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Human Homologue Sequences to the *Drosophila dishevelled* Segment-Polarity Gene Are Deleted in the DiGeorge Syndrome

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Summary

DiGeorge syndrome (DGS) is a developmental defect of some of the neural crest derivatives. Most DGS patients show haploinsufficiency due to interstitial deletions of the proximal long arm of chromosome 22. Deletions of 22q11 have also been reported in patients with the velo-cardio-facial syndrome and familial conotruncal heart defects. It has been suggested that the wide phenotype spectrum associated with 22q11 monosomy is a consequence of contiguous-gene deletions. We report the isolation of human cDNAs homologous to the *Drosophila dishevelled (dsh)* segment-polarity gene. Sequences homologous to the 3′ UTR of these transcripts (*DVL-22*) were positioned within the DGS critical region and were found to be deleted in DGS patients. Human *DVL* mRNAs are expressed in several fetal and adult tissues, including the thymus and, at high levels, the heart. Two transcripts, 3.2 and 5 kb, were detected, in northern blot analysis, with different expression patterns in the surveyed tissues when different cDNAs were used. The isolated cDNAs exhibit high amino acid homology with the mouse and *Xenopus Dvl-1* gene, the only other vertebrate *dsh* homologues so far isolated. The pivotal role of *dsh* in fly development suggests an analogous key function in vertebrate embryogenesis of its homologue genes. Since DGS may be due to perturbation of differentiation mechanisms at decisive embryological stages, a *Dsh*-like gene in the small-region overlap (SRO) might be a candidate for the pathogenesis of this disorder.

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Introduction

DiGeorge syndrome (DGS; MIM 188400) is a developmental defect of the neural crest derivatives from the third and fourth pharyngeal pouches (Kirby et al. 1983, 1985; Bockman and Kirby 1984; Kirby and Bockman 1984; van Mierop and Kutsche 1986). DGS is characterized by thymus and parathyroid gland a/hypoplasia as well as by cardiac defects (Conley et al. 1979). DGS usually occurs sporadically, but it may be inherited as an autosomal dominant condition (Greenberg 1993). The incidence, based on the ascertainment of severe cases, has been estimated at 1/4,000 (Burn et al. 1995). Chromosome studies reveal a cytogenetic anomaly of the 22q11 region in approximately one-fifth of DGS patients (de la Chapelle et al. 1981; Kelley et al. 1982; Greenberg et al. 1988). Molecular cytogenetics detects submicroscopic hemizygosity of 22q11 in ~90% of DGS patients (Scambler et al. 1991; Driscoll et al. 1992*a*). DGS has an extensive phenotypic overlap with the velo-cardio-facial syndrome (VCFS; MIM 19243) (Shprintzen et al. 1978). An etiologic relationship between these two diseases has been confirmed by the demonstration of chromosome 22q11 hemizygosity in approximately two-thirds of VFCS patients (Driscoll et al. 1992*b*). Deletions of 22q11 have also been detected in patients with sporadic heart defects, notably conotruncal abnormalities, suggesting that monosomy at this locus is common in congenital heart diseases (Scambler et al. 1992; Scambler 1993; Goldmuntz et al. 1993; Driscoll et al. 1995). However, the DGS/VCFS phenotype can be wider and may include psychiatric, ocular, and renal abnormalities (Goldberg et al. 1993). The majority of DGS/VCFS patients have deletions of ≥2 Mb with a critical region of deletion overlap (i.e., SRO) contained between the markers scF5 and sc11.1a separated by ~300 kb (Halford et al. 1993; Morrow et al. 1995). These two markers flank a balanced translocation seen in a unique patient (ADU) with DGS/VCFS phenotype (Augusseau et al. 1986). It has been postulated that the translocation breakpoint disrupts a potentially major

gene involved in the pathogenesis of these syndromes (Halford et al. 1993; Wadey et al. 1995). However, it has also been suggested that the ADU translocation may disturb, via position effect, the regulation of a close gene, separating the gene itself from a control region.

DGS abnormalities are compatible with disturbances of a gene(s) important in controlling the rostral neural crest contribution to development (Payne et al. 1995). Several cDNAs have been isolated as mapping into the DGS critical region. Some of them—COMT, LZTR-1, and GpIb—map within the common deleted region but outside the SRO (Grossman et al. 1992; Budarf et al. 1995*b*; Kurahashi et al. 1995). Two additional genes—TUPLE1/HIRA and IDD/DGCR2/LAN—have been positioned within the SRO (Halford et al. 1993; Budarf et al. 1995*a*; Demczuk et al. 1995; Lamour et al. 1995; Wadey et al. 1995). They code for a transcriptional regulator and for an integral membrane protein, respectively, whose role in human embryogenesis remains hypothetical. Neither gene is disrupted by the ADU translocation.

Recently, the ADU translocation breakpoint has been cloned and sequenced (Budarf et al. 1995*a*). It spans a putative exon sequence, rnex40, with weak similarity to rat and mouse androgen-receptor genes. However, no full-length transcript has been isolated and characterized thus far. Therefore, any inference about the functional role of the rnex40 gene in the pathogenesis of DGS and related syndromes must await more-conclusive studies.

We isolated and characterized several human-homologue cDNAs of the *Drosophila dishevelled* polarity gene, which is required for the establishment of fly embryonic segments (Perrimon and Mahowald 1987). A human cDNA 3′ UTR–like sequence (designated "*DVL-22*") was positioned within the DGS SRO and was demonstrated to be deleted in DGS/VCFS patients.

Material and Methods

cDNA Direct Selection

Total yeast DNA was partially digested with *Sau*3A and was subcloned into superCos cosmid vector (Stratagene). Three thousand cosmids were plated and probed with ^{32}P-dCTP–labeled total human genomic DNA. Twenty randomly picked human clones were isolated and digested to completion with *Sau*3A. Fragment protruding ends were filled by use of a deoxynucleotide mix containing biotin-conjugated dUTP (Boehringer-Mannheim) and Klenow fragment of DNA polymerase I. Total RNA was extracted from human fetal brain and sympathetic ganglia by use of the single-step acid-guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). Fetal tissues were obtained according to the current Italian regulation. Poly-A$^+$ RNA was isolated by filtration on oligo-dT cellulose. Poly-A$^+$ RNA (5 μg) was treated with 1 U of RNase-free DNase (Pharmacia) in 20 μl of final solution at 37°C for 1 h. After DNase heat-inactivation, double-strand cDNA was synthesized by use of the TimeSaver cDNA synthesis kit (Pharmacia). One microgram of poly-A$^+$ RNA was retrotranscribed by use of 0.5 μg of oligo-dT and 0.75 μg of random hexamers. Two hundred micrograms of cDNA were ligated to *Not*I linkers. After *Not*I digestion, 200-800-bp cDNA molecules were size selected on 1% agarose gel and ligated to an *Not*I adapter (5′ GGC CGC AAG CAT GCG AAT TCA GGA TCC 3′). One to four nanograms of cDNA was amplified by PCR using an oligonucleotide complementary to the adapter sequence. Amplified cDNA (500 ng) and biotin-labeled cosmid fragments (50 ng) were denatured and hybridized for 48 h at 65°C. The hybridization was performed in 100 μl of 5 × SSPE, 5 × Denhardt's, 0.1% SDS. Ten micrograms of human genomic DNA, 5 μg of yeast DNA, and 1 μg of pBR 322 were used to compete for repetitive sequences. After hybridization, 50 μl of streptavidin-conjugated magnetic beads (DYNABEADS®; Dynal) were added to the mix. The beads/biotinylated YAC fragments/cDNAs complexes were magnetically separated. After being washed in 0.5 × SSC at 65°C for 15 min, the cDNAs were recovered by boiling (5 min at 100°C). cDNAs were amplified by PCR using the oligonucleotide complementary to the *Not*I adapter sequence and were cloned in a T-cloning vector (pBluescript; Stratagene). Single cloned cDNAs were amplified by PCR using the adapter primer. PCR products were blotted onto nylon membrane (Hybond N; Amersham) and hybridized to human genomic DNA, yeast genomic DNA, and pBR 322, in order to rule out clones containing repetitive sequences, vector DNA contaminants, and ribosomal cDNAs. The remaining cDNAs were hybridized to grid-arrayed cosmid clones. Those hybridizing one or more cosmids were sequenced by use of the automated ABI 370A DNA Sequencer and were characterized.

Northern Analysis

Northern blot filters were purchased from Clontech Laboratories and were probed according the manufacturer's instruction. The filters were washed at a final stringency of 0.1 × SSC, 0.1% SDS at 65°C. Human beta-actin (Clontech) was used as an internal standard.

Library Screening

One million cDNA clones of a human adult caudate nucleus library in lambda ZAPII (Stratagene) were plated. Phage were replicated onto nylon membranes, hybridized for 24 h to ^{32}P-dCTP–labeled probes, and were washed at a final stringency of 0.1 × SSC, 0.1% SDS at 65°C. After three rounds of selection, pBluescript phagemids were rescued from the positive clones, according to the manufacturer's instructions. Clone length was checked by PCR amplification of the inserts by use of universal and reverse M13 primers.

FISH

Metaphase chromosomes were prepared from human peripheral blood lymphocytes obtained from DGS/CVFS patients and normal controls, as described elsewhere (Baldini and Ward 1991). Purified cosmid DNA was labeled with biotinylated dATP and digoxigenated dUTP by nick translation (Life Technologies) and hybridized to chromosome spreads, according to routine procedures. Chromosomes were counterstained with DAPI and were visualized as described elsewhere (Baldini and Ward 1991; Calabrese et al. 1994). Fiber-FISH analysis was performed by use of sodium hydroxide–treated slides. Cosmid clones either were biotin-labeled and detected with fluorescein isothiocyanateF–avidin or were digoxigenin-labeled and detected with anti-digoxigenin rhodamine–conjugated antibody. Images were charge-coupled–device captured and were merged through a Vidas Image Analyzer (Zeiss) (Fidlerova et al. 1994).

Results

Isolation and Mapping of Human Sequences Homologous to dishevelled (dsh)

YAC clones 966a8 and 706b10 were isolated from the CEPH YAC library by PCR screening, by use of oligonucleotides designed on the 3′ UTR of the *TUPLE1* gene (TUP1 5′ CAG TCA TCG GGC AGA ACC TCC G 3′ and TUP2 5′ AGC TGG GCT GGC GCT GGT GC 3′). Both YACs were subcloned in cosmids and were used for the direct selection of human fetal CNS and peripheral nervous system (PNS) transcripts. Trapped cDNA sequences were assigned to single cosmid subclones by dot-blot hybridization. Positive cosmids were mapped by FISH. Two overlapping cosmid clones—co29 and co37—hybridizing to a single 200-bp cDNA (D12) were positioned within the DGS SRO. Fiber-FISH experiments showed the cosmid position to be ~10 kb from the *TUPLE1* gene (fig. 1*A*), between the TUPLE 1 gene itself and the more centromeric *IDD* gene. As expected, co29 and co37 revealed the 22q11 hemizygosity in a panel of 7 VCFS patients, previously found deleted by use of *TUPLE1* probes (fig. 1*B*). GenBank homology search revealed that the last 60 bp of cDNA D12, just preceding the polyA stretch, were identical to the 3′ end of the *Dvl-1* cDNA, the mouse homologue of the *Drosophila* segment-polarity gene *dishevelled* (*dsh*) (Sussman et al 1994).

cDNA D12 was used for screening a human adult caudate cDNA library. Four independent cDNA clones—c3, c5, c8, and c10—were isolated. The complete sequence of cDNA c3 and cDNA c5 showed an identical ~700-bp insert, with the PolyA stretch at one end (fig. 2*A*), representing part of the 3′ UTR of a novel human gene. The identity to the trapped cDNA D12 was absolute. The last 60 bp of the cDNAs were identical to the mouse *Dvl-1* gene (fig. 2*B*). cDNA c8 (1,600 bp) covered a whole 1,000 bp of the 3′ UTR and 600 bp of the coding region. cDNA c10 was 1,400 bp long and contained a 115-bp coding region (homologous to the corresponding region of *Dvl-1*) and a complete 1,250-bp 3′ UTR (350 bp longer than c8) (fig. 2*A*).

The homology between human c8 and c10 cDNAs and the mouse *Dvl-1* in the longest open reading frame was >85%. In contrast, the rest of the human *DVL* 3′ UTR did not show any significant homology to the mouse, except in the last 60 bp. Interestingly, the mouse *Dvl-1* 3′ UTR contains a 12-bp tandemly repeated sequence also present in all the isolated human clones, where it is present as a single copy. It is possible that it represents a regulating element, for either RNA transcription or RNA processing. In order to rule out the chimerism of the original YACs, an ~100-kb fosmid contig obtained from a specific chromosome 22 library and mapping centromeric to *TUPLE 1,* was hybridized with three cDNAs—c5, c8, and c10—providing positive signals in a single clone (A7). These data confirm the presence and position of human *DVL* sequences in the DGS/VCFS SRO. Using a synthetic oligonucleotide designed on the very end of the mouse *Dvl-1* cDNA sequence (D12: 5′ CAT TTA CAC AGA AGC AGC TCT ATG 3′), we directly sequenced ~200 bp of both co29 and cosmid A7. The resulting sequences were identical to each other and highly (90%) homologous to the human *DVL* cDNA 3′ UTR sequence (GenBank accession numbers V46461 and V46462).

In order to complete the human *DVL* cDNA sequence, we amplified, by PCR, the 5′ *DVL*-coding region. Oligonucleotides *Dvl-1* (5′ ATG GAC GAG GAG GAG ACG CCG TAC 3′) and *Dvl-3* (5′ GAA ACC ACC CGG CCA TTG AAG CAG 3′) were designed on the mouse *Dvl-1* cDNA sequence and were used in restriction transciption–PCR on human fetal brain cDNA. A 200-bp fragment (*Dvl-1* and *Dvl-3*), starting 27 bp after the first ATG, was amplified, cloned, and sequenced (fig. 2*A*). cDNA *Dvl-1*/*Dvl-3* showed 97% identity to the corresponding mouse sequence. *Dvl-1*/*Dvl-3* was used for cDNA library walking. Several positive cDNAs were isolated. A 1.8-kb positive clone (4B) overlapped the c8 sequences and was completely sequenced (fig. 2*C*).

Figure 2*C* shows the amino acid homologies between the mouse *Dvl-1* and the protein product predicted from the human 4B and c8 cDNAs sequences. As shown, a domain of 25 amino acids, corresponding to positions 377–401 of the mouse sequence, is lacking in the putative human protein derived from the c8 cDNA sequence. According to Sussman et al. (1994), the absence of this domain in the mouse *Dvl-1* transcripts is typical of embryo-derived isoforms. However, cDNA c8 was isolated from a human adult caudate-nucleus library. A region of alternative splicing between clone c8 and 4B is also

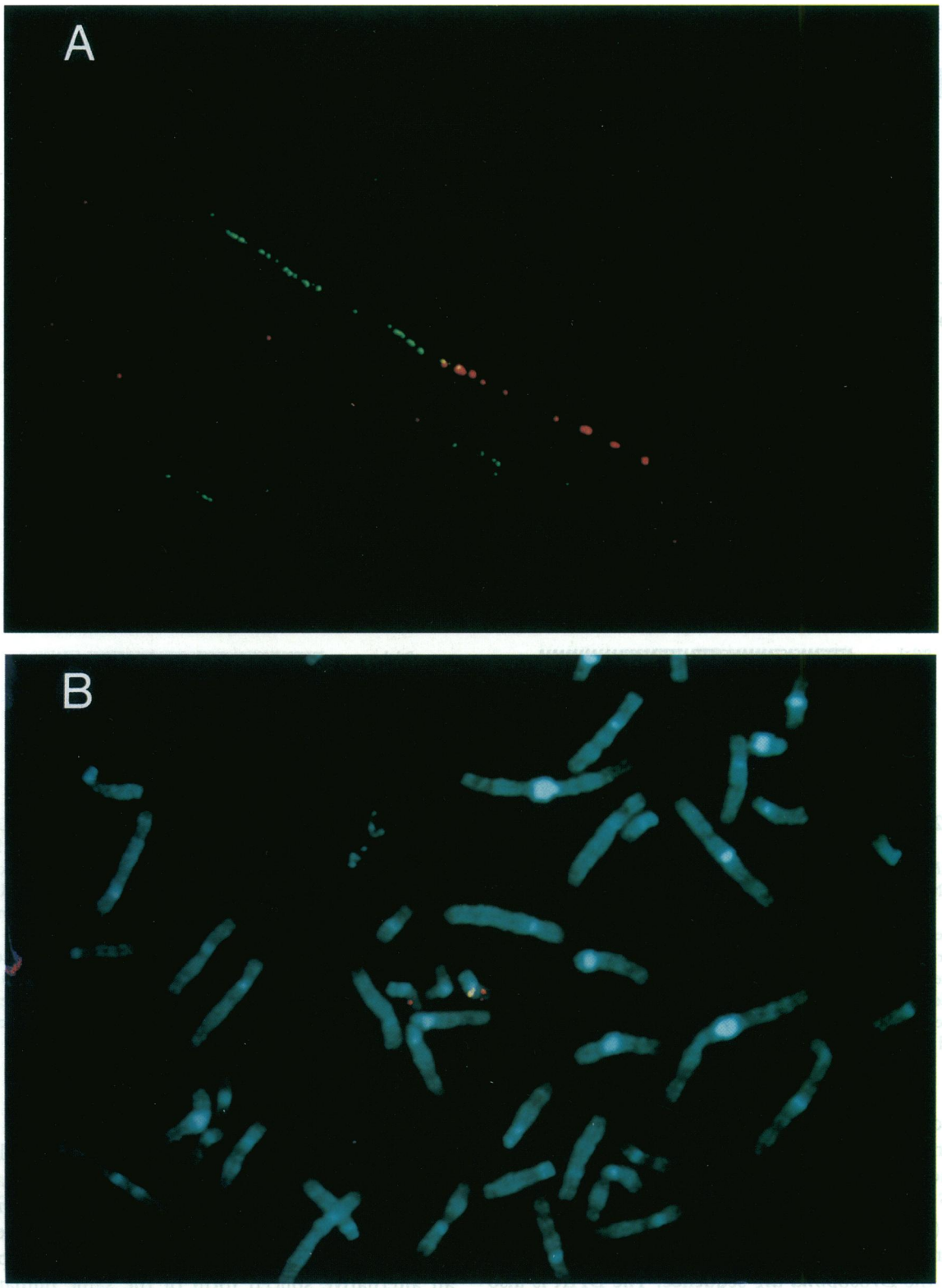

Figure 1 FISH mapping of cosmid clones. *A*, Fiber-FISH analysis with clone co29 (*green signals*) and scF5 cosmid (*red signals*). co29 and scF5 (*TUPLE1*) appear contiguous, with a partial overlap (*yellow signals* [due combined red and green fluorescence]). *B*, Analysis with clone co29 in a partial metaphase of a DGS patient, showing signals on one chromosome 22 only (*yellow spot*). A cosmid clone for the β*ARK2* gene (mapping on 22q11) was used as a control probe and indicates the chromosome 22 pair (*red spots*).

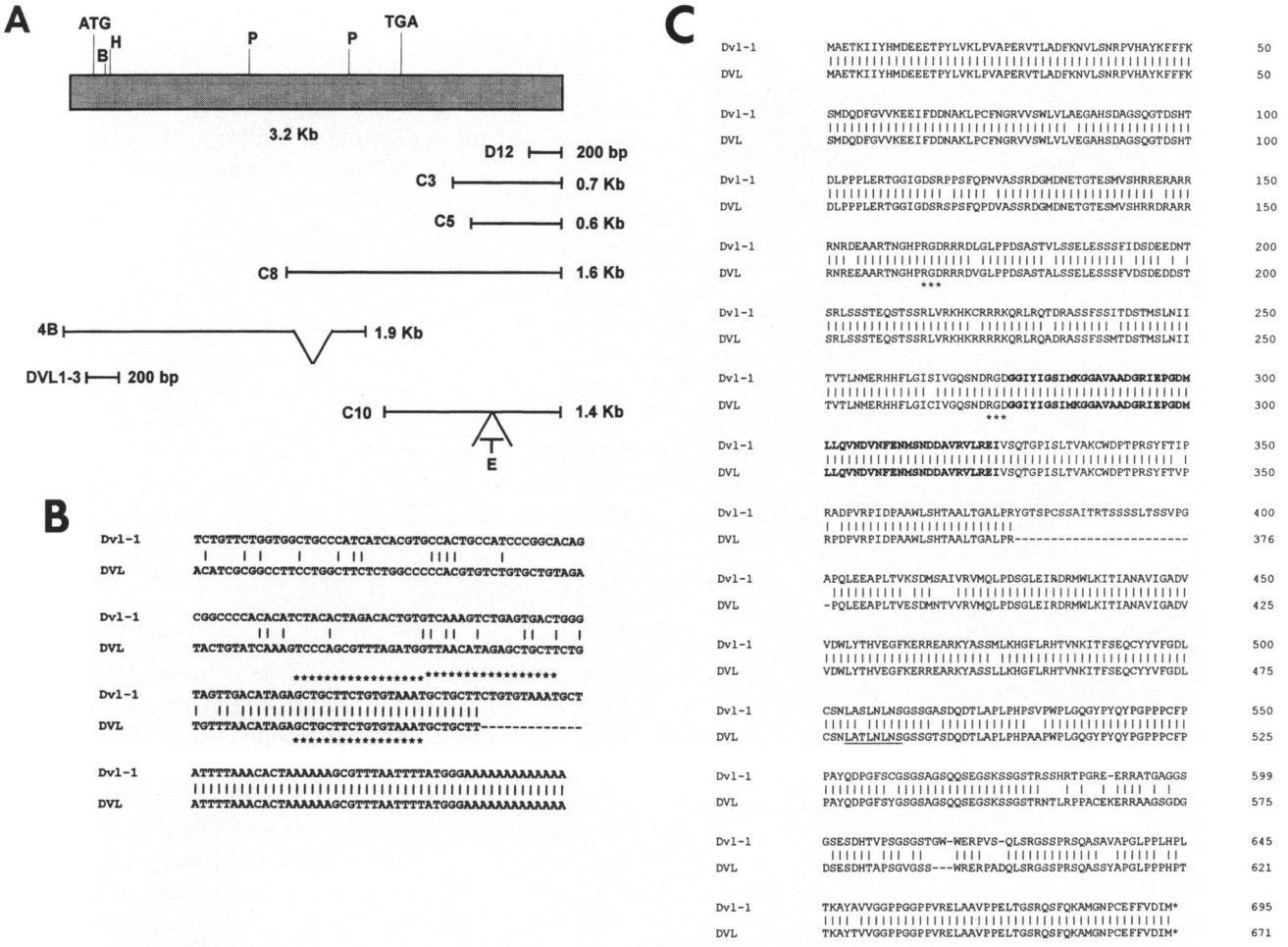

Figure 2 *A*, Graphic representation of the 3.2-kb human *DVL* transcripts. The relative position and the length of the cloned cDNAs are reported. The positions of the start and stop codons are also indicated, as well as some restriction sites. B = *Bam*HI; H = *Hin*dIII; P = *Pst*I; and E = *Eco*RI. The alternatively spliced region of clone 4B is represented by the gap in the straight line. *B*, Alignment of the 3′ end of human *DVL* cDNA to the mouse homologue, *Dvl-1*. A complete identity is evident in the last 60 bp of the sequence. The remaining 3′ UTRs are different. The mouse tandemly repeated motif (5′ TTC TGT GTA AAT CCT GCT 3′) is present in single copy in the human homologous gene. The complete cDNA sequence (*C*) has been deposited in GenBank. Comparison is between the *Dvl-1* and human *DVL1* 1 peptides derived from cDNA c8 and 4B sequences. Amino acid sequences derived from *Dvl-1* and the human cDNAs are aligned, with sequence identity represented by vertical lines. A domain of 25 amino acids (positions 377–401 of the mouse sequence) is lacking from the putative human protein derived from the sequence of cDNA clone C8. The underlined region corresponds to an alternatively spliced exon. The conserved *dlg*-like sequence is shown in boldface. Two RGD recognition sequences are underscored with asterisks. The complete peptide sequence will be deposited in GenBank.

reported from nucleotide 479 to nucleotide 486 of *DVL*. A 48-amino-acid region from position 277 to position 324 shows homology to a sequence known as the "GLGF repeat," or "disk-large homology region" (DHR) (Bryant and Woods 1992). This motif is common to other proteins supposed to be part of cell junctions, such as the *Drosophila dlg* sequence. The DHR is also present as an identical sequence in all *dsh*-related genes known at present. Human *DVL* contains two RGD (arginine-glycine-aspargine) tripeptide sequences, a motif that is associated with recognition systems for cell-surface signaling in proteins shown to play a role in cell adhesion. RGD sites are not present in the *Drosophila dsh* but are conserved in the mouse and *Xenopus* gene.

Expression Analysis

Northern blot analysis using clone c10 revealed two transcripts: one 5 kb and one 3.2 kb (fig. 3*A* and *B*). cDNA c8 reveals only a single band, of 3.2 kb (fig. 3*C*). In a comparison of the sequence identities between c10 and the other cDNA clones, it is likely that it represents an alternative spliced form of the same human *DVL* gene, with a larger 3′ UTR. However, the possibility that c8 and c10 represent the 3′ UTR of strictly related genes cannot be ruled out. There must be more than one *DVL* gene in the human genome. In fact, the library screening performed by use of *Dvl-1*/*Dvl-3* cDNA revealed the presence of other highly homologous *Dvl-1*-like transcripts different from the c8/4B sequences and

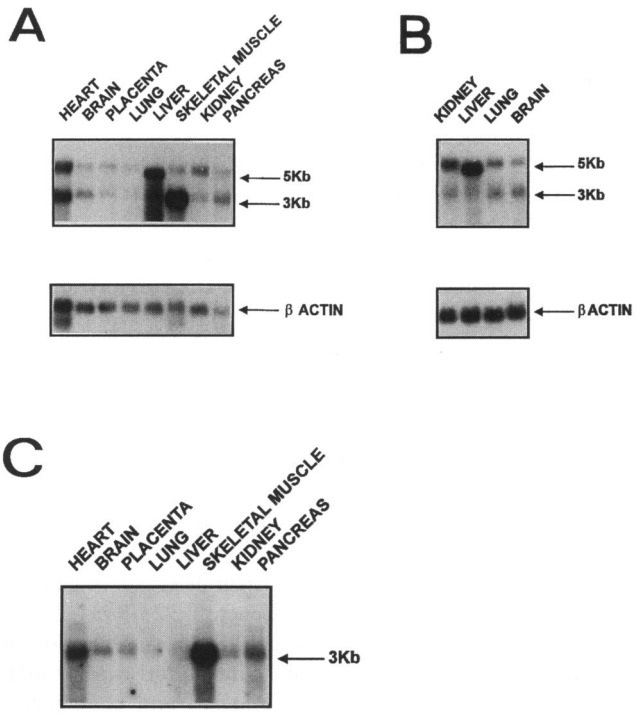

Figure 3 Expression of the human *DVL* gene in adult (*A* and *C*) and fetal (*B*) human tissues. The hybridizations were performed by use of cDNA clone c10 (*A* and *B*) and c8 (*C*) as probes. Two major RNA forms, 3.2 and 5 kb long, are revealed. Clone C8 hybridizes only the 3.2-kb form. Control hybridization to the actin probe is shown.

thus belonging to other gene units—and sometimes also sharing the same 3′ UTR.

Various fetal and adult human tissues express both the 5-kb transcript and the 3.2-kb transcript. Only the 5-kb form is expressed in the liver and in the kidney, whereas the 3.2-kb form is the predominant transcript in the brain, skeletal muscle, and pancreas. Only the 5-kb form is present in the thymus at different stages of differentiation (data not shown).

Discussion

Expressed sequences related to a new human gene family have been isolated from the DGS critical region on chromosome 22 by cDNA direct selection and have been mapped by use of clones deriving from two independent genomic sources. These sequences were demonstrated to be identical to 3′ UTR portions of a homologue of the *Drosophila dsh* segment-polarity gene.

The *Drosophila dsh* is involved in the *Wnt* signaling pathway (Theisen et al. 1994). *Wnt* genes encode secreted glycoproteins that determine cell proliferation and differentiation during normal development in both vertebrates and invertebrates (Dickinson and McMahon 1992). *Wnt* genes have been isolated from different species, including *Caenorhabditis elegans, Drosophila, Xenopus laevis,* mouse, and humans (Nusse and Varmus 1992). They are highly conserved in evolution and include the *Drosophila wingless* (*wg*) and the mouse *Wnt-1* genes (Rijsewijk et al. 1987; Siegfried et al. 1994). The *wg*/*wnt* cascade represents a common strategy, during evolution, for establishing embryonic polarity. In *Drosophila, dsh* is required for *wg* signaling, acting downstream to *wg* itself, as a transducing molecule. *dsh* codes for a cytoplasmic phosphoprotein (Yanagawa et al. 1995). The *wg* function is required for important developmental processes such as segmentation and neuroblast specification (Yanagawa et al. 1995). This suggests that both *wg* homologues and *dsh* homologues may play multiple roles in vertebrate morphogenesis as well. In fact, the demonstration of dorsalizing and neuralizing properties of the *Xenopus* homologue of *dsh* (*Xdsh*) is consistent with this idea. It is therefore possible that human *DVL* sequences act as a cell development–regulating gene in human embryogenesis also. Although there is not direct evidence of involvement of a *DVL*-family gene in DGS/VCFS, functional characteristics of *dsh* suggest that a deficiency in DVL proteins might lead to imbalance of the fate-addressing mechanisms in specific DGS/VCFS-affected cell populations. Interestingly, Wu et al. (1995) have demonstrated that *wg,* whose action is regulated by *dsh,* is required for heart development in *Drosophila.* It is therefore likely that *DVL* gene abnormalities may cause heart-development defects in humans. Northern blot analysis showed that human *DVL*-gene(s) expression (at least for the transcripts that we isolated) is not limited to specific cell lineages. It is possible that different cell populations are specifically sensitive to the dosage effect resulting from hemizygosity at *DVL* locus/loci. On the other hand, some cell populations might rely on the complementing expression of other genes with analogous function. More than one *DVL*-like sequences may exist in the human genome. On the basis of comparative mapping, the mouse *Dvl-1* gene location predicts the presence of a similar gene on human chromosome 1, but it does not exclude the existence of a wider gene family (Beier et al. 1992). We isolated and partially characterized different similar cDNAs, which map on different human chromosomes (authors' unpublished data), confirming the presence of multiple *Dvl*-like sequences in the human genome. The complete-genome characterization of these genes will allow a more accurate definition within this gene family.

DVL-22 is deleted in DGS/VCFS patients, as are the other two genes isolated from the critical region (Halford et al. 1993). Because of its position, the *DVL-22* gene should not be involved in the ADU translocation. However, this does not exclude the possibility that position effects due to the translocation rearrangement may influence the *DVL-22* expression, by separation of control regions located upstream. A similar mechanism has been suggested for cases of Grieg syndrome, campomelic

dysplasia, and aniridia, all associated with translocations placed at a great distance from the gene responsible for them (Vortkamp et al. 1991; Jordan et al. 1992; Foster et al. 1994; Wagner et al. 1994).

DVL-22 may represent an additional example of conserved genes involved in human syndromes. It is notable that it is homologous to regulators of developmental processes in *Drosophila*. It is notable that examples include the *Pax3* gene in Waardenburg 1 syndrome and Waardenburg 3 syndrome, *Pax6* in aniridia, and the *c-kit* oncogene in piebaldism (Jordan et al. 1992; Spritz et al. 1992; Tassabehji et al. 1992). The exact mechanism by which hemizygosity of *DVL-22* is associated with DGS/VCFS abnormalities will be the main target of future studies.

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