Tel-2 Is a Novel Transcriptional Repressor Related to the Ets Factor Tel/ETV-6*

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We report here the isolation of Tel-2, a novel member of the Ets transcription factor family, with high homology to Tel/ETV-6. Tel-2 is the second mammalian member of the Tel Ets family subclass whose prototype Tel is involved in various chromosomal translocations in human cancers. Six differentially expressed alternative splice products of Tel-2 were characterized encoding different Tel-2 isoforms which either contain or lack the amino-terminal Pointed domain and also vary at the carboxyl terminus. In contrast to Tel, which is highly expressed in several different cell types and tissues, Tel-2 is only weakly expressed in a variety of tissues and cell types, including placenta, prostate, spleen, liver, and lung. Tel-2 binds to functionally relevant Ets-binding sites of several genes and only the Tel-2 isoform containing the Pointed domain and the DNA-binding domain acts as a strong repressor of transcription. The retinoic acid receptor α and bone morphogenetic protein-6B (BMP-6) genes are specifically repressed by Tel-2 indicating a function for Tel-2 as an inhibitor of differentiation. Due to the important involvement of Tel in human cancer and the location of Tel-2 within the MHC cluster region, Tel-2 might be involved in chromosomal translocations in human cancer as well.

Deregulated gene expression due to aberrant transcription factor expression is a critical determinant in tumorigenesis. Many oncogenes and tumor suppressor genes encode transcription factors (1, 2). Chromosomal translocations, mutations, gene amplifications, and deletions involving transcription factor genes appear to be frequent in various human malignancies and are specific for a particular tumor type, thereby emphasizing the importance of a stringent control of these factors in normal physiological conditions (1). One particular transcription factor family, the Ets family, has recently gotten a lot of attention with regard to human cancer. The Ets transcription factor family contains almost 30 different mammalian members (3, 4) that function as transcription factors under normal physiological conditions (5–8). All Ets factors share a highly conserved DNA-binding domain, the Ets domain which is sufficient to interact specifically with DNA sequences (5–8).

Various members of the Ets transcription factor family have been shown to cause cellular transformation, when aberrantly expressed. The relevance of Ets transcription factors in human cancer has recently been highlighted by the discovery of several distinct and very specific chromosomal translocations involving various members of the Ets family in different types of human cancer (5–8). The Ets gene Tel/ETV6 on chromosome 12 is involved in many translocations leading to fusion of Tel to different genes including various tyrosine kinases such as abl, Jak2, Ntrk3, and platelet-derived growth factor receptor as well as other transcription factors such as AML1, M1, Cdx2, Mds/evil, and Stl, the fatty acyl-CoA synthetase 2 gene, Ac52, and a gene of unknown function, BTL (9–21). Similarly to Tel, fusion of the Ets factor erg to the fes gene occurs in AML (22). Another type of human cancer, Ewing’s sarcoma, is characterized by translocations of a number of different Ets factors, erg, ETV1, E1-AF, FEV, or fli-1, to the EWS gene (23–27).

Under normal physiological conditions, Ets factors play a critical role in the regulation of genes involved in tissue-development, differentiation, angiogenesis, cell cycle control, and cell proliferation as both transcriptional enhancers or repressors (5–8). The relevance of Ets factors for cell differentiation has been substantiated in knockout mice lacking an Ets factor gene and Tel knockout mice are embryonal lethal due to a yolk sac angiogenic defect (28–30). Tel is the prototype and sole member of a distinct subclass of Ets factors with the closest homologue being the Drosophila Ets factor Yan. A recent deposition of the full-length sequence of a human genomic DNA PAC clone derived from chromosome 6p21.3 within the MHC cluster region indicated the potential existence of a novel Tel-related Ets factor. We now report the cloning and characterization of full-length cDNAs for this new member of the Tel subclass of Ets factors, Tel-2, which is present in at least six alternative splice forms.

MATERIALS AND METHODS

Cell Culture—Human foreskin keratinocytes, CV-1 (green monkey kidney), LNCaP (human prostate), HEK293 (human fetal epithelial kidney), C-33A (human cervical carcinoma), A431 (human vulvar carcinoma), HeLa (human cervical carcinoma), H157 (human large cell lung carcinoma), H249 (human small cell lung carcinoma), HUVEC (human endothelial), U-87 Mg, and U-138 Mg (human glioma cells), U-937 (human monocytes), MG-63 (human osteosarcoma), human synovial fibroblasts, and human chondrocytes were grown as described (31–33).

Isolation and Analysis of cDNA Clones Encoding a Novel Ets-related Protein—To search for novel members of the Ets family human public DNA data bases were searched for sequences homologous to known Ets members as described (32). The human DNA sequence from PAC 50J22

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on chromosome 6p21.3 contained sequences predicting homology to the Ets-related protein Tel. These sequence homologies were spread over several putative exons indicating that it does not encode a pseudogene. PCR primers across two exons spanning the Ets domain were synthesized and used to determine whether we could detect transcripts for this putative gene. RT-PCR amplification of cDNAs derived from human pancreas and prostate as described (34) yielded the expected amplification product of 229 bp. Sequence analysis confirmed that this fragment encoded the new Tel-related cDNA Tel-2.

5′ and 3′ RACE Primer Extension—The 229-bp cDNA amplified from human prostate RNA contained an open reading frame throughout this clone suggesting that part of the 5′ and 3′ end were missing. To determine the 5′ and 3′ end of Tel-2 we performed the RACE method using human adult prostate cDNA ready for 5′-RACE (CLONTECH) and nested primers specific for the 5′ and 3′ ends of the partial Tel-2 cDNA as described (35). Amplified DNA fragments were subcloned and sequenced as described (35). The sequences of the Tel-2 cDNAs were confirmed by repeating amplification using primers specific for both ends of the longest RACE products obtained in the first two rounds of PCR amplification. The 5′ and 3′ end sequences of the Tel-2 cDNAs were confirmed by repeating 5′- and 3′-RACE PCR amplification using primers specific for the 5′ and 3′ ends of the longest RACE products obtained in the first two rounds of PCR amplification. Six alternative splice variants of Tel-2 were isolated and confirmed by full-length cDNA sequencing.

DNA Sequencing and Computer Analyses—Nucleotide sequences were determined at the Beth Israel Deaconess Medical Center DNA sequencing facility using an Applied Biosystems Prism Automatic DNA Sequencer Model 377 using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequence analysis utilized DNA Strider, Lasergene (DNASTAR) and BLAST, BEAUTY, and Clustal W searches (NCBI). All oligonucleotides were purchased from Life Technologies, Inc.

RT-PCR Analysis—cDNAs were generated from 1 μg of mRNA isolated from different cells or tissues using oligo(T)12–18 (priming (Life Technologies, Inc.) and Moloney murine leukemia reverse transcriptase (Life Technologies, Inc.) in deoxyribonuclease I (Life Technologies, Inc.) treated samples. cDNAs derived from different fetal human tissues were obtained from CLONTECH. Each PCR reaction used equivalent amounts of 0.1 ng of DNA, 4 ng/μl of each primer, 0.25 units of Taq polymerase (Promega, Madison, WI), 150 μM of each dNTP, 3 μl of MgCl2, reaction buffer, and water to a final volume of 25 μl and were covered with mineral oil. The sequences of the ubiquitous Tel-2 specific primers were: sense, 5′-CTCTAGGAAGAAGATGTCAGTCG-3′ and antisense, 5′-CCAGGGGCTCTGCTGTCG-3′ with an expected amplification product of 134 bp. The sequences of the Tel-2 specific primers for the 5′ end were: sense, 5′-ACCTGTAACATCGCTGTCCT-3′ and antisense, 5′-CCAGGGGCTCTGCTGTCG-3′ and crossed two introns between exons 6 and 8 with an expected amplification product of 273 bp. The sequences of the Tel-2/Tel-2′ specific primers were: sense, 5′-GCTCTAGAAGATGTCAGTCG-3′ and antisense, 5′-CCAGGGGCTCTGCTGTCG-3′ and crossed three introns between exons 5 and 9 with an expected amplification product of 438 bp. The sequences of the Tel-2 5′ end specific primers were: sense, 5′-CGGCTAACGAGAAACCAGG-3′ and antisense, 5′-CTCGGACACGTTCACATG-3′ and crossed three introns between exons 1 and 4 with an expected amplification product of 418 bp for Tel-2a, 377 bp for Tel-2b, 241 bp for Tel-2c, and 212 bp for Tel-2d. The sequences of the primers for GAPDH were: sense, 5′-CAAGATCTGCTAGATGATCAC-3′ and antisense, 5′-CCATGGGAAAGGCTGGG-3′ with an expected amplification product of 200 bp. The sequences of the primers for retinoic acid receptor α (RARA) were: sense, 5′-GTGTTGCGCCCGCTATCTATG-3′ and antisense, 5′-GTGTTGCGCCCGCTATCTATG-3′ with an expected amplification product of 360 bp. The sequences of the primers for BMP-6 were: sense, 5′-TCCGGGTTCTCAAGTGTTGGA-3′ and antisense, 5′-ACAGCTATACGGGGTCTT-3′ with an expected amplification product of 412 bp.

RT-PCR amplifications were carried out using a PerkinElmer Life Sciences thermal cycler 480 as follows: 20–35 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C followed by 10 min at 72 °C. 10 μl of the amplification product was analyzed on a 2% agarose gel.

Expression Vector and Luciferase Reporter Gene Constructs—The full-length Tel-2 cDNAs were inserted into the EcoRI site of the pCI vector as a COOH-terminal epitope generating pCI/Tel-2a, pCI/Tel-2b, and pCI/Tel-2c expression vectors. The PCR products were subcloned into the SalI site of pCI/HA in-frame with the COOH-terminal HA epitope tag.

Synthetic wild type E74 cDNA site oligonucleotides containing SalI and XhoI ends were inserted into the SalI site of the Δ56-e-fos-pGGL3 plasmid as described (32). The lyn promoter and IgG enhancer pGL3 luciferase constructs are as described (32).

DNA Transfection Assays—Co-transfections of 3 × 105 CV-1 cells were carried out with 3.5 μg of E74/Δ56-pGGL3, lyn/pGL3 or IgH/Δ56-pGGL3 reporter gene construct DNA and 0.5, 1, 1.5, or 2 μg of Tel-2 expression vector DNA using 12.5 μl of LipofectAMINE (Life Technologies, Inc.) as described (32). The cells were transfected 16 h after transfection. Transfections for every construct were performed independently in duplicates and repeated 3 to 4 times with two different plasmid preparations with similar results. Equal amounts of cell extract protein were used to normalize the luciferase assay. Co-transfection of a second plasmid for determination of transfection efficiency was omitted because potential artifacts with this technique have been reported (36) and because many commonly used viral promoters contain potential binding sites for Ets factors.

To compare relative expression levels of Tel-2 isoforms after transfection 3 μg of pCI/HA Tel-2a, Tel-2b, and Tel-2c expression vectors were transfected into COS cells using 12.5 μl of LipofectAMINE. Whole cell extracts were generated 16 h later using a lysis buffer containing 1× phosphate-buffered saline, 5 mM EDTA, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 10 μM leupeptin, 25 μg/ml aprotinin.

In Vitro Transcription-translation—Coupled in vitro transcription-translation reactions with the different untagged and HA-tagged Tel-2 splice variants inserted downstream of the T7 promoter into the pCI vector were performed in TNT rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of [35S]methionine (PerkinElmer Life Sciences) as described (37).

Electrophoretic Mobility Shift Assays (EMSA)—EMSAs and supershift assays were performed as described (33, 37) using 2 μl of in vitro translation product or 3 μl of whole cell extract and 0.1–0.2 ng of 32P-labeled double stranded oligonucleotide probes (5,000–20,000 cpm) in the presence or absence of competitor oligonucleotides (1, 5, 10, 25, 50, and 100 ng) as described above. Competitive EMSA was performed by adding 1 μg of anti-HA tag or anti-mouse IgG antibody and incubating on ice for 10 min. Complexes were then resolved on 10% polyacrylamide gels, containing as buffer 0.5 × TBE as described (32). The anti-influensa HA tag monoclonal antibody was from Roche Molecular Biochemicals and anti-mouse IgG was from Pharmingen.

Oligonucleotides used as probes and for competition studies as follows. 1) Drosophila E74 WT oligonucleotide: 5′-TGCGAGTAAACGAACTCATG-3′ and 3′-ACCTGAGTTCTACATTGACG-5′; 2) Drosophila E74 MUT oligonucleotide: 5′-TGAATACGGGTTGTAATCAG-3′ and 3′-CATTGCGCAACAGGTGCTGAC-5′; 3) murine bHk promoter WT oligonucleotide: 5′-TGACTCCAGAGGACAGGAGTCT-3′ and 3′-CAGCAGCCCTCTCCTGATCAG-5′; 4) Drosophila E74 promoter WT oligonucleotide: 5′-TGACTCCAGAGGACAGGAGTCT-3′ and 3′-CAGCAGCCCTCTCCTGATCAG-5′; 5) polyoma virus early promoter oligonucleotide: 5′-TGAATACGGGTTGTAATCAG-3′ and 3′-ACCTGAGTTCTACATTGACG-5′; 6) HIV-2 LTR WT oligonucleotide: 5′-TGAATACGGGTTGTAATCAG-3′ and 3′-CATTGCGCAACAGGTGCTGAC-5′; 7) HTLV-1 LTR WT oligonucleotide: 5′-TGCGAGTAAACGAACTCATG-3′ and 3′-ACCTGAGTTCTACATTGACG-5′; 8) CAMP responsive element oligonucleotide: 5′-TGAATACGGGTTGTAATCAG-3′ and 3′-CAGCAGCCCTCTCCTGATCAG-5′; 9) C/EBP binding site oligonucleotide: 5′-TGACTCCAGAGGACAGGAGTCT-3′ and 3′-CAGCAGCCCTCTCCTGATCAG-5′; 10) MHC class II enhancer pGL3 luciferase construct.

The abbreviations used are: PCR, polymerase chain reaction; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; HA, hemagglutinin; T7, T7 RNA polymerase; T7 promoter, T7 RNA polymerase and the associated scaffold; 3′UTR, 3′ untranslated region; Ets, erythroblast transformation specific; ets, human immunodeficiency virus, LTR, long terminal repeat; HIC, myosin heavy chain.
human lyn promoter WT oligonucleotide: 5'-TCGAACCAAGAAGT-GCTGACC-3' and 3'-CGGTTGCTCTTACCGACCTGCAG-5'; (13) mouse Enod enhancer WT oligonucleotide: 5'-TCGAAGACTGGA-CAGAAATGAACTGAC-3' and 3'-GGCTGACACTCTGCCTCCTCATC-CTTGTGACAGGGC-5'; (14) human SPRR2A promoter WT oligonucleotide: 5'-TCGACGAGAAGAATGCAGAACCTACCCG-3' and 3'-CTGCTGCTCTCACTTTGATGGGCAGCT-5'.

**RNA Isolation and Northern Blot Analysis—**Poly(A)¹ mRNA were isolated as described by Libermann et al. (31). Total cellular RNA was isolated using guanidine isothiocyanate nucleic acid extraction and cesium chloride gradient ultracentrifugation (38).Northern blots and dot blots containing poly(A)¹-selected mRNA derived from different human tissues (CLONTECH) were hybridized with random prime-labeled Tel-2 full-length cDNA in QuickHyb solution (Strategene) as described (32) and washed at 50 °C with 0.2 SSC, 0.2% SDS.

cDNA Microarrays—10⁶ human osteosarcoma MG-63 cells were transiently transfected in duplicates with 8 µg of pCI-Tel-2b or pCI plasmid using 30 µl of Superfect (Life Technologies, Inc.). Total RNA was isolated separately from each plate 18 and 20 h after transfection using the RNeasy Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. Three types of Atlas human cDNA microarray nylon membranes from CLONTECH Laboratories, Inc. (Palo Alto, CA), each containing 1136 different genes, were hybridized with [³²P]dATP-labeled cDNAs derived from 4 µg of total RNA acquired from the manufacturer at 68 °C in ExpressHyb solution (CLONTECH Laboratories, Inc.) using a Model 2000 Micro Hybridization Incubator (Robbins Scientific). The filters were washed according to the user’s instruction and exposed to Bio-Max MS film (Fisher) for different exposure times. Hybridizations were performed with duplicate experiments.

The spot intensities reflecting gene expression levels on the Atlas human cDNA array were quantified using the Atlas Image 1.5 Software (CLONTECH). The Tel-2b gene expression profiles were compared with the gene expression profile with the parental pCI vector normalizing spot intensities based on the average of the intensities of all spots. Genes up-regulated or down-regulated by Tel-2b were validated by RT-PCR.

**RESULTS**

**Isolation and Characterization of Six Alternative Splice Products of the Human Ets-related cDNA, Tel-2—**To search for novel members of the Ets family the GenBank™ data base was searched for sequences homologous to the Ets domain. A human genomic DNA sequence from PAC 50J22 (Z84484) on chromosome 6p21.3 contained sequences related to the Ets factor Tel and was used for further analysis. Since the Tel-related sequences encoding the DNA-binding domain were spread over several putative exons separated by apparent introns, we assumed that this Ets-related gene does not encode a pseudogene. To test whether this putative Ets-related gene is indeed transcribed into mRNA, we designed PCR primers spanning several putative exons and performed RT-PCR using human genomic DNA. Genomic DNA gave the expected amplification product. Human placenta and prostate-derived cDNA resulted in a smaller amplification product which upon sequencing confirmed the existence of a correctly spliced mRNA expressed in those tissues. We describe here the isolation and characterization of full-length cDNA clones for this new member of the Ets family, the second member of the Tel subclass, which we have named Tel-2. To clone the full-length cDNA encoding Tel-2 we
performed the 5’ and 3’ RACE method as described (35) using RACE ready human prostate Marathon cDNA.

Both strands of the full-length Tel-2 cDNAs were sequenced entirely by double-stranded dideoxy sequencing using T7 and T3 polymerase sequencing primers, and Tel-2-specific primers based on partial DNA sequencing (Fig. 1). Four alternative splice products of Tel-2, Tel-2a, Tel-2b, Tel-2c, and Tel-2d, which differed in their coding regions, were identified. The 5’ end sequences of the Tel-2 cDNAs were confirmed by repeating 5’-RACE PCR amplification using primers specific for the 5’ end of the longest 5’-RACE products obtained in the first two rounds of PCR amplification. The 3’ end of these four Tel-2 splice variants contains a long poly(A) tract which is preceded by a classical polyadenylation site (Fig. 1) at an appropriate distance. All four Tel-2 splice products contain the same 3’ and 5’ ends and are alternative splice forms as was confirmed by PCR amplification and sequencing of full-length cDNAs encoding all four splice products. The length of the Tel-2a full-length cDNA is 1592 bp, of Tel-2b 1551 bp, of Tel-2c 1415 bp, and that of Tel-2d is 1385 bp (Fig. 1). Inspection of the public EST data base identified four ESTs encoding an apparent fifth alternative splice product (Tel-2e and Tel-2f) that diverges at the 3’ end of the cDNA in comparison to the other four Tel-2 splice variants and contains alternative polyadenylation sequences. To clone this putative fifth splice product we used Tel-2e/f-specific primers at the 3’ end of Tel-2e/f and primers from the
FIG. 2. Genomic organization of the human Tel-2 gene. a, the human Tel-2 genomic structure is shown on the top. Exons 1 to 9 are represented by the filled boxes and the introns in between by lines. The lengths of exons (bp) and introns (kilobases) are shown below the exons and above the introns. Functional domains are indicated. The exon configuration of the six alternative Tel-2 splice products is shown below the genomic structure. b, schematic description of the expected proteins encoded by the six alternative Tel-2 isoforms. Functional domains and the location of potential MAP kinase phosphorylation sites (MAPK) are indicated above the figure. c, nucleotide sequence of the immediate 5'-flanking sequence of the human Tel-2 gene. The apparent major transcription start site is indicated by the arrow and 1. Potential regulatory elements in the promoter region are boxed.
5’ end of Tel-2 and performed RT-PCR using the human prostate Marathon cDNA. Two divergent full-length amplification products of the expected sizes containing the 5’ ends of Tel-2b and Tel-2d, respectively, were obtained and fully sequenced confirming the existence of a Tel-2e and Tel-2f splice product (Fig. 1c). Interestingly, the 3’ end of Tel-2c and Tel-2f contains three apparent polyadenylation sites and an L1 LINE repetitive element (Fig. 1c) (39).

**Genomic Organization of the Tel-2 Gene**—Since the full-length sequence of the human genomic PAC clone had been deposited in GenBank™, we were able to align the cDNA sequences of the six Tel-2 alternative splice products to the genomic sequence. A perfect match with the deposited genomic sequence was obtained and enabled us to determine the intron/exon structure of the Tel-2 gene. Sequence analysis revealed that the total length of the transcribed portion of the Tel-2 gene covers more than 33 kilobases and contains 9 exons (Figs. 2a). The first exon, common to all splice products, contains the 5’-untranslated region and the first two amino acids (Figs. 1 and 2). All intron/exon splice junction borders conform with the splice site consensus (G/GT, . . . CA/G) rule (40). Several introns are relatively large with the largest being ~11.5 kilobases between exon 8 and 9 and ~9.5 kilobases between exon 2 and 3 (Fig. 2a). Four of the six cloned Tel-2 isoforms are due to alternative splicing of exons 2 and 3. Both, Tel-2a and Tel-2b are made up of the first 8 exons; however, Tel-2a contains in addition an alternative splice form of exon 3, which extends exon 3 from 166 bp by an additional 41-bp exon 3a. Both alternative splice forms of exon 3 are flanked by consensus splice sites (40). Tel-2c contains exon 1 and exons 3 to 8, but is missing exon 2, whereas Tel-2d contains exons 1 and 2 and exons 4 to 8 and lacks exon 3. The fifth and sixth alternative splice forms Tel-2e and Tel-2f lack exon 8 which has been replaced by a further downstream exon 9. Tel-2e contains the Tel-2b 5’ exons, whereas Tel-2f lacks exon 3 and, thus, is identical to Tel-2d at the 5’ end.

Based on 5’-RACE RT-PCR the major transcription start site for the human Tel-2 gene can be fairly accurately assigned to the nucleotide G as indicated in Fig. 3. However, we cannot rule out the possibility of multiple start sites with shorter 5’-untranslated sequences. A typical TATA box is not found upstream of the apparent transcription start site at the expected distance; instead, a classical initiator Inr element CCAGTT is found 48 bp upstream of the transcription start site (Fig. 2c) (41). Upstream of the transcription start site is a putative E2F-binding site. A potential Ets-binding site partially overlapped the Inr element. Several additional Ets sites as well as NF-κB, AP1, Ikaros-, and HLH-binding sites are present in the immediate upstream region.

**Predicted Amino Acid Sequence of Tel-2**—Sequence analysis of the Tel-2 isoforms revealed a 1023-nucleotide open reading frame encoding a 341-amino acid protein with a predicted molecular mass of 39 kDa for Tel-2b, starting with an ATG at position 76 and terminating with a TGA at position 1101 (Figs. 1 and 2b). Similar to the highly related Ets factor Tel, the Tel-2b protein can be subdivided into several structural and functional domains. The amino-terminal Pointed domain which is potentially involved in protein-protein interactions and is conserved in a subclass of Ets factors is encoded by exons 2 to 4 (Figs. 1 and 2). The COOH-terminal Ets domain which is the DNA-binding domain conserved among all members of the Ets family is divided into three exons (exons 6 to 8) (Figs. 1 and 2). The open reading frame of Tel-2b is interrupted in Tel-2a due to the insertion of the 41-bp long alternative exon 3a. As a result Tel-2a is potentially a bicistronic mRNA which can encode a polypeptide of 111 amino acids containing the amino terminus of Tel-2b including most of the Pointed domain as well as a polypeptide of 282 amino acids starting with an internal ATG in exon 3 created due to the frameshift by the alternative exon 3a containing a new amino terminus, the middle part of Tel-2b and the COOH-terminal Ets domain (Figs. 1 and 2). Sequence analysis of the Tel-2c cDNA which lacks exon 2 revealed a 780-nucleotide open reading frame encoding a 260-amino acid protein with a predicted molecular mass of 30 kDa, starting with an internal ATG in exon 3 and resulting in a protein that lacks the amino-terminal half of the Pointed domain and the amino terminus of Tel-2b (Figs. 1 and 2). Sequence analysis of the Tel-2d cDNA revealed a 858-nucleotide open reading frame encoding a 286-amino acid protein with a predicted molecular mass of 32.6 kDa, starting with the same ATG in exon 1 as Tel-2b and continuing in-frame until the carboxyl terminus (Figs. 1 and 2). However, due to the in-frame deletion of exon 3 the Tel-2d polypeptide lacks most of the Pointed domain, but contains the amino terminus of Tel-2b and the Ets domain. Analysis of the Tel-2e and Tel-2f isoforms revealed a 951-nucleotide open reading frame encoding a 317-amino acid protein with a predicted molecular mass of 36.2 kDa for Tel-2e and a 786-nucleotide open reading frame encoding a 262-amino acid protein with a predicted molecular mass of 29.8 kDa for Tel-2f, starting with the same ATG in exon 1 as Tel-2b (Figs. 1 and 2). However, due to the replacement of exon 8 by a further downstream exon 9 the last amino acid of the Ets domain and all amino acids immediately downstream of the Ets domain are eliminated and replaced by an alternative carboxyl terminus encoded by exon 9. The carboxyl terminus of Tel-2e and Tel-2f encoding 13 amino acids exhibits no homology to any known protein, but is leucine/isoleucine-rich. Tel-2e and Tel-2f isoforms may express proteins with distinct characteristics from the other Tel-2 isoforms.

The ATG initiator codons only partially conform to the consensus eukaryotic translation initiation sequence (42). There are several reasons to believe that the Tel-2b ATG is the translation initiation codon. No additional ATG is found in-frame and an in-frame termination codon is found 70 bp upstream of the ATG. The same ATG is being used in Tel-2a and Tel-2d. However, in Tel-2a it is possible that an internal ATG generated by the frameshift inserted by exon 3a also leads to translation into a protein encoding the COOH-terminal half of Tel-2b. In vitro translation and EMSA analysis indeed indicate that an internal ATG may be used to generate a fragment containing the DNA-binding domain. Tel-2c also apparently uses an internal ATG to generate a protein encoding the COOH-terminal half of Tel-2b as demonstrated by in vitro translation and EMSA. However, we cannot exclude the possibility that translation of some of the Tel-2 isoforms starts either at another more preferable ATG or at a codon different from ATG.

The deduced amino acid sequence of Tel-2b predicts a protein rich in leucine (11%), glutamic acid (7%), glycine (7%), proline (9%), and arginine (8%). A putative PEST sequence with a PEST score of 9.4 is located between amino acids 138 and 155 in Tel-2b. PEST sequences have been implicated in protein degradation and may suggest a low stability of the Tel-2b protein (43). Several potential phosphorylation sites for Akt, p90rsk, p65 ppk, protein kinase C, casein kinase II, cdc2 kinase, and tyrosine kinase are present in Tel-2 (44–48). The predicted Tel-2b protein sequence reveals, furthermore, seven potential MAP kinase phosphorylation sites (Ser/Thr) for ERK, jnk, p38, one of them containing the optimal PXXS/T/P sequence (49, 50). Interestingly three MAP kinase phosphorylation sites and a tyrosine kinase phosphorylation site are conserved in the highly related Tel as well. The importance of MAP kinase
phosphorylation sites for the biological function of Ets-related factors has recently been demonstrated suggesting that at least some of these putative sites might be functionally relevant for Tel-2 as well (51–53).

Sequence Comparison of Tel-2 to Other Members of the Ets Family—Comparison of the deduced amino acid sequence of Tel-2 with other members of the Ets family revealed the highest homology to Tel and to a lesser extent to the Drosophila Ets factor Yan (Fig. 3). Overall, the amino acid homology between Tel-2b and Tel is ~50%. Homologies are clustered in several primary regions, a putative protein-protein interaction domain (Pointed domain) at the amino terminus, the putative DNA-binding domain (ETS domain) which extends over ~85 amino acids at the carboxyl terminus of the gene, a smaller region just upstream of the Pointed domain which includes a conserved optimal Map kinase phosphorylation site, as well as a short acidic domain (C domain, Fig. 2) downstream of the Ets domain (Fig. 3a). Similarly, Yan shows high homology to Tel-2 in the Pointed and Ets domain as well as some lower homology regions throughout the protein (data not shown). Interestingly, the Tel-2/Tel-2f isoforms have an alternative carboxyl terminus that eliminates the acidic C domain, a region of Tel-2 with high homology to Tel. This conserved C-terminal region of Tel and Tel-2 may have functional significance for these factors such as transactivation or protein-protein interaction.

Alignment of the ETS domain of Tel-2 with that of other members of the Ets family reveals highest homology to Tel (88%) and to a lesser extent to Yan (48%) (Fig. 3b). Sequence identity to all other members of the Ets family is 34–44%. Besides the highly conserved ETS DNA-binding domain, the amino terminus of Tel-2 contains a region with significant homology to the Pointed domain present in Tel and a subset of Ets factors (Fig. 3c). Again homology is highest to Tel (63%). Based on the sequence comparisons it is clear that Tel-2 represents the second mammalian member of the Tel subfamily of Ets factors. Expression Pattern of Tel-2 in Human Tissues—To determine the expression pattern of Tel-2 we performed PCR with cDNA reverse transcribed from RNA derived from different human fetal and adult tissues, since Northern blot and dot blot hybridizations with full-length Tel-2b cDNA probes did not result in any specific signal (data not shown) indicating very low abundance of Tel-2 mRNAs in most tissues. A multiple tissue cDNA panel set of normalized (using several housekeeping genes), first-strand cDNA ready for use in PCR analysis and generated using poly(A)" RNA from different human tissues was obtained from CLONTECH. To control for RNA quality and quantity, we performed PCR with primers specific for GAPDH as well (Fig. 4a). Whereas only 20 PCR cycles were applied for GAPDH, 35 cycles were required for Tel-2, further confirming the low abundance of Tel-2 transcripts. Due to the high number of PCR cycles required to obtain a significant amplification product for Tel-2 and the very low abundance of Tel-2 mRNA in most tissues, a relatively high degree of error in the relative quantities of Tel-2 transcripts can be expected. Nevertheless, the PCR reactions were repeated two times with very similar results. Since Tel-2 is expressed in the form of at least six splice variants, we used PCR primers that are either specific for the 5' end alternative splice variants Tel-2a, Tel-2b, Tel-2e, and Tel-2f resulting in differently sized amplification products, primers specific for the 3' end of Tel-2/Tel-2f including exon 9 or primers specific for the 3' end of Tel-2 containing exon 8. The results indicate that the Tel-2 gene is expressed, although with very low abundance in a variety of fetal and adult tissues tested (Fig. 4a). Strikingly, each Tel-2 splice product displays a distinct expression pattern. In adult tissues, placenta expressed the highest levels of Tel-2, followed by liver, prostate, lung, ovary, spleen, thymus, and peripheral blood lymphocytes (Fig. 4a). Lower levels of Tel-2 mRNA were expressed in most other tissues. In human fetal tissues, lung and kidney expressed the highest amounts of Tel-2 (Fig. 4a). Among the different 5' splice forms of Tel-2 Tel-2d, lacking the Pointed domain, was the most frequently expressed transcript which was expressed in the majority of human tissues to some extent.
Highest levels of Tel-2d were observed in liver, prostate, placenta, and lung. Tel-2b, encoding the full-length protein including the Pointed domain, was also highly expressed in placenta, prostate, liver, and lung, and to a lower extent in several other tissues (Fig. 4a). But a significant number of tissues such as testis, brain, and several fetal tissues expressed Tel-2d without any evidence for Tel-2b expression indicating that their expression is regulated in a tissue-specific manner. Only very low levels of Tel-2a and Tel-2c were detected and only in a few tissues suggesting that these isoforms may play a more limited role. Strikingly differential expression was also observed for the alternative 3′ exons 8 and 9. The Tel-2 isoforms containing exon 8 and, thus, the complete Ets domain were expressed in most of the tissues that express Tel-2 except ovary and heart (Fig. 4a). Highest expression was seen in placenta, lung, liver, pancreas, peripheral blood leukocytes, and fetal lung. Tel-2e/Tel-2f, which contain exon 9, miss the last amino acid of the Ets domain and replace a sequence conserved in Tel with an alternative carboxyl terminus were highly expressed in a subset of tissues, with highest expression in placenta followed by spleen, prostate, thymus, and ovary (Fig. 4a). Low levels of Tel-2e/Tel-2f were also observed in the heart, whereas none of the other tissues expressed any detectable levels of Tel-2e/Tel-2f. Tel-2e/Tel-2f expression is much more restricted than expression of the exon 8 containing Tel-2 transcripts. Interestingly, Tel-2e/Tel-2f expression in ovary, prostate, heart, spleen, and thymus appears to be higher than the exon 8 Tel-2 splice forms demonstrating that the unusual Tel-2e/Tel-2f transcripts are likely to be functionally relevant. These results indicate that several of the Tel-2 isoforms are expressed at significant levels in various tissues, although at strikingly different ratios. Unexpectedly, the Tel-2 isoform closest related to Tel, Tel-2b, which contains the Pointed domain and the Ets domain is not the most frequently expressed transcript.

To determine in more detail expression of Tel-2 in different cell types we performed RT-PCR with RNA derived from different cell lines and primers for a common region of Tel-2 that would amplify all splice variants of Tel-2. Like for the fetal and adult tissues most cell lines expressed some Tel-2 mRNA, although in most cell lines expression was very low as well (Fig. 4b). However, some cell lines including A431 squamous carcinoma cells, U-937 monocytic cells, and primary human chondrocytes expressed highly elevated levels of Tel-2. This was particularly...
striking for A431 cells which expressed by far the highest level of Tel-2 from all RNAs tested. Thus, although Tel-2 is weakly expressed in many tissues and cell types, Tel-2 expression might be enhanced in a subset of cell types or cancer-derived cells.

Tel-2 Binds Specifically to Functionally Important Ets-related Binding Sites in a Variety of Genes—To determine whether Tel-2 can bind in a sequence-specific manner to DNA, Tel-2a, Tel-2b, and Tel-2c were in vitro transcribed and translated into protein in a coupled reticulocyte lysate system. Based on the cDNA sequences of the different Tel-2 isoforms we expected to obtain a full-length Tel-2 protein for Tel-2b and truncated forms for Tel-2a and Tel-2c lacking either the amino terminus or the carboxyl terminus of Tel-2b. SDS-polyacrylamide gel electrophoresis analysis of the [35S]methionine-labeled in vitro translation reactions revealed as the major products for Tel-2b two equal intensity proteins with the molecular weight close to the expected molecular weight (Fig. 5a). The lower molecular weight Tel-2b product might be a result of an internal translation start site 17 amino acids downstream of the first ATG. A similar alternative translation start site has been observed for the highly related Tel as well resulting in the translation of two alternative Tel proteins. Tel-2c in vitro translation resulted in the synthesis of a protein with the expected molecular weight, but also in a strong lower molecular weight product which may either encode a degradation product, early termination, or an internal start site. We believe that this lower molecular weight product represents an internal start site, since the Tel-2c ATG which would be used to generate the full-length Tel-2c protein is an internal ATG in Tel-2b and does not conform with the optimal Kozak sequence very well. However, nucleotides surrounding an ATG codon just upstream of the Ets domain are much closer to the Kozak sequence. Tel-2a as described above possibly encodes a bicistronic RNA which could encode either a protein that starts at the original ATG and results in a protein containing the amino

![Diagram](image_url)

**Fig. 4.** Differential expression of Tel-2 and the different isoforms in various human fetal and adult tissues and cell types. a, expression of Tel-2 in different human fetal and adult tissues. PCR analysis of cDNA reverse transcribed from poly(A+) mRNA from human fetal and adult tissues as indicated above the figure using primers specific for the amino-terminal splice variants of Tel-2 (upper panel), the COOH-terminal exon 9 Tel-2e/Tel-2f splice variants (second panel), the COOH-terminal exon 8 Tel-2 variants (third panel), or GAPDH (lower panel). Peripheral blood leukocytes (PBL). The different Tel-2 splice variants are indicated on the right side. Below the figure is a schematic diagram of the intron/exon structure of the Tel-2 gene. Arrows below the exons indicate the location of the different PCR primers (see also "Materials and Methods," "Results," and Fig. 2). b, expression of Tel-2 in various epithelial and nonepithelial cell types. RT-PCR analysis of reverse transcribed poly(A) mRNA from human foreskin epithelium, LNCaP (human prostate), HEK293 (human epithelial kidney), C-33A (human cervical carcinoma), A431 (human vulvar carcinoma), HeLa (human cervical carcinoma), H157 human (large cell lung carcinoma), H249 (human small cell lung carcinoma), HUVEC (human endothelial cells), U-87 Mg and U-138 Mg (human glioma cells), fetal brain, U-937 (human monocytes), human synovial fibroblasts and human chondrocytes using Tel-2 (upper panel), or GAPDH (lower panel) specific primers as described under "Materials and Methods."
terminus of Tel-2b including the Pointed domain or a protein starting from an internal ATG in a different reading frame leading to a protein encoding the COOH-terminal Ets domain of Tel-2b. In vitro translation of Tel-2a revealed indeed two products, although the amino-terminal shorter product was far more abundant than the protein presumably encoding the DNA-binding domain (Fig. 5a). Smaller amounts of additional faster migrating proteins were visible as well in some of the reactions due to partial proteolysis, premature translational termination, or alternative internal initiation codons.

An EMSA was performed using equivalent amounts of in vitro translated full-length Tel-2b as well as the Tel-2a and Tel-2c isoforms to determine their relative ability to bind to an oligonucleotide encoding the Drosophila E74 Ets-binding site, which has previously been shown to bind to several members of the Ets family (54). The E74 oligonucleotide formed several higher molecular weight complexes with both the control reticulocyte lysate (Fig. 5b, lane 1) and reticulocyte lysates expressing Tel-2 proteins (lanes 2–4). A specific protein-DNA complex was formed by the full-length Tel-2b reticulocyte lysate that was not present in either the control reticulocyte lysate or in the Tel-2a or Tel-2c in vitro translation (Fig. 5b). Two additional faster migrating protein-DNA complexes were also specifically formed, which were absent from control lysate, but present in the Tel-2a, Tel-2b, and Tel-2c lysate (Fig. 5b). The exact nature of these two complexes is not clear, since they comigrate in all three Tel-2 isoform lysates, even though each of the isoforms generates proteins with slightly different mo-
Tel-2, a Tel/ETV-6-related Ets Transcriptional Repressor

TABLE I

| Enhancer                  | Binding site                               | Tel-2 |
|---------------------------|--------------------------------------------|-------|
| E74                       | AACCGGAAGTAA                               | ++++  |
| Lyn                       | ACAGGAGAAGTAT                               | ++++  |
| ENDO A                    | GACAGGGAAGTAT                               | ++++  |
| SPRR2A                    | AGCGAGGAGTGA                                | ++++  |
| BLK                       | TCCCGAGGATAT                                | ++++  |
| HIV-2 LTR CD3SR           | GACAGGAGACGC                                | +     |
| HTLV-1 LTR                | GGGAGAATTGG                                 | +     |
| IgH enhancer π site       | GGCAGGAAACAG                                | +/-   |
| IgH enhancer μB site      | TGGGGGAAGGGA                                | +/-   |
| Polymya PEA3              | AGCGAGGATGA                                 | +/-   |
| Fos SRE                   | CACAGGATGTC                                 | -     |
| TCRα enhancer Te2         | CAGCGGATGTC                                 | -     |
| MHC class II promoter     | GTGAGGACCAA                                 | -     |
| Consensus                 | AACAGGGAATAN                                |       |

core. Based on this experiment we have compiled a putative high affinity consensus binding site for Tel-2b (Table I, at the bottom) which is very similar to the consensus recognition sequences for many other Ets factors.

Tel-2b, but Not Tel-2a or Tel-2c Acts as a Strong Repressor of Transcription—Since the alternative splice products of Tel-2 encode different protein isoforms which either lack or contain the Pointed domain or the Ets DNA-binding domain, we were interested to know whether the different splice products of Tel-2 would express any differences in their functions as transcriptional regulators and whether Tel-2 would act as a transcriptional enhancer or repressor. Furthermore, since the highly related Tel had previously been shown to be a repressor of transcription, we compared the activity of Tel-2 to Tel-2a, Tel-2b, Tel-2c, and Tel were inserted into the eukaryotic expression vector pCI and co-transfected into CV-1 cells together with a pGL3 reporter gene construct containing the luciferase gene in which two copies of the E74 Ets-binding site were inserted upstream of the minimal c-fos promoter Δ56 (55). Δ56-pGL3 containing only the minimal c-fos promoter expressed very little luciferase activity above the background of the parental promoterless pGL3 vector (Fig. 6a). Two copies of the wild type E74 promoter Ets site enhanced transcription more than 50-fold as compared with the minimal c-fos promoter due to the action of endogenous Ets factors. Co-transfection with the Tel expression vector resulted in a ~3-fold transcriptional repression of the E74 site (56, 57). Similarly to Tel, the Tel-2b expression vector strongly repressed E74 transcriptional activity by ~6-fold indicating that the full-length Tel-2 protein is an at least as or more potent repressor than Tel (Fig. 6a). Tel-2a and Tel-2c, in contrast, did not significantly affect E74 transcriptional activity, but Tel-2a actually slightly and reproducibly enhanced transcription suggesting that the Tel-2 Pointed domain alone or the Tel-2 Ets DNA-binding domain alone are insufficient to repress transcription. These results also support the notion that Tel-2 repression of transcription is an active process presumably involving corepressors as shown for Tel (57, 58) and both the DNA-binding domain and the Pointed domain and is not just a result of competition for DNA binding. Alternatively, proteins generated by Tel-2a or Tel-2c may not be stable or may not be translocated to the nucleus. To evaluate whether all Tel-2 isoforms generate stable proteins at similar levels after transfection into cells, the HA-tagged Tel-2 isoform expressing vectors were transfected into COS cells. Whole cell extracts of transfected cells were analyzed by EMSA and compared with in vitro-translated Tel-2 demonstrating that Tel-2 b and Tel-2c express large quantities of proteins able to bind to DNA which comigrated with the in vitro translated Tel-2 isoforms, whereas Tel-2a generated significantly lower levels of DNA-binding proteins (Fig. 6b). Indeed although Tel-2c was unable to repress luciferase activity, the level of Tel-2c protein after transfection appeared to be even higher than Tel-2b. Specificity of the protein-DNA complexes was confirmed using the anti-HA tag antibody (Fig. 6b). Since this assay measured the ability of transfected Tel-2 to bind to DNA, these results further support the notion that DNA binding alone (Tel-2c) is not sufficient to act as a repressor of transcription.

A dose curve with differing amounts of Tel-2 expression vectors and constant amounts of the E74 luciferase construct demonstrated that increasing amounts of Tel-2b led to enhanced repression (Fig. 6c). In contrast, increased levels of Tel-2c did not affect E74 transactivation at all. Interestingly, Tel-2a again reproducibly enhanced E74 activity in a dose-dependent manner up to 2.5-fold (Fig. 6c).

To determine whether Tel-2 can repress enhancers or pro-

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TABLE I

The relative DNA binding affinities of full-length Tel-2b towards each site as determined by EMSA (see Fig. 5c) are shown on the right. A potential consensus high affinity binding site for Tel-2b based on comparing the sequences of the high and medium affinity Tel-2 binding sites is summarized at the bottom. Capital letters indicate nucleotides present in high affinity binding sites, whereas small case letters indicate nucleotides in lower affinity binding sites.
FIG. 6. Tel-2b, but not Tel-2a or Tel-2c acts as a potent transcriptional repressor. a, Tel-2b is a strong repressor of transcription. CV-1 cells were co-transfected with the indicated Tel-2 and Tel expression vector constructs or the parental pCI expression vector and a pGL3/c-fos promoter luciferase construct containing the E74 Ets site oligonucleotide or the empty pGL3/c-fos promoter plasmid. Luciferase activity in the lysates was determined as described. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated four times with different plasmid preparations with comparable results.

b, EMSA and supershift of full-length HA-tagged Tel-2a, Tel-2b, and Tel-2c after transfection into COS cells. EMSAs of in vitro translated (in vitro) and transfected (in vivo) HA-tagged Tel-2a, Tel-2b, and Tel-2c or the empty pCI/HA vector incubated with the labeled E74 oligonucleotide probe were carried out with either no antibody or anti-HA tag antibody. The arrows indicate the specific DNA-protein complexes.

c, CV-1 cells were co-transfected with the indicated amounts of Tel-2a, Tel-2b, or Tel-2c pCI expression vector constructs or the parental pCI expression vector and luciferase constructs containing either the E74 Ets site, the lyn promoter, or the IgH enhancer upstream of the minimal c-fos promoter. Luciferase activity in the lysates was determined as described. Data shown are means of triplicate measurements from one representative transfection.
motors of mammalian genes that contain functionally important Ets-binding sites, we co-transfected CV-1 cells with either the lyn promoter luciferase construct (lyn/pGL3) or the IgH enhancer luciferase construct (IgH-Δ56/pGL3) together with increasing amounts of the different Tel-2 expression vectors. Increasing amounts of Tel-2b led to a dose-dependent repression of the lyn promoter similar like for E74 correlating with the ability of Tel-2 to bind with high affinity to the lyn promoter Ets site (Fig. 6c). In contrast, Tel-2b did not significantly repress the IgH enhancer which contains two relevant Ets sites that do not interact with Tel-2 with high affinity (Fig. 6c).

Again Tel-2a slightly enhanced lyn promoter activity at higher doses.

The RARa and BMP-6 Genes Are Target Genes for Tel-2b in MG-63 Osteosarcoma Cells—To understand more about the biological role of Tel-2 and to determine the physiological targets for Tel-2 we used transcriptional profiling methods. Human MG-63 osteosarcoma cells which can be transfected to 80–90% efficiency were transiently transfected with the Tel-2b expression vector (pCI/Tel-2b) or the parental expression vector (pCI). Total RNA was isolated 18 or 20 h after transfection and reverse transcribed into cDNA. 32P-Labeled cDNA probes derived from two independent experiments each were hybridized to the Human 1.2, Human 1.2 II, and Human Cancer 1.2 Atlas Microarray membranes that contain each more than 1100 genes. Autoradiographs were analyzed using the CLONTECH Atlas Image software. The majority of hybridizing genes gave similar relative intensities of hybridization indicating the reproducibility and that expression of most genes did not change significantly upon expression of Tel-2b. Only a small number of genes (RARa, BMP-6, HIV-EP2, and thymosin β-10) were affected by Tel-2b, the majority of them being repressed by Tel-2b as could be expected from our luciferase experiments. A small section of two autoradiographs of the Human 1.2 II Atlas array is shown in Fig. 7a highlighting the similar relative expression pattern of most genes and the differential expression of RARa. Thymosin β-10 was the only gene up-regulated 2.5–3.2-fold in the two experiments, whereas RARa was repressed 4–5-fold, BMP-6 5–10-fold, and interleukin-6 2-fold. To validate the microarray data with an independent method we performed RT-PCR for the RARa and BMP-6 genes with the RNAs derived from the transfected MG-63 cells (Fig. 7b). Whereas the amplification product for the control GAPDH gene did not differ in intensity among the different samples, a drastic reduction in amplification was observed for both RARa and BMP-6 in Tel-2b-transfected cells when compared with the control cells. These results confirm that Tel-2b represses expression of the RARa and BMP-6 genes in MG-63 cells. Since both RARa and BMP-6 are inducers of cell differentiation, their repression by Tel-2b implies a role for Tel-2b in blocking cell differentiation.

**DISCUSSION**

The Ets factor family has evolved as a family of transcription factor genes that play intricate roles in many facets of development and cell differentiation (5–8). Disturbances in Ets factor functions lead invariably to developmental defects and in extreme cases to tumor formation. Indeed the first members of the Ets factor family were derived as transforming oncogenes in retroviruses as well as integration sites of retroviruses raising the notion that defects in Ets factor genes may be involved in human cancer as well (5–8). This hypothesis was confirmed by the findings that at least six different members of the Ets family are involved in various chromosomal translocations in different types of human cancer (9–21). Particularly striking is the involvement of the Ets factor Tel in the majority of 12p13 chromosomal abnormalities present in various human hematological malignancies as well as in certain solid tumors such as fibrosarcomas (9–21, 25–27). Interestingly, Tel translocation partners are derived from various types of proteins including both membrane-bound and cytoplasmic protein-tyrosine kinases as well as nuclear transcription factors. Similarly striking is the variability in the domains of Tel that are involved in these chromosomal translocations and the consequent different mechanisms of Tel mediated transformation. The Tel protein can be divided into two primary functional domains, the amino-terminal Pointed dimerization domain and the COOH-terminal Ets DNA-binding domain. Under normal circumstances Tel appears to act as a transcriptional repressor (57, 58). Chromosomal translocations of Tel to tyrosine kinases such as abl, platelet-derived growth factor-R, JAK2, and NTRK3 invariably involve the fusion of the amino terminus of Tel including the Pointed domain to the carboxyl terminus of the tyrosine kinase including the tyrosine kinase catalytic domain. This fusion leads to constitutive activation of the fused tyrosine kinase due to forced dimerization via the Tel Pointed domain (59). In contrast, fusion of the Tel Pointed domain to the DNA-binding and transactivation domains of the transcription factor AML-1 leads to a switch of AML-1 function from transcriptional enhancement to repression (18, 60). Additional Tel translocations lead to fusion of the COOH-terminal Tel DNA-binding domain to putative transactivation domains of apparent transcription factors such as MN1 and STL which may have the opposite effect of converting the transcriptional repressor Tel into a transcriptional enhancer (15, 21).

Tel has now up to now been the sole mammalian member of its subclass in the Ets family. Tel-2 is a second member of the Tel subclass which may play similar roles as Tel in human cancer. Tel-2 shows high homology to Tel throughout the coding sequence with an overall homology of 50%. Like Tel, Tel-2 con-

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**Fig. 7. Tel-2b represses RARa and BMP-6 gene expression in MG-63 osteosarcoma cells.** a, transcriptional profiling of MG-63 cells transfected with pCI/Tel-2b or pCI. CLONTECH Atlas cDNA microarrays were hybridized to 32P-labeled cDNA probes generated from total RNA isolated from transfected MG-63 cells. Shown are the autoradiographs of a small section of the Atlas Human 1.2 II microarrays from RNA isolated from transfected MG-63 cells after transfection with pCI/Tel-2b or pCI for 18 and 20 h using BMP-6 (upper panel), RARa (middle panel), or GAPDH (lower panel) specific primers as described under "Materials and Methods."
tains an amino-terminal Pointed domain and a COOH-terminal Ets domain which are highly conserved between Tel and Tel-2. In addition, both Tel and Tel-2 contain an optimal phosphorylation site for members of the MAP kinase pathway, PXXSP, immediately upstream of the Pointed domain. No function for this site has been demonstrated in Tel to date, but it is intriguing that this site is highly conserved in both genes at the same position. Furthermore, the repressor activity of the Ets factor closest related to Tel and Tel-2, the Drosophila Yan, is tightly regulated by the MAP kinase pathway (61). Indeed, it has been shown that Tel is phosphorylated in vivo (62). Another similarity between Tel and Tel-2 is the translational initiation of Tel mRNA at two different in-frame ATGs at positions 1 and 43 (62). Our in vitro translation studies with Tel-2b show two equal strength translation products which most likely encode proteins starting at positions 1 and 17. Interestingly, both for Tel and Tel-2, the alternative in-frame starting points eliminate the putative MAP kinase phosphorylation sites. The existence of both translation products for Tel has been demonstrated in vivo using Tel-specific antibodies (62).

Like Tel the homologous Tel-2 isoform Tel-2b acts as a strong transcriptional repressor indicating that Tel and Tel-2 may have related functions. The transcriptional repressor domain of Tel-2 is most likely located at the amino terminus of Tel-2, since the two isoforms of Tel-2, Tel-2a and Tel-2c, which encode proteins lacking either the amino terminus or the carboxyl terminus do not repress transcription. These results also indicate that both the amino terminus including the Pointed domain and the carboxyl terminus including the DNA-binding domain are necessary to generate an active repressor protein. Interestingly, the Tel-2a isoform appears to increase rather than repress transcription slightly in a dose-dependent manner. This is somewhat surprising, since Tel-2a may make two different proteins encoding either the amino terminus only including the Pointed domain or the carboxyl terminus by itself including the Ets domain. A possible scenario may be that one of these proteins binds to a corepressor and, thus, limits the availability of the corepressor for other transcription factors. The repressor domain in Tel has been located to the Pointed domain and the central domain between the Pointed domain and the Ets domain, and it is striking that the Drosophila Ets factor Yan, the closest relative of Tel, also acts as a transcriptional repressor (57, 58, 61). Comparison of the amino acid sequences of Tel-2, Tel, and Yan reveals primarily homologies in the Pointed domain and the Ets domain that are common to all three factors. Nevertheless, additional short stretches of homology between these three repressor Ets factors are seen upstream and downstream of the Pointed domain that may be involved in repressor function. Within the Pointed domain there are several stretches of high homology between these three Ets factors that are less conserved in other Pointed domain containing Ets factors. One sequence G/R/K/A/L/C/L/O/U/L/o/L/T is of particular interest, because a similar sequence is also found in the SAM domain of another repressor factor, the polycomb protein SCM (63). Recent data suggest that Tel acts as a repressor due to recruitment of the corepressor Sin3a which interacts with the amino-terminal 127 amino acids of Tel presumably via the Pointed domain (57, 58). We are now evaluating whether Tel-2 also interacts with Sin3a.

Our transcriptional profiling data also support the notion that Tel-2b acts as a transcriptional repressor in vivo, since the majority of genes affected by Tel-2b in MG-63 osteosarcoma cells were down-regulated. These experiments also give the first clues about the potential biological function of Tel-2. Two of the genes repressed by Tel-2b, BMP-6 and RARα, play important roles in development and cell differentiation and, in particular, regulate bone remodeling and osteoblast differentiation (64–72). Indeed some of the effects of BMP-6 on osteoblast differentiation are similar to the effects of retinoic acid on osteoblasts (66). But whereas BMP-6 induces osteoblast differentiation in all models studied, retinoic acid effects on osteoblast cultures and cell lines have been less straightforward, ranging from the induction to inhibition of differentiation (65, 68, 71–76). Because Tel-2b inhibits BMP-6 and RARα expression in MG-63 cells, Tel-2b is expected to play a critical role as an inhibitor of cell differentiation.

In contrast to Tel, which does not bind efficiently to DNA as a full-length protein, the equivalent Tel-2 isoform Tel-2b binds to a variety of functionally important Ets-binding sites as demonstrated by EMSA. Two faster migrating complexes are also formed that comigrate with complexes formed by the other two isoforms Tel-2a and Tel-2c. In vitro translation reveals the synthesis of a 22-kDa protein common to all isoforms in addition to their expected proteins. This protein may be the result of an internal translation start site using a methionine upstream of the Ets domain with a preferred Kozak consensus sequence. Whether this protein is being synthesized in vivo is not known, but whole cell extracts from cells transfected with expression vectors for the Tel-2 isoforms reveal the same size proteins as in vitro translated Tel-2. The DNA binding specificity does not seem to be significantly different from most other Ets factors, although binding is restricted to sites containing a GGAA core rather than GGAT and Tel-2 strongly prefers a T in the +2 position (Table I).

Tel has been shown to dimerize via the Pointed domain (59). The Pointed domain is present in several other members of the Ets family and in addition to Tel Fli-1, Ets-1, and Ets-2 are apparently able to form homo- and hetero-dimers via the Pointed domain (77). The Pointed domain of Tel-2 is the one closest related to Tel suggesting that Tel-2 may dimerize as well. The Pointed domain is a subclass of the highly conserved SAM domain which is found in various types of proteins from yeast to human including among others the EPH receptors and polycomb proteins (78). The SAM domain has been implicated in both homo- and hetero-dimerization. Since most Ets factors that contain the Pointed domain do not dimerize with each other, it is tempting to speculate that these Ets factors interact with other SAM domain containing proteins such as the polycomb proteins.

The evolutionary relationship between Tel-2 and Tel is also conserved in the genomic organization with both the Tel-2 and Tel genes being encoded by eight exons and exon/intron borders being highly conserved. Whether Tel has the alternative 3′ exon 9 is not known. The Pointed domain of both Tel and Tel-2 is encoded by exons 2, 3, and 4 and the Ets domain by exons 6, 7, and 8 with identical exon/intron borders. The Tel-2 gene has an alternative out-of-frame exon 3a of 41 nucleotides which has not been seen for Tel up to now. We actually have isolated five alternative splice products of Tel-2 which lead to the expression of various Tel-2 isoforms. Only Tel-2b encodes the full-length protein homologous to Tel. The other three isoforms encode proteins in which either the Pointed domain alone or together with the rest of the amino terminus is deleted or proteins which contain only the amino terminus of Tel-2 including the Pointed domain, but lack the Ets DNA-binding domain. The fifth and sixth splice variants Tel-2e and Tel-2f delete the COOH-terminal 38 amino acids immediately downstream of the Ets domain including the last amino acid of the Ets domain. Whether conversion of the last amino acid of the Ets domain from phenylalanine to asparagine affects DNA binding or DNA binding specificity is not known. Nevertheless, this amino acid is part of
the β4 β-sheet based on three-dimensional structure analyses of other Ets factors, a domain critical for DNA binding (79, 80). Tel-2e/Tel-2f also eliminate an acidic domain COOH downstream of the Ets domain which is conserved in both Tel-2 and Tel. No function has been attributed to domain C yet, but its acidic nature may suggest a role in transactivation. Whether similar isoforms exist for Tel is not clear. The function of the different Tel-2 isoforms is not yet clear, since Tel-2b itself appears to act as a repressor. A protein encoding exclusively the Pointed domain of Tel may be able to interact with proteins that normally interact with Tel-2b and, thus, might limit or modulate the function of Tel-2b. However, this protein may also lack a nuclear localization signal and may, thus, not translate to the nucleus. A Tel-2 protein lacking the Pointed domain should still be able to bind to the Tel-2 DNA-binding sites, and may not act as an active repressor, but as a weaker repressor due to competition with other Ets factors or as a transcriptional enhancer. Our co-transfection experiments indeed demonstrate that only Tel-2b is a strong repressor. Interestingly, the Tel-2a isoform appears to enhance transcription slightly, possibly due to competition for a corepressor or elimination of a repressor domain in the truncated protein. Whether Tel-2 contains also a transactivation domain whose activity may be blocked by the repression domain, is not yet clear. It is possible that phosphorylation of Tel-2 may switch Tel-2 from a repressor into an enhancer as has been shown for the ets factor ERP/NET (37, 81).

In contrast to Tel which is abundantly and ubiquitously expressed, Tel-2 expression appears to be very low in all fetal and adult tissues tested. We were so far unable to detect any Tel-2 mRNA by Northern blot or dot blot analysis of poly(A)+ mRNA in a whole set of human fetal and adult tissues. However, using RT-PCR we detected low levels of Tel-2 mRNA expression in many fetal and adult tissues as well as in most human cell lines. No specific pattern of expression was observed which would indicate the role of Tel-2 in a specific organ or cell type. However, the differential expression of the different splice variants of Tel-2 was striking. Surprisingly, the most common splice product was Tel-2d which lacks the Pointed domain, but contains the amino terminus in-frame with the carboxyl terminus. Indeed in several tissues Tel-2d was the sole splice form expressed indicating that Tel-2d may play as important a function as Tel-2b. Tel-2b was also expressed at relatively high levels in several tissues, whereas Tel-2a and Tel-2c expression was very low. Highest levels of Tel-2b mRNA were detected in prostate, liver, lung, and placenta, the same tissues that express the highest levels of Tel-2d. The majority of these splice forms are combined with the Tel-2 isoform containing exon 8 which results in a full-length Ets domain. However, the Tel-2e/Tel-2f isoforms splicing exon 9 rather than exon 8 to the carboxyl terminus are also significantly expressed in a restricted set of tissues. Again placenta and prostate express significant amounts of this splice form. But some tissues such as ovary and heart, and to a lesser extent prostate, spleen, and thymus, almost exclusively express the Tel-2e/Tel-2f isoforms.

By far the highest levels of Tel-2 mRNA were detected in the A431 squamous carcinoma cell line suggesting that Tel-2 may be overexpressed in some cancer cells. This relatively low abundance of Tel-2 is also reflected in the GenBankTM EST data base where only 14 Tel-2-derived ESTs have been deposited. These ESTs are derived from two colon adenocarcinomas, two lung carcinomas, an adrenal adenoma, a chronic B lymphocytic leukemia, a germ cell tumor, two ovarian cancers, an endometrial tumor, and germinal center B cells possibly indicating enhanced expression in human cancers. Interestingly, the Tel-2e/Tel-2f ESTs containing exon 9 were all derived from ovarian and endometrial cancers correlating with our expression analysis, and exon 8 was not present in any entry from these tissues indicating the tissue-specific splicing. In addition, SAGE analysis suggest enhanced expression of Tel-2 in the brain or brain tumors.

Tel-2 is located on human chromosome 6p21.3 in the MHC cluster region. This region has been implicated in a variety of different cancers such as chondroid hamartomas, thyroid adenomas, ductal carcinoma in situ of the breast, B-cell non-Hodgkin’s lymphoma, cervical cancer, astrocytoma, nonsmall cell lung carcinomas, and ovarian carcinomas (82–91). Since chromosomal translocations of Tel are frequent events in various leukemias and fibrosarcoma, it is likely that Tel-2 plays similar roles.

After submission of this manuscript two independent groups reported similar findings (92, 93) on the cloning and characterization of Tel-2 confirming our data about the repressor activity and DNA binding capacity. Both reports, in addition, demonstrate that Tel-2 can homo-dimerize as well as hetero-dimerize with Tel via the Pointed domain (92, 93). Elevated levels of Tel-2 were detected in several hematopoietic tissues including fetal liver and bone marrow (92). Our results add additional information about the various alternative splice products of Tel-2 and possible target genes regulated by Tel-2.

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