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Human and murine *PTX1/Ptx1* gene maps to the region for Treacher Collins Syndrome

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Abstract. *Ptx1* belongs to an expanding family of *bicoid*-related vertebrate homeobox genes. These genes, like their *Drosophila* homolog, seem to play a role in the development of anterior structures and, in particular, the brain and facies. We report the chromosomal localization of mouse *Ptx1*, and the cloning, sequencing, and chromosomal localization of the human homolog *PTX1*. The putative encoded proteins share 100% homology in the homeodomain and are 88% and 97% conserved in the N- and C-termini respectively. Intron/exon boundaries are also conserved. Murine *Ptx1* was localized, by interspecific backcrossing, to Chr 13 within 2.6 cM of *Cam1*. The gene resides centrally on Chromosome (Chr) 13 in a region syntenic with human Chr 5q. Subsequent analysis by fluorescent in situ hybridization places the human gene, *PTX1*, on 5q31, a region associated with Treacher Collins Franceschetti Syndrome. Taken together with the craniofacial expression pattern of *Ptx1* during early development, the localization of the gene in this chromosomal area is consistent with an involvement in Treacher Collins Franceschetti Syndrome.

Introduction

We recently cloned and characterized a murine transcription factor gene, *Ptx1* (Lamonerie et al. 1996). The gene was identified on the basis of its ability to activate pituitary transcription of the pro-opiomelanocortin (POMC) gene. *Ptx1* is a member of an enlarging family of *bicoid*-related genes, another member of which, *Ptx2/PTX2* (RIEG), has recently been cloned and identified as the mutant locus underlying Rieger's Syndrome. This syndrome is a developmental anomaly that has its effects primarily in elaboration of the face where the gene is expressed (Semina et al. 1996; Mucchielli et al. 1996; Gage and Camper 1997).

In the developing head, *Ptx1* is first expressed in the stomodeum. This ectoderm continues to express *Ptx1* as it gives rise to the epithelia of the oral cavity and first branchial arch and subsequently to their derivatives. These include epithelia of the nasopharynx, palate, tongue, adenohypophysis, olfactory epithelium, submandibular glands, and dental epithelium. Secondarily, expression appears to be induced in a streak of mesenchyme in the first branchial arch: muscle and cartilaginous derivatives of this mesenchyme continue to express *Ptx1* (Lanctôt et al. 1997). The gene also displays a posterior pattern of expression in the extraembryonic and lateral plate mesoderm; derivatives of the latter include the muscular tissues in the lower body wall, bladder, hindgut, and genital tubercle. Consequently, *Ptx1* is expressed in the mesen-

chyme of the hindlimb bud, but never in that of the forelimb (Lanctôt et al. 1997). Given its pattern of expression in the "stomodaeal ectomere" (Lanctôt et al. 1997), we wondered if *Ptx1* might, like *PTX2/RIEG*, play a role in craniofacial dysmorphogenesis: the presentation of Treacher Collins Franceschetti (TCOF) Syndrome is consistent with *Ptx1* expression patterns and chromosomal localization in the mouse.

TCOF is an autosomal dominant disorder that occurs with a frequency of 1:50,000 live births (Winter 1996; Fazen et al. 1967). The syndrome affects derivatives of the first branchial arch and is characterized by craniofacial abnormalities (Edery et al. 1994). Expressivity and penetrance are variable, and the defects can include combinations of hypoplasia of the malar bones and mandible, conductive hearing loss, malformation of the external ear, enlarged nose, coloboma of the lower lids, and anti-mongoloid slant. In some pedigrees the disorder worsens with successive generations (Fazen et al. 1967), a trait characteristic of anticipation that occurs where mutations commonly involve the expansion of tri- or di- nucleotide repeats (Warren 1996).

Within the last year, a candidate gene (TCOF1) for TCOF has been characterized (Treacher Collins Syndrome Collaborative Group 1996). In some affected patients, the putative protein would suffer from premature truncation owing to insertion, deletion, or aberrant mRNA (Treacher Collins Syndrome Collaborative Group 1996; Gladwin et al. 1997; Wise et al. 1997). However, TCOF1 was mapped to a location that is more proximal than originally anticipated and that indeed may be incompatible with some recombination data (Dixon et al. 1993, 1994; Treacher Collins Collaborative Group 1996; Edery et al. 1994). Southern blot analysis of DNA from diverse organisms shows that the locus is not highly conserved. Moreover, TCOF1 is ubiquitously expressed and is highly polymorphic (Treacher Collins Collaborative Group 1996; Wise et al. 1997). None of the TCOF1 mutations was of the type associated with the feature of anticipation displayed in some TCOF pedigrees. TCOF1 may be a pseudogene or merely one of two or more genes involved in the pathogenesis of TCOF. The syndrome also may be heterogenic (Arn et al. 1993). The chromosomal position and developmental pattern of *PTX1/Ptx1* expression suggest that it may be involved in the pathogenesis of TCOF.

Materials and methods

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F₁ females and C57BL/6J males as described (Copeland and Jenkins 1991). In total, 205 N₂ mice were used to map the *Ptx1* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, South-

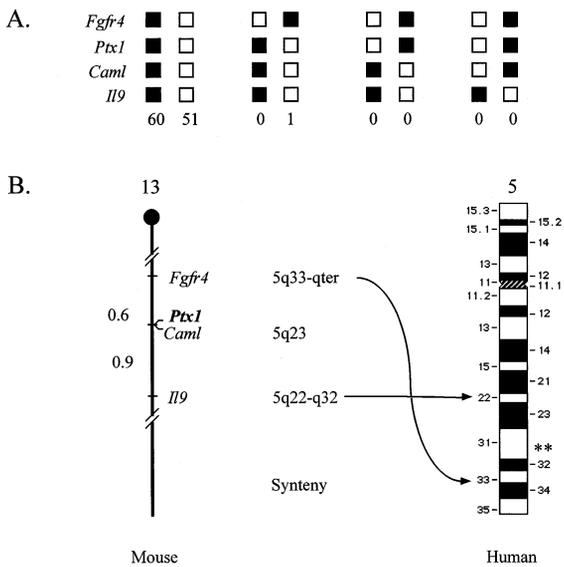


Fig. 1 (A) *Ptx1* maps in the central region of mouse Chr 13. *Ptx1* was placed on mouse Chr 13 by interspecific backcross analysis. The segregation patterns of *Ptx1* and flanking genes in 112 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 112 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F₁ parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. **(B)** A partial Chr 13 linkage map showing the location of *Ptx1* in relation to linked genes is shown. Recombination distances between loci in centiMorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, Md.). Asterisks indicate the location of fluorescent hybridization signal determined by FISH (Fig. 2.)

ern blot transfer, and hybridization were performed essentially as described (Jenkins et al. 1982). All blots were prepared with Hybond-N⁺ nylon membrane (Amersham). The probe, an ~370-bp *EcoRI/NcoI* fragment of mouse cDNA, was labeled with [α^{32} P] dCTP with a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of 1.0 × SSCP (20 × SSCP contains 43.7g Na₂ HPO₄, 12.7₅ NaH₂ PO₄, 88.2g sodium citrate and 140.3g NaCl per liter) 0.1% SDS, 65°C. A major

fragment of 15.0 kb was detected in *HindIII*-digested C57BL/6J DNA, and a major fragment of 10.5 kb was detected in *HindIII*-digested *M. spretus* DNA. The presence or absence of the 10.5-kb *HindIII* *M. spretus* specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Ptx1*, including *Fgfr4*, *Cam1*, and *Il9*, has been reported previously (Avraham et al. 1994). Recombination distances were calculated as described (Green 1981) with the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Isolation of human PTX1 and FISH mapping. Sequences from murine *Ptx1* cDNA were employed to amplify fragments of cDNA from reverse-transcribed human pituitary tumor mRNA (forward primer TCTCTTGTCCCCACAGGTC, reverse primer CCCAGTTGTGTAGGAGTAGCC). The 639-bp fragment derived in this manner was used as a probe to screen a genomic library constructed in λ ZAP. In a screen of 2 × 10⁵ plaque-forming units, one positive clone was isolated that contained only the first two exons of the gene. The missing 3' exon was subcloned from a PAC (courtesy Canadian Genome Analysis and Technology program, Canada) that was isolated with sequences from the 3' end of the genomic clone and the cDNA fragment as probes. Double-stranded DNA sequencing was accomplished with a chain termination protocol (Sanger et al. 1977).

Fluorescent in situ hybridization (FISH) was performed on normal human lymphocyte chromosomes (Lichter et al. 1990). The probe employed was a biotinylated 13-kb genomic fragment of human PTX1 that was detected with avidin-fluorescein isothiocyanate (FITC). Counterstaining was done with propidium iodide and 4',6-diamidino-2-phenylindol-dihydrochloride (DAPI; Heng and Tsui 1993). Separate DAPI and FITC images of metaphase spreads were recorded through a thermoelectrically cooled charge coupled camera (Photometrics, Tuscon, Ariz.) and superimposed digitally (Boyle et al. 1992). Band assignment was determined by measuring the fractional chromosome length and by comparison with the DAPI banding pattern (Heng and Tsui 1993).

The structure of the PTX1 locus was determined by comparison of genomic with cDNA sequences as well as with their murine and chick counterparts. The murine gene structure was determined by mapping genomic restriction fragments with cDNA probes, sequence analysis, and PCR amplification. The genomic clone was also examined for the presence of trinucleotide repeats with oligonucleotides as probe; namely, (CCG)⁶, (CTG)⁶, and (GAA)⁶. Oligonucleotides were labeled by use of terminal transferase and ³²P-dCTP. The human PTX1 sequence was submitted to Genbank (accession numbers AF009648, AF009649, AF009650).

Results

The mouse chromosomal location of *Ptx1* was determined by interspecific backcross analysis. This interspecific backcross map-

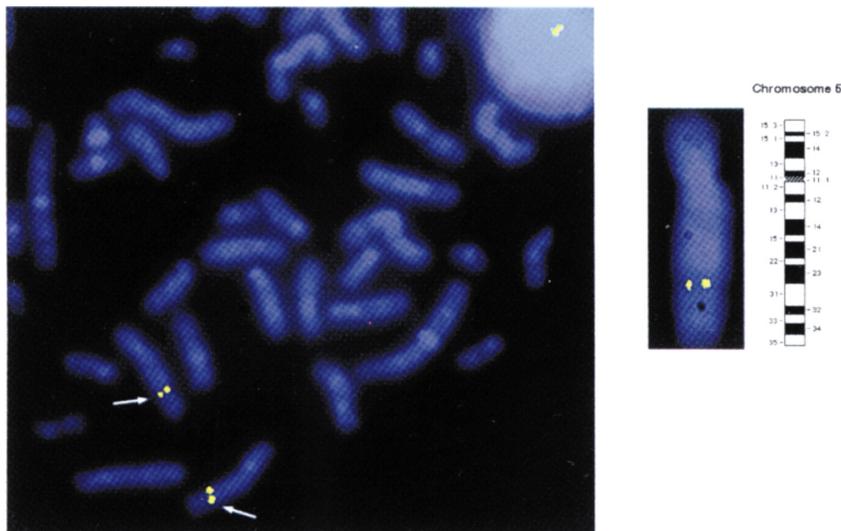
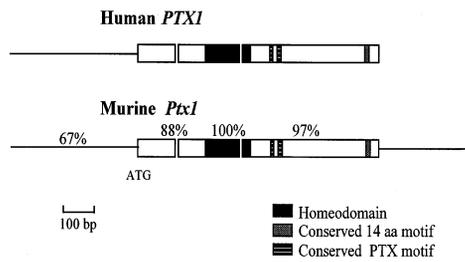
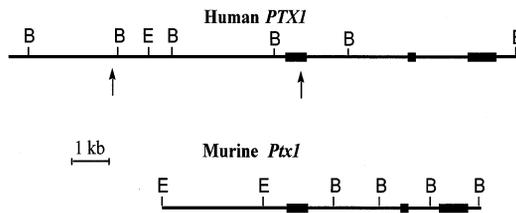


Fig. 2. Fluorescent in situ hybridization links PTX1 to Chr 5q31. Paired copies of PTX1 are detectable on Chr 5 in human lymphocyte metaphase spreads. Arrows indicate sites of hybridization with PTX1 genomic fragments. Positional assignment was made on the basis of fractional chromosome length and DAPI banding patterns.

A.



B.



C.

PTX1	MDAFKGGMSL	ERLPEGLRPP	PPPP.HDMGP	AFHLARPADP	REPLENSASA	S...SDTEL	PEKERGGEPK	GPEDSGAGGT
mPtx1	MDAFKGGMSL	ERLPEGLRPP	PPPP.HDMGP	SFHLARAADP	REPLENSASE	S...SDADI	PKKERGGEAK	GPEDGGAGSA
cPtx1	MDSFKGGMNL	ERLPEGLR..	.PQPSHDMAS	SFHLQRSSEP	RDPIENSASE	S...SDTDV	PEKERSESRK	TR.....
PTX2/RIEG	METNCRKLVs	ACVGL.....	.PQPSHDMAS	PKDKSQQGK	NED.....
mPtx2	METNCRKLVs	ACVQLGVQPA	AVECLFSKDS	EIKKVEFTDS	PKSRKESASS	KLFPRQHPGA	NEKDKGQQGK	NED.....

PTX1	GCGGGADDP	KKKKRRQRT	HFTSQLOEL	EATFQRNRY	DMSMREEI	WTNLTEPRV	VWFKNRRAK	RKRE	RNQQLD
mPtx1	GCGGGAEDP	KKKKRRQRT	HFTSQLOEL	EATFQRNRY	DMSMREEI	WTNLTEPRV	VWFKNRRAK	RKRE	RNQQLD
cPtx1	..TGAADDP	KKKKRRQRT	HFTSQLOEL	EATFQRNRY	DMSMREEI	WTNLTEPRV	VWFKNRRAK	RKRE	RNQMD
PTX2/RIEG	..VGAEDPS	KKKKRRQRT	HFTSQLOEL	EATFQRNRY	DMSTREEI	WTNLTEARV	VWFKNRRAK	RKRE	RNQQAE
mPtx2	..VGAEDPS	KKKKRRQRT	HFTSQLOEL	EATFQRNRY	DMSTREEI	WTNLTEARV	VWFKNRRAK	RKRE	RNQQAE

PTX1	LCKGGYVPQF	SGLVQPYEDV	YAAGYSYNNW	AAKSLAPAPL	STKSFTFFNS	M..SPLSSQS	MFSAPSSISS	MTMPSSMGPG
mPtx1	LCKGGYVPQF	SGLVQPYEDV	YAAGYSYNNW	AAKSLAPAPL	STKSFTFFNS	M..SPLSSQS	MFSAPSSISS	MTMPSSMGPG
cPtx1	LCKNGYVPQF	SGLMQPYDDM	Y.AGYPYNNW	ATKSLTPAPL	STKSFTFFNS	M..SPLSSQS	MFSAPSSISS	MNMPSSMGHS
PTX2/RIEG	LCKNGFGPQF	NGLMQPYDDM	Y.PGYSYNNW	AAKGLTSASL	STKSFPFFNS	M.VNPLSSQS	MFSPPNSISS	MSMSSSMVPS
mPtx2	LCKNGFGPQF	NGLMQPYDDM	Y.PGYSYNNW	AAKGLTSASL	STKSFPFFNS	MNVNPLSSQS	MFSPPNSISS	MSMSSSMVPS

PTX Family Motifs

<u>14 aa Motif</u>								
PTX1	AVPGMPNS..	GLNNINNL	GSSLNSAM.S	PGACPYGTPA	SPYSVYRDT	NSSLASLRLK	SKQHSSFGYG	GLQGPASGLN
mPtx1	AVPGMPNS..	GLNNINNL	GSSLNSAM.S	PGACPYGTPA	SPYSVYRDT	NSSLASLRLK	SKQHSSFGYG	GLQGPASGLN
cPtx1	AVPGMANS..	.GLNNINNI	GSSLNSAMSS	P.ACYPYPPG	SPYSVYRDT	NSSLASLRLK	SKQHSSFGYS	SLQSPGSSLN
PTX2/RIEG	AVTGVPGSSL	NSLNNLNLS	SPSLNSAVPT	P.ACPTAPPT	PPT.VTRDTC	NSSLASLRLK	AKQHSSFGYA	SVQKPASNLS
mPtx2	AVTGVPGSSL	NSLNNLNLS	SPSLNSAVPT	P.ACYPYAPT	PPY.VYRDT	NSSLASLRLK	AKQHSSFGYA	SVQNPASNLS

PTX1	ACQYNS....
mPtx1	ACQYNS....
cPtx1	ACQYNS....
PTX2/RIEG	ACQTAVDRPV
mPtx2	ACQYAVDRPV

Fig. 3 (A) Conservation of human and mouse *PTX1*. The positions of introns are shown by discontinuities in the diagram, and the positions of consensus sequence motifs are indicated by boxes. One represents a motif shared by other homeobox genes (Semina et al. 1996), while the other appears to be unique to the *PTX* sub-family. Within coding sequences, the percentage homology shown is that of the amino acid sequences, whereas nucleotide sequences were compared in the 5' untranslated region. **(B)** Mouse and human *Ptx1/PTX1* gene organization. Both genes share identical intron/exon boundaries. The human *PTX1* gene has two regions containing trinucleotide repeats, which are indicated by arrows. **(C)** Comparison of amino acid sequences in human, mouse, and chick *Ptx* homologs. Boxed residues comprise the homeodomain, underlined sequence marks a region conserved in the *Ptx* family, and the overlined sequence is the 14 residue domain conserved in a subset of homeobox genes expressed during craniofacial development.

ping panel has been typed for over 2300 loci that are well distributed among all the autosomes as well as the X Chr (Copeland and Jenkins 1991). The mapping results indicated that *Ptx1* is located in the central region of mouse Chr 13 linked to *Fgfr4*, *Camf1*, and *I19*. Although 112 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 1a), up to 179 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies with the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere-*Fgfr4*-1/179-*Ptx1*-0/115-*Camf1*-1/115-*I19*. The recombination frequencies [expressed as genetic distances in centiMorgans

(cM) ± the standard error] are $-Fgfr4-0.6 \pm 0.6-[Ptx1, Camf1-0.9 \pm 0.9-I19]$. No recombinants were detected between *Ptx1* and *Camf1* in 115 animals typed in common, suggesting that the two loci are within 2.6 cM of each other (upper 95% confidence limit). *Ptx1* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown); however, an extinct mouse mutation, dumpy (*dpy*), that affects development of the snout and hindlimbs was localized to Chr 13 (Hollander 1981). The central region of mouse Chr 13 shares a region of homology with human Chr 5q (Fig. 1b). In particular, *Camf1* has been placed near human 5q23. The tight linkage between *Ptx1* and *Camf1* in mouse suggested that *Ptx1* resides on 5q, as well.

A 13-kb human genomic probe was used to identify the PTX1 locus on chromosomal spreads. FITC-labeled probe was localized to 5q31 by analyzing the DAPI banding patterns and measuring the fractional chromosomal length. Analysis of 20 metaphase spreads demonstrated that positive fluorescent in situ hybridization signals were observed in 14 out of 20 spreads, and signals could be identified on both homologs in 9 of these (Fig. 2.). The chromosomal localization of PTX1 places the gene proximal to TCOF1 but within the region of critical interest for TCOF (Edery et al. 1994; Dixon et al. 1994; Treacher Collins Syndrome Collaborative Group, 1996).

PTX1, like its murine and chick homologs, possesses a homeodomain with a *bicoid*-class third helix. The homeodomain is highly conserved between mouse and human (100%), as are the C- and N- termini (88 and 97% respectively; Fig. 3a,b). This conservation is also evident at the level of gene structure as the murine and human genes both have similar organization: the intron/exon borders are identical. Human PTX1 is also conserved when compared with sequences obtained from chick (Fig. 3c). Like products of the PTX2 gene family, the PTX1 homologs encode a conserved C-terminal sequence of 14 residues noted to be present in other homeobox genes that share periods of expression in craniofacial structures during development (Semina et al. 1996). Moreover, there appear to be two short consensus sequences just C-terminal of the homeodomain that are present in members of the PTX family. Finally, a trinucleotide repeat (GCC)⁵ is present in the open reading frame of the first exon. Similar repeats are also present upstream of the gene. We have not yet identified signs of repeat expansion in TCOF probands; however, genomic Southern blots have not always proven reliable when repeat expansion alleles are chimeric in length and representation. In summary, the fine mapping of murine *Ptx1* and human PTX1 indicates conservation in sequence, organization, and chromosomal localization on syntenic regions of mouse and human Chr 13 and 5 respectively.

Discussion

Taken together with the localization of the PTX1 gene in the area critical for TCOF, the murine pattern of *Ptx1* expression in the stomodeum and its derivatives is highly suggestive of an involvement of PTX1 in the clinical manifestations of TCOF. PTX1 and TCOF1 are close on Chr 5 and would probably cosegregate: mutations in the PTX1 locus might account for TCOF in families that did not exhibit TCOF1/Treacle mutations, or it might itself be the causative gene if TCOF1 is a pseudogene. Further, the ubiquitously expressed TCOF1/Treacle gene product, if it exists, must mediate its effect through another agent that exhibits tissue specificity, like *Ptx1*. PTX1 is a member of the same gene subfamily that encodes PTX2 (RIEG) and displays a similar yet distinct expression pattern consistent with a role in facial development (Semina et al. 1996; Mucchielli et al. 1996; Lanctôt et al. 1997). TCOF, like Rieger's Syndrome, is autosomal dominant and could also exert its effect through haploinsufficiency. Finally, the localization of PTX1 expression to the pituitary anlage and organ is not inconsistent with an involvement in TCOF because PTX2 (RIEG) has a similar period of activity in Rathke's pouch. Neither Rieger's nor TCOF is usually associated with endocrine symptoms: PTX1 and PTX2 may be redundant in the pituitary, whereas their differences in craniofacial mesenchyme expression may account for the differences between the two syndromes.

Sporadic references to ancillary anomalies appear throughout the TCOF literature (Hunt and Smith 1955; Rogers 1964; Szlazak 1953), and these anomalies occur in regions where *Ptx1* is transcribed in mouse. For example, some TCOF patients have abnormalities of dentition, feet, digestive, and reproductive tracts that are all domains of early murine *Ptx1* expression. Furthermore, abnormal expression or function of PTX1 in the foregut endoderm

may result in posterior displacement of the soft palate and epiglottis during buccal/pharyngeal fusion in TCOF patients.

We are currently assessing TCOF families for the presence of mutations in the PTX1 locus.

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