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JAZ Requires the Double-stranded RNA-binding Zinc Finger Motifs for Nuclear Localization*

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We have cloned and characterized a novel zinc finger protein, termed JAZ. JAZ contains four C_2H_2-type zinc finger motifs that are connected by long (28–38) amino acid linker sequences. JAZ is expressed in all tissues tested and localizes in the nucleus, primarily the nucleolus. JAZ preferentially binds to double-stranded (ds) RNA or RNA/DNA hybrids rather than DNA. Mutation of individual zinc finger motifs reveals that the zinc finger domains are not only essential for dsRNA binding but are also required for its nucleolar localization, which demonstrates a complex trafficking mechanism dependent on the nucleic acid-binding capability of the protein. Furthermore, forced expression of JAZ potently induces apoptosis in murine fibroblast cells. Thus, JAZ may belong to a class of zinc finger proteins that features dsRNA binding and may regulate cell growth via the unique dsRNA binding properties.

Interactions between nucleic acids and proteins are fundamental to many cellular processes. Many nucleic acid binding proteins interact preferentially with a specific DNA sequence or a particular RNA molecule (1). Sequence-specific DNA-binding proteins generally interact with the B-form of DNA, whereas RNA duplex forms an A-helix (1–3). Among RNA species, dsRNA[1] has many peculiar features (4). Various dsRNA species can result from a number of biosynthetic pathways, including normal gene transcription and following virus infection (4). Moreover, intramolecular RNA duplex can occur as the result of either local hairpin structures or long distance base pairing. Recently a, ~65-amino acid dsRNA binding motif has been found in a number of proteins that can specifically recognize dsRNA (5). For example, dsRNA-dependent protein kinase (PKR) contains two such dsRNA binding motifs within the regulatory domain (4). Thus, identifying and characterizing dsRNA-binding proteins may help to advance our understanding of the fundamental processes regulating cell growth.

C_2H_2 type zinc finger (ZF) proteins comprise one of the largest gene families (6, 7). Development of molecular biological techniques and structural studies have further promoted cloning of novel ZF proteins (8). For most of these proteins the biological function(s) has not been determined, but a role as a DNA-binding protein in gene transcription is considered a likely mechanism of action. However, *Xenopus* TFIIIA is a unique protein requiring the first three ZF domains to bind 5 S DNA but can also bind helical stems of 5 S RNA with the next three finger domains (6, 9). The recognition of RNA is thought to be primarily dependent on the 5 S RNA structure (10). Some ZF proteins are reported to bind RNA homopolymers (11). Recently, *Xenopus* dsRBP-ZFa has been characterized as a unique C_2H_2-type ZF protein that can specifically bind dsRNA and RNA-DNA hybrids (12).

In eukaryotic cells, the nucleus is separated from the cytoplasm where protein synthesis occurs. Therefore, all nuclear proteins must be selectively transported into the nucleus. Protein transport across the nuclear envelope is usually mediated by specific nuclear localization signals (NLS) (13, 14). Over the past several years, remarkable progress has been made in ordering the events and factors involving in the nuclear transporting systems (13, 14). However, it is not clear whether all nuclear proteins use common factors and pathways to gain entrance. Here, we have identified a mammalian dsRNA-binding ZF protein, JAZ, that requires ZF domains not only for dsRNA binding but also for its nuclear localization.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 cells and COS7 cells were obtained from American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. The mouse interleukin-3-dependent NFS/N1.H7 cells were grown and maintained as described previously (15).

Yeast Two-hybrid Screening—By using PCR-based mutagenesis, the *Bam*HI fragment of mouse PKR cDNA (16) cloned in pBluescript KS was first mutated at lysine 271 to arginine to generate the catalytically inactive PKR[K271R], the mouse equivalent to human PKR[K296R] mutant (17), and then subcloned into a GAL4-DNA-binding domain vector pGBT9 (CLONTECH). A random primed NFS/N1.H7 cell cDNA library was generated in GAL4-activation domain vector pGAD10 (CLONTECH). The yeast two-hybrid screening of the cDNA library was performed according to the Matchmaker Two-hybrid System protocol (CLONTECH). The host yeast HF7c cells were sequentially transformed with PKR[K271R]/pGBT9 and pGAD10 library plasmids and plated on medium lacking tryptophan, leucine, and histidine. A total of 4×10^6 clones was screened for growth in the selection medium and assayed for β-galactosidase activity. The plasmids harvested from positive clones were used to transform *Escherichia coli* HB101 ($leuB^-$), cells and the library plasmids were recovered from the *E. coli* colonies grown on the plates lacking leucine.

RACE—The cDNA synthesis and the adapter ligation for RACE was performed using Marathon cDNA Amplification Kit (CLONTECH) and poly(A) RNA extracted from either mouse NFS/N1.H7 cells or human placenta (CLONTECH). Based on the sequence of clone 112 obtained from the yeast two-hybrid screening, an antisense primer 5′-CTGACCAGGCAGGGACAGCTTTAGT-3′ and a sense primer 5′-GAGCAGGTCTTCCAGGAAGTATTCC-3′ were prepared for use in the 5′- and

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AF083339 and AF083340.

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[1] The abbreviations used are: ds, double-stranded; NLS, nuclear localization signal; PKR, double-stranded RNA-dependent kinase; ZF, zinc finger; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; GST, glutathione *S*-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; bp, base pair; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; ss, single-stranded; GFP, green fluorescent protein.

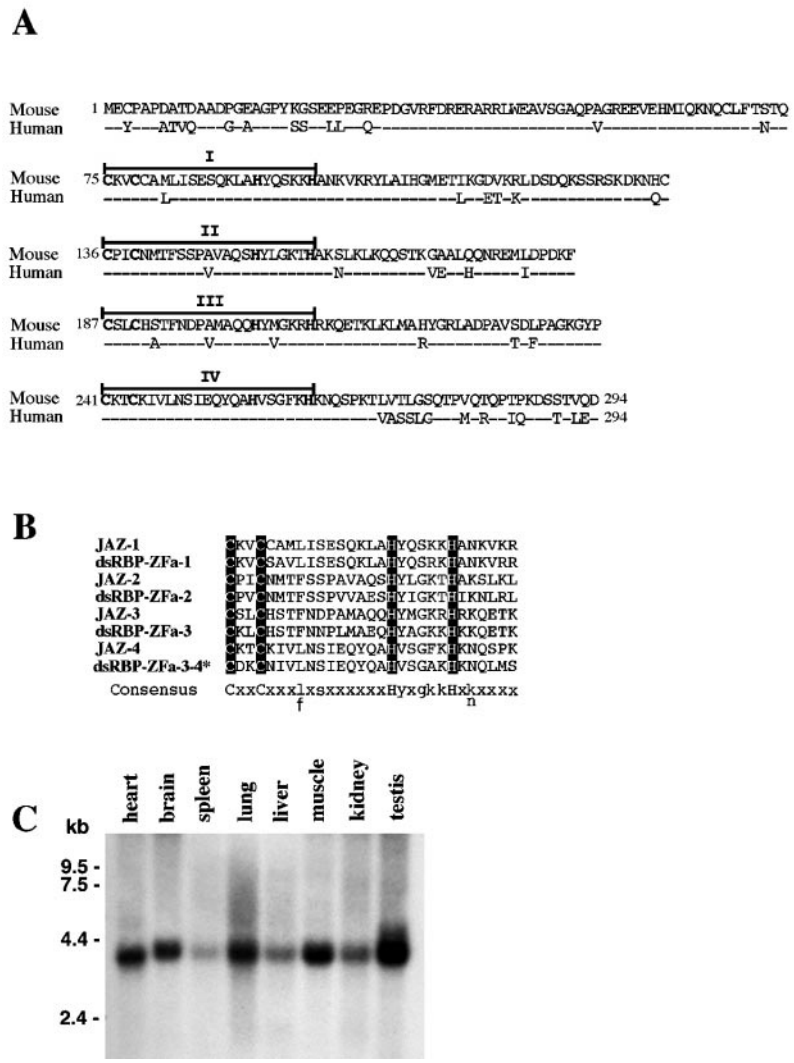

FIG. 1. *A*, predicted amino acid sequences of mouse and human JAZ. Only those amino acids that differ in the two proteins are indicated for human JAZ. The four C_2H_2 ZF domains (*I–IV*) are aligned. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF083339 and AF083340. *B*, ZF domains of JAZ and dsRBP-Zfa. The consensus indicates residues shared by at least five sequences or two alternatives among eight sequences. *, the last three (5th to 7th) ZFs of dsRBP-Zfa are almost identical to the 4th ZF. *C*, expression of JAZ mRNA in various mouse tissues. Northern blot analysis was carried out using a radiolabeled JAZ cDNA. Each lane contains 2 μg of poly(A) RNA from eight different mouse tissues.

3′-RACE, respectively, to obtain full-length mouse JAZ cDNA. Using the National Center for Biotechnology Institute BLAST search, a human expressed sequence tags clone (GenBank™ accession number F06769) was identified that is homologous to an area in the middle of mouse JAZ cDNA, whereas another clone (GenBank™ accession number R56173) was found to be homologous to the 3′-end of the coding region for mouse JAZ. For 5′-RACE of human JAZ, an antisense primer 5′-CCTTAGTGGACTGCTGCTTCAGCTT -3′ was used since the complementary sequence was found in both mouse JAZ and the F06769 clone. To obtain the full coding sequence for human JAZ, PCR was performed between the sequence 5′-AGCAGCAGAAGCAAAGACAAGAACC-3′, found in both mouse JAZ and F06769, and 5′-GGTGATTCTGCCCAGCATCCCATATT-3′, found in R56173. Each PCR was performed by the step down method (18) using Takara LA *Taq* DNA polymerase (PanVera, Madison, WI) pretreated with TaqStart Antibody (CLONTECH). The cycling condition consisted of 1 min denaturation at 94 °C, 3 min elongation at 68 °C with the annealing temperature progressively lowered from 68 to 45 °C by 3 °C every fourth cycle, followed by an additional 35 cycles at 55 °C. The PCR product was gel-purified and ligated into the pCRII vector (Invitrogen) which was used to transform *E. coli* DH5α. Multiple clones were selected to analyze the sequence of each RACE product.

Northern Blot Analysis—A mouse multiple tissue blot was obtained from CLONTECH and probed with a 2-kilobase pair JAZ cDNA fragment (clone 112) that was radiolabeled using RadPrime DNA labeling system (Life Technologies, Inc.).

Gel Shift Assays—To produce a glutathione *S*-transferase (GST)-JAZ fusion protein, JAZ cDNA (clone 112) was subcloned into pGEX1λT vector (Amersham Pharmacia Biotech). The fusion protein was expressed in *E. coli* DH5α and purified by glutathione-agarose affinity chromatography according to the manufacturer's instruction. The 130-mer dsRNA and 160-mer RNA-DNA hybrid probes were prepared as described (19). The pBluescript II SK vector (Stratagene) was digested with *Bss*HII to produce a 172-bp DNA fragment containing the T7 and T3 promoters and intervening template sequences. This *Bss*HII fragment was also used as a DNA competitor or a radiolabeled 170-mer DNA probe after 5′-end-labeling using T4 polynucleotide kinase (Life Technologies, Inc.) and [γ-^{32}P]ATP. The 160-nucleotide single-stranded (ss) RNAs were synthesized using T7 and T3 RNA polymerase MAXIscript *in vitro* transcription kits (Ambion) in the presence or absence of [α-^{32}P]UTP. To make a 130-bp dsRNA, equimolar amounts of the two transcripts were mixed and hybridized in binding buffer (80% formamide, 40 mM PIPES at pH 6.7, 400 mM NaCl, and 1 mM EDTA) (19). For competition experiments, a large quantity of ssRNA was prepared using the MEGAshortscript *in vitro* transcription kit (Ambion). The 160-mer RNA/DNA hybrid was synthesized by mixing the T7 transcript (100 pmol), T3 primer (300 pmol), and SuperScript RT II (Life Technologies, Inc.), gel-purified, and used as a cold competitor or radiolabeled probe following the 5′-end-labeling (12). The quality was verified by sensitivity to ribonuclease H (Life Technologies, Inc.). The indicated amounts of GST-JAZ and each nucleic acid substrate were incubated in 50 μl of binding buffer (20 mM Hepes, pH 7.3, 20 mM KCl, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM dithiothreitol, 10% glycerol, 100 μg/ml bovine serum albumin, and 0.1% Nonidet P-40) at room temperature for 1 h as described (12). Since a kinetic experiment showed no differences in binding following incubation times of 15 min up to 2 h, a 1-h incubation time was chosen (data not shown). Although we used the zinc-containing binding buffer previously used for the analysis of dsRBP-ZFa (12), similar results were obtained using a buffer containing 5 mM EDTA, suggesting that exogenous zinc is not required in this assay (data not shown). The samples were loaded onto an 8% native polyacrylamide gel and run for 2 h at 150 V in buffer containing 45 mM Tris borate, pH 8.0, and 1 mM EDTA. The gel was dried and subjected to autoradiography.

Antibody Production and Immunoblotting—JAZ cDNA was cloned

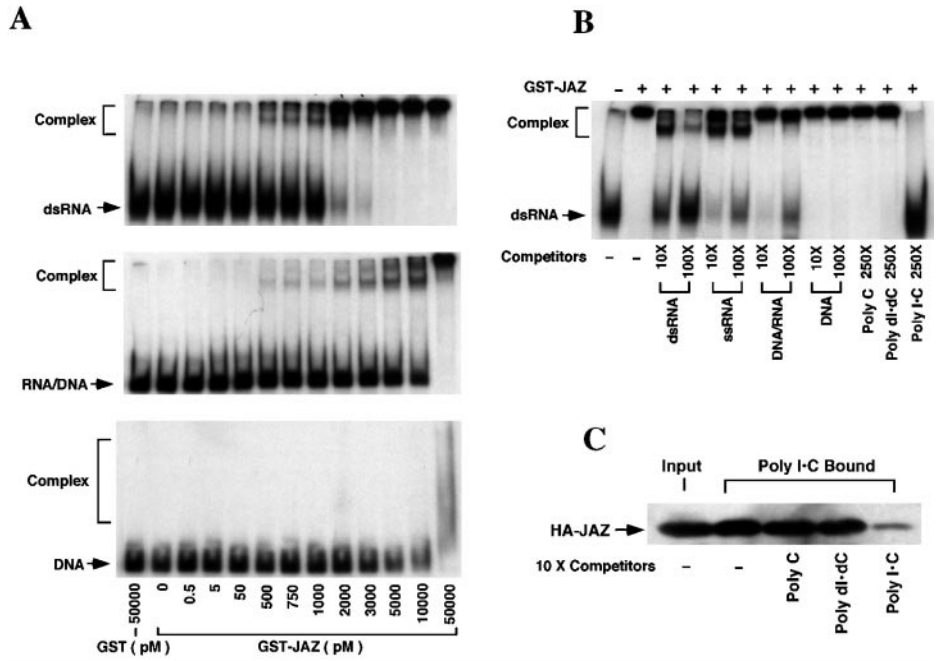

FIG. 2. **JAZ preferentially binds to dsRNA and RNA/DNA hybrids.** *A*, 50 pM of either a 130-mer dsRNA, a 160-mer RNA/DNA hybrid, or a 170-mer DNA was incubated with varying concentrations of GST-JAZ and subjected to PAGE analysis. GST was used as a control. *B*, competition studies. A ^{32}P-labeled dsRNA probe (130-mer, 50 pM) was incubated with GST-JAZ (5,000 pM) in the presence of the unlabeled dsRNA (130-mer), RNA/DNA hybrid (160-mer), or DNA (170-mer) at the indicated times higher concentration than the dsRNA probe. Poly(C), poly(dI·dC), and poly(I·C) were also tested at 250× concentrations of the probe. *C*, poly(I·C) beads binding assays. COS7 cells were transfected with HA-JAZ expression plasmid. After 2 days, the cells were lysed and aliquots (50 μg) of lysates were incubated with poly(I·C) immobilized on agarose beads in the absence or presence of competitors (poly(C), poly(dI·dC), and poly(I·C)). The bound proteins and 50 μg of the cell lysate (the input) were analyzed by immunoblotting using an anti-HA antibody.

into the pRSET vector (Invitrogen) in order to generate a polyhistidine-tagged JAZ. The protein was induced in *E. coli.* BL21 (DE3) pLysS (Novagen, Madison, WI) and partially purified with TALON metal affinity resin according to the manufacturer's instruction (CLONTECH). The fraction containing JAZ was incubated with poly(I·C)-agarose beads (Amersham Pharmacia Biotech), and the protein was dissociated by boiling in SDS-PAGE sample buffer and further purified by SDS-PAGE. The gel slice containing the recombinant JAZ was used to immunize a rabbit to raise anti-JAZ antiserum (COVANCE Research Products Inc., Denver, PA). The antibody was purified from the serum using protein A-agarose beads as described (20).

Subcellular Fractionation—NIH3T3 cells were collected from six 100-mm dishes following trypsinization and washed with phosphate-buffered saline. Nuclei and the nuclear lysate were prepared as described (21). A preliminary screening of optimal protease inhibitors using a protease inhibitor set (Roche Molecular Biochemicals) and lysates from HA-JAZ-transfected NIH3T3 cells revealed that pepstatin and metalloprotease inhibitors were essential in order to protect JAZ from degradation by cellular proteases. For subcellular fractionation, the cells were suspended in lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.7 μg/ml pepstatin, 400 μg/ml phosphoramidone) and lysed using a Dounce homogenizer (type B pestle). The cell lysate was centrifuged for 10 min at 2000 rpm to pellet the nuclei. The supernatant was further centrifuged for 90 min at 150,000 × *g* (Beckman XL-80 rotor) to obtain the cytosolic fraction (supernatant). The resulting pellet was dissolved in TBS containing 1% Triton X-100 to obtain the particulate fraction. The nuclei were resuspended in extraction buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 0.7 μg/ml pepstatin, and 400 μg/ml phosphoramidone) and incubated on ice for 1 h. The nuclear extract was passed through cotton wool to remove insoluble materials and used for the nuclear fraction. The poly(I·C) beads (150 μl) were added to each subcellular fraction and incubated for 1 h at 4 °C. The beads were washed three times with TBS containing 1% Triton X-100, and the bound proteins were eluted by boiling in Laemmli buffer. The samples were loaded and run on an SDS-PAGE gel (10%) and transferred to a nitrocellulose membrane that was incubated with a 5% milk solution for blocking. The specificity of the JAZ antibody was examined by antigen competition. Duplicate filters were prepared, and each filter was incubated for 1 h with the antibody solution that had been incubated with either GST-JAZ or GST protein alone that had been bound to glutathione-agarose beads. After incubation with secondary antibody for 1 h, the immunoreactive proteins were detected using the ECL system (Amersham Pharmacia Biotech),

Immunofluorescence Staining—The hemagglutinin (HA) epitope-tagged JAZ cDNA was subcloned into the pcDNA3 vector (Invitrogen) and transfected into COS7 cells using LipofectAMINE (Life Technologies, Inc.). Forty eight hours later, the cells were fixed in 4% paraformaldehyde for 30 min at 4 °C and subsequently permeabilized with 0.2% Triton X-100 for 20 min. After blocking with 3% bovine serum albumin in phosphate-buffered saline for 1 h, the slips were incubated with 5 μg/ml rhodamine-conjugated 12CA5 antibody (Roche Molecular Biochemicals) for 1 h. The slides were washed with phosphate-buffered saline, mounted with mounting medium (95% glycerol, 2.5% *n*-propyl gallate, 2.5% diazabicyclo[2.2.2]octane), and analyzed under a fluorescence microscope (Nikon) supplemented with a Micromax digital camera (Princeton Instruments).

Mutation Analysis—All site-directed mutagenesis experiments were performed using the Transformer Kit (CLONTECH). An *Eco*RI/*Xba*I fragment of HA-JAZ cDNA was cloned into pUC19. A new *Bam*HI site was created at the end of the JAZ-coding region. After the first histidine codon of each ZF domain was mutated to an alanine codon, the *Eco*RI/*Bam*HI cDNA fragment was cloned into pEGFP-N1 vector (CLONTECH) to create JAZ fused with the amino terminus of GFP (termed Wt-1). The carboxyl-terminal GFP fusion protein was constructed by inserting an *Xho*I/*Bam*HI fragment containing the coding region of JAZ into pEGFP-C3 (Wt-2). ΔII–IV mutant was created by deleting the *Nco*I-*Bam*HI fragment followed by blunt-end ligation. Each plasmid was transfected into NIH3T3 cells grown on 6-well plates. After 24 h, subcellular localization of GFP fusion proteins was analyzed using an inverted fluorescence microscope.

Poly(I·C) Beads Binding Assays—Each plasmid was transfected into COS7 cells as described above. After 48 h the cells were lysed in buffer A (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1 μg/ml pepstatin). Aliquots (50 μg) of lysates were incubated with 20 μl of poly(I·C)-agarose beads (Amersham Pharmacia Biotech) in the absence or presence of nucleic acids (poly(C), poly(dI·dC), and poly(I·C), Amersham Pharmacia Biotech) for 1.5 h at 4 °C. The beads were washed three times with buffer A before boiling in SDS-PAGE sample buffer. The eluted samples and 50 μg of the whole cell lysates were then loaded onto a 10% SDS-PAGE gel followed by immunoblotting using an anti-HA antibody (12CA5, Roche Molecular Biochemicals) for HA-JAZ or an anti-GFP antiserum (CLONTECH) for GFP fusion proteins. For quantitative analysis, the blot was incubated with ^{125}I-protein A (Amersham Pharmacia Biotech) after the primary antibody incubation, and the immunoreactive signal was measured by InstantImager™ (Packard, Meriden, CT).

RESULTS

Cloning of Mouse and Human JAZ—In order to identify proteins that interact with the dsRNA-dependent protein kinase PKR, we screened a mouse NFS/N1.H7 cell cDNA library using the yeast two-hybrid method. One of the positive clones, c122, contained 1926-bp cDNA with an open reading frame of 885 nucleotides that encoded a putative 294 amino acid polypeptide. To obtain the full-length cDNA, we employed RACE using poly(A) RNA from NFS/N1.H7 cells. Although the

longest murine 5′-RACE clone obtained did not have a stop codon in the coding frame prior to the putative initiating methionine codon, there is a stop codon 18 bp upstream of the corresponding methionine codon in a human 5′-RACE clone (see below). This suggests that c112 likely contains the entire coding region of the mRNA. The sequence analysis of the 3′-RACE product revealed that JAZ cDNA has a long (2.4-kilobase pair) 3′-untranslated region that precedes a typical polyadenylation signal AATAAA. By using the National Center for Biotechnology Institute BLAST search, we found two human expressed sequence tags sequences in GenBank™ that are homologous to the c112 sequence. The F06769 sequence is homologous to the middle of the coding region, whereas the R56173 sequence is homologous to the 3′-end of the coding region. Based on these expressed sequence tags sequences, we applied RACE to clone the human homologue from human placental RNA. The human cDNA also obtained codes for a putative polypeptide of 294 amino acids with 89% homology to the mouse protein (Fig. 1).

Structural Analysis and Tissue Distribution of JAZ—Examination of the protein sequence revealed the presence of four homologous C_2H_2-type ZF domains (ZF domain I–IV in Fig. 1*A*). Hereafter, the protein is referred to as JAZ (for just another zinc finger protein). Interestingly, these domains are connected by unusually long linker sequences (*i.e.* 28–38 amino acids) compared with those of other known ZF proteins that have short (6–8 amino acids), well conserved linkers (7). These unique linkers begin with basic residues and are predicted to form helix-loop structures, in contrast to ZF domains in the other finger proteins that form sheet-helix structures.

A National Center for Biotechnology Institute BLAST search found that JAZ is homologous to the amino-terminal half of *Xenopus* dsRBP-ZFa (12). The peptide sequences of the four C_2H_2 domains and the following 6 residues of JAZ are particularly homologous to each other (Fig. 1*B*). The dsRBP-ZFa also has long (34–44 amino acids) linkers, but the homology within this region is limited to the first 6 residues. JAZ is missing the last three ZF domains that follow a serine-, proline-, and glycine-rich region present in dsRBP-ZFa (12). Therefore, JAZ may not be the mammalian counterpart of dsRBP-ZFa but apparently belongs to the same protein family.

Next, the expression and tissue distribution of JAZ was assessed by Northern blot analysis of poly(A) RNA prepared from different mouse tissues. The radiolabeled JAZ cDNA probe was found to hybridize to a single 3.6-kilobase pair species present in all tissues examined (Fig. 1*C*). These results indicate that JAZ is a ubiquitously expressed gene.

Characterization of the Nucleic Acid Binding Properties of JAZ—To determine whether JAZ contains nucleic acid binding properties, we conducted gel shift assays. Different types of nucleic acid duplexes were incubated with GST-JAZ and subjected to non-denaturing PAGE analysis. We found that JAZ can bind to dsRNA, RNA/DNA hybrids, and DNA with vastly different affinities. Delayed mobility of the radiolabeled dsRNA or RNA/DNA hybrid probe could be detected following incubation with 500 pM GST-JAZ. Further shifts were observed in a dose-dependent manner, suggesting that the dsRNA (130-mer) and RNA-DNA hybrid (160-mer) probes used have multiple JAZ-binding sites. In contrast, greater than 100 times more JAZ was required to detect any shift of the DNA probe. These results indicate that JAZ has a relatively high affinity for an A-form duplex, especially dsRNA, *in vitro* (Fig. 2*A*).

To confirm this substrate specificity, the radiolabeled dsRNA probe was incubated with GST-JAZ in the presence of increasing concentrations of nonradioactive dsRNA, RNA-DNA, DNA, or ssRNA. Results show that JAZ binding to the dsRNA is unaffected by DNA but can be greatly reduced by excess dsRNA (Fig. 2*B*). An RNA/DNA hybrid can also compete but less potently than dsRNA. Furthermore, slight competition was observed with ssRNA, whereas an RNA homopolymer, poly(C), failed to affect the dsRNA binding. These results suggest that JAZ has no or very low affinity for single-stranded RNA but may bind to intra- or intermolecular duplex portions of the 160-mer ssRNA tested. Furthermore, recombinant JAZ expressed in COS7 cells can efficiently bind dsRNA poly(I·C)-agarose beads, and the binding was competed with free poly(I·C) but not with poly(C) or poly(dI·dC) (Fig. 2*C*). These results indicate that JAZ expressed in mammalian cells also specifically binds to dsRNA.

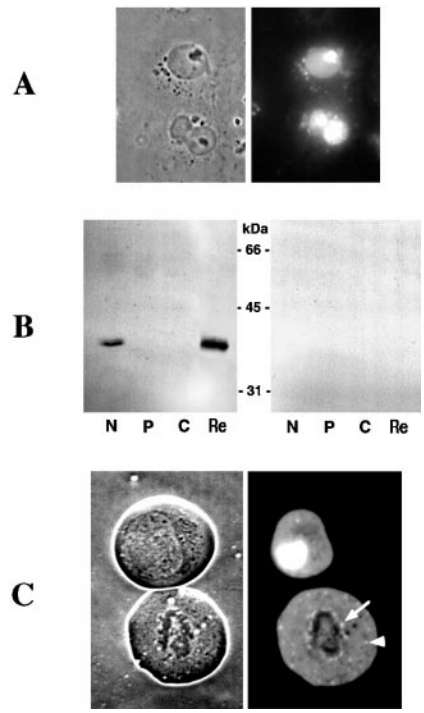

FIG. 3. **JAZ localizes in the nucleus.** *A*, COS7 cells were transfected with the expression plasmid carrying the HA-JAZ cDNA sequence. The expressed HA-JAZ protein was analyzed by immunofluorescence staining using rhodamine-conjugated anti-HA antibody (*right*). *Left*, the phase-contrast image. *B*, immunoblotting analysis of JAZ in subcellular fractions from NIH3T3 cells. Subcellular fractionation was performed as described under "Experimental Procedures." The duplicate filters containing the nuclear (*N*), the cytosol (*C*), and the particulate (*P*) fractions were probed with the anti-JAZ antibody before (*left*) and after (*right*) incubation with excess amount of the antigen. *Re*, the lysate from NIH3T3 cells transfected with JAZ/pECG2 plasmid as described in Fig. 6. *C*, localization of JAZ-GFP protein in a cell at metaphase. The JAZ-GFP expression plasmid was transfected into NFS/N1.H7 cells by electroporation. The *right panel* shows the expressed green fluorescence signals from two cells at interphase and anaphase observed 8 h after electroporation. *Left*, the phase-contrast image. An *arrowhead* points to the prenucleolar body, and an *arrow* points to the perichromosomal layer.

JAZ Localizes to the Nucleus—Since JAZ is a nucleic acid binding protein, we wanted to determine its intracellular localization. The hemagglutinin (HA) epitope-tagged JAZ cDNA was transfected into COS7 cells, and the expressed protein was detected by immunofluorescence microscopy using an anti-HA monoclonal antibody conjugated with rhodamine. Fluorescence was observed exclusively in the nucleus, primarily in the nucleolus (Fig. 3*A*).

To investigate the localization of endogenous JAZ, we produced a polyclonal antibody using the recombinant JAZ fusion protein as described under "Experimental Procedures." Initial attempts to detect a specific signal by immunoblotting using

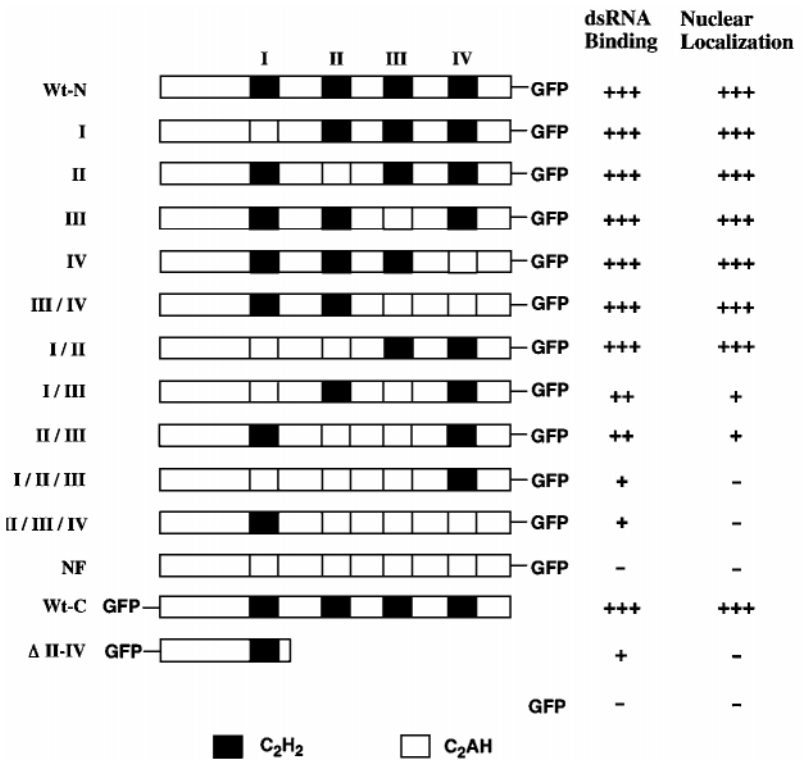

FIG. 4. **ZF mutation analysis.** The cDNAs encoding wild-type (*Wt*) or JAZs containing a $C_2H_2 \rightarrow C_2AH$ mutation at the indicated ZF domains (*I–IV*) were fused with GFP and expressed in COS7 cells. The poly(I·C) binding assay was performed as shown in Fig. 2*C,* and the dsRNA binding efficiency was compared with that of the wild type. The summarized average data are indicated by +++ (90–100%), ++ (40–60%), + (5–10%), and − (not detected). The number of cells showing the nuclear-localized JAZ-GFP, as observed in Fig. 5, was scored as the percentage of the total number of green fluorescent positive cells. +++ (90–100%), + (1–5%), and − (0%).

whole cell lysates from NIH3T3 cells were unsuccessful although an intense signal could be detected in 3T3 cells transiently transfected with JAZ plasmid. These data suggest that endogenous JAZ protein levels may be quite low. Since JAZ efficiently binds dsRNA, nuclear lysates from 1×10^8 cells were incubated with poly(I·C)-agarose beads to concentrate JAZ (Fig. 2). The eluate from the beads was found to contain a 36-kDa protein that was recognized by the antiserum (Fig. 3*B, left panel*). The apparent molecular mass of the band, 36 kDa, corresponds well with the calculated molecular mass of 33 kDa for JAZ. Any slight difference may suggest post-translational modification of the protein. However, this immunoreactive protein could not be detected in either the cytosolic or the particulate fraction of cells even following concentration with poly(I·C) beads. Moreover, no hybridizing band was detected when the antiserum was adsorbed with an excess amount of the recombinant JAZ before blotting (Fig. 3*B*, *right panel*), indicating that the antiserum does specifically recognize JAZ. Collectively, these findings strongly indicate that JAZ is localized to the nucleus.

The nuclear/nucleolar localization of JAZ was further studied in living cells using a green fluorescent protein (GFP)-tagged JAZ construct. When the JAZ-GFP expression plasmid was transiently transfected into NFS/N1.H7 cells, the green fluorescence localized exclusively in the nucleus, particularly in the nucleolus, indicating that GFP fusion does not affect the localization of JAZ (Fig. 3*C*). Interestingly, in a metaphase cell in which the nucleolus is absent, the fluorescence signal of JAZ-GFP diffused into the cytoplasm with some patchy accumulations at the prenucleolar bodies and the periphery of mitotic chromosomes (Fig. 3*C*, *arrowhead* and *arrow*, respectively). This finding indicates that JAZ may bind to chromosome-associated RNA and/or DNA and thus segregate with the dividing chromosome.

Mutational Analysis of JAZ Zinc Finger Domains—JAZ contains four C_2H_2-type ZF domains that appear essential for dsRNA binding. To investigate the contribution of individual ZF domains, site-directed mutagenesis was performed on each domain by changing the first histidine to alanine ($C_2H_2 \rightarrow C_2AH$), which destroys the ZF structure (22). The mutant JAZ cDNA was subcloned into a GFP fusion expression vector. To perform the dsRNA binding assay, lysates from COS7 cells transfected with either wild-type or mutant JAZ-GFP were incubated with poly(I·C) beads, and the binding efficiency was analyzed by immunoblotting. Results show that destruction of one ZF domain does not significantly reduce the dsRNA binding capacity of JAZ (*I–IV* in Fig. 4). However, the dsRNA binding efficiency was reduced by 40–60% when either the first and third (I/III) or the second and third (II/III) ZF domains were simultaneously destroyed. Furthermore, mutation of three domains (I/II/III or II/III/IV) even further decreased the binding, and a mutant where all four fingers were mutated (NF) showed no dsRNA binding. On the other hand, ΔII-IV, a mutant that has only one ZF domain and the first 10 amino acids of the HC-linker sequence, showed weak but detectable dsRNA binding. These results suggest that each ZF domain has an affinity for dsRNA. The mutants I/III and II/III retain two intact ZF domains that are separated by a much longer sequence than original linker sequences due to the destruction of the middle domains. Interestingly, mutants retaining two consecutive ZF domains (I/II and III/IV) exhibited much stronger binding than the I/III or II/III mutant. These results suggest that the length of the linker is also important for efficient dsRNA binding.

Next, we transfected NIH3T3 cells with wild-type or individual ZF mutant cDNAs in order to analyze the effect of a specific ZF mutation on intracellular JAZ localization. The GFP signal from the vector alone control showed GFP to be distributed diffusely throughout the cell (Fig. 5). As observed in NFS/N1.H7 cells (Fig. 3), the wild-type JAZ-GFP construct showed a nuclear, especially nucleolar, localization pattern (data not shown). This pattern was not altered by mutation of a single ZF domain (see Fig. 5, domain *II* mutant as an example), and mutants that retained two consecutive fingers still exhibited nucleolar localization (Fig. 5, *I/II* as a example). However, after transfection with either the I/III or II/III construct (*I/III*

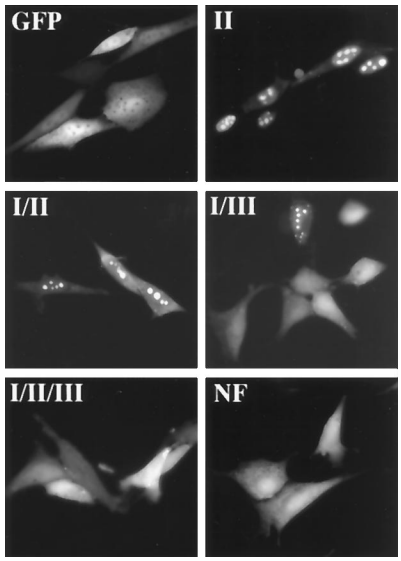

FIG. 5. **Cellular localization of JAZ mutants.** NIH3T3 cells were transfected with the plasmid encoding GFP alone (*GFP*) or JAZ-GFP fusion proteins containing the $C_2H_2 \rightarrow C_2AH$ mutation at domain II (*II*), domains I and II (*I/II*), domains I, II, and III (*I/II/III*), or all four domains (*NF*). After 24 h, green fluorescence signals from GFP fusion proteins were analyzed using an inverted fluorescence microscope.

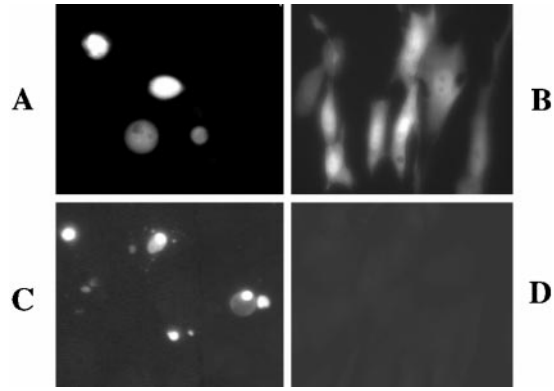

FIG. 6. **Forced expression of JAZ causes apoptosis.** NIH3T3 cells were transiently transfected with either the JAZ/pEIG2 (*A* and *C*) or the JAZ NF/pEIG2 (*B* and *D*). At 72 h after transfection, the morphological change in the transfected cells was visualized by the signal from the co-expressed GFP (*A* and *B*), and the apoptotic cells were evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay using tetramethylrhodamine-6-dUTP (*C* and *D*).

in Fig. 5), less than 5% of the fluorescence-positive cells showed a nucleolar pattern, and virtually no nucleolar fluorescence was observed with mutant constructs in which three or more fingers had been destroyed (Fig. 5, *I/II/III* and *NF*). Thus, a strong correlation exists between dsRNA binding efficiency and nuclear/nucleolar localization, indicating that JAZ requires ZF domains not only to bind dsRNA but also for nuclear localization (Fig. 4).

Forced Expression of JAZ Causes Apoptosis—In order to determine a functional role for JAZ, we attempted to overexpress JAZ in NIH3T3 cells. However, we were unable to obtain stably expressing cells after multiple transfection attempts, suggesting that exogenous expression of JAZ, at least when expressed at high levels, may interfere with normal cell growth and/or viability. Therefore, we developed a unique transient expression vector, pEIG2, as described under "Experimental Procedures." The pEIG2 plasmid contains an internal ribosomal entry site from the encephalomyocarditis virus, which permits the simultaneous translation of two proteins (*i.e.* JAZ and GFP) from a single mRNA. Immunoblotting analysis using the anti-JAZ antibody confirmed a high level of expression of JAZ 48 h after transfection of NIH3T3 cells with the JAZ/pEIG2 plasmid (Fig. 3*A, lane Re*). However, after 72 h the green fluorescent-positive cells began to shrink in size and became rounded and detached from the culture dish, suggesting that these cells were undergoing apoptotic cell death (Fig. 6*A*). In contrast, cells transfected with JAZ cDNA in which all four fingers were mutated (NF) did not show any such morphological changes (Fig. 6*B*). The cells were further analyzed by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling to determine DNA fragmentation, the molecular hallmark of apoptosis (23). Results reveal that JAZ/pEIG2- but not JAZ-NF/pEIG2-transfected cultures contained dUTP-labeled cells that confirmed apoptotic cell death (Fig. 6, *C* and *D*). These results indicate that expression of exogenous JAZ can potently induce apoptosis and, furthermore, that intact ZFs are required for this apoptosis-inducing property.

DISCUSSION

Although many RNA-binding proteins have been isolated and characterized, only a few ZF proteins have been reported to bind RNA (not necessarily dsRNA-specific) (3). Although the interaction of ZF proteins such as Sp1 and ZF-QQR with RNA-DNA hybrids has been reported, these proteins have virtually no affinity for dsRNAs (24). Recently, a C_2H_2-type ZF protein was cloned by screening a *Xenopus* ovary cDNA expression library using dsRNA as probe (12). This protein, termed dsRBP-ZFa, contains seven C_2H_2-type ZF motifs and specifically binds dsRNA and RNA-DNA hybrids (12). Our results show that JAZ also has a higher affinity for an A-form duplex dsRNA than a B-form duplex DNA, demonstrating that mammalian cells also express dsRNA-binding ZF proteins.

The four ZF domains in JAZ and the seven in dsRBP-ZFa are homologous to each other (Fig. 1*B*). In addition, the ZF domains in these proteins are widely separated by long H-C linkers that distinguish them from other DNA-binding ZF proteins. To determine how these unique ZFs and linkers contribute to the unique dsRNA binding property, we conducted site-directed mutagenesis and deletion studies. It has been reported that the dsRBP-ZFa protein fragment containing either the first three or the last three ZF domains can bind to dsRNA as strongly as the full-length protein (25). Our results show that each ZF domain has an affinity for dsRNA, indicating that the amino acid sequence comprising these ZF domains forms a structure that preferentially binds an A-form helix. Furthermore, mutants with two consecutive ZF domains (*i.e.* III/IV and I/II mutants) exhibit stronger dsRNA binding than mutants with two separated ZF domains (*i.e.* I/III and II/III), suggesting the distance between ZF domains is essential for efficient binding. In contrast to the ZF sequence, there is no sequence homology between the linkers of JAZ and dsRBP-ZFa, and the peptide length of the linkers varies between 28 and 38 amino acids for JAZ and 34 and 44 amino acids for dsRBP-Zfa. The two ZF domains of I/III are isolated by 71 amino acids because of the destruction of the middle finger; therefore, the optimal length of the linker for efficient binding appears to be shorter than this. Thus, two domains connected by an appropriate length of linkers may coordinately bind to the minor groove of a single dsRNA molecule.

Sequence-specific DNA-binding proteins generally interact with the B-form of DNA, whereas RNA duplex forms an A-helix (1, 2). JAZ binds an A-form dsRNA like poly(I·C) in a sequence-independent manner (Fig. 2). Recent studies have suggested that the highly conserved dsRNA binding domain, which is present in many dsRNA-binding proteins including PKR, recognizes the minor groove of A-helix that is formed in a relatively sequence-independent manner (4, 26). Since an RNA/

DNA helix has an intermediate A/B conformation that is more similar to dsRNA than DNA, JAZ appears to have a higher affinity for an RNA/DNA hybrid than DNA (Fig. 3). Our results indicate that each ZF domain has an affinity for dsRNA, suggesting that the ZF domain of JAZ may form a similar structure to that of the common dsRNA binding motif. Further mutagenesis experiments within the ZF sequence and NMR analysis may reveal an analogy between these two types of dsRNA binding domains.

Subcellular fractionation studies and immunocytochemical analysis revealed that both endogenous and recombinant wild-type JAZ proteins localize to the nucleus. It is known that macromolecules larger than 30–40 kDa cannot diffuse from the cytoplasm into the nucleus and appear to be actively transported by carrier proteins that recognize NLS in the cargo protein (13, 14). Although endogenous JAZ is a 36-kDa protein (Fig. 3*A*), the JAZ-GFP fusion protein used for the localization studies has a molecular mass of 67 kDa (data not shown) and thus would apparently require such a transport mechanism. A computer program, PSORT (27), found two overlapping putative NLSs, called a 4-residue pattern, at amino acid positions of 206 (KRHR) and 207 (RHRK). Surprisingly, site-directed mutagenesis analysis reveals that JAZ constructs mutated at ZF domains fail to localize to the nucleus, although these point mutants retain all additional peptide sequences intact, including any potential NLSs. Moreover, the loss of nuclear/nucleolar localization of the mutants is highly correlated with a decreased affinity for dsRNA. These results indicate that, in contrast to other nuclear proteins, any NLSs are not sufficient and that the dsRNA-binding ZFs are essential for nuclear localization of JAZ. On the other hand, the dsRNA binding capacity alone may not be sufficient for nuclear localization since many dsRNA-binding proteins are expressed in the cytoplasm (28). Therefore, it remains possible that the putative NLS sequences may play a role in the trafficking of JAZ. Further mutagenesis experiments are required to test this possibility. Alternatively, JAZ may require ZF domains to associate with other nuclear shuttling proteins. In either case, nuclear localization of JAZ clearly depends on intact ZF domains.

The nuclear, and particularly nucleolar, localization pattern of JAZ may be a clue to its physiological function. Forced expression of exogenous JAZ causes apoptosis in NIH3T3 cells, suggesting a potential growth regulatory function for JAZ. This toxic effect is most likely specific since forced expression of other nucleolar localizing proteins or dsRNA-binding proteins is not generally toxic (29). Therefore, the apoptosis-inducing properties of JAZ appear to be another unique feature of this novel ZF protein. The ZF-deficient JAZ does not induce apoptosis, suggesting that the dsRNA-binding capability may be associated with the cytotoxicity. Interestingly, PAG608, a recently identified p53-inducible gene, also contains three C_2H_2-type ZF domains with long (54–77 amino acids) H-C linkers, localizes in the nucleolus, and induces apoptosis when overexpressed in human osteosarcoma and lung adenocarcinoma cells (30). Moreover, the amino acid sequences of the first and second ZF domains of PAG608 are homologous to those of JAZ (52 and 65% identical, respectively), suggesting that PAG608 may be another member of this new class of ZF proteins. Therefore, it will be important to determine whether PAG608 can bind dsRNA and whether dsRBP-ZFa can also induce apoptosis. If so, then JAZ, dsRBP-Zfa, and PAG608 may be members of a new class of dsRNA-binding ZF proteins functionally related to each other.

It has been found that a significant fraction of mammalian cellular heterogeneous nuclear RNA exists in the double-stranded form (4). The fact that the nucleolus is the site of transcription and processing of rDNA and the formation of pre-ribosomal particles (31, 32) suggests that JAZ may be involved in ribosomal biogenesis. The nucleolus contains many different proteins, including pre-ribosomal proteins and enzymes such as RNA polymerase I, topoisomerases, nucleases, kinases, and phosphatases (31–33). At metaphase, the JAZ-GFP fusion protein disperses throughout the cytoplasm with some accumulation in prenuclear bodies (32) and in the area surrounding the chromosomes called the chromosomal periphery (34) (Fig. 3*C*). Many nucleolar proteins that are involved in rRNA synthesis are also found to accumulate in this vicinity during mitosis, but the biological significance and the mechanism(s) of such accumulation are not yet clear (34). We postulate that forced expression of JAZ may disturb growth regulation at critical steps in macromolecular biosynthesis, such as ribosomal synthesis. Inhibition of such critical metabolism may then result in apoptosis. Formal proof of this exciting possibility awaits further analysis.

JAZ was identified during yeast two-hybrid screening using PKR, a dsRNA-binding protein, as bait. However, it is currently unknown whether JAZ functionally associates with PKR in mammalian cells. The interaction between PKR and JAZ in yeast cells appears to be dependent on dsRNA since PKR mutants lacking the dsRNA binding motif fail to interact with JAZ (data not shown). This suggests that dsRNA species present in the host yeast may facilitate the interaction of the two dsRNA-binding proteins and indicates an indirect binding mechanism dependent on dsRNA. It is of note that most of the yeast strains used in laboratories are infected with dsRNA viruses (35). Since a positive signal in the yeast two-hybrid assay is generally considered to indicate the direct interaction between two proteins, the interaction between JAZ and PKR may represent a unique example of potential indirect interactions that may also occur in this assay system.

In conclusion, our results suggest that JAZ is the first mammalian member of a family of dsRNA-binding ZF proteins. JAZ is an example of a nuclear protein whose localization requires the nucleic acid binding capability of the zinc finger domains. Although the physiologic function of such proteins is not yet clear, their tissue-independent nuclear expression may suggest that these dsRNA-binding ZF proteins also play a fundamental role in eukaryotic cell growth and survival.

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