitated with a rabbit serum directed against a recombinant RIZ protein KG7.1S (residues 245–573). The immunoprecipitated proteins were analyzed by immunoblot using the monoclonal antibody 2D7 specific for the rat RIZ protein (amino acids 215–244). As shown in Fig. 1, two protein products of 250 and 280 kDa were specifically recognized by the RIZ antibodies. The identification of the 250-kDa protein has been described; the 280-kDa protein has also been noted previously but was not characterized (13). Protein products of identical size were also found in human Y79 cells by immunoblot using mouse antisera KGSE (13). That these proteins could be recognized by two different RIZ antibodies suggests that they are candidate cellular RIZ protein products. Also, the 280-kDa protein could be recognized by another monoclonal antibody, P4E1 (see below).

To show that our full-length RIZ cDNA could encode a 280-kDa protein, we transfected into human HT1080 cells an expression construct of rat cDNA p3RIZr. The transiently ex-
AAACATGA (rat and mouse) or AAATATGA (human) (13). To determine whether ATG201 could serve as an initiation codon, we made a construct p3RIZrKK, which deleted residues 1–92 containing the normal initiation codon and the first two internal ATG codons. As shown in Fig. 2A, upon transient transfection of p3RIZrKK into human HT1080 cells, a polypeptide was produced which was recognized by 2D7 and comigrated with the cellular 250-kDa RIZ. The recognition of the recombinant polypeptide by 2D7 shows that ATG201, rather than a downstream ATG, must be the initiation codon for it. The result further suggests that ATG201 is the most likely initiation codon for the cellular 250-kDa RIZ protein if it is to be produced from internal initiation; the other two candidate ATG codons are ~100 residues upstream and are expected to give rise to a distinguishably larger product in SDS gels.

We next examined whether the 250-kDa cellular protein might indeed lack the NH2-terminal region of full-length RIZ1. We generated a monoclonal antibody P4E1 that was raised against the NH2-terminal PR domain region of the rat RIZ protein. Cell extracts from rat B50 cells and HCR1 cells were immunoprecipitated by 2D7 serum (KG7.1S), and the immunoprecipitated proteins were analyzed by immunoblot using either 2D7 or P4E1 monoclonal antibody. As shown in Fig. 2B, although both proteins could be recognized by 2D7, only the 280-kDa protein was recognized by P4E1, suggesting that the 250-kDa protein lacks the NH2-terminal PR domain region. Taken together with the above described results, the data were consistent with internal initiation for the 250-kDa cellular protein. We will hereafter call the 280-kDa polypeptide RIZ1 and the 250-kDa polypeptide RIZ2.

We have shown previously that the 250-kDa cellular RIZ2 protein is a nuclear protein (13). To confirm this, we determined whether the cDNA-derived 250-kDa protein is also a nuclear protein. The 250-kDa construct p3RIZrKK and the 280-kDa construct p3RIZr were transfected into SAOS2 cells seeded on glass coverslips. The cells were analyzed by immunofluorescence using 2D7 monoclonal antibody. Nuclear staining was observed in either p3RIZr or p3RIZrKK transfected cells but not in pcDNA3 vector transfected cells, suggesting that both the 280-kDa and 250-kDa products are located in the nucleus (Fig. 3).

The RIZ2 Promoter—The above data showed that an internal initiation codon of RIZ1 was capable of initiating the synthesis

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**Fig. 2. Structure of cellular RIZ protein products.** Panel A, RIZ cDNA-derived products comigrated with cellular RIZ proteins. To compare the endogenous proteins with cDNA-derived products, human fibrosarcoma HT1080 cells were transfected with full-length rat cDNA expression construct p3RIZr (RIZ 1–1706), vector pcDNA3 (mock), or p3RIZrKK that contains ATG201 as the first in-frame ATG codon (RIZ 201-1706). After transfection, the cells were lysed and the extracts immunoprecipitated with RIZ antiserum KG7.1S. The immunoprecipitated products were analyzed on SDS gel followed by 2D7 immunoblot. Immunoprecipitation products of rat B50 cells extracts were run as comparison. Panel B, the 250-kDa RIZ lacked the NH2-terminal PR domain region. B50 cell and HCR1 cell extracts were immunoprecipitated using RIZ serum KG7.1S. The immunoprecipitated products were divided into two parts and were loaded into two separate lanes on the same gel. A prestained protein marker (M) was loaded between the sample lanes. Following transfer to an Immobilon P filter, the filter was cut into two pieces along the marker lane. One piece was incubated with 2D7 antibody (left) and the other with P4E1 antibody (right).

**Fig. 3. Nuclear localization of RIZ cDNA-derived polypeptides.** Human osteosarcoma SAOS2 cells were transfected with RIZ1 expression vector p3RIZr, the PR domain-minus expression vector p3RIZrKK, or vector pcDNA3. The cells were stained with DAPI and monoclonal antibody 2D7 and visualized by fluorescent microscopy.
of a 250-kDa polypeptide. Is this how RIZ2 actually generated in nature? Could RIZ2 be produced from the same transcript that gives rise to the 280-kDa RIZ1 polypeptide? Although ATG201 can serve as an initiation codon when placed as the first in-frame ATG, could it also do so in the context of RIZ1 transcript where it would be the fourth in-frame ATG? The data shown in Fig. 2B suggest that this is unlikely; the full-length cDNA produced only the 280-kDa product. The same data also suggest that proteolytic cleavage is unlikely involved in generating RIZ2.

The data were consistent with the existence of RIZ2-specific transcripts. To study this further, we performed RNase protection analysis. An antisense RIZ probe was synthesized by T3 polymerase on the Apo1-restricted plasmid pBSK96–102. This probe covered the region between amino acids 111 and 208 of the human cDNA. As shown in Fig. 4A, we found three major protected fragments by RNA from human B cell acute lymphoblastic leukemia line BALL1, indicating three different RIZ transcripts. Although the longest, full-length protected fragment of ~284 nucleotides represented RIZ1, the smaller fragments of 172 and 155 nucleotides might represent RIZ2-specific transcripts. The same size smaller fragments were also observed when using a 3’ extended longer antisense probe (generated from BglII-restricted plasmid pBSK96–102), showing that they were derived from the 5’ portion of the probe. The results suggested that the 5’ ends of the putative RIZ2 transcripts were covered by these antisense probes and mapped to amino acid residue 156 and 150. Identical results were also found in several other human cell lines and tissues (not shown).

We also analyzed rat tissue RNA using rat cDNA-derived probe and observed similar results (see below). The presence of the smaller transcripts was also confirmed by primer extension analysis (Fig. 4B). An antisense oligomer starting at residue 179 was used for the extension reaction using BALL1 RNA.

Two major extended products of 63 and 87 nucleotides were observed, indicating that these transcripts started at amino acid residues 144 and 156, which was in general consistent with the result of RNase protection analysis. We concluded that transcripts specific for the RIZ2 protein are normally expressed in the cells that contain ATG201 (rat) or ATG202 (human) as the first in-frame initiation codon.

We next examined how these RIZ2 transcripts might be generated. We first partially characterized the genomic DNA of the human, mouse, and rat RIZ gene. We isolated the full-length human RIZ cDNA of 7,943 nucleotides through ’5’ rapid amplification of cDNA ends and making use of the Expressed Sequence Tag data base (dbest) and the IMAGE consortium cDNA clones (26). The cDNA was then used to screen the chromosome 1-specific cosmid library (27). We found that the human RIZ cDNA was encoded by 10 exons (Fig. 5). The exon structures of the ’5’-coding regions of mouse and rat cDNAs were also analyzed and were found to be identical to human. Based on this organization of exons, the smaller transcripts as revealed by RNase protection and primer extension analysis all have their 5’ ends located in the middle of exon 6 or coding exon 5 (Fig. 6). Because these transcripts did not start at the exon boundary, they appear unlikely to be generated by alternative splicing. If these transcripts were to be generated by alternative promoter, their 5’ ends must be located within exon 6. We would predict that a promoter might exist near the boundary of intron 5 and exon 6.

To prove this prediction, we characterized the human, mouse, and rat genomic DNA containing the boundary of intron 5 and exon 6. Sequence analysis of the region revealed three boxes of sequences which are conserved among species (Fig. 7). These conserved boxes are located within 100 base pairs of the boundary and include putative binding sites for several transcription factors such as SP-1, WT1, EGR1, C/EBP, and other CCAAT-binding proteins, and E-box binding proteins (29, 30)

The region is typically GC-rich; no TATA box was found. The observation of at least two different length transcripts as shown by RNase protection and primer extension analysis was also consistent with a TATA-less promoter, many of which are known to initiate transcription at multiple sites. Further, we have found in exon 6 the consensus sequence of MED-1 (multiple start site element downstream) GCCCTCC/G, recently described for those TATA-less promoters with multiple start sites (31). These sequence features therefore predict a TATA-less promoter at the boundary of intron 5 and exon 6.

To show activity for this putative promoter, we generated the pCAT186 plasmid, which contained the conserved elements of intron 5 and the intact exon 6 linked to the CAT gene. Strong CAT activity was observed when pCAT186 was transfected into 3T3 cells (Fig. 8). The corresponding fragment of rat genomic DNA also conferred promoter activity (not shown). Deletion of part of exon 6 (pCAT186S) abolished promoter activity.

Having identified the RIZ2 promoter and its transcripts, we next determined whether the relative level of RIZ1 and RIZ2 mRNA may vary among different cell types. We examined a number of rat tissues by RNase protection analysis; the pattern given by rat tissue RNA was similar to that of human RNA (Fig. 9). A similar ratio (1:1) of RIZ1 to RIZ2 transcripts was found in most tissues examined including brain, heart, skeletal muscle, kidney, liver, and spleen. The pattern found in testis was unique where RIZ1 transcript was at a 5–10-fold higher level then RIZ2.

**DISCUSSION**

In this paper we showed that the *RIZ* gene encodes a full-length protein product of 280 kDa and an alternative smaller product of 250 kDa, designated RIZ1 and RIZ2, respectively.
FIG. 5. RIZ gene exon structure. The full-length human RIZ cDNA sequence was assembled from overlapping fragments. Exons are marked. The initiation codon of RIZ2 is underlined. The GenBank™/EBI Data Bank accession number is U17838.
Both products could be recognized by three different antibodies, rabbit serum KG7.1S, mouse serum KGESE, and monoclonal 2D7, which were raised against two different regions of RIZ. RIZ1 was also recognized by the monoclonal P4E1 directed against the NH2-terminal PR domain region of RIZ1. RIZ2 was not recognized by P4E1, suggesting that it lacks the NH2-terminal region of RIZ1. Also, the protein product expressed from the full-length RIZ cDNA comigrated with RIZ1, whereas a PR domain-deleted mutant cDNA with ATG201 serving as the first in-frame initiation codon produced a protein that comigrated with RIZ2. The data suggest that RIZ2 lacks the PR domain of RIZ1.

We investigated the mechanisms of production of RIZ2. We showed that internal initiation from the RIZ1 transcript and proteolytic cleavage of RIZ1 were unlikely involved because the transfected full-length RIZ1 cDNA comigrated with RIZ1, whereas a PR domain-deleted mutant cDNA with ATG201 serving as the first in-frame initiation codon produced a protein that comigrated with RIZ2. The data suggest that RIZ2 lacks the PR domain of RIZ1.

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ated with therapy-related acute myeloid leukemia and myelodysplastic syndrome as well as with chronic myeloid leukemia in blast crisis (32). The protooncogene EVI1 was first identified in the mouse and is activated in murine myeloid leukemia by proviral insertion in the EVI1 common integration site (4). In humans the expression of this gene can be activated in myeloid leukemias and myelodysplastic diseases by chromosomal rearrangements at either 5q or 3q of the gene (32–34). Activation of EVI1 can also occur as part of the fusion mRNA, AML1-EVI1 or AML1-MDS1-EVI1. Abnormal expression of EVI1 has also been detected in patients with myeloid leukemia and cytogenetically normal karyotype (35).

The MDS1-EVI1 fusion gene encodes an intact PR domain, wherein MDS1 encodes one-third of the PR sequence and EVI1 the remaining two-thirds (22). The EVI1 transcript is generated by an internal promoter within the MDS1-EVI1 gene (36). An in-frame internal ATG codon just 3' of the PR domain in MDS1-EVI1 becomes the first ATG and the initiation codon in the EVI1 transcript. Thus, the MDS1-EVI1 gene can produce at least two different products, MDS1-EVI1 and EVI1, which are differentiated by the PR domain. The EVI1 gene, but not the MDS1-EVI1 gene, may function to promote transformation as it is often overexpressed in leukemia cells.

The similarity between RIZ and MDS1-EVI1 as shown by this study further raises interest in the function of PR domain and the role of RIZ as a candidate cancer gene located on 1p36.
What functional difference could the PR domain make between RIZ1 and RIZ2 or between MDS1-EVI1 and EVII? Apparently, it was not involved in the nuclear localization of either gene product. Given the example of deregulation of EVI1 expression as a consequence of 3q26 abnormalities, could deregulation of RIZ1 or RIZ2 expression also occur in human cancer as a consequence of 1p36 abnormalities? This study should provide the means and reagents for addressing these important questions.

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FIG. 9. RNase protection analysis of RIZ1 and RIZ2 transcripts in various adult rat tissues. Panel A, antisense RNA probe was generated from AccI-restricted plasmid pSK12.1SX. 30 µg of total RNA from various adult rat tissues was analyzed. The major protection products are marked. Panel B, schematics of RNase protection. Partial rat RIZ cDNA is schematically shown with exons provisionally numbered as in the human gene. Antisense RIZ RNA probes are shown as straight lines, and the vector sequence is shown as a curved line.