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Mouse TCOF1 Is Expressed Widely, Has Motifs Conserved in Nucleolar Phosphoproteins, and Maps to Chromosome 18

William A. Paznekas,* Nian Zhang,† Thomas Gridley,† and Ethylin Wang Jabs*,[1]
**Department of Pediatrics, Medicine, and Surgery, Center for Medical Genetics, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, Maryland 21287-3914; and*
†The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609-1500

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Mutations in the human *TCOF1* gene have been identified in patients with Treacher Collins syndrome (Mandibulofacial Dysostosis), an autosomal dominant condition affecting the craniofacial region. We report the isolation of the entire mouse *Tcof1* coding sequence (3960 bp) by performing a computer-based search for mouse cDNA clones homologous to *TCOF1* and generating overlapping RT-PCR products from mouse RNA. Tcof1 is a 1320 amino acid protein of 135 kd with 61.4% identity to TCOF1 and displays repeating motifs enriched for serine- and acidic amino acid-rich regions with potential phosphorylation sites and putative nuclear localization signals. *Tcof1* maps to the mouse chromosome 18 region syntenic with human chromosome 5q32→q33 which contains the *TCOF1* locus. Northern blot hybridization indicates *Tcof1* expression is ubiquitous in adult tissues and in the embryonic stage, is elevated at 11 dpc when the branchial arches and facial swellings are present in mouse. Our results are consistent with TCOF1 mutations leading to the Treacher Collins syndrome phenotype.

We have isolated the mouse *Tcof1* coding sequence to determine conserved and, therefore, important functional regions in it and its human homologue. The human *TCOF1* gene is widely expressed, and codes for a putative nucleolar phosphoprotein (1-3). Northern blot analysis indicates that a transcript of approximately 5.3-5.8 kb is detected in human placenta; fetal brain, lung, liver, and kidney; and adult heart, brain, lung, liver, kidney, and pancreas. A larger transcript of 6.3 kb is present in adult skeletal muscle. Translation of the 4,233 nucleotide open reading frame predicts a 1,411 amino acid protein of approximately 144 kd. The predicted TCOF1 protein is of very low complexity, and 57.6% of its amino acids residues are alanine, serine, lysine, glutamine, and proline. The protein has repeated motifs for putative casein kinase phosphorylation sites (S/T-X-X-D/E) and binding sites for nuclear and nucleolar localization signals (K-K/R-X-R/K) (1). Its amino acid sequence resembles those of phosphoproteins, such as rat Nopp140, that shuttle from the cytoplasm into the nucleolus (4) suggesting that TCOF1 may be a chaperone protein.

The multiple stretches of serine- and acidic amino acid-rich sequences in TCOF1 not only resemble stretches of phosphoprotein sequences such as those in Nopp140 (e.g., SSSDSSEDSSEEE) (4), but also those in several other viral and cellular transcription factors such as ICP4 of HSV-1 (e.g., SSSSSASSSSSSDEDEDDD), IE180 of pseudorabies virus (e.g., SSSSSGGSDSD), IE62 of VZV, Sox-4, and PC4(p15) (5). In the case of ICP4, the serine- and acidic amino acid-rich sequence is important for its interaction with TFIIB, a general transcription factor (6). Rat Nopp140 has been shown to function as a transcriptional coactivator of the α-1 acid glycoprotein (agp) gene by physically interacting with AGP/EBP (enhancer binding protein) and TFIIB (5). These findings suggest that TCOF1 may also function as a transcription factor.

Mutations in the *TCOF1* gene have been identified in Treacher Collins syndrome patients (1,3,7). The phenotype is highly variable with features of downslanting palpebral fissures, lower eyelid colobomas, malar and mandibular hypoplasia, and dysplastic ears with conductive hearing loss. Mutations found in Treacher Collins syndrome patients are predicted to cause a premature termination codon or haploinsufficiency. Defects or deficiencies in transcriptional activation, nucleolar trafficking, and/or TCOF1-shuttled protein(s) may lead to the Treacher Collins syndrome phenotype.

[1] To whom correspondence and reprint requests should be addressed. Fax: (410) 955-0484. E-mail: ewjabs@welchlink.welch.jhu.edu.

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METHODS

cDNA clones. A BLASTN search ((8); http://www.ncbi.nlm.nih.gov:80/cgi-bin/BLAST/nph-blast) of the Non-redundant Database, GenBANK EST Division, was performed using the human *TCOF1* sequence, comprising 26 exons with the termination codon in exon 25 as previously elucidated by our laboratory ((1); GenBank accession nos. U76366, U84640-U84665). GenBank sequences from nine mouse clones with the smallest sum probability (P(N)) ranging from 1.2e-71 (N=1) to 1.0e-15 (N=2) were analyzed. Five such clones were obtained (Genome Systems Inc., St. Louis, MO) and sequenced by the Johns Hopkins Genetic Resources Core Facility using an Applied Biosystems 373 automated DNA sequencer (Foster City, CA).

Derivation of the coding sequence of mouse Tcof1. Reverse-transcriptase polymerase chain reaction, RT-PCR (GeneAmp RNA PCR Kit; Perkin-Elmer, Foster City, CA), was used to amplify cDNA from outbred mouse liver total RNA, to confirm sequences from the above cDNA clones and to obtain intervening sequences. First strand cDNA synthesis was performed with random hexamers or a gene specific primer (GSP1, 5′CTTCTGCTTGCCATCC3′), and RT-PCRs were performed using primer sets based on sequences from either clones or overlapping RT-PCR products. The reaction conditions were according to the manufacturer's instructions except per 20 ul reaction volume, the total RNA template ranged in amount from 0.033 to 1 ug; $MgCl_2$ concentration was either 3.75 or 5.00 mM; and the reaction time at 42°C was extended to 60 min. RT-PCR-1, -2, and -3 products were generated using forward primer (fp) GSP2 5′TCAGAGCTTGGCCAGAA3′ and reverse primer (rp) GSP1 with initial denaturation at 95°C for 2 min., then 30 cycles with denaturation at 95°C for 1 min., annealing at 54°C for 30 sec. and elongation at 68°C increasing in duration from 3.5 to 10 min., and final elongation at 72°C for 7 min. The product of this reaction was used as a template for amplification with fp GSP3 5′CCCAAGACCGACACCTGTCA3′ and rp GSP4 5′GGCATCATGGGGAGTGTTTC3′ under the above conditions except the annealing temperature was 64°C. The second PCR yielded a 2550 bp product. It was either TA cloned (Invitrogen, San Diego, CA) or reamplified with primers GSP3 and GSP4 and an enzyme mixture containing Taq and Pwo DNA polymerase (Boehringer Mannheim Corp., Indianapolis, IN), gel purified (QIAquick Gel Extraction Kit; Qiagen, Chatsworth, CA), and sequenced directly as described above.

The PCR primers used to generate the following cDNA products are as follows: RT-PCR-A (320 bp), fp 5′CGGCACGAGGCGGCTA3′ and rp 5′CTGAGCTGCTAACAGGATC3′; RT-PCR-B (565 bp), fp 5′CAGGAAGCGGCGGGAG3′, rp 5′GCCCGCTGACACCGTTC3′; RT-PCR-C (562 bp), fp 5′CAGAACCAGTGTGACGACGC3′, rp 5′CTTCTGCTTGCCATCC3′; RT-PCR-D (675 bp), fp 5′CAGCAGTGCCGTGGAAAC3′, rp 5′TCCAGAAGCTCTGTCAGGACC3′; and RT-PCR-E (649 bp), fp 5′CGCCTCAGAAGCCCAAGA3′, rp 5′CAAGATTCTGCTCTATGCG3′. The conditions for PCRs were as above except the annealing temperatures were primer specific and ranged from 55-66°C and elongations were at 72°C.

Chromosomal localization. Hybridization of Southern blots of PvuII-digested DNA from C57BL/6J and *Mus spretus* with a 2550 bp *Tcof1* partial cDNA probe (RT-PCR-1: nucleotides 312 to 2861, a region homologous to human *TCOF1* exons 4 to 20) revealed a restriction fragment length polymorphism that was used to map the *Tcof1* locus. *Pvu*II digested fragments of 4.0 and 2.0 kb were detected in C57BL/6J DNA, while fragments of 3.5, 2.4, and 2.0 kb were detected in *Mus spretus* DNA. The 4.0 kb informative PvuII RFLP was used to follow segregation of the *Tcof1* gene in 94 N2 animals [(C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei)] from the Jackson Laboratory BSS interspecific backcross panel (9). The presence or absence of the 4.0 kb C57BL/6J-specific PvuII fragment was followed in backcross mice. Recombinational distances, most likely marker order, and statistical analyses were performed with the program Map Manager (10). Complete raw data for the Jackson BSS backcross are available (http://www.jax.org/resources/documents/cmdata).

Northern blot analysis. Northern blots containing poly A+ RNA from fetal Swiss Webster/NIH mice of 7 to 17 dpc and from adult BALB/c mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (CLONTECH Laboratories, Palo Alto, CA) were probed with the random-primed α-^{32}P-dCTP-labeled, 2550 bp *Tcof1* partial cDNA (RT-PCR-1) and *β-actin* cDNA (CLONTECH Laboratories) probes using the Hybrisol I hybridization solution (50% formamide, 6×SSC; Oncor, Gaithersburg, MD) at 45°C and wash conditions of high stringency at 65°C with 0.1×SSC, 0.1%SDS. The level of *Tcof1* expression was quantitated by standardization relative to *β-actin* expression using the STORM860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Our initial strategy to obtain mouse *Tcof1* cDNA clones utilized a computer-based search, rather than a hybridization approach to screen cDNA libraries. Our BLAST search using the human *TCOF1* sequence (1) yielded homologous, mouse cDNA sequences from nine clones. The four clones with regions of highest identity to TCOF1 were sequenced. The Soares mouse 19.5 dpc total fetus cDNA clone 473404 (GenBank accession no. AA038551) is 76.4% identical to the 5′ end of the human *TCOF1* sequence (nucleotides 1 to 377 of exon 1 through a portion of exon 4). The other three clones contain overlapping sequences and are homologous to the 3′ end of the human *TCOF1* sequence. The Stratagene mouse embryonic carcinoma cDNA clone 532131 (GenBank accession no. AA068319) has 71.1% identity to *TCOF1* exon 19 through a portion of exon 23 (nucleotides 3043 to 3800). The Soares mouse 13.5-14.5 dpc total fetus cDNA clone 386990 (GenBank accession no. W65599) and Soares mouse 4 weeks cDNA clone 621553 (GenBank accession no. AA178758) are homologous to *TCOF1* exons 20 to 23 and exons 21 to 25, respectively. We performed BLASTN searches using sequences from these clones to detect additional overlapping clones. The sequence from clone 621553 is homologous to the Soares mouse 13.5-14.5 dpc total fetus cDNA clone 390622 (GenBank accession no. W71736). This clone extends the mouse sequence homology to the end of the *TCOF1* open reading frame.

We used RT-PCR to amplify cDNA from mouse total RNA, to confirm the sequences from these clones and to obtain the intervening sequence (Fig. 1). *Tcof1* cDNA was generated for five regions homologous to human *TCOF1* 5′ untranslated region (5′ UTR) through exon 3 (RT-PCR-A), exons 1 through 6 (RT-PCR-B), exons 18 through 22 (RT-PCR-C), exons 20 through 23 (RT-PCR-D), and exon 23 to the 3′ UTR (RT-PCR-E) using primer sets (see Methods section) based on sequences from either clones or overlapping RT-PCR products.

Mouse cDNA sequence homologous to human *TCOF1* exons 4 through 20 was also obtained by RT-PCR. After first strand synthesis, PCR was performed on cDNA with GSP1 from the mouse cDNA

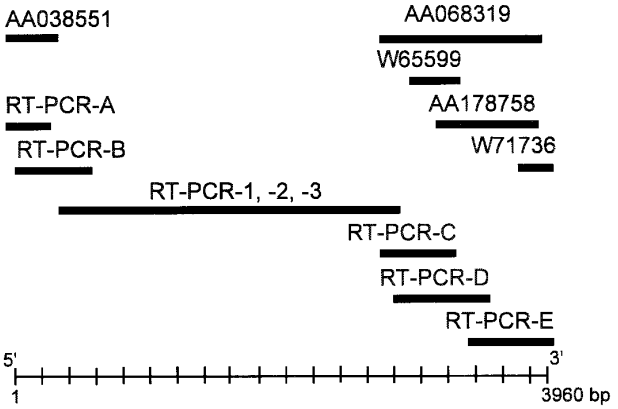

FIG. 1. Diagram of the clones (designated by their GenBank accession no.) and RT-PCR products used for sequencing. Their size and location are shown relative to the 3960 bp open reading frame of *Tcof1*. The scale is marked in 200 bp intervals.

sequence that is homologous to human *TCOF1* exon 22 and forward GSP2 homologous to human *TCOF1* exon 3. The product of this reaction was used as a template for amplification with forward GSP3 homologous to *TCOF1* exon 4 and reverse GSP4 homologous to *TCOF1* exon 20. The second PCR yielded a 2550 bp product. The sequences from three independently derived clones or PCR products (RT-PCR-1, -2, -3, Fig. 1) were obtained in both directions. A consensus sequence was generated to exclude errors introduced by PCR, cloning, or sequencing.

The mouse *Tcof1* open reading frame is 3960 bp in length (GenBank accession no. AF001794). It is 273 nucleotides shorter than and is 74.3% identical to human *TCOF1* (BESTFIT; Genetics Computer Group Software, Madison, WI). Translation of the nucleotide sequence predicts a 1320 amino acid protein of approximately 135 kd (GENE RUNNER for Windows; Hastings Software, Inc., Hastings, NY) (Fig. 2). It displays 61.4% identity and 71.6% similarity with human TCOF1. Tcof1 is also of low complexity, consisting primarily of serine (14.9%), alanine (14.2%), lysine (11.7%), proline (9.6%), and glutamic acid (8.2%) residues. It is a soluble, basic protein with a theoretical pl of 9.35 (ExPASy: http://expasy.hcuge.ch/cgi-bin/protparam) and average hydrophobicity of −0.924016 (SOSUI: http://www.tuat.ac.jp/cgi/~mitaku/sosui2.script).

Amino acid alignments assisted in the visual inspection and identification of repeating motifs and putative phosphorylation, nuclear, and nucleolar localization sites. As in the human protein, the carboxyl-terminal end of Tcof1 contains several nuclear localization signal motifs (Fig. 2). The central portion of the mouse Tcof1 protein has a repeated amino acid motif unit, -KP-GK--Q--A-S--K--GA--PGK--GPVA--Q--K--ED-s-SssEE--sDsE--P--PA-Q, that contains serine- and acidic-rich sequences (Fig. 2) that are potential casein kinase phosphorylation sites (lower case letters) similar to those seen in human TCOF1. Each of these amino acids occur in at least five of the ten repeat units in mouse Tcof1. All, but seven of these conserved amino acids are also present in at least five of the ten repeat units in the human gene. Human *TCOF1* exons 7 to 16 are each translated to yield one of the ten repeat units of relatively conserved array of amino acids (1). The function of this interesting repeating motif is unknown. Neither the repeat unit coded by exon 14 in human TCOF1 or the homologous repeat unit in mouse contains the serine- and acidic-rich sequences (Fig. 2). The interspecies amino acid identity is less among their respective repeat units in the central portion of the protein, than between either their amino- or carboxyl-terminal ends (Fig. 3). Mouse Tcof1 amino acid residues 1 to 211 are 75.1% identical and 81.8% similar to the human protein sequence coded by its exons 1 through 6. Mouse Tcof1 amino acid residues 840 to 1320 are 60.8% identical and 72.3% similar to the human protein sequence generated from its exons 17 through 25. The region of the ten repeat units in mouse and humans are 56.9% identical and 67.3% similar. Conservation in these regions is similar to that seen for other nucleolar phosphoproteins. For example, the human (11) and rat (4)Nopps are 89.9% identical at the amino terminal end (NOPP amino acid no. 1-79 and Nopp amino acid no. 1-79) and 88.3% identical at the carboxyl terminal end (Nopp amino acid no. 557-704 and NOPP amino acid no. 553-699), but only 68.3% identical at the repeats from the central region.

The *Tcof1* cDNA sequence maps to a conserved region on mouse chromosome 18 syntenic with human chromosome 5q32→q33 where its human homologue has been mapped (12). We have confirmed its location to the midportion of mouse chromosome 18 by interspecies backcross analysis. C57BL/6J and *Mus spretus* DNAs were digested with several enzymes and analyzed for informative restriction fragment length polymorphisms (RFLPs) by Southern blotting using a Tcof1 cDNA probe (RT-PCR-1, Fig. 1). The most likely marker order and recombination frequencies (expressed as genetic distances in centimorgans +/− the standard error; Map Manager, (10)) are centromere--[*Gk-rs1*]--3.19+/−1.81--[*Atq1, Lmnb1, D18Wsu181e*]--1.06+/−1.06--[*Tcof1, li*]--2.13+/−1.49--[*D18Hun11, D18Bir6*]. The *Tcof1* gene showed no recombination with the Ia-associated invariant chain gene (Ii) or with several anonymous DNA markers (*D18Bir5, D18Ucl1, D18Xrf246, D18Hun10, code 443*). Our raw typing data for this cross, as well as for all other markers typed using the Jackson BSS backcross, are available at http://www.jax.org/resources/documents/cmdata.

Determination of the temporal and tissue-specific

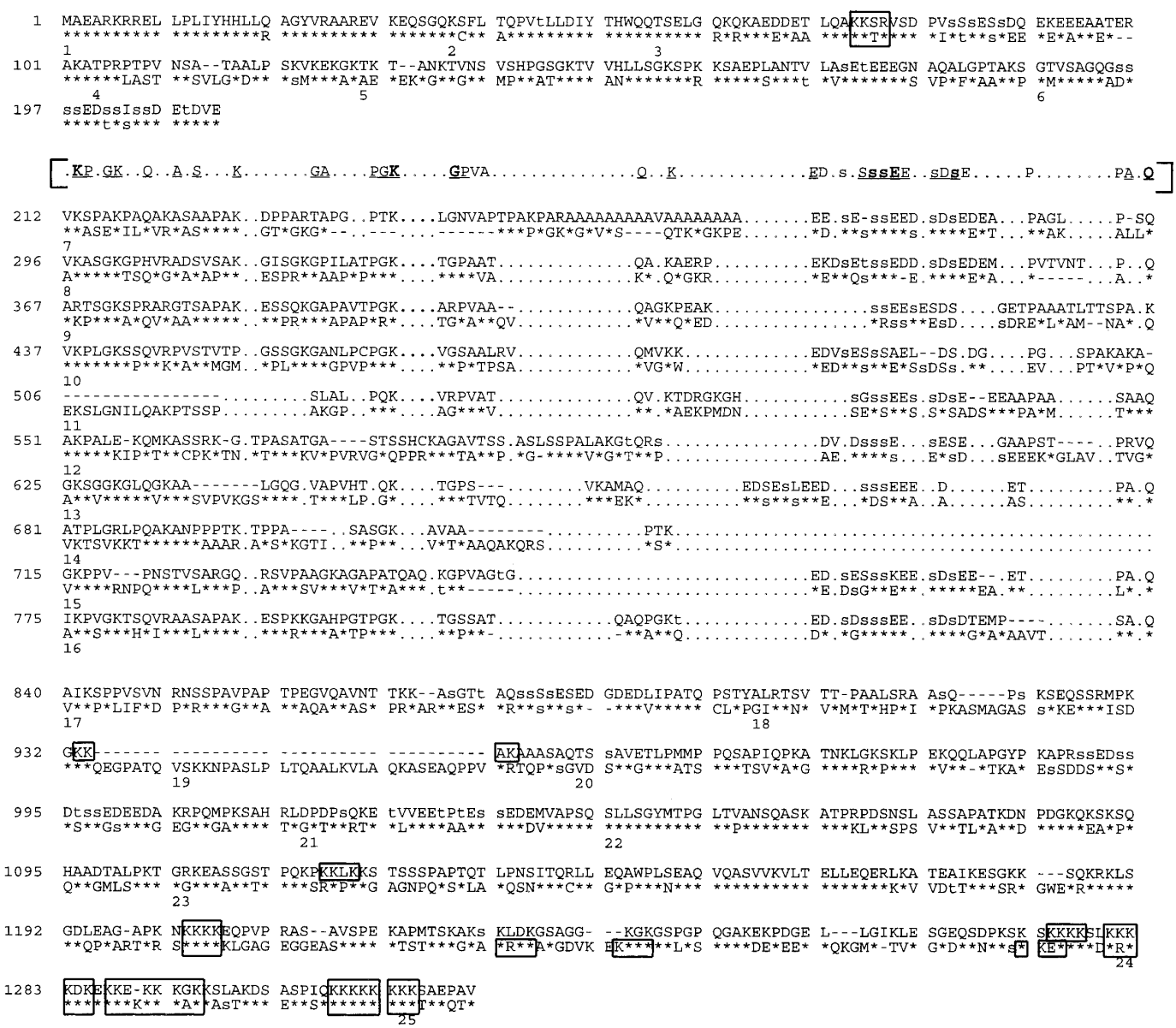

FIG. 2. The Tcof1 amino acid sequence is presented. The amino acid residues are numbered on the left. The mouse protein was aligned to the human TCOF1 using the computer program BESTFIT. The mouse sequence is above the human sequence. Asterisks indicate identity. The dashes (−) indicate gaps introduced by the program to produce the best alignment between the mouse and human protein. Regions of human TCOF1 coded by each of its 25 exons are indicated by the exon number under the first amino acid coded by the exon. The repeated domains are aligned among each other. Dots (. . .) were introduced to achieve the best alignment. Above the ten repeats, the conserved amino acid motif for the units is shown after amino acid 211 and between the brackets. These amino acids occur in at least five of the ten of the repeats in mouse and in human, as well, if underlined. The bold-typed amino acids (-K--K--G--ssE--s--Q) are conserved in at least seven of the ten repeats in both species. There are 82 amino acids in mouse and 87 in human that are potential phosphorylation sites, shown in lower case, and nuclear and nucleolar localization signals are boxed.

expression of *Tcof1* by Northern blot hybridization revealed an approximately 4.2-4.6 kb band in all lanes showing it is expressed in embryonic and adult stages of development and has relatively, ubiquitous tissue expression in adults (Fig. 4). The human and mouse *Tcof1* appear to be expressed in the same adult tissues tested (1,3). The expression of mouse adult *Tcof1* is increased in lung and testis by 1.3-1.5 times and decreased in liver and kidney 0.4-0.6 times relative to the other adult tissues. Mouse embryos of 11 dpc express approximately 2.80, 1.96, and 2.15 times as much *Tcof1* relative to earlier (7 dpc) and later (15 dpc and 17 dpc) embryonic stages of development, respectively.

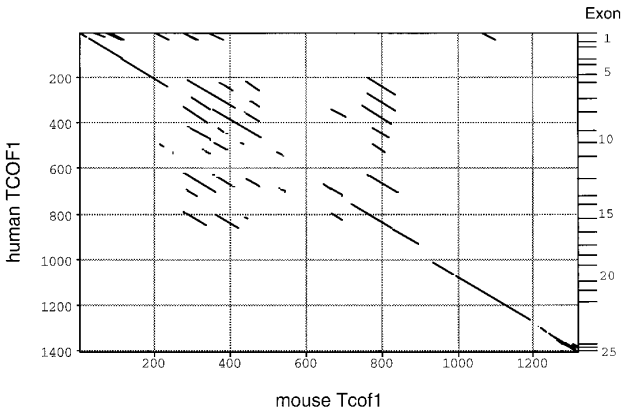

FIG. 3. Homology matrix between mouse and human TCOF1 (Pustell Protein Matrix, MacVector; IBI, New Haven, CT). The x-axis is the mouse amino acid sequence (1320 amino acids) and the left y-axis is the human amino acid sequence (1411 amino acids). The right y-axis shows the regions of the human protein that are coded by each of its 25 exons. A point is plotted for a window of 60 amino acids in mouse and human TCOF1 that yields greater than 30% identity. Note amino acid sequences at the amino- and carboxyl-terminal ends of the protein are the most identical regions between mouse and humans. Multiple offset lines demonstrate less identity among the repeat units.

DISCUSSION

We have isolated and sequenced the entire coding region of mouse *Tcof1*. The conserved, potential phosphorylation and nuclear localization signal sites and the mapping of mouse Tcof1 to a chromosomal 18 region syntenic with the human chromosomal assignment of *TCOF1* to 5q32-q33 confirm that this protein is the mouse homologue to human TCOF1. Like the human protein, it may function as a transcription co-activator and/or a protein involved in nucleolar trafficking. Our mouse *Tcof1* sequence differs from another report (12; GenBank accession number U81030). After nucleotide 381 (A of the start codon ATG is nucleotide number 1), our *Tcof1* nucleotide sequence contains 54 bps that are not found in published sequence from Dixon and colleagues and considered by them to be one of the larger gaps in alignment between human and mouse *Tcof1*. This additional sequence was detected in three independently derived, RT-PCR products sequenced in both directions and codes for 18 amino acids (KTANKTVNSVSHPGSGKT) that are 55.5% identical to a region from amino acid 130 to 147 coded by human *TCOF1* exon 5 and does not alter the subsequent amino acid sequence. This cDNA sequence does not result from alternative splicing because no intron or splice sites are present in the sequence of PCR products generated from this region in mouse genomic DNA (BALB/c) (data not shown). Therefore, we conclude that the full length cDNA contains these 54 bps. However, we concur that there are still two relatively large gaps in alignment between mouse Tcof1 and the human protein coded between exons 10 and 11 and also between exons 18 and 20. The exon structure in the latter region is not conserved between species. The primers predicted to be homologous to the 5′ end of exon 18 (5′CTCAGAACCAGTGTGACGACGC3′) and to exon 19 (5′ACGCCGCCGCCTTTGC3′) of human TCOF1 generated a PCR product from mouse genomic DNA that demonstrated the absence of an intervening intron (data not shown). This suggests that in mouse there may not be an exon equivalent to exon 19 in *TCOF1*. Our sequence also differs at amino acid residue 35, a glycine (codon GGC), from the reported alanine (GCC) (12). The corresponding residue in the human TCOF1 is also a glycine (Fig. 2).

It has been hypothesized that *Tcof1* should be expressed as early as 8-9 day post-conception (dpc) in mice or 4-5 weeks post-conception in humans when the first and second branchial arches appear. The branchial arches are largely populated by cranial neural crest cells which form the facial structures (orbits, zygomatic, malar, middle ear ossicles, and mandible) which are affected in Treacher Collins syndrome (13,14). The higher level of expression of *Tcof1* at the time when the branchial arches arise could be a possible explanation as to why a ubiquitously expressed gene when mutated would result in a mandibulofacial dysostosis phenotype.

Specific spatiotemporal *in situ* hybridization studies have revealed that *Tcof1* is expressed, at an especially high level, in the craniofacial region (12). Apparently, its peak expression level is at 9 dpc, and expression is reduced to near background level by 10 dpc as assessed by whole mount *in situ* hybridization on whole embryos. Our quantitative data, however, suggests that

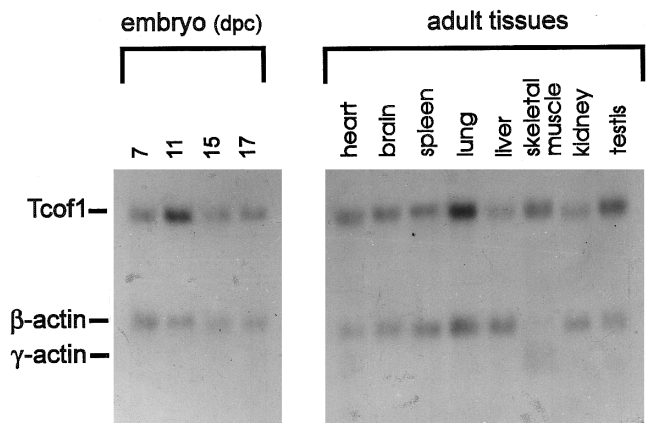

FIG. 4. Expression of *Tcof1* in embryonic (7-17 dpc) and adult tissues by Northern blot hybridization. The blot was simultaneously probed with *Tcof1* partial cDNA (RT-PCR-1) (4.2-4.6 kb band) and *β-actin* cDNA probes. The level of *Tcof1* expression was compared to that of *β-actin* (2 kb band). For skeletal muscle, there is more *γ-actin* (1.8 kb band), than *β-actin* expression which are both detected by the *β-actin* probe.

Tcof1 expression level for the whole mouse embryo is still elevated at 11 dpc and gradually decreases from day 15 to 17. The higher level of expression during 11 dpc embryonic stage of development is consistent with the presence of the branchial arches and facial swellings during organogenesis.

Because *Tcof1* is expressed in the craniofacial region, creating a mouse heterozygous (+/−) for a deleted *Tcof1* allele could result in a mouse with mandibulofacial dysostosis, especially because TCOF1 mutations are predicted to cause haploinsufficiency in Treacher Collins syndrome. Moreover, several animal models created in this fashion have shown direct phenotypic correlation with the human condition. For example, the heterozygous *GLI3* or *TWIST* deleted allele in mice results in a phenotype similar to that seen in the human craniofacial conditions, Greig cephalopolysyndactyly and Saethre-Chotzen syndrome, respectively (15,16). A search of the Mouse Genome Database (http://www.informatics.jax.org/mgd.html) revealed that the only uncloned mouse mutation in this region of chromosome 18 is shaker-with syndacytlism (*sy*), a recessive radiation-induced mutation. The *sy* homozygous mutant phenotype bears little resemblance to that of Treacher Collins syndrome other than deafness due to differing structural etiologies, so that it is unlikely that *sy* represents a mutation in the *Tcof1* gene.

Therefore, it will be important to make a mouse model for mandibulofacial dysostosis which will allow developmental and genetic studies that are not possible in man. Also, even if a "true" mouse model is not created, phenotypic examination of such a mouse and, if viable, a homozygous null *Tcof1* mouse (−/−) could yield information about the biological function of TCOF1. Additional cellular and biochemical assays will determine if TCOF1 acts as a transcription factor and/or a chaperone protein.

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