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## A novel human SCAN/(Cys)<sub>2</sub>(His)<sub>2</sub> zinc-finger transcription factor *ZNF323* in early human embryonic development

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### Abstract

The C<sub>2</sub>H<sub>2</sub> zinc-finger motif found in many transcription factors is thought to be important for nucleic acid binding and/or dimerization. Here, we have identified and characterized a novel zinc-finger gene named *ZNF323* using degenerate primers from an early human embryo heart cDNA library. The predicted protein contains six different C<sub>2</sub>H<sub>2</sub> type zinc fingers and a SCAN box. *ZNF323* maps to chromosome 6p22.1–22.3. The expression levels were different during different development stages of human embryo between 15 and 23 weeks. Northern blot analysis shows that a 3.2-kb transcript specific for *ZNF323* was expressed at high levels in the lung, liver, and kidney, while weakly expressed in intestine, brain, muscle, cholecyst, heart, and pancreas. In adult tissues, *ZNF323* is expressed at high levels in liver and kidney, weakly in lung, pancreas, brain, placenta, muscle, and heart. Taken together, these results indicate that *ZNF323* is a member of the zinc-finger transcription factor family and may be involved in the development of multiple embryonic organs. © 2002 Elsevier Science (USA). All rights reserved.

**Keywords:** C<sub>2</sub>H<sub>2</sub> zinc finger; SCAN box; Novel transcription factor *ZNF323*; Gene expression

Eukaryotic transcription factors are classified according to the structural motifs that are responsible for interacting with the DNA sequence. The most well-known motifs are the helix-turn-helix, helix-loop-helix, and zinc finger. The sequence, number, and organization of the zinc-finger motifs are important for the biological function of proteins. During cell differentiation and development, zinc-finger domains are involved in the binding of transcription factors to their cognate DNA recognition site, resulting in the specific activation or repression of gene expression [1]. Many zinc-finger proteins have been demonstrated to function as transcriptional regulators and zinc-finger genes are frequently targeted for disruption in many human diseases and cancers.

Since the identification of the first zinc-finger domains in *Xenopus* transcription factor *TFIIIA* [2,3], hundreds of

proteins possessing these domains have been described. This large zinc finger family may be divided into many subfamilies such as glucocorticoid receptor, ring finger, *GATA-1* type, GAL4 type, LIM family, and C<sub>2</sub>H<sub>2</sub> type [4–6]. It is estimated that in the C<sub>2</sub>H<sub>2</sub> zinc finger family, about one-third of the members are *krüppel*-like genes, as characterized by the presence of highly conserved connecting sequences “TGEKPYX” between the last histidine of the preceding finger motif with the first cysteine of the next finger (H–C link) [7]. Substantial evidence indicates that the *krüppel*-like genes play an important role in many physiological processes as transcriptional regulators [8,9].

Many *krüppel*-like zinc-finger proteins contain highly conserved amino-terminal motifs such as the *krüppel*-associated box (KRAB), the finger-associated box (FAX), the poxvirus and zinc finger (POZ) domain, and the SCAN box or leucine-rich region (LER) [10–12]. These conserved domains play distinct roles in terms of transcription regulation of target genes. The SCAN

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domain was originally derived from the first four proteins found to contain this domain (SRE-ZBP, CT-fin-51, AW-1, and number 18 cDNA) [13–16]. This domain is an 96-residue, leucine-rich region that contains three segments predicted to be  $\alpha$ -helices. The primary amino acid sequence of this domain is not similar to any of the other zinc finger-associated domains. Several lines of evidence show that the SCAN domain plays an important role in the assembly and function of this SCAN/ $C_2H_2$  subgroup of transcriptional regulators.

In the current studies, we describe the identification and characterization of a novel human gene encoding a 406-amino acid *krüppel*-like zinc-finger protein and termed *ZNF323*. This widely expressed gene encodes a zinc finger protein containing six different  $C_2H_2$  type zinc fingers and a SCAN box. Northern blot analysis shows that *ZNF323* is expressed at high levels in the embryonic liver, secondly in embryonic kidney and lung, and weakly expressed in the intestine, brain, muscle, cholecyst, and pancreas. The expression levels were different at different stages during human embryo development ranging from 15 to 23 weeks. The *ZNF323* transcript is expressed at high levels in adult liver and kidney, weakly in lung, pancreas, brain, placenta, and muscle. Taken together, these results indicate that *ZNF323* is a transcription factor involved in development of embryonic multiple organs.

## Materials and methods

**Preparation of RNA.** All of the human embryonic tissues were obtained from 15- to 23-week abortive fetus, which were provided by the Health Center of Women and Children Hospital, Changsha, People's Republic of China, with the consent of the patients and the regulation of university policy. Total RNAs from these embryonic tissues were prepared using improved method of extracting total RNA with phenol–chloroform as described in Molecular clone. Pellets of total RNA were dissolved in diethyl pyrocarbonate-treated water and stored at  $-80^\circ\text{C}$  prior to use.

**Construction of a human fetal heart cDNA library.** The total RNA in hearts from 20-week human embryos was extracted using standard methods, which were pretreated with DNase I (RNase free) to eliminate DNA contamination. mRNA preparation and reverse transcrip-

tion reaction were performed using a cDNA PCR Library Kit and cDNA Synthesis Kit according to manufacturer's protocol (TaKaRa). Briefly, 500  $\mu\text{g}$  embryonic heart total RNA was used for preparing mRNA with columns. Reverse transcription reactions were performed with 5  $\mu\text{g}$  embryonic heart mRNA and oligo(dT)-RA primer according to cDNA Synthesis Kit protocol. After Cassette Adaptor Ligation reactions using cDNA PCR Library Kit, cDNA amplification reactions were performed with RA primer, CA primer, and TaKaRa Ex Taq.

**Cloning of zinc finger-containing cDNA.** According to a highly conserved “knuckle” region (TGEKPFQC) and conserved  $C_2H_2$  zinc-finger sequence (CRECGKAF) of *krüppel*-like type zinc-finger gene, we designed a pair of degenerate oligonucleotide primers D1 (Table 1) as described previously [17]. The PCR amplification protocol was  $94^\circ\text{C}$  for 4 min,  $94^\circ\text{C}$  for 30 s,  $50^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 2 min for 5 cycles;  $94^\circ\text{C}$  for 30 s,  $55^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 2 min for 30 cycles. The PCR products were subcloned into T-vector and then sequenced. The partial cDNA sequences of this novel gene were assembled from ESTs of databases. Three pairs of gene-specific primers were designed, based on the sequences of ESTs for PCR reaction (Table 1). The heart cDNA library was used as template and the PCR products were cloned into T-vector and sequenced.

**RACE PCR.** A rapid amplification of cDNA ends (RACE) technique was performed using total RNA of human fetal heart. The 5' upstream and 3' downstream sequences of the *ZNF323* gene were amplified by RACE PCR using the SMART cDNA Amplification Kit (TaKaRa Biotechnology) according to manufacture's protocols. The RACE RT-primers were phosphorylated at 5' end for the following ligation. The primers specific for the 5' and 3' end of the cDNA were R7/R8 and R9/R10, respectively (Table 1). The amplified cDNA fragments were subcloned into T-vector. After these cDNA fragments of RACE had been sequenced, they were assembled into contigs to complete the full-length cDNA.

**Northern blot analysis.** Total RNA (20  $\mu\text{g}$ ) of multiple tissues from 15- to 23-week human fetus was prepared previously and separated through 1% formaldehyde–agarose gel electrophoresis. These RNAs were then transferred onto nylon membranes as described in Molecular Clone. The Multiple Tissue Northern (MTN) blot membrane containing mRNA from eight adult tissues was purchased from Clontech Company.

The *ZNF323* probe was labeled in the presence of [ $^{32}\text{P}$ ]dCTP using a random primer labeling kit (TaKaRa). [ $^{32}\text{P}$ ]dCTP was bought from Ya Hui Company of China. The labeled probe was hybridized with prepared MTN blot membranes at  $42^\circ\text{C}$  overnight. A human 2-kb  $\beta$ -actin cDNA probe was also hybridized as the control. The membrane was washed twice under high-stringency conditions with  $2\times$  SSC, 0.1% SDS for 15 min each time at  $42^\circ\text{C}$ , and then with  $0.1\times$  SSC, 0.1% SDS for 10 min at  $42^\circ\text{C}$ , and then exposed to X-ray film at  $-80^\circ\text{C}$  using an intensifying screen.

Table 1  
Set of specific oligonucleotide primers

Primer	Sense primer	Anti-sense primer
D1	ACIGG(C/A)GAGAAGCC(T/A)TTCCA(A/G)TGT	GAAIC(A/T)CTT(G/A)CCGCATTTCGTT(A/G)CA
P2	TATGGGCTGGAACCTGACACT	CTTGCTTTGATGCCAACTCC
P3	GCCTATGGTGACACAGCTCAGA	GGACCAAAGGCTAGGGAATAAG
P4	GGCACCAAATCTTATCTGG	CAAGAATGTCAGCAGAGGCA
P5	GTACTCGAGCCAGGAGTTGGCATCAAAGCA	CGAGAGCTCAGGTTGCAATGATGACTGAAGGC
ORF6	GGAAGAATAGCCAGAGTAAGCC	GTTCTGCTGGAACAGTATGGA
R7	AGTTGTTCCCTCGAAGGTG	
R8	TTCTTGGTCCCAGATAGG	
R9	CCATTTGTGAGCTGTGGGACT	
R10	GACACTTATTGCCAGGGT	

## Results

### Sequence analysis of the *ZNF323* gene

Since nucleotide sequences of the *krüppel*-like zinc finger connecting region are highly conserved, it was feasible to isolate homologous genes of this family using PCR amplification from a cDNA library of human early embryo. We designed specific degenerate primers as described under Materials and methods. We obtained 26 homologous sequences, including novel and known ones. A novel 324-bp fragment of a putative *krüppel*-like zinc-finger cDNA was isolated. The sequence obtained was subjected to human homology searching against expressed sequence tag (EST) database using Blast (<http://www.ncbi.nlm.nih.gov/blast>). An EST BQ006383 was found to match the cDNA clone. A number of ESTs representing the same novel gene were identified in a further search. The partial cDNA sequences of this novel gene were assembled from ESTs BG438146, BF676393, BI559355, BQ006383, BM722371, BI438976, and BM985167. To confirm the cDNA sequence from the database, three pairs of gene-specific primers were designed based on the sequences of ESTs for PCR: primer P2 (nucleotides 122–1102), P3 (nucleotides 999–2031), and P4 (nucleotides 2054–3087). The heart cDNA library was used as template and the PCR products were cloned into T-vector and sequenced. This approach, together with bioinformatic analysis, RACE PCR, and Northern blot analysis, was an efficient system to clone novel *krüppel*-like zinc-finger C<sub>2</sub>H<sub>2</sub> type zinc-finger genes.

To obtain the full-length cDNA, the 5' upstream and 3' downstream sequences of the gene were amplified by RACE PCR. The primers specific for the 5' ends and 3' ends of the cDNA were R7/R8 and R9/R10, respectively (Table 1). These procedures yielded a 600-bp DNA for 5'-RACE fragments and a 200-bp DNA for 3'-RACE fragments. An analysis of these two fragments suggested that they were cDNA fragments from the novel zinc-finger gene. So, they were assembled into contigs to complete the full-length cDNA. The full-length cDNA was 3197-bp in length and contained a region encoding a SCAN domain. The gene is named *ZNF323*, as approved by HUGO Nomenclature Committee. The nucleotide sequence data reported here are available in GenBank with Accession No. AF513019.

The complete sequence of the *ZNF323* cDNA is 3179-bp in length and contains an open reading frame (ORF) of 1221 nucleotides. The protein predicted from the open reading frame has a calculated relative molecular mass of 44,660 Da. The cDNA has a relatively long 3'-untranslated region with a consensus polyadenylation signal (AAATAAA). The complete *ZNF323* nucleotide and deduced amino acid sequences

are shown in Fig. 1. Sequence analysis and database comparisons indicate that the predicted protein contains six different C<sub>2</sub>H<sub>2</sub> zinc finger domains in tandem arrays at the COOH terminus, characteristic of transcription factor proteins of this family. The zinc-finger motif (ZF1–ZF6) matches the consensus sequence for members of the C<sub>2</sub>H<sub>2</sub> family of DNA-binding proteins (Fig. 2A). The amino acid sequences of this region were aligned with similar domains in several other zinc-finger transcription factors such as *ZFP38*, *ZNF139*, *ZNF192*, *ZNF202*, *ZNF263*, and *ZNF306* (Fig. 2B). Interestingly, there are highly conserved consensus sequences TGEKPYX (X representing any amino acid) between adjacent zinc-finger motifs (Fig. 2A). The sequence, number, and organization of the zinc-finger motifs are important for the biological function of the family of proteins. From these features, it is reasonable to predict that *ZNF323* could encode a DNA-binding protein with transcriptional regulatory properties.

Besides the putative zinc-finger DNA-binding domain, the deduced amino acid sequence also contains a novel element upstream of the zinc-finger domain designated the SCAN box. This element is a 95-residue, leucine-rich region that contains three segments strongly predicted to be  $\alpha$ -helices. This region is homologous with similar elements in several other zinc-finger transcription factors such as *ZNF174*, *ZNF165*, *ZNF192*, *ZNF202*, *ZNF12*, *ZFP38*, *ZNF232*, and *ZNF306* (Fig. 3). The amino acid regions between the SCAN box and the zinc-finger domains vary in length and do not share extensive sequence homology.

### The genomic organization of the *ZNF323* gene

We performed PCR using adult brain cDNA library with specific primers ORF6 (Table 1) for ORF of *ZNF323* and we obtained a 1.2-kb cDNA fragment. After this fragment had been subcloned and sequenced, BLAST analysis showed this sequence to be identical to the part of the draft genomic sequences of PAC RP5-874C20 overlapping the chromosome 6p22.1–22.3, NT-007592 of the GenBank database. And the *ZNF323* gene is adjacent to *ZNF187* and *ZNF306*. So we concluded that the *ZNF323* gene maps to chromosome 6p22.1–22.3. We compared the human genome databases to the cDNA sequence of *ZNF323* to explore the genomic organization of the *ZNF323* gene. According to the results of alignments of two sequences, the intron/exon boundaries in the mRNA were determined. *ZNF323* spans approximately 29.45 kb and is organized into eight exons. All exon–intron junctions contain the gt/ag consensus splice site (Table 2). The last three exons encode the open reading frame. Exon VIII also encodes the 3'-untranslated long sequence of the *ZNF323* gene (Fig. 1B).

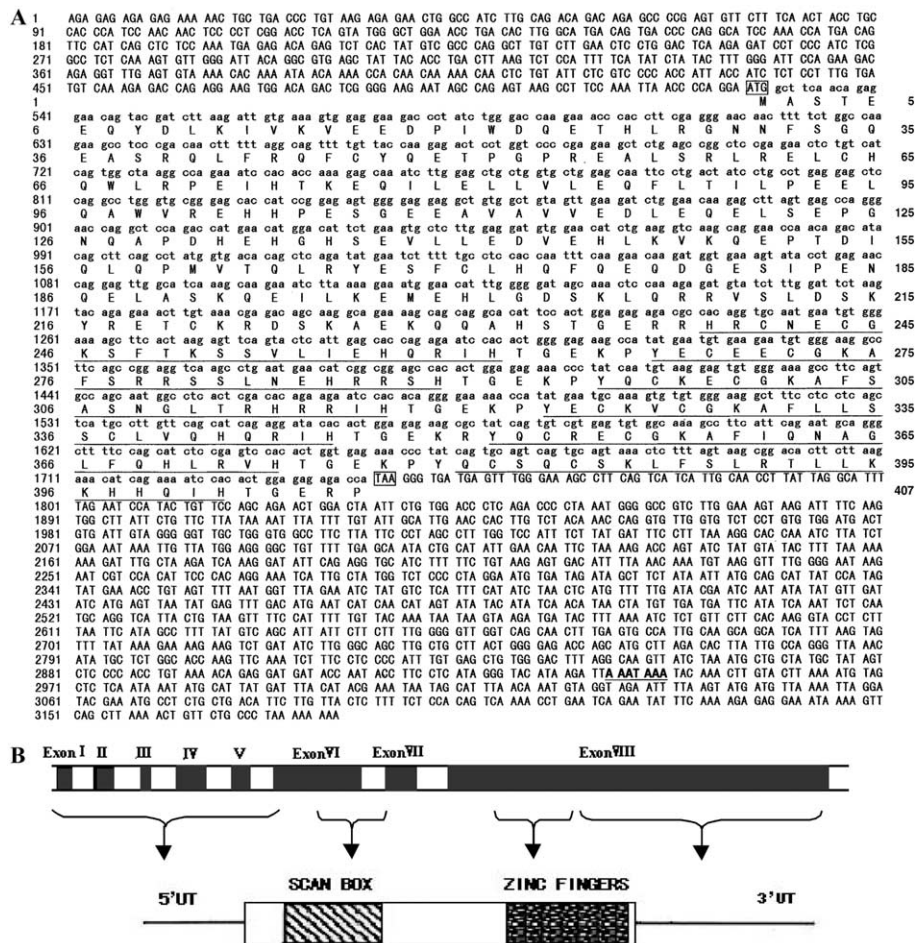


Fig. 1. (A) Nucleotide and deduced protein sequences of the human *ZNF323* gene. *ZNF323* encodes a polypeptide of 406 amino acids, and the initiation ATG and termination TAA codons are boxed. Amino acids are identified by their one-letter code. The six zinc finger regions are underlined. Both nucleotides and amino acids are numbered at the left and right sides of each line, respectively. The putative polyadenylation signal sequence **AAATAAA** is underlined and is given in bold characters. (B) Genomic organization of the *ZNF323* gene from 6p22.1 to 22.3. Eight exons corresponding to different structural regions of the *ZNF323* gene are shown.

#### Tissue expression pattern of the *ZNF323* gene

To understand the expression profiles of *ZNF323* gene in various tissues, we designed specific primers P5 (Table 1) to amplify nucleotides 457–1249 of *ZNF323* gene as the probe to examine its expression in multiple tissues at different stages during human embryo development ranging from 18 to 23 weeks. As shown in Fig. 4, Northern blot analysis detects an expected transcript of about 3.2 kb in all the major embryo tissues. The *ZNF323* gene is widely expressed in many embryonic organs, with the high levels found in liver and secondly in kidney and lung. The gene is expressed weakly in intestines, brain, muscle, cholecyst, heart (18 weeks), and pancreas. In the heart, there is no detectable expression except that of 18 weeks. The *ZNF323* transcript is expressed at high levels in adult liver and kidney, weakly in intestine, brain, heart, and muscle. The control 2.0 kb  $\beta$ -actin mRNA was present in all tissues.

When the cDNA of the *ZNF323* gene was used to search against the UniGene database, the UniGene cluster was obtained. This cluster included 44 ESTs that came from liver, intestines, heart, lung, brain, kidney, muscle, spleen, and cholecyst as well as some tissues not used in our Northern blot, such as uterus, testis, and ovary. This result was consistent with our Northern blot analysis. Our data suggest that *ZNF323* is a transcription factor in human embryo development.

#### Upstream finger-associated SCAN box

The deduced amino acid sequence contains a novel element of the zinc finger domain that consists of about 95 amino acids near the amino terminus. This region, originally derived from the first four proteins found to contain this domain (SRE-ZBP, CT-fin-51, Aw-1, and number 18 cDNA) and designated as the SCAN box, was identified by its homology with similar elements in several other zinc-finger transcription factors such as



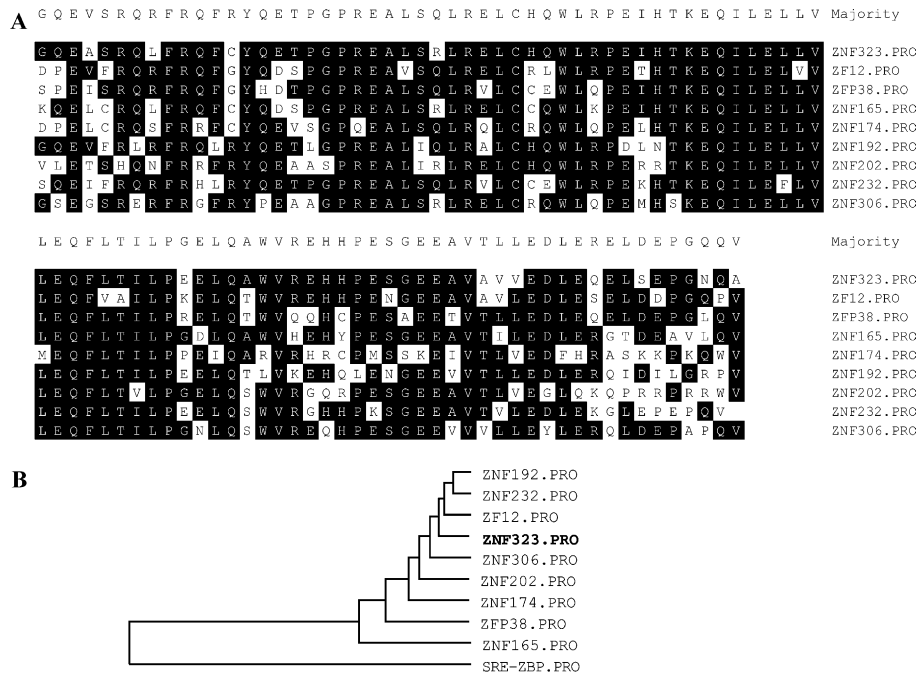


Fig. 3. Amino acid alignment of SCAN domain. (A) The amino acid sequences of the SCAN domains from the following genes are aligned: *mZF-12* (GenBank Accession No. BM055705), *ZFP38* (XM056169), *ZNF165* (X84801), *ZNF174* (U31248), *ZNF192*, *ZNF202*, *ZNF232* (Y15067), and *ZNF306*. A consensus sequence for the SCAN domain is presented underneath the alignment. Residues that are highly conserved among these sequences are indicated within the majority sequence in boldface type. Identical residues fitting the SCAN consensus have been boxed and are shaded in dark. (B) Uprooted phylogenetic tree relating SCAN domain sequences. All genes shown in (A) and *SRE-ZBP* (Z11773) were used. All genes originate from *Homo sapiens*, except that *ZF-12* and *ZFP38* originate from mouse.

The zinc-finger motifs are involved in the DNA binding. The SCAN box may play an important role in the assembly and function of this subclass of transcriptional regulators. Although the biological function of *ZNF323* is unknown, the predicted sequence contains conserved motifs that provide insight into its potential role in regulating the transcription factor function. The *ZNF323* gene contains an open reading frame of 1221 nucleotides, encoding a predicted protein of about 44,660 Da. The amino acid sequence of the *ZNF323* protein has two characteristic structural features. At the NH<sub>2</sub> terminus, the *ZNF323* protein contains a con-

served SCAN domain. Like many other SCAN domain proteins, *ZNF323* also contains six different C<sub>2</sub>H<sub>2</sub> type zinc fingers in tandem arrays at the COOH terminus.

C<sub>2</sub>H<sub>2</sub> type zinc-finger gene family is one of the largest gene families and each member has several repeated zinc-finger motifs. Recently, it has been estimated that about 35% of this family members are *krüppel*-like zinc finger genes, which are characterized by highly conserved connecting sequences "TGEKPYX" between adjacent zinc-finger motifs. Several lines of evidence indicate that *krüppel*-like zinc-finger genes are important transcriptional regulators involved in many physiologi-

Table 2  
Genomic organization of the *ZNF323* gene from 6p22.1 to 22.3

Intron	Exon		Intron	Intron size
	Exon number	Exon size		
gaataaaatcatattttacctgcctacagCCCCGAGTGT	1	65	ACAGACAGAGgtgagccaggaattaacaagcatgcc	178
gctacccaactttttgttttttagcagACCCAGGCA	2	93	TGGCATGACAggtgtaagtaccatatacatgttggtctaa	12070
ggataatattttttatttttcagagacagAGTCTCACTA	3	48	TCCAAATGAGgttaagatagaaccagtccttgggcca	3852
tgggattccagaagacagaggtttgagTGTA AAAACAC	4	140	TCATATCTATgtttgtacttgaattttgtattccttttctcaa	883
ttttaaatcacagtattccctttacttaaaagCCAGAGTAAG	5	85	GGAAGAATAGgttaagtattagacagaaaaagtatga	7075
aatatgcctacctgccttcaggctocagACCATGAACA	6	415	AGGGAACCAGgtgagaggagaaagtggactctagac	1767
attacattgtttgttcattttatttccagATGGTGAAAG	7	154	CAAGAACAAAgtaaggattttgacaggtcccttcaggag	528
	8	1904		

Exon and intron sizes are given in base pairs. Intronic and exonic sequences are shown in lower and uppercase characters, respectively. The last and first two bases of introns (ag and gt for acceptor- and donor-splice sites, respectively) are shown in bold.

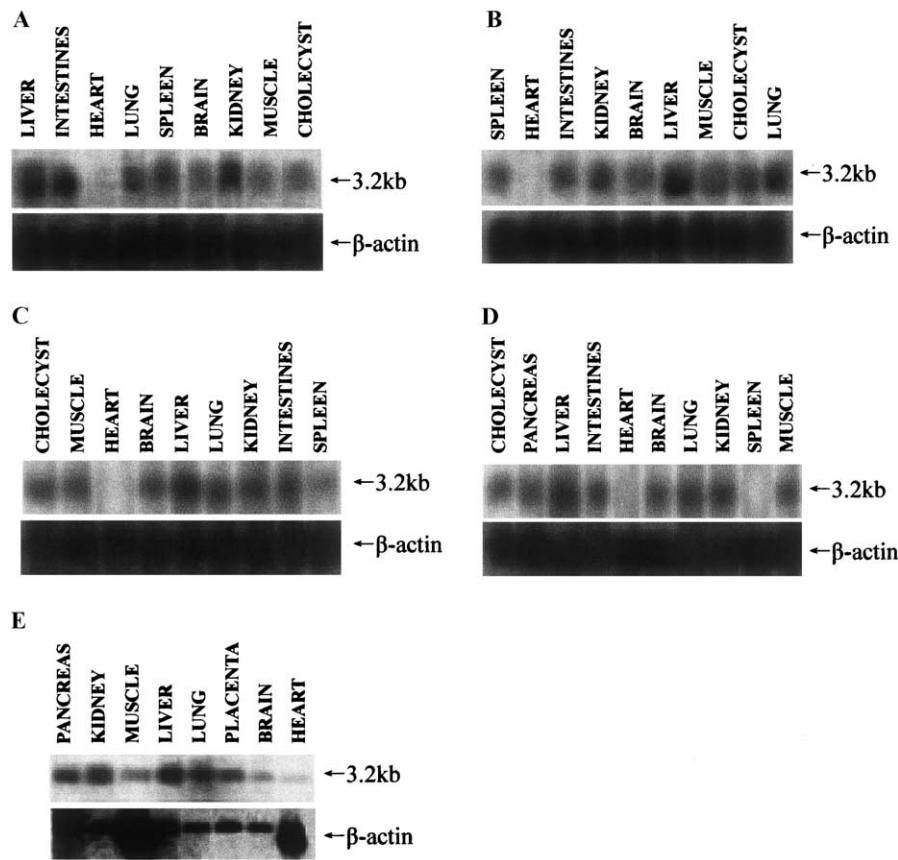


Fig. 4. Expression of *ZNF323* in human embryonic and adult tissues analyzed by Northern blot analysis. Membranes containing 20  $\mu$ g per lane RNAs from multiple human embryo tissues were hybridized with a  $^{32}$ P random-labeled probe, which contains ORF nucleotides 457–1249 of *ZNF323*. The same membranes were also hybridized with  $\beta$ -actin to normalize for loading differences. (A) Eighteen-week human embryo tissues. (B) Twenty-week human embryo tissues. (C) Twenty-three-week human embryo tissues. (D) Twenty-five-week human embryo tissues. (E) Human adult tissues.

cal processes. For example, in *Drosophila*, these genes are essential transcriptional repressors during development and growth. In mouse and human, *PLZF* gene is associated with the development of rhombomere in hindbrain [18,19] and involved in the pathogenesis of acute promyelocytic leukemia with t(11;17) chromosomal translocation [20,21].

$C_2H_2$  type zinc-finger proteins are reported to bind DNA in monomeric form. Recently, some research results suggest that SCAN domain-containing zinc-finger protein requires specific homo- or hetero-dimerization for DNA binding and transcriptional modulation [22,23]. For example, the SCAN domain of *ZNF202* can mediate selective protein oligomerization and the zinc-finger motifs to bind to specific DNA elements [24,25]. The SCAN box was derived from the first four proteins found to contain this domain (*SRE-ZBP*, *CT-fin-51*, *Aw-1*, and number 18 cDNA). Protein sequence analysis showed that the SCAN box was predicted to form two or three amphipathic helices, which would permit interactions with other proteins containing this domain. To date, many zinc-finger proteins containing this do-

main have been identified. *SRE-ZBP* is a human transcription factor, containing seven zinc fingers and binding to the *c-fos* serum response element, and is recognized as a repressor of *c-fos* transcription. *ZNF165* is a zinc finger gene located on chromosome 6p21, which is expressed specifically in the testis [26]. *ZNF174*, containing three zinc finger and a SCAN domain, selectively repressed reporter activity driven by the platelet-derived growth factor- $\beta$  chain and transforming growth factor- $\beta$  promoters and bound to DNA in a specific dimerization manner [16]. Experiments have demonstrated that the SCAN box is not an independent transactivation or repression domain and may function to recruit co-repressors and transactivators necessary for transcriptional regulation. Although the function of the SCAN box has not yet been elucidated clearly, the conservation of this module and its  $\alpha$ -helical structure suggests that this module may serve *ZNF323* as a dimerization domain or a site that interacts with other transcriptional factors, resulting in the repression of gene expression.

In summary, we have cloned and characterized a novel human SCAN/ $C_2H_2$  zinc-finger transcription

factor, *ZNF323*. The biological function of the *ZNF323* gene is to await for further study, but we can get a fundamental understanding from this report.

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