The Human TCF-1 Gene Encodes a Nuclear DNA-Binding Protein Uniquely Expressed in Normal and Neoplastic T-Lineage Lymphocytes

By Jan Castrop, Dick van Wichen, Marieke Koomans-Bitter, Marc van de Wetering, Roel de Weger, Jacques van Dongen, and Hans Clevers

The TCF-1 gene encodes a putative transcription factor with affinity for a sequence motif occurring in a number of T-cell enhancers. TCF-1 mRNA was originally found to be expressed in a T cell-specific fashion within a set of human and mouse cell lines. In contrast, expression reportedly occurs in multiple nonlymphoid tissues during murine embryogenesis. We have now raised a monoclonal antibody to document expression and biochemistry of the human TCF-1 protein. As expected, the TCF-1 protein was detectable only in cell lines of T lineage. Its expression was always restricted to the nucleus. Immunohistochemistry on a panel of human tissues revealed that the TCF-1 protein was found exclusively in thymocytes and in CD3+ T cells in peripheral lymphoid tissues. Western blotting yielded a set of bands ranging from 25 kD to 55 kD, resulting from extensive alternative splicing. The TCF-1 protein was detectable in all samples of a set of 22 T-cell malignancies of various stages of maturation, but was absent from a large number of other hematologic neoplasms. These observations imply a T cell-specific function for TCF-1, a notion corroborated by recent observations on Tcf-1 knock-out mice. In addition, these results indicate that nuclear TCF-1 expression can serve as a pan-T-lineage marker in the diagnosis of lymphoid malignancies.

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TCF-1 is uniquely expressed in T lymphocytes

Fig 1. Mapping of the αTCF-1 epitope. Graphic representation of the MBP/human (h)TCF-1A fusion protein, two common human splice forms, hTCF-1A and hTCF-1B protein, a truncated version of hTCF-1 (hTCF 1 trunk), murine (m) TCF-1B, and the related LEF-1 cDNA. Relevant amino acid positions are indicated. The HMG bars represent the DNA-binding domain of the TCF-1 protein. Single amino acid differences between mTCF-1 and hTCF-1 are indicated with small vertical bars below mTCF-1B. Reactivity with the fusion protein was scored by enzyme-linked immunosorbent assay (ELISA). Reactivities with the other proteins were tested in the COS cell-screening assay. All the human TCF forms are positive, while the mTCF and LEF are negative. The epitope of the MoAb αTCF is located directly N-terminal to the HMG box.

**Materials and Methods**

Production and purification of the TCF-1 fusion protein. The 900-bp EcoRI insert of phage TCF-1, which encodes the TCF-1A splice form from amino acid 102 to the C-terminus, was ligated into the EcoRI-digested pH902 plasmid (New England Biolabs, Beverly, MA) to yield pH-TCF. After transformation into bacterial strain DH5α, bacteria were grown on Luria-Bertani Medium (LB) agar plates. Colonies were picked, and plasmids were screened for a correct TCF-1 insert by sequence analysis. The pH-TCF was then transformed into TB1 bacteria. Cells were grown in LB containing 100 µg/mL ampicillin. After reaching mid-log phase, the bacteria were induced by isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 100 µmol/L NaCl, and 0.05% Tween 20, pH 7.4. Lysis was achieved by sonification (three times for 3 minutes), after which the cellular debris was removed by centrifugation (10 minutes at 15,000g, 4°C). The MBP/TCF fusion protein was purified in a single affinity chromatography step according to the manufacturer’s instructions. Amlose-coated resin beads (New England Biolabs) were used to bind the fusion protein, and the protein was subsequently eluted with maltose (10 µmol/L). Purification of the fusion protein was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a PhastSystem (Pharmacia, Uppsala, Sweden) using precast 8% to 25% gradient gels. Protein bands were visualized by silver staining.

Gel retardation and methylation interference footprinting assay. Experiments were performed as described elsewhere. In brief, oligonucleotide probes were labeled either at the positive or negative strand with [γ-32P]adenosine triphosphate (ATP) using T4-polymerase kinase. Probes were purified on a sequencing gel. After annealing, the probes were purified on a nondenaturing acrylamide gel. The labeled probes were partially methylated using dimethylsulfate. Methylated probe (100,000 cpm) was used in a gel retardation reaction. In a binding reaction, recombinant protein (50 ng) and 25 ng poly(dIdC) were incubated in a volume of 75 µL containing 10 mmol/L HEPES, 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), and 15% glycerol. After addition of probe (equaling 2.5 ng), the reaction was left at room temperature for 30 minutes. Samples were electrophoresed through a 4% nondenaturing polyacrylamide gel in 0.25 × Tris-borate/EDTA (TBE) at room temperature.

Oligonucleotides used were as follows: Mw-1: GGGAGACTGAGC-CCCGGTTGCTCCTCACAC annealed to CCCGTGAGGA-CGCGCCCTTTGTTCCTCACAGTC; and Mw-1S: GGGAGACTGAGC-CGCGGTTGCTCCTCACAC annealed to CCCGTGAGGAC-GCACCGGCTCCTAGTC. Oligonucleotides were from Isogen (Amsterdam, Holland).

After fractionation by gel retardation assay, the gel was subjected to autoradiography. Bound and free probes were cut out and recovered by electroelution. After cleavage at the G and A residues using NaOH, the reaction products were analyzed on a 12.5% polyacrylamide–8 mol/L urea sequencing gel.

Generation of MoAbs. Six-week-old Balb/c mice were immunized by subcutaneous injection of 100 µg fusion protein in Freund’s complete adjuvant on day 1, followed by a similar injection in Freund’s incomplete adjuvant at day 10, followed by repeated intraperitoneal injections at weekly intervals of 100 µg fusion protein in phosphate-buffered saline (PBS). When an anti–TCF-1 titer of 1:100 was obtained in the COS screening assay (see below), the spleen was isolated, and 1 × 10^8 splenocytes were fused to an equal number of Ag8 myeloma cells using the standard polyethylene glycol protocol. Selection in hypoxanthine/aminopterin/thymidine was initiated directly after replating the cell suspension into 15 96-well, flat-bottom plates. Supernatants were screened 10 to 14 days after the hybridoma fusion. Positive hybridomas were repeatedly subcloned.
Hybridoma screening assay. As the substrate for the screening of hybridoma supernatants, COS cells were transiently transfected with pCDM7 or pCDM8 vectors encoding human TCF-1A or TCF-1B, mouse TCF-1 (not shown), or human LEF-1 (not shown) using diethyl aminoethyl (DEAE)-dextran, according to standard procedure. Briefly, COS cells were trypsinized from petri dishes, washed in PBS, and incubated for 60 minutes at 37°C in RPMI containing 0.4 mg/mL DEAE/dextran (Pharmacia) and 1 μg/mL of the relevant expression plasmid. Cells were then washed once and plated in 96-well, flat-bottom culture plates at a concentration of 10^5 per well. After 48 hours, the cells were washed once with PBS, fixed in the plates with 100% methanol for 20 minutes at room temperature (RT), and stored under methanol at −20°C.

For screening, the plates were washed once with PBS, and then 50 μL of hybridoma supernatant was added. After overnight incubation in PBS, the plates were washed once with PBS and incubated for 1 hour at RT with horseradish peroxidase-conjugated rabbit-antimouse Ig reagent (1:300 in PBS; RAMPO, Dakopatts, Glostrup, Denmark). After two more washes in PBS, a color reaction was performed with 0.02% 9-amino-3-ethylcarbazol/0.1% hydrogen peroxide in 0.1 mol/L sodium acetate, pH 4.8. Individual wells were then examined for staining using an inverted microscope.

Western blotting. Approximately 3 × 10^6 cells were used as protein sample. The cells were prepared as protein samples by washing them with PBS and resuspending them in 30 μL sample buffer (15 mmol/L Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.005% bromophenol blue [BPB], 4% β-mercaptoethanol). After sonification for 20 seconds and boiling for 5 minutes, the protein samples were separated on SDS-PAGE. Gels were blotted to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were blocked with 5% milk in washing buffer (PBS, 0.1% Tween-20) for 2 hours, incubated with the monoclonal αTCF-1 (culture supernatant) for 2 hours, washed for 15 minutes in washing buffer, and incubated with RAMPO (diluted 1:2,000) for 2 hours. The specific antibody signals were detected using an enhanced chemiluminescence kit (ECL; Amersham, Buckinghamshire, UK).

Immunohistochemistry. Twelve organs of human origin (jejunum, heart, liver, lung, muscle, stomach, skin, kidney, spleen, thymus, tonsils, lymph node), originally presented to the Department of Pathology (University Hospital Utrecht, Utrecht, The Netherlands) for histopathologic diagnosis, were of normal architecture and cellular composition. Frozen sections (8 μm) were prepared from these tissues and dried overnight. Fixation was performed in absolute acetone for 10 minutes, and then the sections were rinsed in PBS. Sections were incubated with the MoAb for 1 hour at RT. Dilutions of the MoAb were made in PBS supplemented with 1% human serum albumin (PBS/HSAS). The sections were then washed in PBS for 30 minutes. The second step was performed in RAMPO for 30 minutes at RT, followed by a 30-minute rinse in PBS. A third step was performed using horseradish peroxidase-labeled swine antirabbit Ig antibody (SWARPO, Dakopatts) for 30 minutes at RT. After a final rinse in PBS, peroxidase was visualized by incubation in a diaminobenzidin (DAB) solution with Ni-ammonium sulfate as an enhancer for 5 minutes. Sections were counterstained with nuclear fast red for 3 minutes. Slides were dehydrated using ethanol, cleared in xylol, and embedded in synthetic resin (DPX). For immunoenzymatic double-labeling, incubation with αTCF-1 in PBS/HSAS was followed by RAMPO and SWARPO as described above. A second staining was then performed with biotinylated anti-CD3, -CD4, or -CD8 antibodies, followed by alkaline phosphatase-labeled streptavidin. TCF-1 was subsequently visualized using DAB and nickel enhancement, resulting in a black nuclear stain. Alkaline phosphatase was detected using Naphtol AS-BI phosphate as substrate and Neufchaisin as chromogen.

Immunofluorescence. Cells were cytopun onto a glass slide, air-dried, and fixed in absolute, ice-cold methanol. After 20 minutes, the slides were transferred to PBS, and excess PBS was removed, followed by incubation with the first antibody for 1 hour at RT. The slides were then washed in PBS and incubated with a fluorescein isothiocyanate (FITC)-labeled rabbit-antimouse Ig (Becton Dickin-
Fig 3. Immunohistochemical detection of TCF-1 in COS cells. (A) COS cells transiently transfected with pCDM8-TCF-1 and stained with the αTCF-1 antibody. The arrows point to brightly staining COS cell nuclei. (B) COS cells transfected with pCDM8-LEF-1 do not stain with the αTCF-1 MoAb.

RESULTS

Production of an anti-TCF-1 MoAb. To produce recombinant TCF-1 antigen for immunization, we cloned the original insert of phage TCF-1 into the plasmid pH902 (see Materials and Methods). The resulting plasmid encoded a fusion protein consisting of approximately two thirds of the human TCF-1A protein fused to the carboxy terminus of MBP (see Fig 1). The MBP moiety allowed affinity purification and potentially served as an immunogenic carrier. The fusion protein was still capable of binding to DNA in a sequence-specific fashion as determined by gel retardation and subsequent methylation interference footprinting (Fig 2). This indicated that the recombinant TCF-1 was correctly folded.

Several attempts at raising polyclonal antibodies against human TCF-1 in rabbits and chicken remained unsuccessful, although anti-MBP responses were always readily detectable. This was likely due to the extreme conservation of TCF-1 between species. Therefore, we pursued the generation of MoAbs by continued reboosting of the same mice and screening tail blood samples for anti-TCF-1 reactivity at low serum dilutions. Subsequent screening of large numbers of hybridomas would then allow the rescue of rare anti-TCF-1 antibody-secreting clones. As a screening assay, we chose immunoperoxidase staining of methanol-fixed, TCF-1-transfected COS cells. The advantage of this screening assay was twofold. First, specificity of the antibodies could readily be determined by transfection of various expression constructs into COS cells. Second, antibodies positive in this screening assay were likely to be reactive with histologic samples.

Of four mice immunized with the fusion protein, one eventually developed an anti-human TCF-1 response at low titer (1:100). Splenocytes were isolated from this mouse and subjected to hybridoma fusion. Among approximately 1500 hybridomas tested, one scored positive in the COS cell-screening assay (Fig 3). The MoAb produced by this hybridoma was of IgG1 subclass and was termed αTCF-1.

Mapping of the αTCF-1 epitope. We next localized the epitope on the TCF-1 molecule. This was important for the
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characterization of the MoAb, as at least four alternative splice products of TCF-1 exist, some of which might not be recognized. As expected, the MoAb was found to be reactive with the MBP/TCF fusion protein, which mapped the recognized epitope to the C-terminal two thirds of the TCF-1 molecule. Immunohistochemistry of transfected COS cells revealed that αTCF-1 was reactive with both the TCF-1A and TCF-1B splice forms of human TCF-1, indicating that the epitope was located in the common stretch of amino acids 1 to 243. 

Expression in COS cells of a small fragment of TCF-1 containing the DNA-binding domain (residues 118 to 235; see Fig 1) again resulted in strong nuclear staining. The antibody did not react with murine TCF-1, transfected into COS cells, or with endogenous TCF-1 in nuclei of murine thymocytes (not shown). Murine TCF-1B and human TCF-1B differ only by four amino acids in the relevant region. As indicated in Fig 1, these sequence differences are clustered directly N-terminal to the HMG box. We, therefore, concluded that the epitope mapped to this region of the molecule. Importantly, this region is common to all splice alternatives of human TCF-1. The experimental data pertaining to the epitope mapping are summarized in Fig 1. To further characterize the specificity of the MoAb, we tested its reactivity with the most closely related member of the HMG box family, human LEF-1, transfected into COS cells. LEF-1, which is also expressed in T lymphocytes, is 98% identical to TCF-1 in the HMG box region, but diverges significantly from TCF-1 in the remainder of the molecule. The αTCF-1 MoAb did not react with transfected LEF-1 in the peroxidase staining assay, whereas a polyclonal anti-LEF serum readily yielded a bright nuclear staining (not shown).

Western blotting. The antibody was found to be reactive in Western blotting of extracts prepared from T cell lines (Fig 4A), as well as from thymus and peripheral blood T cells (Fig 4B). All non–T-lineage cell lines tested were negative, as were control tissue extracts. Two clusters of bands were consistently observed, one of approximately 25 kD to 32 kD and another of approximately 42 kD to 49 kD. Significantly, the predicted molecular weight of the various splice forms of TCF-1 were all in the order of 26 kD to 28 kD. The banding pattern was insensitive to reduction of the samples before electrophoresis, excluding that some of the bands represented disulfide-linked homo- or heterodimers (not shown). The origin of the complexity of the banding pattern was, thus, not directly apparent. Reexamination of the known C-terminal splicing alternatives revealed that a hypothetical combination of alternative exon XI with exon XB would result in a much longer open reading frame of approximately 45 kD (Fig 4C). Indeed, this splicing alternative (termed TCF-1E) could be closed and was found to represent a rather abundant form of TCF-1. Subsequently, additional alternative exons in the N-terminal region of the protein were cloned. These novel splice alternatives are currently being characterized in more detail. Theoretically, the TCF-1 gene can encode over 100 protein isoforms (M. van de Wetering and H. Clevers, unpublished data, January 1995), all of which are predicted to react with the αTCF-1 antibody. Although the analysis is still ongoing, the presently available data indicate that the complexity of the Western blot pattern results from extensive alternative splicing of the TCF-1 pre-mRNA molecule.

TCF-1 is a nuclear protein uniquely expressed in T lymphocytes. To define the subcellular localization of the TCF-1 protein, several T cell lines were subjected to immunofluorescent staining. In all cases, a clear nuclear staining pattern was observed. This was in line with observations made on the transfected COS cells, where the TCF-1 protein also accumulated in the nucleus (Fig 3). Fluorescence confocal laser-microscopy allowed a high resolution analysis of the nuclear expression. A diffuse punctate pattern was observed in interphase nuclei. Neither nucleoli nor metaphase chromosomes stained (Fig 5).

The tissue-specific expression pattern of the TCF-1 protein was subsequently determined in a panel of adult human tissues. Consisting of jejunum, heart, liver, lung, muscle, stomach, skin, kidney, thymus, spleen, tonsil, and lymph node. Frozen sections of the relevant tissues were stained with the α-TCF-1 MoAb. As demonstrated in Fig 6, a nuclear staining pattern was readily detectable in lymphoid cells in normal thymus and in tonsils, spleen, and lymph nodes. In the latter three organs, TCF-1–positive nuclei were concentrated in the T-cell areas. Morphologically nonlymphoid cells in these tissues (eg, stromal elements) never displayed any staining. Double-staining with CD3, CD4, and CD8 antibodies confirmed that TCF-1–positive cells were always of T lineage (not shown). Interestingly, TCF-1 expression in individual thymocytes varied dramatically, but appeared particularly high in a subset of cortical thymocytes.

The TCF-1 protein was never detected in nuclei of nonlymphoid cells in any of the other tissues examined. In particular, lung and kidney, the two most prominent sites of TCF-1 mRNA expression in the mouse embryo, did not display any nuclear staining. Some extracellular staining (most likely of the basement membrane) was observed in the glomerulus of the kidney (not shown). This was interpreted as crossreactivity to a basement membrane component. No other sites of such nonspecific reactivity were observed.

TCF-1 protein expression appeared to be completely restricted to the T lineage. TCF-1 expression could, thus, potentially serve as a useful diagnostic lineage marker.
next analyzed expression of the TCF-1 protein in thymocytes of four independent thymuses by immunofluorescence on cytospin preparations and found that greater than 98% of the cells expressed TCF-1 (Fig 7). This indicated that TCF-1 was expressed throughout the thymic differentiation pathway and should be considered a pan-thymocyte marker. Immunofluorescent analysis of a panel of 22 well-characterized T-lineage malignancies (see Table I) extended these observations. TCF-1 expression was found in all samples, including leukemias representing immature T-lineage cells (CD2⁺, surface CD3⁻). A control panel of 57 non-T hematologic malignancies was negative in all cases.

DISCUSSION

The present study provides the first description of the expression pattern and subcellular localization of the TCF-1 protein. To obtain a MoAb specific for human TCF-1, we generated a fusion protein, persistently immunized mice, performed a hybridoma fusion, and screened a large number of subsequent hybridomas on COS cells transiently expressing TCF-1. The single MoAb we obtained was subsequently found to be reactive with a region of the protein common to all splice forms of human TCF-1. Furthermore, the epitope was not lost after methanol fixation of COS cells, Western blotting, or immunohistochemistry on frozen sections.

Using the αTCF-1 MoAb, we subsequently found that the TCF-1 protein was located in the nuclei of normal as well as malignant T cells. Although most transcription factors localize to the nucleus, this is certainly not true for all. In several cases, transcriptional control is brought about by the regulated transfer of transcription factors from the cytoplasm.
Fig 6. Immunohistochemical staining performed with αTCF-1 MoAb on tonsil (A and B) and thymus (C). (A) Nuclear staining is readily detectable in cells in the paracortical (P) region, the predominant site for T cells. Limited numbers of cells are stained in the germinal center (GC) and mantle (MZ), the predominant B-cell sites. (B) A magnification of panel A illustrating the nuclear staining in positive T cells. (C) Nuclei of T cells in the cortex (C) and medulla (M) of the thymus stain brightly with the αTCF-1 antibody.
Table 1. Expression of TCF-1 in Various Hematologic Malignancies

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The presence of Tcf-1 protein in malignant cells was assessed by immunofluorescence in blood, bone marrow, or both, as indicated. Positivity in T-lineage malignancies was seen in 100% of the tumor cells in all cases.

Abbreviations: ALL, acute lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome.

The availability of the MoAb allowed us to study the expression of the TCF-1 protein at the single-cell level in normal human adult tissues. The human TCF-1 protein was found to be uniquely expressed in T cells, while no expression was observed in any of the nonlymphoid tissues examined. This observation fits with the lack of mRNA observed after Northern blotting analysis of postnatal murine nonlymphoid tissues. However, it is in stark contrast with TCF-1 gene transcription in mouse embryos, which occurs in complex and rapidly changing expression patterns throughout embryogenesis. We have not determined the expression patterns of TCF-1 mRNA or protein during human embryogenesis but believe it will resemble that in mouse embryos. These data indicate that the TCF-1 gene is dynamically expressed during embryogenesis, but after birth, its expression occurs uniquely in T lymphocytes. This fits with the notion that, at least postnatally, TCF-1 fulfils a regulatory function in T-cell differentiation. In line with this, we have recently generated TCF-1 knock-out mice, whose only phenotypic abnormality is a severe impairment in thymocyte differentiation.
In addition to the expected molecular weight of 26 kD to 28 kD, an unexpected, prominent cluster of bands around 42 kD to 48 kD was consistently observed in Western blot analysis of primary T cells and T cell lines. This led to the identification of numerous novel splice forms of TCF-1. One of the prominent novel forms resulted from the opening of a previously unidentified reading frame in exon X conserved between mouse and man (M. van de Wetering and H. Clevers unpublished data, January 1995). We are currently evaluating the transcriptional control properties of these novel TCF-1 forms, some of which are highly abundant. Polymerase chain reaction (PCR) analysis of mouse embryonic tissues and thymocytes, as well as stage-specific Western blotting of sorted thymocyte subpopulations, have so far not revealed a differential expression of the various splice alternatives.

This study illustrates that the expression of TCF-1 is completely restricted to the T lineage and, vice versa, that all T-lineage cells express TCF-1. As revealed by gene disruption in the mouse germline, the expression of TCF-1 is a prerequisite for normal T-lineage differentiation. It is, therefore, to be expected that not only normal but also malignant T cells will express the TCF-1 gene. This implies that the expression of TCF-1 should be useful as a diagnostic marker for the T lineage. As demonstrated in Table 1, this notion was confirmed within a panel of hematologic neoplasms. It appears to be a rational approach to determine cell lineage by directly assaying for proteins that control differentiation of that lineage. Thus, the availability of reagents such as the αTCF-1 MoAb should refine lineage determination of hematologic malignancies.

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