The Human TRIDENT/HF1-11/FKHL16 Gene: Structure, Localization, and Promoter Characterization

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We recently identified the winged-helix/fork head transcription factor Trident in mouse and described its expression in cycling cells. Here we report the isolation and characterization of the human TRIDENT (HGMW-approved symbol FKHL16) cDNA and gene. Homology between the human and the mouse Trident proteins was 79%. The gene consists of 10 exons and is located on chromosome 12 band p13. The winged-helix DNA-binding domain is encoded on three exons. Analysis of the promoter in synchronized Rat-1 fibroblasts revealed a fragment of 300 bases responsible for the cell cycle-specific expression of the TRIDENT gene. © 1997 Academic Press

INTRODUCTION

One of the largest and most diverse classes of DNA-binding proteins are the transcription factors that regulate gene expression. Many of these proteins can be grouped into families that use related structural motifs for recognition. Among these are the helix–loop–helix, the helix–turn–helix, the leucine zipper, the zinc finger, and the homeodomain families of proteins (reviewed in Pabo and Sauer, 1992). A novel DNA-binding motif was identified by comparison of the DNA-binding domains of the Drosophila homeotic fork head (fkh) and the HNF-3 proteins (Weigel and Jäckle, 1990). Based on the “butterfly-like” co-crystal structure of the fork head motif on DNA, members of this family are also referred to as winged-helix proteins. The winged-helix domain was detected in many proteins of different species ranging from yeast to human. Members of this class of transcription factors act as key regulators in both embryogenesis and differentiated tissues (for a recent review, see Kaufmann and Knöchel, 1996).

Recently, we described the isolation and characterization of Trident, a member of the winged-helix family in mouse (Korver et al., 1997a). In adult mice, Trident expression is restricted to the thymus, whereas in the mouse embryo expression is ubiquitous. Trident is expressed uniquely in cycling cells, showing an expression pattern similar to factors responding to proliferative signals. Expression is absent in resting lymphocytes derived from peripheral blood, but could be induced upon stimulation with mitogens in vitro (Korver et al., 1997b). Whenquirescent fibroblasts are stimulated to enter the cell cycle by the addition of growth factors, the Trident gene is transcriptionally activated upon entry into S phase. However, when exponentially growing cells progress through the cell cycle, the Trident mRNA and protein levels appear to be invariant. In M phase the Trident protein is phosphorylated, presumably by p34CDC (Korver et al., 1997a; Westendorf et al., 1994).

A recent report by Ye et al. (1997) describes the cloning and expression of the human TRIDENT/HF1-11 cDNA. Using in situ hybridization, the authors show a broad expression in proliferating cells in many embryonic organs. Expression was found in the cycling cells in the crypts of the intestine and in the proliferating cells in the testis during spermatogenesis. The winged-helix DNA-binding domain of Trident/HF1-11 is necessary and sufficient for sequence-specific DNA binding. The protein is a transcriptional activator on selected binding sites, while insertion of an alternative exon sequence in the C-terminal region represses this activity (Korver et al., 1997a; Ye et al., 1997).

Here we report the exon/intron organization, the chromosomal localization, and the characterization of the promoter of the human TRIDENT gene.

MATERIALS AND METHODS

Isolation of cDNA and genomic clones. A murine Trident cDNA clone (Korver et al., 1997a) was used to screen a human cDNA library derived from peripheral blood lymphocytes of a leukemia patient (HPB-ALL; Korinek, Kalousova, and H. Clevers, unpublished) under
<p>FIG. 1. (A) Alignment of the human and mouse Trident protein sequences, showing 79% homology. The winged-helix DNA-binding domain is boxed. (B) Exon/intron organization of the <em>TRIDENT</em> gene. Exons are indicated by black boxes and roman numerals. <em>ApoA1</em> (A) and <em>SacI</em> (S) sites are indicated. Two alternative exons, as reported by <em>Ye et al.</em> (1997), are designated exons <em>Va</em> and <em>VIIa</em>. (C) Sequence of the <em>TRIDENT</em> gene. Exon sequences appear in capital letters. Amino acids are indicated in single-letter code. An asterisk indicates the start of the cDNA clone, designated +1. Putative E2F and Myc binding sites are in boldface. Alternative exons <em>Va</em> and <em>VIIa</em> are in italics. Restriction sites used for pFlax constructs are underlined. The sequence of the promoter region (<em>SacI</em>–<em>BamHI</em>, 2.4 kb) has been deposited with GenBank (Accession No. Y12773).<br/><br/>low-stringency conditions (final wash 40°C, 2× SSC). A 3.5-kb clone was obtained, containing the complete coding region for the <em>TRIDENT</em> protein.<br/>The human cDNA clone was used to screen a genomic P1 library at GenomeSystems (St. Louis, MO). An 80-kb clone containing the <em>TRIDENT</em> gene was isolated (DMPC-HFF#1-1000-C5). After restriction site mapping and Southern analysis using the human cDNA as a probe, <em>ApoA1</em> and <em>SacI</em> genomic fragments were subcloned into pBluescript SK for sequence analysis. Oligonucleotides were from Isogen (Maarsen, The Netherlands).<br></p>
Chromosomal localization. Fluorescence in situ hybridization (FISH) was performed on normal human lymphocyte metaphase chromosomes, using the human cDNA as a probe. Dioxygenin-labeled cosmid DNA (200 ng) was precipitated with 50× Cot-1 DNA (Gibco BRL, Gaithersburg, MD) and dissolved in 12 μl of a hybridization solution containing 2× SSC, 10% dextran sulfate, 1% Tween 20, and 50% formamide. The probe mixture was heat-denatured at 80°C for 10 min, followed by incubation at 37°C to allow reannealing of highly repetitive sequences. Hybridization of this probe to heat-denatured chromosome spreads under a 18 × 18-mm coverslip was done for 40 h at 37°C.

After hybridization, the slides were washed according to regular FISH protocols (Pinkel et al., 1988). The hybridized probe was detected immunohistochemically using one layer of sheep anti-dioxygenin-conjugated fluorescein isothiocyanate (FITC; dilution 1:20; Boehringer Mannheim). Afterward, the preparations were counterstained with an anti-fade solution, supplemented with 0.5 mg/ml 4,6-diamino-2-phenylidole (DAPI).

Metaphase spreads were captured by a cooled high-performance CCD camera (Photometrics) coupled to a Macintosh Quadra 950 computer. Separate images of both TRIDENT-hybridizing signals (FITC) and DAPI-counterstained chromosomes were recorded and transformed into pseudocolored images using the image analysis software package Oncor Image (Oncor, Gaithersburg, MD). The identification of the individual chromosomal regions was deduced from converted DAPI staining patterns.

PCR-based analysis of somatic hybrids exclusively containing human chromosome 12 or fragments thereof (Sinke et al., 1992) was performed using the primers TRI-MAP-UP and TRI-MAP-DO as indicated in Fig. 1C.

Primer extension analysis. Total RNA was prepared in RNAzol according to the manufacturer's instructions (Cinna-Biotech, Houston, TX) from the human T cell line Jurkat, followed by phenol/chloroform extraction and 2-propanol precipitation. Fifty micrograms of RNA was coprecipitated with 5 × 10⁴ cpm 32P-labeled oligonucleotide TRI-PE (bp 144–177) and subsequently resuspended in 30 μl of hybridization solution containing 40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide. After 16 h of hybridization at 30°C samples were ethanol precipitated and resuspended in 20 μl of 1× AMV reverse transcriptase buffer, 40 units of AMV reverse transcriptase, and 35 units of RNase inhibitor (all from Pharmacia, Uppsala, Sweden), and samples were incubated for 1 h at 42°C. Reactions were stopped by the addition of 1 μl of 0.5 M EDTA followed by 1 μg of RNase A. Reaction products were phenol extracted, ethanol precipitated, and subjected to electrophoresis on 6% polyacrylamide, 8 M urea gels.

Transfections and luciferase assays. Fragments of the TRIDENT promoter were subcloned into the luciferase reporter construct pFlash (Fig. 4A). Rat-1 cells were transfected by electroporation. Cells (1.5 × 10⁶) were transiently transfected with 5 μg of the reporter construct in a volume of 250 μl. Pulse conditions were 960 μF and 250 V using a Gene Pulser apparatus (Bio-Rad, Hercules, CA). The cells were plated on two 10-cm dishes. After overnight culturing on 10% FCS, cells were serum starved for 48 to 72 h on 0.25% serum to synchronize them in G₀. One of the plates was harvested (time point t = 0); cells on the other plate were allowed to reenter the cell cycle by addition of 10% serum and were harvested after 16 h (t = 16), when the cells were in S phase (Korver et al., 1997a). Samples were lysed in 250 μl lysis buffer (1 mM DTT, 1% Triton X-100, 15% glycerol, 25 mM Tris, pH 7.8, and 8 mM MgCl₂). Luciferase activity was determined on a Lumac/3M biocounter. Values were corrected for amounts of protein, and the fold induction in S phase was calculated.

Gel retardation assay. Annealed oligonucleotides were labeled by T4 kinase with [γ-32P]ATP. All probes were purified by nondenaturing polyacrylamide electrophoresis. For a typical binding reaction, nu-
clear extract (5 μg protein) and 1 μg of poly(dI–dC) were incubated in a volume of 15 μl containing 10 mM Hepes, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 12% glycerol. After 5 min of preincubation at room temperature, probe (20,000 cpm) was added and the mixture was incubated for an additional 20 min. The samples were then electrophoresed through a nondenaturing 8% polyacrylamide gel run in 0.25× TBE at room temperature.

RESULTS AND DISCUSSION

Isolation and Genomic Organization of the TRIDENT cDNA and Gene

Low-stringency screening of an HPB-ALL library with the mouse cDNA coding for Trident (Korver et al., 1997a) revealed a positive human cDNA clone. By comparison with the murine sequence, the human cDNA clone appeared to encode the TRIDENT/HFH-11 protein (747 amino acids). The coding region of the cDNA clone was identical to one of the differentially spliced isoforms (FH-11B) reported by Ye et al. (1997). Overall sequence homology with murine Trident was 79% at the amino acid level (Fig. 1A). The human cDNA was used to screen a P1 genomic library, resulting in a positive plasmid with an insert of approximately 80 kb containing the complete human TRIDENT gene. Figures 1B and 1C depict the result of the structural analysis of the TRIDENT gene. Ten exons were identified, spanning approxi-
mately 25 kb. The first exon was preceded by a region of high C and G content, characteristic of a CpG island (Bird, 1986). Two alternative exons, present in isoform HFH-11A (Ye et al., 1997), were designated exon Va and exon VIIa. Interestingly, intron 4 is present in the region encoding the winged-helix domain at the same position as was reported for another member of this family, FKHL13 (Murphy et al., 1997).

The TRIDENT Gene Maps to Chromosome 12 Band p13

To determine the chromosomal localization of the TRIDENT gene, nonradioactive in situ hybridization, using the human cDNA as a probe, was performed on normal human lymphocyte metaphases. Using this approach, the TRIDENT gene could be assigned to band 12p13 (Fig. 2A). The localization on 12p13 was confirmed by PCR-based analysis of a mouse–human hybrid cell line (M28; Zhang et al., 1989) which contains a human i(12p) chromosome as its only human constituent (Fig. 2B, lane 5). Further analysis of radiation-reduced hybrids containing fragments of chromosome 12p strongly suggested a localization for TRIDENT telomeric to the FGF-6 locus on 12p13.3 (Sinko et al., 1992; data not shown).

Chromosomal abnormalities involving this region on chromosome 12 are found in a broad spectrum of cancers (Mitelman, 1994). The genes coding for the ETS-like transcription factor TEL, and the cyclin-dependent kinase inhibitor p27 (KIP1), are likely candidates for playing a role in these malignancies. However, in several studies no mutations in either of these genes were found, suggesting the presence of yet another tumor suppressor gene in 12p13 (Takeuchi et al., 1996; Steg-
maier et al., 1996; Wlodarska et al., 1996). The cell cycle-specific expression pattern of TRIDENT suggests its involvement in the control of proliferation, and therefore TRIDENT is a candidate gene for association with cancers. Further work will be required to establish whether genetic aberrations in the TRIDENT gene are involved in these malignancies.

Characterization of the TRIDENT Promoter

As a prelude to transcription regulation studies of TRIDENT, its promoter was characterized. To map the transcription start site of TRIDENT, we performed primer extension analysis on total RNA extracted from the T cell line Jurkat. A 34-mer oligonucleotide representing antisense sequence in exon I (bp 144–177) was used as a primer. Figure 3A depicts the results of a representative experiment. Several extended products were obtained (arrows); the start sites as predicted from the primer extension experiments are indicated in Fig. 3B. This prediction was corroborated by the observation that the longest cDNA clone started in the vicinity of the predicted cap sites. No potential TATA box was found in this region.

To analyze the transcriptional regulation of the TRIDENT gene, the promoter region was cloned and characterized. A 2.4-kb fragment (SacI–BamHI) upstream of the putative transcription starts was analyzed for promoter activity (Fig. 4A). The fragment was cloned into a luciferase reporter plasmid (pFlash) and was transfected transiently into Rat-1 cells. Significant activity of this promoter was observed, approximately 100-fold higher than background levels (not shown).

The cell cycle-specific activity of the TRIDENT promoter was studied in transiently transfected Rat-1 cells. Rat-1 cells were used for these experiments because they are readily transfected and can be synchronized following transfection. As expression of TRIDENT mRNA in synchronized cells is known to be induced upon entry into S phase (Korver et al., 1997a), we asked whether the observed promoter activity was responsive to serum stimulation and, if so, whether such responsiveness was conferred by a specific region within this promoter fragment.

The Rat-1 cells were transfected with constructs containing TRIDENT promoter sequences and control fragments in the luciferase reporter plasmid pFlash (see Fig. 4A), and the cells were subsequently serum starved. A comparison was made between the activity of these reporter constructs in quiescent cells and that in cells cultured for 16 h following restimulation. Rat-1 cells were previously shown to be in the middle of S phase after 16 h of restimulation, as determined by [3H]thymidine incorporation, whereas TRIDENT expression is induced at the onset of S phase (Korver et al., 1997a). The fold induction in S phase is given in Fig. 4B. The 2.4-kb promoter construct (Tri, −2436 to +60) was inducible fourfold. Deletion of the upstream 2.0 kb of the promoter did not lead to loss of this inducibility, as demonstrated by the activity of both the Hind (1.4 kb, −1411 to +60) and the Pvu (0.43 kb, −437 to +60) constructs after 16 h of serum stimulation. However, the activity of Apa (0.3 kb, −296 to +60) showed the highest induction (eightfold). Activity of the 5′ antisense control plasmid, which is likely to result from the presence of promoter-like sequences in the antisense strand that function in a cell cycle-independent manner, was observed. This activity did not show any response to serum stimulation (Fig. 4B).

These data indicate that sequences between −296 and the transcription start site are essential for the induction of promoter activity following serum stimulation. A gel shift assay was performed with nine 30-bp oligonucleotides spanning this region upstream of nucleotide +1, as indicated in Fig. 3B. We asked whether nuclear extracts of Rat-1 cells contain proteins binding to these oligonucleotides and, if so, whether
this binding pattern changed during the cell cycle. To this end, the radioactively labeled oligos (1–9) were incubated with nuclear extracts from either asynchronously growing Rat-1 cells (Fig. 5, lanes A) or Rat-1 cells synchronized in G0 (lanes 0) or in S phase (lanes S). As is clear from Fig. 5, shifted bands were detected with all of the oligos tested, some of which appeared in distinct phases of the cell cycle only (indicated by the arrows). The proteins responsible for these shifts are likely candidates for playing a role in the regulation of the TRIDENT promoter, although we have not yet identified the nature of these complexes.

One site that could be important in the regulation of TRIDENT expression is an E box motif (located at position −49 to −44, see Fig. 1C). This motif is often found in transcriptional enhancers, which can be bound by a number of transcription factors, including the products of the myc proto-oncogenes (Marcu et al., 1992). The expression of this small family of immediate-early response genes occurs early during G0 to G1 transition, and its levels are, like that of TRIDENT, invariant throughout the cycle in continuously proliferating cells. The protein products of these genes are believed to facilitate a cell’s progression through the cycle to eventually achieve DNA synthesis in S phase. Myc proteins are therefore considered candidates for involvement in the regulation of TRIDENT expression. However, based on previously reported data, it is not likely that the Myc proteins are solely responsible for the induction of the TRIDENT promoter (Marcu et al., 1992). No other obvious binding sites for transcription factors were found in the 296-bp ApaI fragment, suggesting a role for proteins interacting with as yet unidentified binding sites.

In conclusion, the promoter data in this study in combination with the expression of TRIDENT/HHF-11/FKHL16 in all cycling, but not in resting, cells (Korver et al., 1997a; Ye et al., 1997) indicate that this transcription factor is likely to play a role in the control of proliferation, rather than in the differentiation of specific cell types.

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