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Isolation and Characterization of a Novel Gene from the DiGeorge Chromosomal Region That Encodes for a Mediator Subunit

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Hemizygous deletions on chromosome 22q11.2 result in developmental disorders referred to as DiGeorge syndrome (DGS)/velocardiofacial syndrome (VCFS). We report the isolation of a novel gene, *PCQAP* (PC2 glutamine/Q-rich-associated protein), that maps to the DiGeorge typically deleted region and encodes a protein identified as a subunit of the large multiprotein complex PC2. PC2 belongs to the family of the human Mediator complexes, which exhibit coactivator function in RNA polymerase II transcription. Furthermore, we cloned the homologous mouse *Pcqap* cDNA. There is 83% amino acid identity between the human and the mouse predicted protein sequences, with 96% similarity at the amino- and carboxy-terminal ends. To assess the potential involvement of *PCQAP* in DGS/VCFS, its developmental expression pattern was analyzed. *In situ* hybridization of mouse embryos at different developmental stages revealed that *Pcqap* is ubiquitously expressed. However, higher expression was detected in the frontonasal region, pharyngeal arches, and limb buds. Moreover, analysis of subjects carrying a typical 22q11 deletion revealed that the human *PCQAP* gene was deleted in all patients. Many of the structures affected in DGS/VCFS evolve from *Pcqap*-expressing cells. Together with the observed haploinsufficiency of *PCQAP* in DGS/VCFS patients, this finding is consistent with a possible role for this novel Mediator subunit in the development of some of the structures affected in DGS/VCFS. © 2001 Academic Press

INTRODUCTION

Major clinical features characterizing DiGeorge syndrome/velocardiofacial syndrome (DGS/VCFS) patients are congenital heart defects, impaired cellular immunity due to thymus aplasia or hypoplasia, absence of parathyroid gland function, and facial dysmorphism (Ryan *et al.*, 1997; Vantrappen *et al.*, 1999). These abnormalities are attributed to defective early neural crest cell migration and/or differentiation in the pharyngeal arches, where neural crest cells contribute to the morphogenesis of the heart great vessels, thymus, parathyroid gland, and craniofacial structures (Kirby and Waldo, 1995). Most DGS patients have a large hemizygous interstitial deletion spanning approximately 3.0 Mb, the typically deleted region (TDR) (Driscoll *et al.*, 1992). However, no relationship between the size or site of the deletion and the complexity of the clinical phenotype was demonstrated so far. Moreover, the heterogeneity of deletions found in a few patients with atypical, shorter deletions (McQuade *et al.*, 1999; O'Donnell *et al.*, 1997) and the description of several nonoverlapping critical regions (Amati *et al.*, 1999) suggest a more complex molecular mechanism underlying the DGS/VCFS.

The DiGeorge chromosomal region has been completely sequenced (Dunham *et al.*, 1999) and several genes mapping to the TDR have been isolated and further characterized. The spatial and temporal distribution during mouse and human embryonic development of two predicted transcription regulators, *Hira* (Wilming *et al.*, 1997) and *ZNF74* (Ravassard *et al.*, 1999), respectively, and of the T-box transcription factor *TBX1* (Chieffo *et al.*, 1997) suggested a potential involvement of these genes in the morphogenesis of neural crest cell and pharyngeal arch derivatives and hence a contribution to some of the features of the DGS/VCFS phenotype. On the other hand, Yamagishi *et al.* (1999) proposed that haploinsufficiency of *UFD1L* is the cause for the DGS/VCFS phenotype. This assumption was based on the developmental expression pattern of *Ufd1* and the identification of a single patient showing all the classical symptoms of DGS and carrying a minideletion comprising *UFD1L* together

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with *CDC45L*. However, the possibility still remains that this particular patient's phenotype is a combined effect of mutations in *UFD1L* and *CDC45L*. Extensive mutational analysis in DGS patients without apparent 22q11 deletions have provided evidence for the absence of *UFD1L* point mutations (Wadey *et al.*, 1999). These data and further analysis of engineered chromosome mouse models argue against a "single gene" hypothesis for the complex DGS/VCFS pathology.

Transcription of protein-coding genes by RNA polymerase II in eukaryotes is a highly regulated process that requires the assembly of multiprotein complexes, the so-called transcriptosome, at the enhancer and promoter regions of class II genes (Halle and Meisterernst, 1996). These multiprotein entities consist of at least three different classes of factors. The minimal set of proteins necessary to properly initiate transcription on TATA box-containing promoters *in vitro* consists of RNA polymerase II and the general transcription factors (GTFs), which together form the basal machinery (reviewed in Roeder, 1996). Activators are mainly sequence-specific DNA binding proteins that interact with *cis*-regulatory elements on RNA polymerase II genes, thereby creating regulatory surfaces that are crucial but not sufficient for transcriptional activation. To achieve full activation of gene transcription, eukaryotic cells harbor a large number of accessory factors, the positive cofactors or coactivators, which mediate the response of the RNA polymerase II basal transcription machinery to activators. These cofactors act by altering the gene structure in the chromatin (Orphanides and Reinberg, 2000; Vignali *et al.*, 2000), creating regulatory networks and enhancing the formation of basal initiation complexes (Malik and Roeder, 2000). Among these cofactors, a large complex, the Mediator or SRB/Mediator, was first identified in yeast and shown to be a coactivator required for activated transcription in purified *in vitro* systems in the presence of GTFs (Flanagan *et al.*, 1991; Kim *et al.*, 1994; Koleske and Young, 1994). More recently, mammalian Mediator-like complexes have been identified. TRAP/SMCC, the so far best characterized complex, was shown to contain homologues of some yeast Mediator subunits in addition to other mammalian-specific factors (Gu *et al.*, 1999; Ito *et al.*, 1999). Other Mediator-like complexes, including ARC (Näär *et al.*, 1999), DRIP (Rachez *et al.*, 1999), hSRB/Mediator (Boyer *et al.*, 1999), CRSP (Ryu *et al.*, 1999), NAT (Sun *et al.*, 1998), and the murine Mediator (Jiang *et al.*, 1998), share similar subunit composition so that they may represent very similar cellular entities. PC2 was isolated as a positive cofactor from the human USA fraction (Meisterernst *et al.*, 1991) and shown to behave as a high-molecular-weight structure, indicative of a multisubunit complex (Kretzschmar *et al.*, 1994). Indeed, a recent study (Malik *et al.*, 2000) reported the purification of PC2 and its structural and functional characterization as a Mediator-like complex containing a subset of the subunits of the larger TRAP/SMCC complex.

The present study describes the isolation, molecular cloning, and characterization of the human *PCQAP* (PC2-glutamine/Q-rich-associated protein), a novel gene that encodes a subunit of the large Mediator-like complex PC2 and maps to the DiGeorge TDR on chromosome 22. We further cloned the corresponding mouse *Pcqap* cDNA and showed that human and mouse proteins are highly homologous. *PCQAP* is ubiquitously expressed in human fetal and adult tissues. Mouse *Pcqap* is also ubiquitously expressed during embryogenesis but high transcript levels are found in the frontonasal mass, pharyngeal arches, and limb buds. Derivatives of these structures are affected in the DGS/VCFS, thus rendering *PCQAP* an interesting gene, individually or together with genes controlling highly regulated developmental pathways, with regard to abnormal embryonic development.

MATERIALS AND METHODS

Library screening, human PCQAP and mouse Pcqap cDNA cloning, and sequencing. The *PCQAP* minimal cDNA (clone DG1.1) was isolated from a human HeLa cDNA library (Clontech, Palo Alto, CA) following the manufacturer's instructions. The library was screened with the probe pep1 corresponding to nucleotides 88 to 379 of the clone IMAGp998E13267 (GenBank Accession No. AL046886). The *PCQAP* full-length cDNA was produced by PCR using the DG1.1 clone as a template. The PCR product containing exons 1 and 2 was cloned into the *Not*I and *Xho*I sites of a modified pcDNA3.1 vector (Invitrogen, Gronongen, The Netherlands), from which the *Hin*dIII site was previously removed. A 2.7-kb fragment was excised from the clone IMAGp998E13267 and inserted into the *Hin*dIII (present in the PCR product) and *Xho*I sites of the former construct. The 5′ terminal end of the mouse *Pcqap* cDNA was produced by PCR using the clone IMAGp998O215146 (GenBank Accession No. AI787536) as a template and an oligonucleotide encompassing bp 88 to106 of the EST clone AI787982. The PCR product of 630 bp was cloned into the *Not*I and *BamH*I sites of the above-mentioned pcDNA3.1 modified vector. A 3.3-kb fragment was excised from the clone IMAGp998O215146 and inserted into the *Xho*I and *Hin*dIII (present in the PCR product) sites of the former construct. Constructs were analyzed by DNA sequencing.

PCQAP genomic organization. The intron/exon structure was defined comparing the human *PCQAP* cDNA and the genomic PAC m11 sequences (GenBank Accession No. AC004033). Most of the exon/intron boundaries were determined by direct sequencing of cosmid/fosmid DNA with primers designed on the *PCQAP* cDNA sequence. Further, intronic primers flanking each exon were designed to verify intron/exon boundaries by PCR and sequencing (Table 1). PCRs were performed to amplify each exon separately from PAC m11.

Total RNA preparation and primer extension analysis. Human primary T-lymphocytes were isolated from buffy coats (Red Cross Blood Donor Service, Munich, Germany) by separation on a Ficoll–Paque gradient following the manufacturer's instructions (Amersham–Pharmacia Biotech Europe GmbH, Freiburg, Germany). For primer extension analysis, human T-Jurkat cells and T-lymphocytes were treated with or without 20 ng/ml of the phorbol ester PMA for 24 h. Total RNA was prepared as previously described (Chomczynski and Sacchi, 1987). End-labeled ATG-rev primer (5′-GAAACGTCCATGCCTGTTCC-3′) (0.5 pmol) was annealed to 50 μg of total RNA. Primer:RNA hybrids were collected and used for reverse transcription according to Sambrook *et al.* (1989). A reference sequence-reaction was prepared using the T7 DNA polymerase sequencing kit (USB Corp., Cleveland, OH). Samples were finally analyzed by elec-

TABLE 1

Exon Boundaries and Intronic Primers Used to Amplify Each Exon of the Human *PCQAP* Gene

Exon	Exon size	Exon boundaries	cDNA position		Primer sequence	Intron size
1	68	AGCTGG. . .GTGAGT	1	F	-GGCCTGGCTCTGTGACTG	29376
				R	-CTAAGGAGGAAGGTCCCACAAT	
2	88	TTTCAG. . .GTAAGG	69	F	-GTGTGTGCAAACGTCTCTTCTC	14232
				R	-TACACCCTAGGACACCCAACAG	
3	52	TTTTAG. . .GTAAGT	157	F	-AAGCGTCTGAATCTGCCTTG	1658
				R	-ACAACCCAGAAATGCAAAATCT	
4	30	ATATAG. . .GTAAGA	209	F	-TGCCTACAGAACTGACCTACCC	1762
				R	-ATGAGGCATGCACTATGTCATT	
5	213	CCACAG. . .GTGAGT	239	F	-TCCCACAGATCCTATGAATGC	9299
				R	-CAAATCCTCCAAGAAGTGTGGT	
6	239	TCTCAG. . .GTACCA	452	F	-CTGGCGACTCTGGTCTTTTC	1775
				R	-AGGCCAAGAGCAGGAACAG	
7	345	CTCTAG. . .GTGAGT	797	F	-ACCAATTCAGCAGCCACC	1707
				R	-CCAAAGAGCCTAGAAACTACTGC	
8	115	CTCAAG. . .GTGAGG	912	F	-CATTTCCTAAGCTTCCACAGGA	13985
				R	-ACATGCACCTGCTGATGACTAT	
9	137	TTCCAG. . .GTAGGC	1049	F	-CACTTCCAGGTCAGCCAGAG	93
				R	-CTGCAGCCAAGTGAGAGAAA	
10	124	CTGCAG. . .GTAAGT	1173	F	-CGGCTCACATGTTTCTCTCA	146
				R	-AGAGCTCGCACACCCAGTAT	
11	135	CTGCAG. . .GTAGGC	1306	F	-GGCCCTCAGAGCTCAAGTT	83
				R	-TGCAGCCTACCTTCGTTCTTGTCG	
12	64	TCCCAG. . .GTGAGC	1372	F	-CGACAAGAACGAAGGTAGGC	966
				R	-ATCATCACCAAGTGCCCATC	
13	69	CTGCAG. . .GTGAGT	1441	F	-GAGGTTTCTGATGGCTGAGG	431
				R	-GCTCTTAGGAGAGCCCTGGT	
14	159	CTCCAG. . .GTATGT	1600	F	-GTGTGAAGGCCCCCTAAATG	86
				R	-CCCAGCTGGACATACGTGA	
15	167	CCCCAG. . .GTGAGT	1767	F	-GTGTGCCAGGTGTGGTCA	461
				R	-TCTGGACACTCACCCAGCTT	
16	99	TTGCAG. . .GTAGGT	1866	F	-GTGGAGTCCTGTTCCAGAGC	742
				R	-AAGCCTGGTTTTCCACAATG	
17	137	TTGCAG. . .CCAAGA	2003	F	-CACTGCTCTGTTGCAGACG	
				R	-ACCCCAGGCTCTCTAAGGAA	

trophoresis through a 7% polyacrylamide/7 M urea gel and autoradiography.

Chromosomal mapping. The chromosomal localization of *PCQAP* was determined using fluorescence *in situ* hybridization (FISH) with the *PCQAP*gen probe. The probe was generated by PCR using the primers *PCQAP*gen1 (5′-ATTGTGGGACCTTCCTCCTT-3′) and *PCQAP*gen2 (5′-AATCTGAGCCCTGAAAAGCA-3′), which amplify the genomic region from bp 45097 to bp 48096 of the PAC m11. FISH analysis was performed on metaphase chromosomes according to previously described protocols (Novelli *et al.*, 1999; Pizzuti *et al.*, 1996).

Production of antibodies. The N-terminus PCQAP peptide MDVSGQETDWRSTAF*C* linked to KLH via the additional C-terminal cysteine was used for the production of a rabbit polyclonal peptide antibody (Eurogentec, Brussels, Belgium). Antibody purification from the serum was performed according to standard immunological techniques.

The production of a rat monoclonal antibody against the C-terminal epitope of PCQAP has been described elsewhere (G. Mittler *et al.*, manuscript submitted).

Isolation of nuclear extracts and Western blotting. Human primary T-lymphocytes were separated from buffy coats as described above. To obtain enrichment of naive T-cells, the cell suspension was further purified on commercial human T-cell enrichment columns (R&D Systems GmbH, Wiesbaden, Germany) according to the manufacturer's protocols. Magnetic separation was performed using anti-CD45RO-conjugated microbeads and VS+ separation columns (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Human B-lymphocytes (Raji), Jurkat T-lymphocytes and HeLa cervical carcinoma cells, HEK-293 cells, mouse NIH3T3 fibroblasts, F9 testicular carcinoma, and P19 cells (kindly provided by Dr. H. Stunnenberg, University of Nijmegen, The Netherlands) were used to prepare nuclear extracts as described (Olnes and Kurl, 1994). Protein (100–200 μg) was either directly separated on a 15% SDS–polyacrylamide gel or first immunoprecipitated with the rat monoclonal antibody, subjected to SDS–PAGE, and transferred to nitrocellulose filters (Bio-Rad, Munich, Germany). Filters were then probed either with the rat monoclonal antibody or with the polyclonal peptide antibody.

Northern blot and RNA Master dot blot. Northern blot and RNA Master dot blot filters were purchased from Clontech and probed according to the manufacturer's instruction. The filters were hybridized with the probe pep1. Human ubiquitin (Clontech) was used as an internal standard for the RNA Master dot blot.

Whole mount in situ hybridization. For *in situ* hybridization of C3H wild-type embryos a 528-bp *Not*I–*Bam*HI fragment of *Pcqap* subcloned into pBluescript (Stratagene, La Jolla, CA) was used as template to synthesize riboprobes using the DIG-RNA labeling system (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's directions. *In situ* hybridization was performed using the *InsituPro* robot from ABIMED (Langenfeld, Germany) following a protocol previously described (Spörle and Schughart, 1998). RNA hybrids were visualized with an alkaline-phosphatase substrate color reaction mixture (Roche Molecular Biochemicals).

TABLE 2

Mutation Analysis of *PCQAP*

Position	Nucleotide change	Patient	Control
2076 bp (exon 16)	G>A	1\22	1\43
IVS11–16	C>T	5\22	8\34
IVS3+54	A/G	5\22	5\22
1526 bp (exon 11)	C>T	5\22	12\40

SSCP analysis and sequencing. Twenty-two nondeleted Italian patients who had one of the common conotruncal cardiac lesions observed in DGS/VCFS were used for SSCP analysis. The patients were initially screened for 22q11 and 10p13 deletions by FISH analysis as previously described (Wadey *et al.*, 1999). To detect point mutations and small insertions and deletions, genomic DNA from these patients was screened by SSCP for the entire *PCQAP* coding region. Local ethical review and consenting procedures were followed. Any electrophoretic variation observed was further evaluated by DNA sequencing. PCR products were sequenced with both forward and reverse PCR primers (Table 2).

RESULTS

Isolation and Cloning of Human PCQAP

In our efforts to characterize components of the PC2 Mediator-like complex, two protein bands of apparent molecular weight of 105 and 108 kDa were identified and termed PCQAP (G. Mittler *et al.*, manuscript submitted). The PCQAP doublet showed a similar migration pattern in SDS–PAGE compared to the ARC105/TIG-1 component of the recently published human ARC complex (Näär *et al.*, 1999). Using the peptide sequence available for ARC105/TIG-1 (Näär *et al.*, 1999), a BLAST search of the GenBank database (NCBI) was performed to obtain useful information about the gene encoding PCQAP. Several expressed sequence tags (ESTs) and a BAC and a PAC clone (GenBank Accession No. AC007731 and AC004033, respectively) from the human chromosome 22 that perfectly matched the published sequence of the ARC105/TIG-1 peptide were found. One of the EST clones (GenBank Accession No. AL046886) contained a sequence encoding two successive polyglutamine stretches, frequently seen in transcription factors, which were found also in PCQAP. This result opened the possibility to perform an *in silico* cloning of the virtual cDNA of *PCQAP*.

Further BLAST search the GenBank databases allowed us to generate a set of overlapping ESTs containing an ORF for a glutamine-rich protein of 746 amino acids. To corroborate these predictions, a human HeLa cDNA library was then screened with a probe generated by RT-PCR amplification of a 291-bp fragment (pep1) corresponding to nucleotides 88 to 379 of the EST clone AL046886. A clone harboring an insert of 2053 bp was pulled out: full sequencing further revealed that it contained the 5′-terminal part of the virtual cDNA (minimal cDNA) and an additional ORF coding for an unknown PCQAP-related protein. In parallel, sequencing of the EST clone AL046886 gave a sequence that perfectly corresponded to almost the full length of the *PCQAP* virtual cDNA but that lacked 76 bp (starting from the translation initiation codon) at the 5′ end. The full-length cDNA was generated by fusing the 5′ end of the *PCQAP* minimal cDNA with the cDNA insert of the clone AL046886: this cDNA encodes an ORF identical to the predicted PCQAP protein (GenBank Accession No. AF328769).

PCQAP Homology to a Murine Glutamine-Rich Protein

To confirm the identity of the predicted PCQAP sequence with the 105/108-kDa protein doublet isolated from the PC2 Mediator-like complex, two antibodies, a polyclonal peptide antibody directed against the first 15 amino acids of the deduced amino-terminus (GenBank Accession No. AF328769) and a rat monoclonal antibody raised against the C-terminal epitope of PCQAP, were generated and used in immunoblot experiments. Nuclear extracts were prepared from human B- and T-lymphocyte cell lines and from naive T-lymphocytes. Equal amounts of protein were separated by SDS–PAGE and probed with the monoclonal antibody after electroblotting. This antibody recognized a doublet of apparent molecular weight of 105/108 kDa in nuclear extracts of human lymphocytes (Fig. 1A), confirming the presence of the predicted carboxy-terminal epitope in the endogenous protein. In parallel, nuclear extracts from human HeLa and HEK-293 cells, together with those obtained from mouse NIH3T3 fibroblasts, F9 cells, and teratocarcinoma P19 cells, which have been used as a model for neural crest cells (McBurney *et al.*, 1982), were immunoprecipitated with the rat monoclonal antibody raised against the

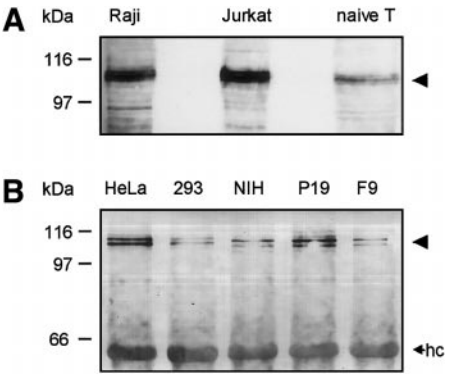

FIG. 1. Western blot analysis of nuclear extracts. (**A**) 100 μg of nuclear extract proteins from the human B- (Raji) and T- (Jurkat) lymphocyte cell lines and naive T-lymphocytes (naive T) were separated on a 15% SDS gel and after electrotransfer were probed with the rat monoclonal antibody raised against the C-terminal part of PCQAP. (**B**) 200 μg of nuclear extracts prepared from HeLa cells, HEK-293 cells, NIH3T3 fibroblasts (NIH), and F9 and P19 carcinoma cells were immunoprecipitated with the rat monoclonal antibody. Immunocomplexes were subjected to SDS–PAGE, transferred to nitrocellulose filters, and hybridized with the polyclonal peptide antibody. Arrows indicate the protein doublet corresponding to PCQAP; hc, heavy chain.

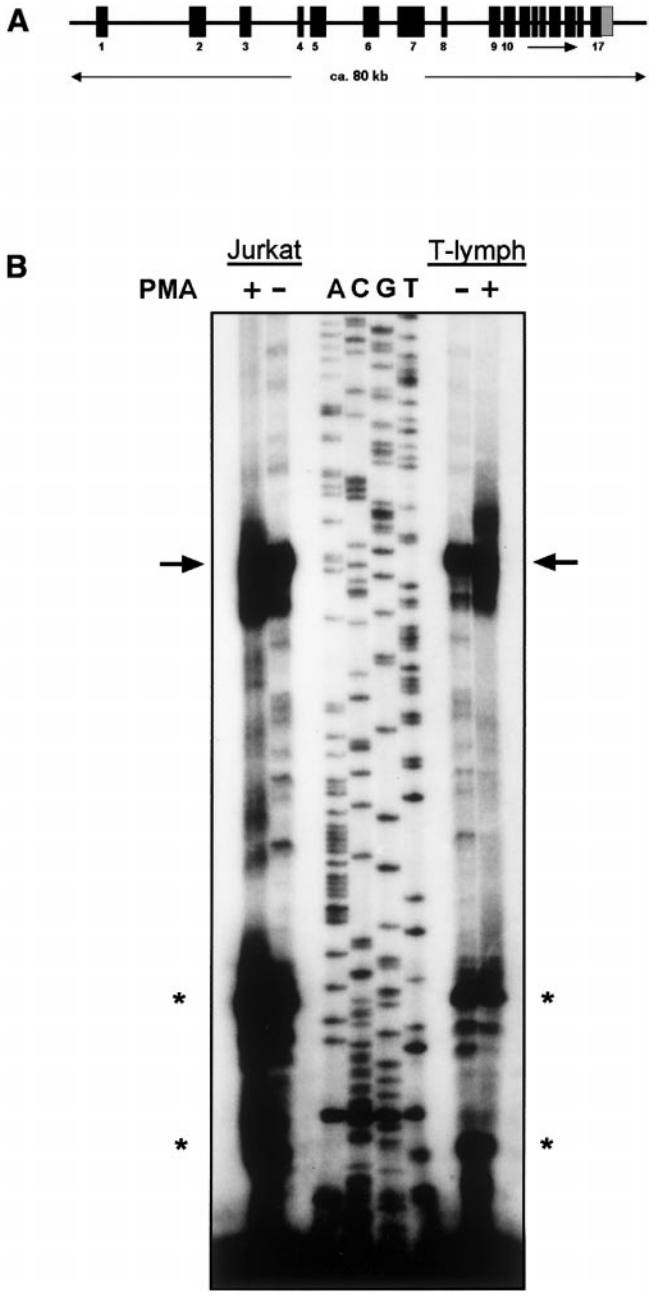

FIG. 2. Genomic organization of *PCQAP*. (**A**) Schematic diagram representing the genomic structure of the human *PCQAP* cDNA. The exons are represented by numbered boxes and are drawn to scale. The gray box represents the 3′UTR. The intronic regions are not to scale. (**B**) Primer extension analysis of total RNA from human Jurkat and primary T-lymphocytes. 50 μg of total RNA was prepared from T-lymphocytes previously treated with or without 20 ng/ml PMA for 24 h, hybridized with an end-labeled primer, and subjected to reverse transcription. Labeled cDNA was analyzed by electrophoresis on a 7% denaturing gel and autoradiography. Main transcription start site is indicated by arrows. Asterisks indicate nonspecific start sites. A, C, G, and T indicate the reference sequence reaction.

C-terminal epitope of PCQAP and probed with the polyclonal peptide antibody. The peptide antibody detected the protein doublet in the nuclear extracts of the human cell lines analyzed (Fig. 1B). A protein doublet with mobility identical to that of the human *PCQAP* was recognized in nuclear extracts from mouse cells (Fig. 1B), suggesting the existence of a murine *PCQAP* homologue. As a control, *in vitro* translation of the cloned *PCQAP* cDNA was also performed. The generated protein had the same apparent molecular weight as the endogenous PCQAP in a standard SDS gel (data not shown).

Genomic Organization of Human PCQAP

The possible exon/intron structure of the gene encoding for PCQAP was further determined by comparing the *PCQAP* cDNA with the genomic sequence of the PAC m11 (GenBank Accession No. AC004033). The computer approach identified 17 putative exons and 16 introns spanning over 80 kb. To verify the correct gene structure, primers were designed around the possible exon/intron boundaries (Table 1) and PCRs were performed to amplify each exon separately using the PAC m11 as a template. The exon sizes range from 30 (exon 4) to 345 bp (exon 7). The intron length varies between 83 (intron 11) and 29376 bp (intron 1) (Fig. 2A). The donor and acceptor splice sites at each exon/intron junction follow essentially the consensus roles.

To identify the promoter region and map the possible transcription initiation site(s), primer extension analysis was performed on total RNA prepared from the human Jurkat T-cell line and primary T-lymphocytes treated with or without the phorbol ester PMA for 24 h. An unambiguous signal was detected on the sequencing gel using an end-labeled oligonucleotide complementary to positions 44992–45011 of the PAC clone m11 and the RNA of untreated lymphocytes as a tem-

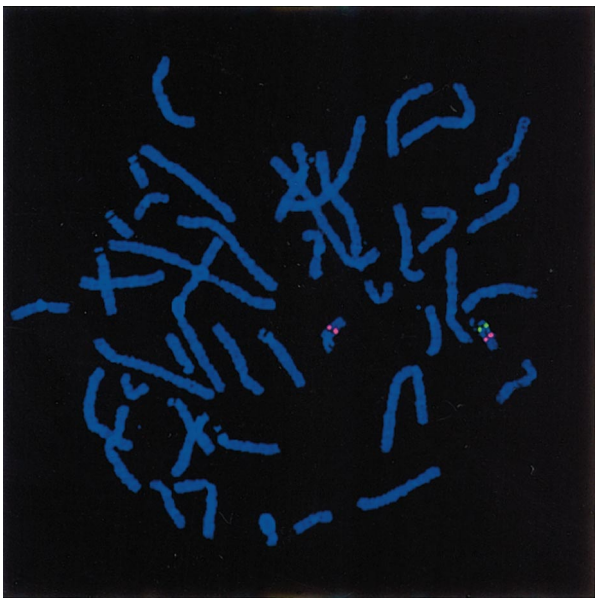

FIG. 3. FISH analysis of metaphase chromosomes of a representative DiGeorge patient with the *PCQAP*gen probe harboring the *PCQAP* gene. Only one signal (green) could be detected on the undeleted chromosome 22, as it was on 24 additional metaphases analyzed. A cosmid clone for the ARK2 gene (Calabrese *et al.*, 1994) mapping at 22q11 was used as a control probe. The signal (red) for the control probe was detected on the chromosome 22 pair.

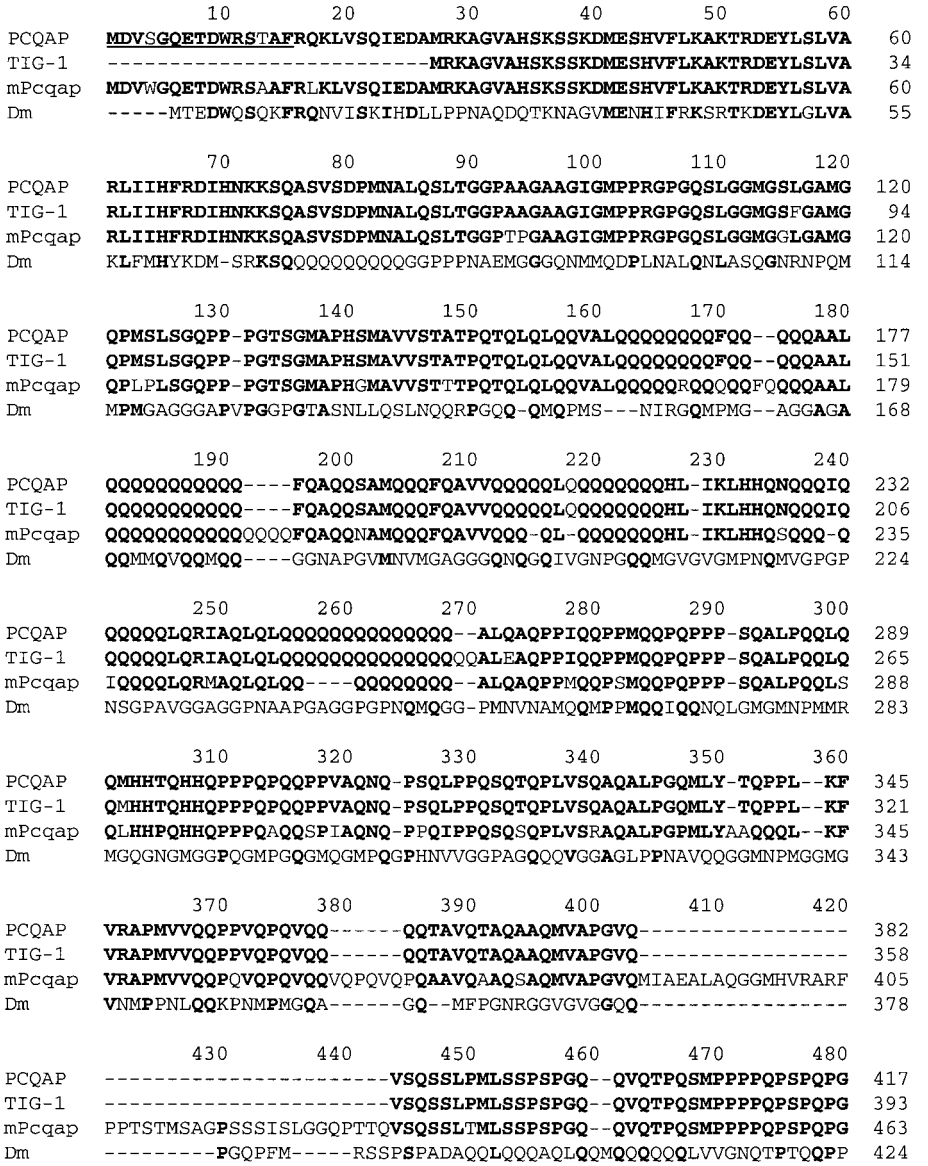

FIG. 4. Alignment of the deduced amino acid sequences of human PCQAP, TIG-1 (GenBank Accession No. AF056191), and mouse mPcqap with the *Drosophila melanogaster* (Dm) glutamine-rich protein (GenBank Accession No. AAF51490). Amino acids identical in human, mouse, and fly proteins are shown in boldface. Underlined is the PCQAP peptide sequence used for generation of the polyclonal peptide antibody.

plate (Fig. 2B). If the total RNA from PMA-treated lymphocytes was primed, the observed band was diffuse and the intensity of the signal even more pronounced (Fig. 2B), suggesting that expression of *PCQAP* is up-regulated by PMA in human T-lymphocytes. Based on a reference sequence reaction, the main transcription start site was mapped at position 44863 of the PAC m11 genomic sequence. The first in-frame translation initiation codon is located at position 45001 of the genomic sequence and in exon 1 of *PCQAP*. Sequence analysis of the 5′ flanking region of the *PCQAP* gene using TRANSFAC and TFD databases revealed the presence of multiple putative transcription factor binding sites, among them AP-2, Sp1, TFIID, GATA-1, and GATA-2, suggesting a promoter function for this region.

PCQAP Maps to the DiGeorge Chromosomal Region to the 22q11.2 Locus

A physical mapping of *PCQAP* was obtained by a computational approach, profiting from the fact that the sequence of the human chromosome 22 was available by the time of the present study. *PCQAP* overlaps with the genomic sequence of PAC m11 and partially with that of BAC 32 (GenBank Accession Nos. AC004033 and AC007050, respectively), which are physically mapped to the chromosomal region between bp 4,000,001 and bp 5,000,000 (http://www.sanger-.ac.uk). This sequence comparison demonstrated that *PCQAP* is located 74 kb distal to *ZNF74* (Aubry *et al.*, 1993) and 194 kb proximal to *HCF2* (Herzog *et al.*, 1991) and is transcribed on the same strand of these

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                490        500        510        520        530        540
PCQAP   QPSSQPNSNVSSGPAPSPSSFLPSPSPQPSQSPVTARTPQNFSVPSPGPL--NTPVNP--  473
TIG-1   QPSSQPNSNVSSGPAPSPSSFLPSPSPQPSQSPVTARTPQNFSVPSPGPL--NTPVNP--  449
mPcqap  ---SQPNSNVSSGPAPSPSSFLPSPSPQPSQSPVTARTPQNFSVPSPGPL--NTPVNP--  516
Dm      TP-QMPTPNMIPSPALVPQSSPQMMQMQNSQRNIRQQSP-SASINTPGQVTGNSPFNPQE  482

                550        560        570        580        590        600
PCQAP   --------SSV---------M----SPAGS--SQA-------E-----------E-----  487
TIG-1   --------SSV---------M----SPAGS--SQA-------E-----------E-----  463
mPcqap  --------SSV---------M----SPAGS--SQA-------E-----------E-----  530
Dm      EALYREKYKQLTKYIEPLKRMLAKISNDGTNVEKMTKMSKLLEILCNPTQRVPLETLLKC  542

                610        620        630        640        650        660
PCQAP   QQYLDKL-------KQLSKYIEPLRRMINKIDKNEDRKKDLSK-MKSLLDILTDPSKRCP  539
TIG-1   QQYLDKL-------KQLSKYIEPLRRMINKIDKNEDRKKDLSK-MKSLLDILTDPSKRCP  515
mPcqap  QQYLDKL-------KQLSKYIEPLRRMINKIDKNEDRKKDLSK-MKSLLDILTDPSKRCP  582
Dm      EKALEKMDLISYSGQQFGKSSNPLLEVINTTLQSPVANHTLYRTFRPTLELLFGTDITAP  602

                670        680        690        700        710        720
PCQAP   LKTLQKCEIALEKLKNDMAVPTPPPPPVPPTKQQYLCQPLLDAVLANIRSPVFNHSLYRT  599
TIG-1   LKTLQKCEIALEKLKN-------------------DMR---------------------  534
mPcqap  LKTLQKCEIALEKLKN-------------------DMA---------------------  601
Dm      VPA-KRPRVEEKSTSF--------------------EQE---------------------  620

                730        740        750        760        770        780
PCQAP   FVPAMTAIHGPPITAPVVCTRKRRLEDDERQSIPSVLQGEVARLDPKFLVNLDPSHCSNN  659
TIG-1   -CP-------------------------------LPH--RPR-------------------  542
mPcqap  -VP--TP-PPPPV-------------------LPT--KQQ------------D-------  617
Dm      -VP--HV-LQGEI---------------AR--LDT--KFK---------VKLDTTSQINN  648

                790        800        810        820        830        840
PCQAP   GTVHLICKLDDKDLPSVPPLELSVPADYPAQSPLWIDRQWQYDANPFLQSVHRCMTSRLL  719
TIG-1   ------CH----------RPNS-STYAS----------RSW---MPSWPTS----------  563
mPcqap  -----LCQ----------PLLD-AVLAN---------IRSP---V--FNHSLYRTFVPAMM  648
Dm      KAIRLICCLDDKRLPSVPPVSVSVPEEYPWQAPDCSLAEQEYSATPFLQTVQQALIARIS  708

                850        860        870        880
PCQAP   QLPDKHSVTALLNTW------AQSVHQACLSAA--------  746
TIG-1   --------AHLSSTI------PCTAHSFQP-----------  579
mPcqap  AI---HG-PPIVSPV------VCSRKSPV------------  667
Dm      KLPKNYSLSHLLDTWEMAVRQACSPQSKPRAVCELSTLLGV  749
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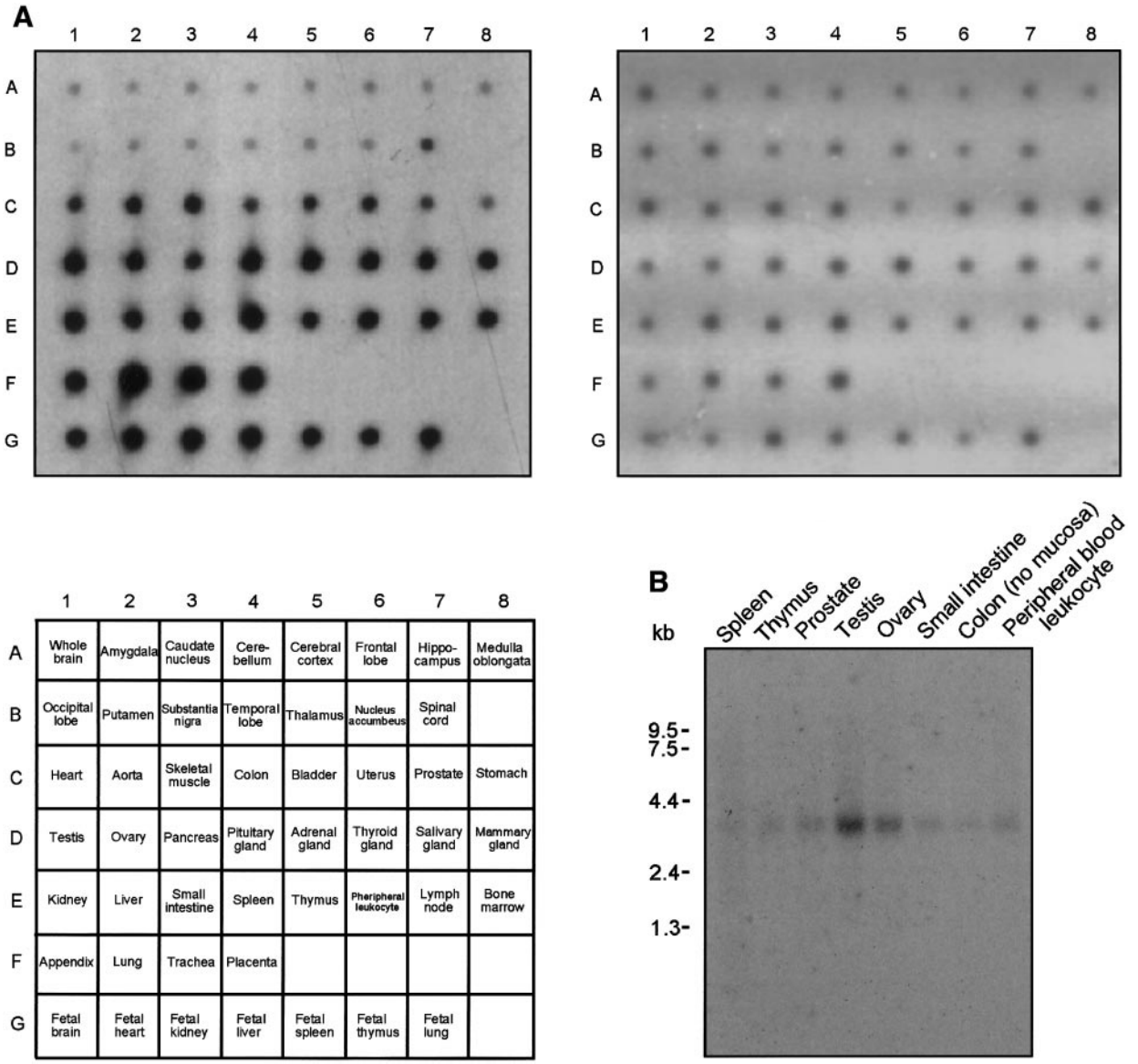
FIG. 4—*Continued*

genes. The position of the *PCQAP* gene was further examined by FISH analysis using a PAC clone probe (PCQAPgen) generated by PCR and the control probe scF15 (Halford *et al.*, 1993). Metaphase chromosomes from 25 DiGeorge patients carrying a typical 22q11.2 deletion and previously characterized according to Novelli *et al.* (1999) and those from 25 unrelated controls were analyzed. In all DiGeorge patients tested, the *PCQAP*gen probe failed to hybridize to the chromosome 22 carrying the interstitial deletion, whereas both signals, one for the *PCQAP*gen and one for the control probe, were detected on the undeleted chromosome (Fig. 3). The *PCQAP*gen and the control probes hybridized to both chromosomes 22 in unaffected individuals (data not shown). Thus, *PCQAP* is deleted in DiGeorge patients carrying the major hemizygous deletion of the TDR.

Cloning of the Mouse Pcqap cDNA

BLAST search of the GenBank database using the *PCQAP* cDNA sequence produced two mouse EST clones matching the 5′-terminal region of *PCQAP* cDNA. Sequencing of the EST clone IMAGp998O215146 (GenBank Accession No. AI787536) revealed that it contains a cDNA insert of approximately 3.3 kb, with high homology to the *PCQAP* cDNA, but which lacks 19 bp at the 5′ end, as determined by comparison with the sequence of the EST clone AI787982. The full-length mouse *Pcqap* cDNA was then generated by fusing an oligonucleotide corresponding to bp 1 to 19 of the EST clone AI787982 to the clone IMAGp998O215146. Translation of the predicted ORF produced a protein consisting of 667 amino acids (GenBank Accession No. AF328770). Sequence analysis (BLASTN and BLASTP) revealed that the *Pcqap* coding sequence is highly homologous to the human *PCQAP* sequence, with 89% nucleotide and 83% amino acid identity. The amino- and carboxy-terminal parts of the human PCQAP and mPcqap proteins share up to 97% sequence identity (Fig. 4). Search of the protein database (BLASTP) led to identification of a human homologue (TIG-1, GenBank Accession No. AF056191) and a homologue glutamine-rich protein (GenBank Accession No. AAF51490) of unknown function in



	1	2	3	4	5	6	7	8
A	Whole brain	Amygdala	Caudate nucleus	Cere-bellum	Cerebral cortex	Frontal lobe	Hippo-campus	Medulla oblongata
B	Occipital lobe	Putamen	Substantia nigra	Temporal lobe	Thalamus	Nucleus accumbeus	Spinal cord	
C	Heart	Aorta	Skeletal muscle	Colon	Bladder	Uterus	Prostate	Stomach
D	Testis	Ovary	Pancreas	Pituitary gland	Adrenal gland	Thyroid gland	Salivary gland	Mammary gland
E	Kidney	Liver	Small intestine	Spleen	Thymus	Pheripheral leukocyte	Lymph node	Bone marrow
F	Appendix	Lung	Trachea	Placenta				
G	Fetal brain	Fetal heart	Fetal kidney	Fetal liver	Fetal spleen	Fetal thymus	Fetal lung	

FIG. 5. Human mRNA Master dot blot and multiple-tissue Northern blot. (**A**) A commercial RNA Master blot filter from Clontech was hybridized with the pep1 probe (left); as a control the filter was reprobed with a ubiquitin cDNA fragment (right). At the bottom is the blot diagram showing the tissues analyzed. (**B**) Multiple adult tissue Northern blot was hybridized with the pep1 probe. One band of approximately 3.7 kb corresponding to the *PCQAP* transcript was detected in all tissues examined.

Drosophila melanogaster (Fig. 4). No homologues were found in *Xenopus laevis*, *Caenorhabditis elegans*, or *Schizosaccharomyces pombe.*

Comparative mapping experiments using a *PCQAP*-specific probe localized the mouse *Pcqap* gene on the chromosome MMU16 in the region syntenic to the human DiGeorge critical region between the genes *Znf74l* and *HcfII* (data not shown).

Expression in Human Fetal and Adult Tissues

Hybridization of a 291-bp fragment of the human *PCQAP* cDNA (pep1) to a human mRNA Master dot blot revealed the presence of *PCQAP* mRNA in all the adult and the 18- to 24-week-old fetal tissues examined (Fig. 5A). Further analysis of an adult multiple-tissue Northern blot revealed a distinct band of approximately 3.7 kb in all the mRNA (Fig. 5B), which is consistent with the length of the *PCQAP* transcript isolated. Slightly higher levels of mRNA were detected in testis and ovary.

Expression of Pcqap during Mouse Embryogenesis

The expression of *PCQAP* in human fetal heart and thymus raised the possibility that *PCQAP* may play a role in the development of the heart and the pharyngeal region of the head. Therefore, we determined the spatial distribution of *Pcqap* mRNA during mouse embryogenesis. Whole-mount *in situ* hybridization was performed on mouse embryos from day 9.5 to day 12.5 of gestation, a period during which critical events in organogenesis take place. For these experiments a mRNA probe complementary to bp 78 to bp 606 of the

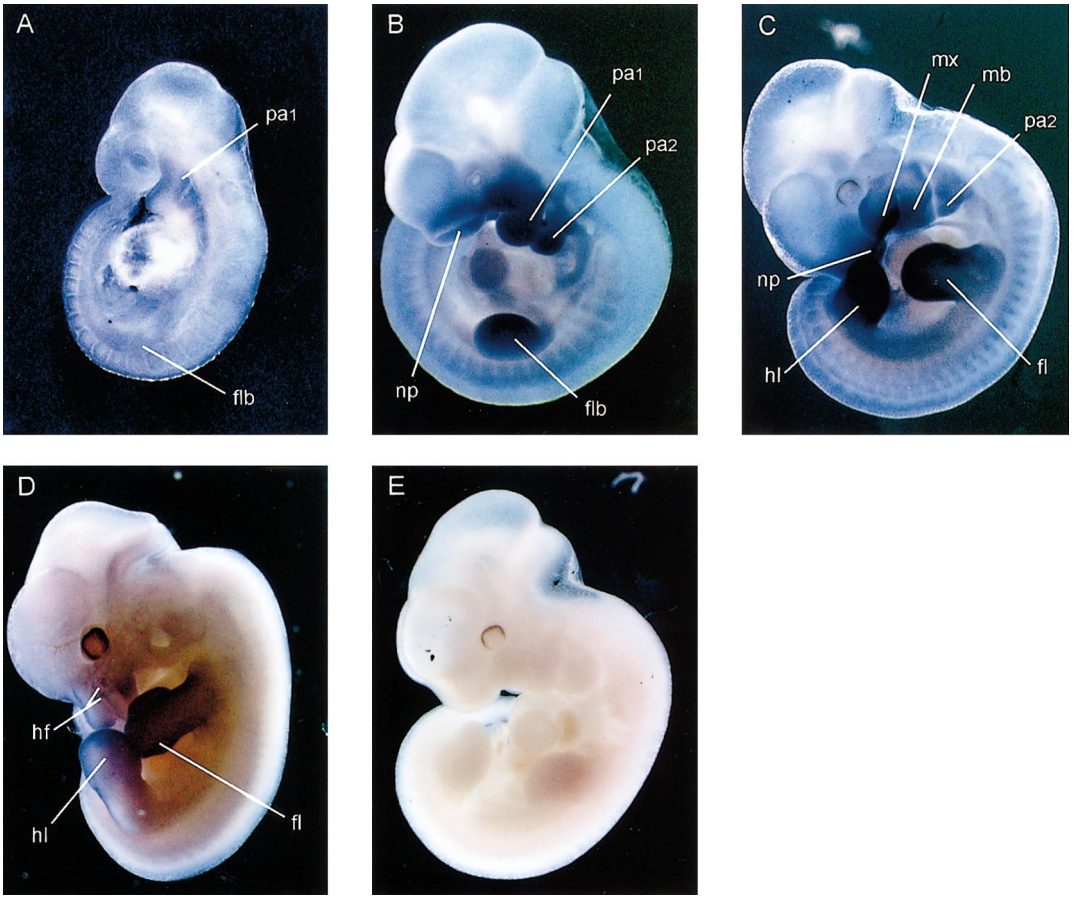


FIG. 6. Whole-mount *in situ* hybridization with *Pcqap* DIG-labeled probe. (**A**) Lateral view of an E9.5 embryo. *Pcqap* is ubiquitously expressed at low levels, with slightly elevated levels in the first pharyngeal arch and in the forelimb bud. (**B**) At E10.5 *Pcqap* transcripts are clearly detected in the frontonasal region, first and second pharyngeal arches, and forelimb bud (also in the hindlimb bud, not visible from this side). (**C**) Lateral view of E11.5 embryos shows enhanced expression in the maxillary and mandibular components of the first pharyngeal arch, in the second pharyngeal arch, and in the distal part of the limbs. (**D**) Lateral view of an E12.5 embryo reveals *Pcqap* transcripts in the hair follicles of the vibrissae and still in the distal part of the limbs. (**E**) E11.5 embryo as a negative control. Abbreviations: pa1, first pharyngeal arch; pa2, second pharyngeal arch; flb, forelimb bud; mx, maxillary component of the first pharyngeal arch; mb, mandibular component of the first pharyngeal arch; np, nasal process; fl, forelimb; hl, hindlimb; hf, hair follicle.

mouse *Pcqap* cDNA was used. At E9.5 *Pcqap* was found to be expressed ubiquitously at low levels, whereas slightly elevated signals were observed in the pharyngeal arches and in the forelimb buds (Fig. 6A). At E10.5 *Pcqap* expression was more pronounced in the first and second pharyngeal arches, the nasal processes, and the limb buds (Fig. 6B). At E11.5 high transcript levels were detected in the frontonasal region, the maxillary and mandibular processes of the first pharyngeal arch, the second pharyngeal arch, and the developing fore- and hindlimbs (Fig. 6C). From E11.5 to E12.5 *Pcqap* continues to be expressed in the distal parts of the developing limbs and in the facial region. At E12.5 *Pcqap* transcripts were also found in the developing hair follicles of the vibrissae (Fig. 6D). No specific signal was detected in hybridization experiments with a noncomplementary riboprobe (Fig. 6E).

Mutation Analysis

Mutational analysis performed in 22 DiGeorge undeleted patients (Wadey *et al.*, 1999) revealed mobility shift in the amplicons representing exons 14 and 16 and introns 3 and 11. Further sequence analysis using both forward and reverse primers was performed in each of the cases showing mobility shift, as well as in unrelated controls (Table 2). Two SNPs were detected, in exons 14 (1692G/A) and 16 (2076G/A). No amino acid exchange is expected following these substitutions. The 1526C/T SNP modifies a restriction site for *Ban*II and *Bgl*I and adds a restriction site for *Hin*fI, while the 2076G/A SNP modifies a restriction site for *Msp*A1I and *Nsp*BII. Screening of 42 unrelated controls revealed a heterozygosity rate of 0.3 for 1526C/T, whereas 2076G/A was found in only 1 control over the 42 analyzed. The intronic SNPs (IVS3 + 54A > G) and (IVS11 − 16T > C) were also detected in 2 unrelated controls, suggesting that they may represent a rare population polymorphisms or variations from the published sequence.

Because of the presence of glutamine-rich regions within the *PCQAP* gene and the intrinsic nature of these regions to mediate expansion and/or recombina-

tion, we examined seven CAG homopolymeric stretches, five located inside exon 6 and two in exon 7, in 61 unrelated parents of DGS/VCFS-deleted patients. PCR analysis of the selected regions showed repeat number polymorphism in normal individuals for CAG stretch located in exon 7 from bp 738 to bp 789 of the *PCQAP* cDNA, whereas the other CAG stretches seem not to be polymorphic. Alleles contained from 15 to 18 trinucleotide repeats with 22.4% heterozygosity in the Italian population. The distribution of the alleles in control individuals was unimodal, with similar patterns among the parents of DGS/VCFS patients (data not shown).

DISCUSSION

Deletions within 22q11.2 cause profound developmental defects in humans (Driscoll *et al.*, 1992). More than 90% of affected individuals have similar overlapping deletions spanning a region of about 3.0 Mb in length (TDR), while a few of them have unique small-sized 22q11.2 deletion endpoints (Amati *et al.*, 1999; Driscoll *et al.*, 1992). Despite attempts to define a critical region for DiGeorge syndrome and characterization of at least 27 genes mapping to the TDR, no gene by itself was proved so far to be responsible for the complex pathological phenotype.

We isolated the human *PCQAP* gene and showed that it maps to the TDR on the 22q11.2 locus; furthermore, we cloned the mouse homologue *Pcqap*. The two genes have 87% nucleotide identity and are 83% identical over the currently available protein-coding region, where the amino- and the carboxy-terminal parts are well conserved. Accuracy of the sequence prediction and homology between human and mouse proteins were confirmed by using antibodies raised against PCQAP conserved epitopes. This allowed the analysis of various human and mouse cell lines, in which PCQAP was detected as a protein doublet of apparent molecular weight of 105/108 kDa. At the present time, it is not possible to explain whether the two bands correspond to differentially modified forms of the same protein or if they rather represent the product of different spliced transcripts, even though there is no indication, based on the sequence information, for the latter possibility. PCQAP was found to be a novel subunit of the previously described USA-derived coactivator PC2 (Kretzschmar *et al.*, 1994; G. Mittler *et al.*, manuscript submitted), which was recently shown to be a Mediator-like complex containing a subset of the polypeptides of the human TRAP/SMCC coactivator complex (Malik *et al.*, 2000). Careful analysis of the SDS–PAGE profile of the PC2 complex revealed high similarity to the activator-recruited cofactor ARC (Näär *et al.*, 1999) and the related DRIP (vitamin D reporter interactory proteins) complexes (Rachez *et al.*, 1999). Whereas PCQAP was not identified in TRAP/SMCC, DRIP, CRSP, and the PC2-core complexes (reviewed in Malik and Roeder, 2000), it appears to be related to the subunit ARC105, which proved to be identical to TIG-1 (standing for TPA-inducible gene) (Näär *et al.*, 1999). The observation that *PCQAP* levels are up-regulated by the phorbol ester PMA, which triggers nonspecific activation of T-lymphocytes, further suggested a close relationship to *TIG-1*. Cloning of *TIG-1* was finished during the completion of the present study (Abraham and Solomon, 2000) and comparison of the cDNA sequences revealed high degree of similarity to *PCQAP*. However, the *PCQAP* cDNA appears to be more expanded and complete than the *TIG-1* cDNA, which lacks 78 nucleotides at the 5′ end and encodes a protein with a less extended carboxy-terminal part, the presence of which in the endogenous protein was confirmed by using both the peptide and the monoclonal antibodies in the present study. We therefore assume that *PCQAP* and *TIG-1* both originate from the same gene mapping to the TDR on human chromosome 22, although our primer extension and biochemical data (present study and G. Mittler *et al.*, manuscript submitted) rather support the hypothesis that *PCQAP*, and not *TIG-1*, represents the proper full-length gene.

Yeast Mediator has been shown to function by integrating positive and negative regulatory signals at the enhancer level, thus recruiting RNA polymerase II and associated proteins to promoters (reviewed in Malik and Roeder, 2000). Although the mechanisms by which Mediator influences transcription are not fully determined yet, a similar function has been hypothesized for the metazoan Mediator complexes based on the observation that different subunits of TRAP/SMCC interact with different activators and may lead to combined control of RNA II polymerase activity (Ptashne and Gann, 1997). This implicates tissue- and gene-specific transcription regulation mechanisms, i.e., differential modulation and also synergistic activation of a given gene by more activators. The exact composition of the PC2 Mediator-like complex as well as the components directly interacting with PCQAP or ARC105/TIG-1 within the multiprotein complex remains to be determined. Similarly, neither activators nor negative regulatory proteins that target PCQAP or ARC105/TIG-1 have been found, thus leaving the function of these novel subunits still obscure. However, we assume that the PC2 Mediator-like and the closely similar murine Mediator (Jiang *et al.*, 1998), as well as the ARC complexes, control expression of multiple, different genes, among others of genes involved in highly controlled developmental pathway(s). Concentration-dependent changes in PCQAP or TIG-1 may therefore result in disrupted stoichiometry of the complex itself and hamper its biological function. In this context, the chromosomal localization of human *PCQAP* to the DiGeorge locus on chromosome 22, together with the observation that 25 patients with defined 22q11.2 deletion were all deleted also for *PCQAP*, suggested the importance of further expression analysis of this gene.

PCQAP is ubiquitously expressed in human fetal and adult tissues. This is consistent with a general

function of the Mediator-like coactivator PC2 complex that can be recruited by RNA polymerase II to various gene promoters *in vitro* (G. Mittler *et al.*, manuscript submitted). Expression of *Pcqap* in mouse embryos at various midgestation stages is also ubiquitous, as shown by whole-mount *in situ* hybridization. However, higher expression was detected in the frontonasal mass, in the pharyngeal arches, and in the limb buds. Most of the mesenchyme of the pharyngeal arches derives from cranial neural crest cells and will later differentiate into specific organs and structures of the head and the neck (Kirby and Waldo, 1995). The thymus, thyroid, and parathyroid glands are derived from the third pharyngeal pouch that is colonized by cardiac neural crest cells from the time of its formation (Kirby and Waldo, 1990). Moreover, the neural crest cell populations of the pharyngeal arches 4/6 migrate in the outflow tract of the developing heart, where they participate in the formation of aorticopulmonary and truncal septa (Jiang *et al.*, 2000; Kirby and Waldo, 1995). The elevated *Pcqap* expression levels in those embryonic structures are suggestive of a potential involvement of *Pcqap*, and hence of Mediator, in the complex regulation of developmental pathways underlying the morphogenesis of the derivative organs. For instance, *Pcqap* could play a role in the development of the palate and related structures, which derive from the first pharyngeal arch. *Pcqap* expression is also seen in the developing limbs, a structure in which many genes are differentially expressed and distinct but interdependent pathways are activated during the embryonic development (Manouvrier-Hanu *et al.*, 1999). Limb anomalies are rare in DiGeorge patients, but have been described in some cases of patients carrying atypical deletions (Cormier-Daire *et al.*, 1995; Prasad *et al.*, 1997; Saitta *et al.*, 1999).

The temporal and spatial distributions of *Pcqap* during mouse embryogenesis show general regions of overlap with *Hira* (Wilming *et al.*, 1997), *Ufd1* (Pizzuti *et al.*, 1997; Yamagishi *et al.*, 1999), *Tbx1* (Chapman *et al.*, 1996), and *ZNF74* (Ravassard *et al.*, 1999), suggestive of a possible role of all these genes, independently or in an as yet unknown common pathway, in the abnormalities observed in people with DGS/VCFS. However, mice heterozygous for *Hira* (Scambler, 2000) or *Ufd1* mutations (Lindsay *et al.*, 1999) are apparently normal and although the heterozygous deletion *Df1* (Lindsay *et al.*, 1999) or *Tbx1* haploinsufficiency (Jerome and Papaioannou, 2001; Lindsay *et al.*, 2001; Merscher *et al.*, 2001) causes cardiovascular abnormalities reminiscent of DGS/VCFS, these mice do not show all the phenotypes reported in DGS/VCFS patients. Evidence for the absence of *UFD1L* (Saitta *et al.*, 1999; Wadey *et al.*, 1999), *TBX1* (Chieffo *et al.*, 1997; Lindsay *et al.*, 2001), and *PCQAP* (present study) point mutations in patients without obvious 22q11.2 deletions exclude these genes as responsible by themselves for the DiGeorge phenotype, at least in those patients. It is possible therefore that the full phenotype of DGS/VCFS is caused by haploinsufficiency of more than one gene acting in a common developmental pathway. Alternatively, it may be that regulatory elements or modifier genes located at a distance can affect the activity of *UFD1L* or *TBX1*. In this context one might consider the interesting possibility that *PCQAP* is involved in regulation of *TBX1* or a *TBX1*-responsive gene(s) and thereby causes a DGS/VCFS phenotype in the rare patients having intact *TBX1* alleles. Analysis of PCQAP at the molecular level will shed light on the function of this gene within the PC2–Mediator complex and in transcription regulation. Creation of a mouse model with targeted disruption of *Pcqap* may further help to unravel genes targeted by PC2–Mediator and also address the complex mechanism(s) underlying pathologies associated with 22q11 deletions.

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