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Molecular cloning and expression analysis of a novel gene *DGCR8* located in the DiGeorge syndrome chromosomal region

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Abstract

We have identified and cloned a novel gene (*DGCR8*) from the human chromosome 22q11.2. This gene is located in the DiGeorge syndrome chromosomal region (DGCR). It consists of 14 exons spanning over 35 kb and produces transcripts with ORF of 2322 bp, encoding a protein of 773 amino acids. We also isolated a mouse ortholog *Dgcr8* and found it has 95.3% identity with human DGCR8 at the amino acid sequence level. Northern blot analysis of human and mouse tissues from adult and fetus showed rather ubiquitous expression. However, the in situ hybridization of mouse embryos revealed that mouse *Dgcr8* transcripts are localized in neuroepithelium of primary brain, limb bud, vessels, thymus, and around the palate during the developmental stages of embryos. The expression profile of *Dgcr8* in developing mouse embryos is consistent with the clinical phenotypes including congenital heart defects and palate clefts associated with DiGeorge syndrome (DGS)/conotruncal anomaly face syndrome (CAFS)/velocardiofacial syndrome (VCFS), which are caused by monoallelic microdeletion of chromosome 22q11.2.

Keywords: *DGCR8*; 22q11.2; DiGeorge syndrome chromosomal region; DiGeorge syndrome; Conotruncal anomaly face syndrome; Velocardiofacial syndrome; Gene structure; Expression analysis; Genome sequencing

Deletion of chromosome 22q11 (del22q11) is the most frequent chromosomal deletion found in human with an incidence of 1:4000 live births [1]. This deletion has been associated with a complex clinical phenotype, including DiGeorge syndrome (DGS)/conotruncal anomaly face syndrome (CAFS)/velocardiofacial syndrome (VCFS) [1–5]. Although these diagnoses are highly variable, the most common clinical findings include learning disabilities, characteristic facial appearance, submucous cleft palate, conotruncal heart defects, thymic hypoplasia or aplasia, hypocalcemia, and psychiatric illness [6–8].

Most patients with these syndromes have a monoallelic microdeletion within chromosome 22q11 [9,10]. Ninety percent of patients have a 3.0-Mb deletion and about 7% of patients have a 1.5-Mb deletion, of which proximal breakpoint is the same as that of the 3.0-Mb deletion, but the distal breakpoint is more nested [11–13]. Thus, most patients have the 1.5-Mb deletion in common, so-called "DiGeorge syndrome chromosomal region" (DGCR). However, there are patients whose deletion has no overlap with those two types of deletion [14,15] or patients who have no detectable deletion in the 22q11 [16–18]. In the 22q11.2, there are seven low copy repeat elements (LCR22s), two of which appear to be involved in generating the 1.5-Mb deletion through unequal recombination events during meiosis [19,20]. Thus, there have been enormous efforts made to identify genes locating in the 1.5-Mb deletion region and to correlate with any one of complex clinical phenotypes [11–13].

We determined the entire euchromatin sequence of human chromosome 22 and identified 545 genes including 247 known genes and 298 probable genes [21]. Since the initial computational annotation was performed mainly with the homology search against existing transcript database, all of probable genes must be

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confirmed for their existence by the experimental method. It is also quite possible that substantial number of genes might have not been found by the computational method. Hence, we have carried out manual evaluation of computational results followed by experimental isolation of cDNAs. The *DGCR8* was found indeed as a novel gene in the DGCR. Here, we describe initial characterization of human *DGCR8* and its mouse ortholog *Dgcr8* in terms of the genomic structure, protein motifs, transcript variants, and expression profiles in the human/mouse tissues and developing mouse embryos. Based on these observations, we postulated a possible involvement of *DGCR8* in the molecular pathogenesis of the DGS/CAFS/VCFS.

Materials and methods

Computer analysis of human genome sequence and protein structure. The nucleotide sequence of human BAC clone p158l19 located in the DGCR was obtained through the GenBank/EMBL/DDBJ database (Accession No. AC006547). The nucleotide sequence homology was searched against nr, EST, and HTGS databases using BLAST through the NCBI WWW server (http://www.ncbi.nlm.nih.gov/BLAST/) after masking the repetitive elements by RepeatMasker2 (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). Protein motif was predicted by Pfam (http://www.sanger.ac.uk/Software/Pfam/) and SMART (http://smart.embl-heidelberg.de/).

cDNA cloning. The cDNA cloning was performed using PCR-amplified products from brain cDNA library of Multiple Tissue cDNA (MTC) Panels (Clontech, Palo Alto, CA). Two pairs of PCR primers hF1 (5′-TGCGGCGGTCGGTCGGTGAG-3′) and hR1 (5′-CCTACCCCGTCACCAACACTC-3′), hF2 (5′-GGCAATTCAATCGGGAAATG-3′) and hR2 (5′-CGCCATGACGTATTCACTCTTCT-3′) were designed, which were later identified to correspond to exons 1 and 2, exons 5 and 11, respectively. PCRs were performed using both KOD plus and KOD Dash PCR systems (Toyobo, Japan). The PCR-amplified products were cloned into *Hin*cII site of pUC118 plasmid vector (Takara, Japan) and used as templates for nucleotide sequencing. The sequence information was deposited in the DDBJ database (Accession No. AB050770).

To identify human *DGCR8* and mouse *Dgcr8* transcripts, both human fetal heart cDNA library and mouse 7-day embryo cDNA library (Clontech, Palo Alto, CA) were screened using the appropriate probes labeled with [α-^{32}P]dCTP. The hybridization was performed at 65 °C for overnight. The filter was washed once at 65 °C in 2× SSC/0.5% SDS, and then once at 65 °C in 0.5× SSC/0.5% SDS. Positive recombinant phage plaques were purified and phage DNA was isolated. DNA inserts were obtained by PCR and cloned in the *Eco*RV site of pBluescript II KS (-). Nucleotide sequences were obtained on a Perkin–Elmer ABI 377 DNA sequencer. Assembly and analysis of DNA sequences were performed using the gap4 program (Staden Package http://www.mrc-lmb.cam.ac.uk/pubseq/) [22]. All PCR primers were designed using OLIGO software package (National Bioscience, Plymouth, MN).

Northern blot hybridization. The probe for human transcripts was PCR product amplified using primers hF1 and hR1. The probe for mouse tissues was amplified using primers mF1 (5′-AGAGGCGAATGGAGGAAAAA) and mR2 (5′-CTTCATCTCACGGTTGAACTGA-3′) from 7-day embryo cDNA library. Amplification was carried out using KOD plus PCR system (Toyobo, Japan) following the cycling conditions: denaturation at 94 °C for 2 min followed by 30 cycles of 95 °C for 10 s, 58 °C for 2 s, and 72 °C 15 s, with a final 5-min incubation at 72 °C. Multiple Tissue Northern (MTN) blots (Clontech) prepared from the human adult and fetal tissues as well as mouse adult and embryo tissues were hybridized under the following conditions: probes were labeled with [α-^{32}P]dCTP using nick translation labeling kit (Vysis, Downers Grove). The hybridization was performed at 42 °C for overnight. The blot was washed once at 65 °C in 2× SSC/0.5% SDS and then once at 65 °C in 0.5× SSC/0.5% SDS. Autoradiogram was carried out using IP plate (Fujifilm, Japan) for 24 h and then hybridization signals were detected by FLA-3000G system (Fujifilm, Japan).

Collection of mouse embryos. Embryos were obtained from a breeding colony of Jcl:ICR mice (CLEA Japan, Tokyo) maintained on an 8:00 to 20:00 light–dark cycle, with morning of the day of observation of a vaginal plug defined as E0.5 [23]. Mothers were killed and the embryos were harvested, fixed, and subjected to whole-mount in situ hybridization at varying time points.

In situ hybridization. In situ hybridization was performed using digoxigenin (DIG)-labeled anti-sense riboprobes synthesized from the mouse cDNA clone *Dgcr8*. The cDNA clone was obtained from I.M.A.G.E. clones (Accession No. AA217940), linearized with restriction endonucleases, and riboprobe was synthesized by T3, T7 RNA polymerase (Ampliscribe T3/T7 High Yield Transcription Kits, Epicentre Technologies, Madison, WI).

The mouse embryos at various stages were treated with proteinase K (20–30 μg/ml) for 15–30 min at 37 °C depending on their stage of development. Pre-hybridization was performed at 70 °C for 2 h in a solution containing 50% formamide, 5× SSC, 2% blocking solution (Roche Diagnostics, GmbH, Mannheim, Germany), 0.1% Triton X-100, 10 mM Pipes, 5 mM EDTA, 50 μg/ml heparin, and 1 μg/ml yeast tRNA. Hybridization was performed in the same solution with the DIG-labeled riboprobes for 15 h. After a series of washes, embryos were treated in the 2% blocking solution for 1 h and incubated with anti-DIG antibody (Roche Diagnostics) for overnight at 4 °C. After washing by KTBT Buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM KCl, 0.1% Triton X-100, and 2 mM levamisole), the color reaction was performed at room temperature in the substrate color reaction mixture (NBT/BCIP) for 1 h and terminated by washing embryos five times with PBS-0.1% Tween 20, and passing 50% glycerol/PBS-0.1% Tween 20 buffer. In situ hybridization on sections was performed as described previously [24].

Results

Isolation of human DGCR8 cDNA

In the most distal part of DGCR, an interval between *T10* and *HTF9C* was a relatively long (>30 kb) region without genes. Although no transcript data were found by homology search in the database, a set of gene prediction programs suggested a novel gene in this region, where a high value of GC and CpG contents was found just before the predicted first exon (Fig. 1A). This prediction was assured by PCR analysis with a primer pair designed to cover two predicted exons using various cDNA sources as templates. The PCR amplified a unique DNA fragment from tissues including fetal heart, testis, and brain. This DNA fragment was later identified to contain a sequence covering exon 5 to exon 11 of the novel gene. Then, we attempted to isolate cDNA clones for this gene (designated *DGCR8*) from fetal heart cDNA library using PCR-amplified products as probes. Consequently, we have successfully isolated

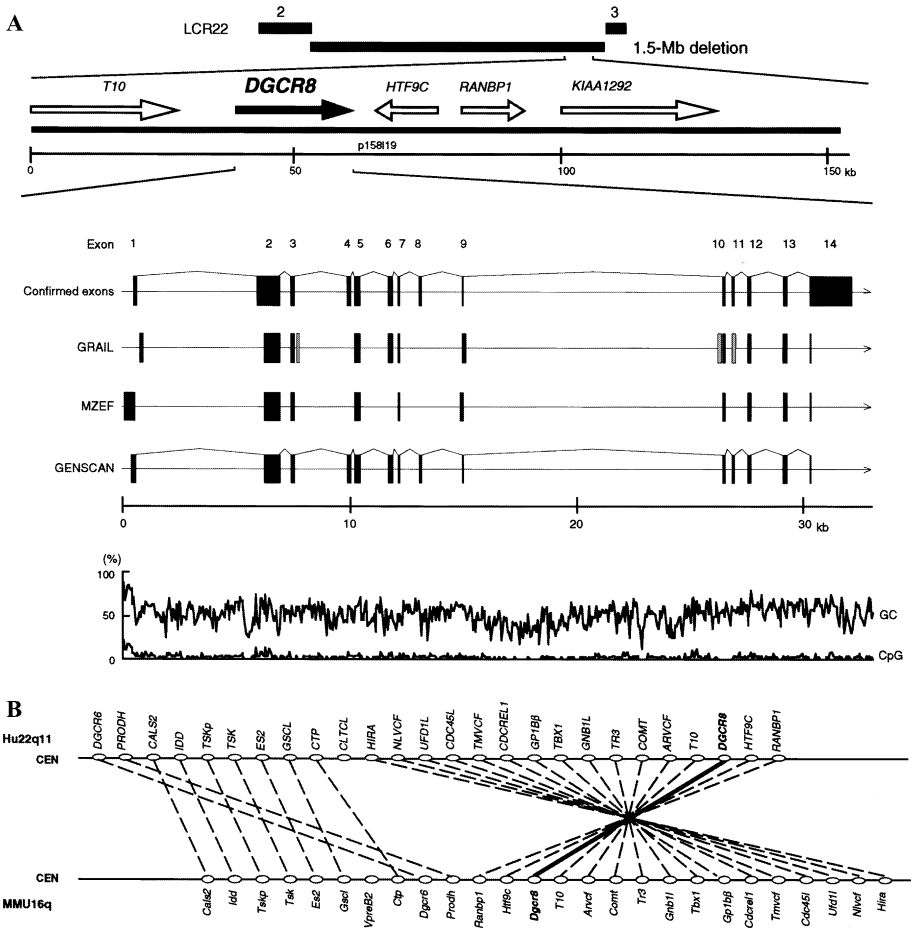

Fig. 1. A novel human gene *DGCR8* and mouse syntenic region including *Dgcr8*. (A) Location of a novel human *DGCR8* in the 1.5-Mb deletion region of chromosome 22 (top), results of gene prediction software (GRAIL, MZEF, and GENSCAN) (middle), GC and CpG contents (bottom). In the middle panel, solid and gray boxes in GRAIL lane stand for 'excellent' and 'good' scores, respectively. GC and CpG contents were calculated by window/scroll sides of 50 bp. (B) Comparison of the gene maps of the human chromosome 22q11.2 and mouse chromosome 16q.

cDNA clones covering the entire transcript. The full-length cDNA was 4466 bp in size and its open reading frame (ORF) was 2322 bp encoding a protein of 773 amino acids. Comparison of the cDNA sequence with the genomic sequence of human BAC clone p158l19 indicated that the *DGCR8* consists of 14 exons, spanning over 35 kb in size.

Isolation of mouse Dgcr8 cDNA

Next, we attempted to isolate the mouse ortholog of human *DGCR8*. Homology search using human *DGCR8* cDNA sequence identified that mouse ortholog *Dgcr8* resides in the mouse BAC clone 72k21 (Accession No. AC003060) located on mouse chromosome 16q, particularly the region syntenic to the human chromosome 22q11.2. Therefore, we designed PCR primers based on the results of comparison between the genomic sequence of mouse BAC clone 72k21 and cDNA sequence of human *DGCR8*. Then, PCR-amplified product was used as probe for the screening of the mouse 7-day embryo cDNA library. Consequently, we have isolated cDNA clones for a mouse *Dgcr8*. The full-length cDNA was 4226 bp in size and its ORF was 2322 bp, encoding a protein of 773 amino acids. Comparison of the cDNA sequence with the genomic sequence of mouse BAC clone 72k21 revealed that *Dgcr8* also consists of 14 exons like human *DGCR8* and its genome size is about 35 kb (data not shown). Comparison of the ORF nucleotide sequences between mouse *Dgcr8* and human *DGCR8* showed 90.6% identity and the deduced amino acid sequences between mouse and human proteins showed 95.3% identity.

Chromosomal mapping of human DGCR8 and its mouse ortholog

We analyzed the 1.5-Mb deletion region of chromosome 22q11.2 associated with the DGS/VCFS/CAFS and confirmed the location of the novel human *DGCR8* in this small region between two genes *T10* and *HTF9C*. Similarly, mouse ortholog *Dgcr8* was located between two genes *T10* and *Htf9c* on the syntenic region of mouse chromosome 16q (Fig. 1B). Interestingly, the

neighboring 16 genes (*HIRA* to *RANBP1*) are arranged in the same order for both human and mouse. However, the chromosomal segments containing these genes are flipped in mouse as compared to humans, so that the orientation of human and mouse genes is opposite.

Motif analysis of human DGCR8 and mouse Dgcr8 proteins

To identify protein motifs, the amino acid sequences of both human DGCR8 and mouse Dgcr8 proteins were analyzed by the program Pfam through WWW server. We found that the novel protein possesses two double-stranded (ds) RNA binding motifs (DSRMs; Pfam Accession No. PF00035) in the C-terminal half and one WW motif (Pfam Accession No. PF00397) in the N-terminal half.

Expression analysis of human DGCR8 and its mouse ortholog

Northern blot analysis was performed using the PCR product as a probe, which was derived from a region spanning exon 1 and exon 2 of the human *DGCR8* cDNA. Three different transcripts of 1.5, 3.5, and 4.5 kb were found (Fig. 2A). The major transcript of 4.5 kb was found in all the adult and fetal tissues tested, whereas two additional minor transcripts of 1.5 and 3.5 kb were found only in the testis. However, when the probe covering exon 5 to exon 11 was used, only 4.5-kb transcript was found (data not shown).

We also analyzed the expression profile in mouse using the probe spanning exon 4 to exon 6 of mouse *Dgcr8* cDNA. Two transcripts of 3.5 and 4.5 kb were found in all the adult mouse tissues as well as mouse embryos at different developmental stages except that 7-day embryo produced only 4.5-kb transcript (Fig. 2B).

Expression of Dgcr8 in mouse embryos

We performed in situ hybridization on tissue sections and whole-mount of mouse embryos using anti-sense and sense RNA probes which were derived from a region between exon 11 and exon 14 of mouse *Dgcr8*. In situ hybridization on the tissue sections indicated that *Dgcr8* expression is detectable at stage E12.5 in the vascular mesenchymal cells surrounding palatine shelves and vessels of aorta and pulmonary trunk (Figs. 3A, C,

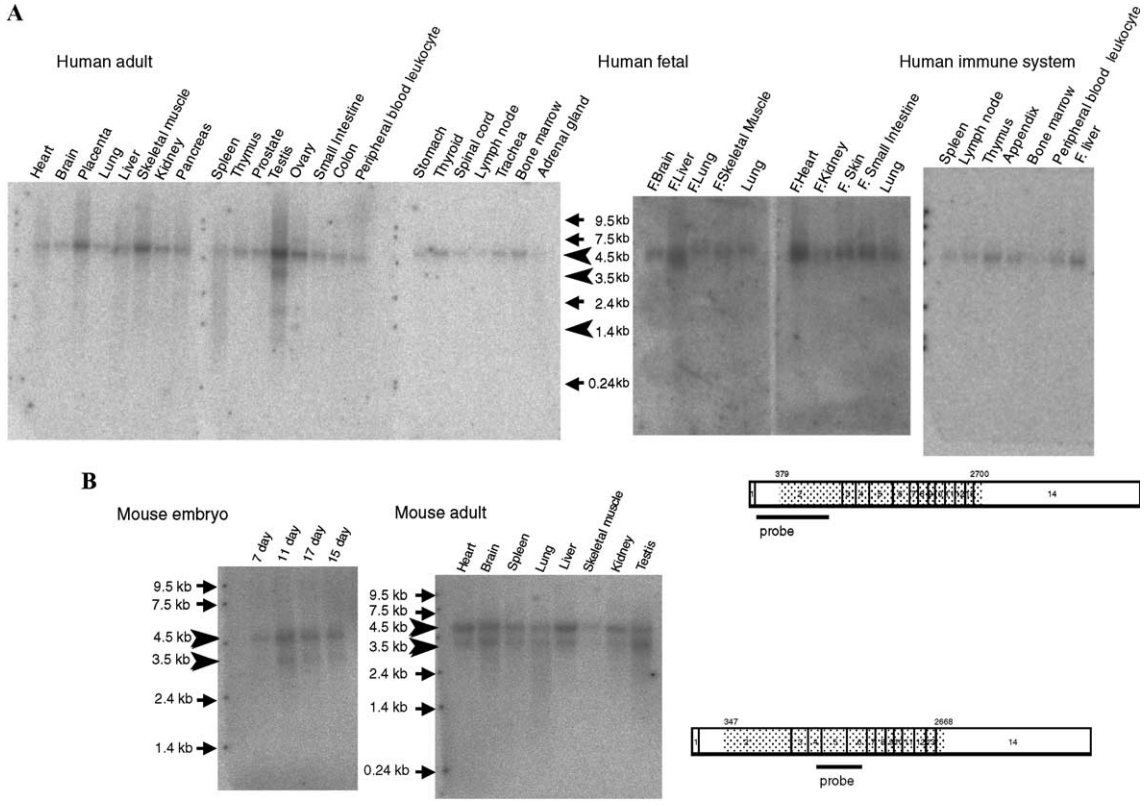

Fig. 2. Northern blot analysis of *DGCR8*/*Dgcr8* gene expression with multiple tissue panels of human and mouse. A set of autoradiograms for *DGCR8*/*Dgcr8* and a schema of the cDNA are shown. Gray area in the schema represents a coding region with nucleotide numbers of the start codon and stop codon. The position of each probe is shown as a bar. (A) Human *DGCR8*: a single 4.5-kb transcript is seen in all the human adult and fetal tissues except testis, in which three transcripts of 1.5, 3.5, and 4.5 kb are seen. (B) Mouse *Dgcr8*: two transcripts (3.5 and 4.5 kb) are seen in all the tissues of adult mouse and embryos at different stages except 7-day embryo. Arrowheads indicate the position of transcripts and arrows indicate markers.

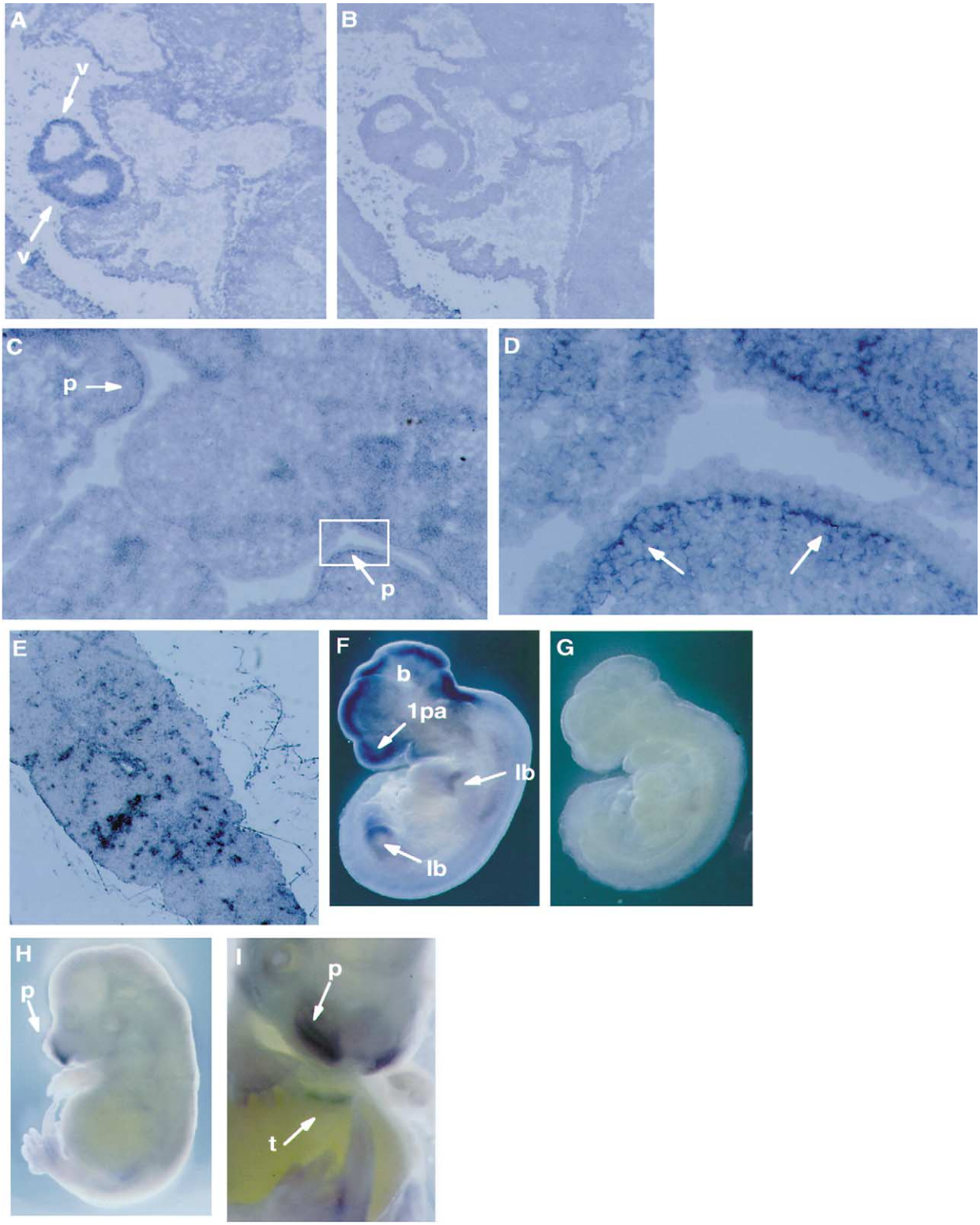

Fig. 3. In situ hybridization analysis of mouse *Dgcr8* gene expression. (A) Sagittal section of mouse embryo at E12.5. Expression is seen in vessels (v) with an anti-sense probe. (B) A negative control with a sense probe using the section adjacent to that used for (A). (C) Horizontal section of mouse embryo at E12.5. Expression is seen in palatine shelves (p; 20×). (D) Horizontal section of mouse embryo at E12.5. Same as (C) with higher magnification (50×). Arrows indicate palatine shelves. (E) Horizontal section of adult mouse shows expression of *Dgcr8* in thymus. (F) At E11.0, expression is seen in neuroepithelium at primary brain (b), pharyngeal arch first region (1pa), and limb buds (lb). (G) A negative control with a sense probe using the section adjacent to that used in (F). (H) Lateral view of mouse embryo at E14.0. Expression is seen in the palate (p) precursor. (I) Frontal view of mouse embryo at E14.0. Expression is seen in the palate (p) and thymus (t) precursors.

and D). The adult thymic medulla also showed the expression of *Dgcr8* (Fig. 3E). The whole-mount in situ hybridization also exhibited this *Dgcr8* expression in the pharyngeal arch first region including palate precursors at stage E11.0 (Fig. 3F). Additionally, the embryos of this stage showed signals in the developing limb bud and neuroepithelium of primary brain (Fig. 3F). Furthermore, at stages E13.5 to E14.0, expression was more specific in the palate (Figs. 3H and I) as well as thymus precursors (Fig. 3I).

Discussion

We have isolated and characterized a novel human gene *DGCR8* which is located in the DGCR of chromosome 22. We also isolated a mouse ortholog *Dgcr8* from the syntenic region of mouse chromosome 16q. Both human and mouse genes consist of 14 exons in a similar genomic structure spanning over 35 kb and encode a protein of 773 amino acids possessing one WW motif and two DSRM motifs.

The DSRM motif consists of ~70 amino acids in which basic and hydrophobic amino acid residues are dispersed throughout. The DSRM motif is found in many proteins that bind dsRNAs or single-strand RNAs with extensive duplex secondary structure [25–27]. In general, the protein family with DSRM motif is known to play important roles in translation, RNA processing, and RNA editing and appears to be involved in the embryonic pattern formation [26,27].

The WW motif consists of 38–40 amino acids and is widely found among various proteins involved in the structural, regulatory, and signaling roles [28,29]. The WW motif is named after two highly conserved tryptophan (W) residues spaced ~20–22 amino acids apart. The WW motif is implicated in mediating protein–protein interactions by binding to proline-rich motif, such as PPxY [30]. Furthermore, the WW motif and its interacting ligand, which include viral Gag proteins, sodium channels, interleukin receptors, and a subgroup of serine/threonine kinases, have been implicated as playing roles in various disease states including Liddle's syndrome (a genetic form of hypertension) and muscular dystrophy [28]. Interestingly, the novel DGCR8 and Dgcr8 proteins possess both DSRM motif and WW motif and hence we assume that they must play a significant biological role. Further study is necessary to obtain direct evidence for RNA interaction with DSRM motif and ligand binding with WW motif of these novel proteins.

Northern blot analysis of human and mouse tissues showed three different transcript variants: a major transcript of 4.5 kb is found ubiquitously in many tissues and two minor transcripts of 3.5 and 1.5 kb are found solely in the testis (Fig. 2A). A cDNA clone of 907 bases was found to be registered in the GenBank/EMBL/DDBJ database (Accession No. AF165527) which is composed of the same sequence exon 1 to exon 2 and initial 92 bases of intron 2 containing a stop codon and a potential poly(A) signal. We believe this registered cDNA is probably a 1.5-kb transcript found in the testis. Since the 3.5 and 1.5-kb transcripts were not detected by the probe for exon 5 to exon 11 but detected by the probe for exon 1 to exon 2, it is likely that the 3.5-kb RNA is also a splicing variant. It remains to be clarified whether these minor transcripts may play any distinct roles in the testis.

The whole-mount in situ hybridization of mouse developing embryos revealed that mouse *Dgcr8* transcripts are detectable in neuroepithelium of primary brain, limb bud, vessels of aorta and pulmonary trunk, thymus, and around the palate. Regarding these observations, it is worthy to note that a number of candidate genes for the DGS/CAFS/VCFS including *CRKL*, *UFD1L*, *HIRA*, and *TBX1* and their mouse orthologs have been reported [31–35]. Each of these genes accounted for only a part of the DGS/CAFS/VCFS phenotypes and none of them are definitively proven. However, the expression profile of *Dgcr8* in developing mouse embryos seems consistent with the clinical phenotypes associated with the DGS/CAFS/VCFS. Especially, the prominent expression of *Dgcr8* in the limited part of palate strongly suggests that this gene is likely to be involved in the palatine shelves and the hemizygosity of the gene would possibly cause the cleft palate associated with the DGS/CAFS/VCFS. Taking all these observations together, we postulate that the *DGCR8* should be considered as an additional candidate gene for the DGS/CAFS/VCFS, especially for the palate-related symptoms. Further analysis of *DGCR8* and its mouse ortholog may provide new clues to better understand the complex clinical phenotypes of the DGS/CAFS/VCFS.

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