Cloning and characterization of a human DEAH-box RNA helicase, a functional homolog of fission yeast Cdc28/Prp8

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ABSTRACT

During the splicing process, spliceosomal snRNAs undergo numerous conformational rearrangements that appear to be catalyzed by proteins belonging to the DEAD/H-box superfamily of RNA helicases. We have cloned a new RNA helicase gene, designated DBP2 (DEAH-box protein), homologous to the Schizosaccharomyces pombe cdc28+/prp8+ gene involved in pre-mRNA splicing and cell cycle progression. The full-length DBP2 contains 3400 nucleotides and codes for a protein of 1041 amino acids with a calculated mol. wt of 119 037 Da. Transfection experiments demonstrated that the GFP-DBP2 gene product, transiently expressed in HeLa cells, was localized in the nucleus. The DBP2 gene was mapped by FISH to the MHC region on human chromosome 6p21.3, a region where many malignant, genetic and autoimmune disease genes are linked. Because the expression of DBP2 gene in S.pombe prp8 mutant cells partially rescued the temperature-sensitive phenotype, we conclude that DBP2 is a functional human homolog of the fission yeast Cdc28/Prp8 protein.

INTRODUCTION

The excision of introns from nuclear precursors to messenger RNAs (pre-mRNAs) by a splicing reaction requires two distinct transesterification reactions. In the first step, the 5′ splice site is cleaved to form a branched intron–exon 2 lariat. In the second step, cleavage of the 3′ splice site and ligation of the two exons occur. This splicing reaction is catalyzed by a macromolecular complex called a spliceosome that consists of five small nuclear RNAs (snRNAs), U1, U2, U4/U6, U5, and a large but undetermined complex called a spliceosome that consists of five small nuclear RNAs (snRNAs), U1, U2, U4/U6, U5, and a large but undetermined number of proteins (1–3).

Helicases catalyze the unwinding of double-stranded DNA and RNA sequences by disrupting the hydrogen bonds between the two strands (4). They are thought to be involved in both DNA and RNA metabolisms, including RNA splicing. Helicases are structurally characterized by several consesus amino acid sequence motifs in the molecule that define specific superfamilies (5–8). Previous, several putative RNA helicase proteins containing a typical superfamily II DEAD/H box were identified as splicing factors of the budding yeast, Saccharomyces cerevisiae. These proteins are Prp5 and Prp28 (9,10), Prp2 and Prp16 (11,12), Prp22 (13) and Prp43 (14), which are involved in the spliceosome assembly (Prp5, Prp28), the catalytic reactions (Prp2, Prp16), the mRNA release from the spliceosome (Prp22), and the last disassembly process of the spliceosome (Prp43), respectively.

Recently, a cdc28+/*prp8+ gene encoding a DEAH-box protein was identified in the fission yeast Schizosaccharomyces pombe (15). The cdc28+ gene was initially identified in a screen for temperature-sensitive mutants that exhibit a cell-division cycle arrest and was found to be required for mitosis (16). The cdc28+/prp8+ gene product is most similar to the Prp2, Prp16, Prp22, Prp43 proteins of S.cerevisiae, which are members of the DEAH-box protein family of putative RNA helicases. All these results suggest that DEAH-box proteins may be involved in both RNA splicing and cell cycle progression (15).

We have previously identified two new genes, the members of the DEAH-box helicase gene family, designated DBP1 (DEA-H-box protein; 17) and DBP2. They were cloned by PCR using several degenerated oligonucleotide corresponding to box I and box V of the DEAH-box helicase family as primers. In this study, we describe one of these putative human RNA helicase genes. The possible role of this putative RNA helicase in pre-mRNA splicing and progression through the cell cycle is discussed.

MATERIALS AND METHODS

Oligonucleotide primers and reverse transcription PCR

The sequences of the degenerated primers used for gene cloning were: the sense primer 5′-GAGACXGGXTXGGXARACXAC-3′ and the antisense primer 5′-GAXGYCTCXGCXAXRTTXGTG-3′ (where X represents any base, Y represents a pyrimidine, R represents a purine), which were designed from the conserved amino acid sequences proximal to the DEAH-box corresponding to ETGSGKTT (box I) and TNIATS (box V), respectively (16). One µg of human testis poly(A)+ RNA was used for reverse transcription (SuperScript\textsuperscript{TM} HRT, BRL) primed by oligo d(T)\textsuperscript{12–18} and random hexamers as described by Imamura et al. (17). The DEAH-box containing the sequences was subsequently amplified from the mixture of cDNAs by the polymerase chain reaction (PCR) using the above primers. The PCR conditions were: initial denaturation at 94°C for 5 min,

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followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 1 min, and a final elongation at 72°C for 5 min. The amplified products, including a DNA fragment (800 bp), were separated by electrophoresis in 1–2% agarose gels, were visualized by staining with ethidium bromide, and were isolated by electrophoresis.

cDNA library screening

A total of 1 × 10⁶ clones of a human pancreas cDNA library in λgt11 (Clontech Palo Alto, CA) were screened by using the DBP2 (800 bp) PCR fragment as a probe. Screening was made under stringent conditions with hybridization in 50% formamide, 5× SSPE, 5× Denhardt’s, 0.1% SDS and 100 μg/ml sheared salmon sperm DNA at 42°C overnight. The filters were rinsed in 2× SSC–0.1% SDS at room temperature and twice in 0.2× SSC–0.1% SDS at 60°C for 15 min, and then were subjected to autoradiography.

DNA sequence analysis

The DNA sequence was determined by cycle sequencing using a Prism Sequencing Kit (Perkin Elmer) and an automated DNA sequencer (Model ABI 373, Applied Biosystems, Inc.). The nucleotide sequences reported in this paper were submitted to the GenBank/EMBL Data Bank with accession number AB001601. The sequence homologies with known genes were analyzed by a program from Intelligenetics, and by FASTA and TFASTA available from a GCG database search program package (18).

Northern blot analysis

The tissue-specific expression of DBP2 was studied using a series of human multiple-tissue northern (MTN) blots I and II (Clontech, Palo Alto, CA), which contained 2 μg of poly(A)+ RNA from various tissues and organs. The filters were hybridized with 2 × 10⁶ c.p.m./ml 3²P-radiolabeled C-terminal region (752 bp fragment, nucleotide number 2468–3220) of DBP2 cDNA, which was used as a probe. Hybridization and washing were as suggested by the manufacturer and then the filter was exposed to X-ray film with intensifying screens at –70°C. Rehybridization of the same filters by a β-actin probe showed that a comparable amount of RNA was loaded in each lane.

Zoo blot analysis

The presence of DBP2 gene homologs in other organisms was examined by Southern blot analysis with a series of Zoo blot filters (Clontech, Palo Alto, CA). which contained 2 μg of genomic DNA from various organisms. In the first method, the DNA was digested with EcoRI, run on a 0.7% agarose gel, transferred to a charged-modified nylon membrane, and were fixed by UV irradiation. The 3²P-labeled full-length DBP2 cDNA was used as a probe. Hybridization and washing were as suggested by the manufacturer and the filters were subjected to autoradiography.

Transfection and fluorescence microscopy

The coding region of full-length DBP2 was placed downstream of the enhanced green fluorescent protein (EGFP) (19) in the pEGFP-C3 (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. The structure of the plasmid construct was confirmed by direct sequencing. HeLa cells were seeded onto a poly-l-lysine-coated cover glass (Iwaki Co., Tokyo), were cultured overnight and then were transfected with 5 μg of expression plasmid DNA mixed with 10 μg lipofectin (Gibco-BRL, NY) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde and were mounted under a cover glass with 0.2 μg/ml DAPI (Sigma, St Louis, MO) in antifade solution (Wako, Tokyo). The EGFP fusion proteins and nuclei were visualized by fluorescence microscopy with a Nikon Optiphot-2 microscope fitted with a 60× Nikon PlanApo oil immersion objective and a double-pass filter set for fluorescein/DAPI. Fluorescent images were collected using a high performance CCD camera and were processed by MacProbe (Perceptive Scientific instruments, League City, TX).

Isolation of P1 phage clones

P1-#14162 and -#14163 clones containing 80 and 100 kb fragments of genomic DNA encoding the human DBP2 gene were obtained by screening a P1 phage library by PCR using two specific primers (Genome Systems, St Louis, MO). The forward primer AG170 5′-GGCAGAAGGTTTGTTGGCAACGAAT-3′ and the reverse primer AG197 5′-CTAAAGCCTTGAGCACGGACAAA-3′ were designed from the human DBP2 encoding sequence described in this paper. The following amplification cycles were used: 30 cycles of 94, 50 and 72°C for 30 s each and one cycle of 72°C for 7 min. These conditions yielded one specific PCR product of ~506 bp from human placental genomic DNA. The P1-#14162 DNA was used for further investigation.

Fluorescence in situ hybridization (FISH)

The regional chromosomal localization was determined by FISH using fragments of P1-#14162 DNA containing the human DBP2 gene as a probe (20,21). Two different methods were used. In the first method, DNA was labeled with digoxigenin-dUTP by nick-translation, and the labeled probe was combined with sheared salmon sperm DNA and was hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. Specific hybridization signals were detected by incubating the hybridized slides with fluorescein-labeled anti-digoxigenin antibodies followed by counterstaining with 4′,6-diamidino-2-phenylindole. In the second method, the labeled P1-#14162 DNA was cohybridized with a biotin-labeled probe specific for the centromere of chromosome 6. This second method results in the specific labeling of the centromeric region of chromosome 6 after incubation with Texas red-labeled avidin. Of a total of 80 metaphase cells analyzed, 71 showed specific labeling.

Complementation test

To construct a plasmid expressing DBP2 in S.pombe, the end of the SalI fragment containing the full-length DBP2 cDNA (C11) was bluntly treated with the Klenow fragment of DNA polymerase I and was inserted into the BalI site of the pREP3 expression vector (22) consisting of the inducible nmt1 promoter ars1 and the LEU2 gene as a selectable marker. The resultant plasmid was then introduced into the S.pombe pre-mRNA splicing mutant ppb8 (leu− leu1-32 prp8-1) (15) by the method of Okazaki et al. (23). Transformants were selected in the synthetic minimum medium (MM) containing 10 μg/ml of thiamine. Isolated leu+ transformants were then streaked on the MM plates without thiamine to induce
the expression of DBP2 from the nmt1 promoter, and were incubated at 26 or 36 °C to assess the complementing activity of DBP2 for the temperature-sensitive phenotype of prp8.

RESULTS AND DISCUSSION

Identification of the putative human DEAH-box RNA helicase

To isolate human RNA helicase genes of the DEAH-box protein family that may be required to splice mRNA precursors, various combinations of primers were tested for RT–PCR amplifications from the information of the conserved motif sequences of a putative RNA helicase (15). The RT–PCR was made using 1 µg poly(A)+ RNA of human testis to make a cDNA template. Only the primer pair set used to obtain the longer PCR fragments (800 bp), among the successful combinations, are described in Materials and Methods. Cloning and sequencing of these fragments revealed that the 800 bp fragments contained two different kinds of cDNA sequences. Because the two DNA sequences showed a high homology to the conserved motifs of the RNA helicase gene of the DEAH-box protein family, they were designated DBP1 (17) and DBP2. The DBP2 gene had a high homology (>72%) to the fission yeast S.pombe cdc28+/prp8+.

Figure 1. Homology of DBP2 to other DEAH-box protein family. (A) Alignment of the amino acid sequences of DBP2 and Cdc28/PpP8. Boldface characters indicate identical positions. Roman numerals indicate the position of the motifs found in the DEAH-box RNA helicase. (B) Phylogenetic tree derived from the alignment of the DEAH-box RNA helicase family. The tree was computed using the unweighted pair group method with an arithmetic mean (UPGMA; Geneworks, IntelliGenetics Inc.).
gene, which is involved in the cellular processes of pre-mRNA splicing and cell cycle progression (15).

Cloning and characterization of DBP2 cDNA

To examine if the homology between Cdc28/Prp8 and DBP2 extends outside the DEAH-box region, an attempt was made to isolate a full-length cDNA of DBP2. The full-length cDNA clones C2, C4, C5 and C11 containing the poly(A)^+ tail region were obtained from the λgt11 library by screening 1 x 10^5 recombinant phage plaques of the original human pancreas cDNA library using a 32P-labeled DBP2 PCR fragment as a probe. Of the positive clones, the C11 clone containing a long insert (3.4 kb) was further analyzed. Sequence analysis revealed that the C11 clone contained an open reading frame of 3.1 kb that encoded a polypeptide of 1041 amino acids with a predicted molecular mass of 119 037 Da. GenBank/EMBL Data Bank with accession number AB001601. The ATG at nucleotide residues 140–142 is likely to be the translation initiation codon because the nucleotide sequence surrounding the first methionine codon is GCCATGG, which agrees well with the consensus sequence proposed by Kozak (AG/CCATGG (24,25) and contains two in-frame stop codons at 44–46 and 131–133. A polyadenylation signal AAATAA (nucleotides 3349–3354) is found in the C-terminal region (residues 729–1041), but no CTA (arginine) codon is found in the N-terminal region of the DBP2 product (residues 422–728), which is conserved in many RNA helicases, shows a 72% identity with the DBP2 and fission yeast Cdc28/Prp8 RNA helicases. The central domain of the DBP2 product (residues 422–728), which is conserved in many RNA helicases, shows a 72% identity with Cdc28/Prp8. In addition, an extensive homology (48% identity) is conserved in the N-terminal region (residues 729–1041), but no significant homology was found in the C-terminal region of the coding sequence. Figure 1B shows a phylogenetic tree showing these relationships. We assume that the DBP2 is a homolog of Prp2 of S.cerevisiae and Prp8 of S.pombe, based on sequence similarities and a functional complementation for Prp8 shown later.

Northern and Southern blot analysis

The multiple tissue northern blot analysis was made to determine the size and tissue distribution of DBP2 mRNA in humans. Northern blot analysis was made using 32P-labeled 752 bp fragment as a probe, which encodes the C-terminal region of DBP2. Figure 2 shows the expression profiles of the DBP2. High expression levels of DBP2 mRNA, with a major band corresponding to ∼3.4 kb, was detected in many tissues and organs. The DBP2 is expressed strongly in heart, skeletal muscle and testis. The size of transcripts detected in the majority of organs was 3.4 kb, but two additional bands longer than 3.4 kb were also seen in many organs in addition to the major 3.4 kb band. The DBP2 is also expressed strongly in several tumor cell lines (data not shown). From this result, the DBP2 appears to be ubiquitously transcribed, although the levels of expression were different from each other. The ubiquitous expression of DBP2 in many tissues and organs suggests that the protein encoded by the DBP2 has an important function in these tissues and organs. Rehybridization of the same filters by a β-actin probe assured that a comparable amount of RNA was loaded in each lane.

To evaluate the evolutionary conservation of the DBP2 sequence, the full-length cDNA was hybridized to genomic DNA blots, i.e. ‘Zoo-Blot’, prepared from a variety of eukaryotic species (Fig. 3). Signals were obtained in humans, monkey, rat, mouse, dog, cow, rabbit and yeast, but no homologous DNA to DBP2 was detected in chicken, suggesting that the DBP2 gene seems to be conserved in the genomes of most eukaryotes, except birds.

Expression of DBP2 in HeLa cells

Because DBP2 is a candidate of ATP-dependent RNA helicase involved in mRNA splicing and/or cell cycle progression, its
location in the cell is of interest. The specific antibody against DBP2 is unavailable, so we used the EGFP-tagged system. The full-length DBP2 cDNA was placed downstream of the EGFP sequence in the pEGFP-C3 DNA as described in Materials and Methods, and the subcellular distribution in the transfected HeLa cells was analyzed by fluorescence microscopy. The result clearly shows that the EGFP-tagged DBP2 gene product is exclusively in the nuclei when expressed in HeLa cells (Fig. 4a). The EGFP itself does not contain the nuclear localization signal, and was dispersed in the cell (Fig. 4c). A control plasmid encoding DBP2 without a EGFP tag gave no fluorescence (data not shown).

**Chromosomal location of DBP2**

The chromosomal location was determined by FISH using P1 phage DNA that contains human DBP2 gene as a probe. P1-#14162 DNA was labeled with digoxigenin and was hybridized to the metaphase chromosomes derived from PHA-stimulated human peripheral blood lymphocytes. The results of this analysis indicated that DBP2 gene is located on human chromosome 6 (Fig. 5A). A more detailed determination of the DBP2 location was made by analysing the fractional chromosome length; 10 specifically hybridized chromosomes were measured.
calculations indicated that the DBP2 gene is in the position 43% of the distance from the centromere to the telomere of chromosome 6p, an area that corresponds to 6p21.3. Figure 5B shows an idiogram indicating the chromosomal location of human DBP2. Various genetic alterations involving the 6p21 locus have been identified with many malignant, genetic and autoimmune diseases (27). Future studies must determine the role of DBP2 gene in diseases.

**Functional complementation of S.pombe prp8 by human DBP2**

DBP2 shows the highest homology in amino acid sequence with S.pombe Cdc28/Prp8. To test if DBP2 is a functional homolog of human S.pombe prp8-1 mutant could complement the temperature-sensitive phenotype of the mutation. The full-length human S.pombe Cdc28/Prp8, we examined if the expression of DBP2 in the S.pombe Cdc28/Prp8. To test if DBP2 is a functional homolog of DBP2 shows the highest homology in amino acid sequence with DBP2 functional complementation of DBP2 by DBP2.

REFERENCES