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Nucleic Acids Research, 1997, Vol. 25, No. 9 **1715–1719**

The winged-helix transcription factor Trident is expressed in cycling cells

Wouter Korver*, Jeroen Roose and Hans Clevers

Department of Immunology, University Hospital Utrecht, PO Box 85500, 3508 GA, Utrecht, The Netherlands

Received February 7, 1997; Revised and Accepted March 19, 1997

DDBJ/EMBL/GenBank accession no. Y11245

ABSTRACT

We describe the cloning and characterization of *Trident*, a novel member of the fork head/winged-helix family, from murine thymus. In the mouse embryo, the gene was expressed in all tissues, whereas in adult mice expression was only detected in the thymus. Further analysis revealed that Trident expression strictly correlated with cell cycling, independent of cell type. Timing of [^{3}H]thymidine incorporation showed that mRNA and protein expression were strongly upregulated upon entry into the S phase of the cell cycle. Moreover, the protein was phosphorylated in M phase. PCR-mediated selection of optimal binding sites yielded a consensus motif resembling that of other family members. These results identify Trident as a transcription factor, which is likely involved in cell cycle-specific gene regulation.

INTRODUCTION

The family of fork head (fkh) or 'winged-helix' proteins comprises a large number of transcription factors, defined by a conserved DNA binding domain. The prototype of this group is the product of the homeotic *Drosophila melanogaster* gene *fork head* (1), which is required during the terminal development of the *Drosophila* embryo. Comparison of the sequences of fkh and the hepatocyte nuclear factor-3α, a protein involved in liver-specific gene expression in rat (2), revealed a novel, highly conserved DNA-binding protein motif of ~110 amino acids. After the elucidation of the butterfly-like co-crystal structure of HNF-3γ bound to DNA (3, see below), the members of this family are also referred to as 'winged-helix' proteins.

In the last few years, over 80 new members of this family have been identified in species ranging from yeast to man. Individual members perform various biological functions: their expression patterns are usually temporally and spatially restricted and some of these genes appear to play a central role in embryonic development. Examples are genes involved in notochord formation in mouse development (*HNF-3*β; 4,5), development of the neural axis in *Xenopus laevis* (*pintallavis*; 6), thymus development and hair growth (*winged-helix nude*, the gene responsible for the nude phenotype in mice; 7), brain development in rat (*Brain factor 1*; 8) and vulval cell fate in *Caenorhabditis elegans* (*lin-31*; 9).

Two genes of the family have been shown to play a direct role in tumorigenesis. A chromosomal translocation frequently found in alveolar rhabdomyosarcoma results in a fusion between the genes coding for PAX3, a transcriptional regulator during early neuromuscular differentiation and ALV, a member of the fork head family (10). The oncogenic potential of the resulting chimeric protein suggests that transcriptional deregulation is the mechanism underlying the tumor development. Another winged-helix protein, encoded by the retroviral oncogene *qin*, found in the genome of the avian sarcoma virus 31, induces oncogenic transformation in cell culture and causes tumors in chickens (11).

The DNA-binding specificity of several members of the fork head family has been determined using selection of protein recognition sites from a pool of random sequence oligonucleotides. Single core sequences of 6 or 7 nt were found for four human and three rat proteins (12,13). Subtle differences at the 3′ and 5′ termini of the consensus sites allow distinct binding and transcriptional activation of different family members. The promoter of the lung specific gene *CC10*, for example, allows various winged-helix family members to bind *in vitro* and *in vivo*. From the proteins tested, however, only FREAC-1 appears to be a strong activator of the *CC10* gene in a lung cell line (14).

The three dimensional structure of the fork head motif of HNF-3γ bound to DNA was determined in an X-ray crystallographic analysis (3). Two loops accompanying an α-helical thorax form a butterfly-like structure, referred to as 'winged-helix'. From the organization of α-helices and β-turns the winged-helix motif can be classified as a variant of the helix–turn–helix motif (reviewed in 15).

Here we describe the isolation and characterization of *Trident*, a new member of the fork head/winged-helix gene family in mouse.

MATERIALS AND METHODS

Isolation of cDNA clones

Total RNA was prepared from mouse thymus using RNAzol according to the manufacturer's instructions (Cinna-Biotecx). Poly(A$^+$) RNA was isolated with oligo(dT) Dynabeads. cDNA was made using random hexamers according to the protocol of the Perkin Elmer Cetus corporation. The degenerate primers (Isogen, Maarssen, The Netherlands) 5′-AA(G/A)CC(A/T/C)CC(A/T/C)TA(A/T)TC(G/A/T/C)TA(T/C)AT-3′ and 5′-(G/A)TG(C/T)GT(G/A)AT(G/A/T/C)GA(G/A)TTCTGCCA were used in PCR (94, 60 and 72°C all for 1 min, 35 cycles) on 100 ng cDNA. Products of the expected size were blunted using T4 DNA polymerase, cloned into pBluescriptSK and sequenced. The product encoding the fork head DNA binding domain was used as a probe to screen an EL-4 cDNA library in λZAP under standard conditions (16).

*To whom correspondence should be addressed. Tel: +31 30 250 7674; Fax: +31 30 251 7107; Email: w.korver@lab.azu.nl

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TRIDENT   DSVSERPPYSYMAMIQFAINSTERKRMTLKDIYTWIEDHFPYFKHIAKPGWKNSIRHNLSLHDMFVR-ETS---ANGKVSFWTIHPSANRHLTLDQVFKPLEPGSPQSPEHLESQQKRPNPE
HNF-3γ    PLTHAKPPYSYISLITMAIQQAPGKMLTLSEIYQWIMDLFPYYRE-NQQRWQNSIRHSLSFNDCFVKVARSPDKP-GKGSYWALHPSSGNMFE-NGCYL--------------RRQKRFKLE 46 %
fkh-1     PKDMVKPPYSYIALITWAIQNAPDKKITLNGIYQFIMDRFPFYRD-NKQGWQNSIRHNLSLNECFVKVPRDDKKP-GKGSYWTLDPDSYNMFE-NGSFL--------------RRRRRFKKK 44 %
ILF       PKDDSKPPYSYAQLIVQAITMAPDKQLTLNGIYTHITKNYPYYRT-ADKGWQNSIRHNLSLNRYFIKVPRSQEEP-GKGSFWRIDPASESKLIEQAFRK--------------RRPRGVPCF 44 %
HTLF      KSATSKPPYSFSLLIYMAIEHSPNKCLPVKEIYSWILDHFPYFAT-APTGWKNSVRHNLSLNKCFQKVERSHGKVNGKGSLWCVDPEYKPNLI-QA-LK--------------KQPFSSASA 43 %
FREAC-2   LRRPEKPPYSYIALIVMAIQSSPSKRLTLSEIYQFLQARFPFFRG-AYQGWKNSVRHNLSLNECFIKLPKGLGRP-GKGHYWTIDPASEFMFE-EGSFR--------------RRPRGFRRK 42 %

CONS           *PPYS*  *I  AI   * K +**  IY  * + *P+++  +  *W NS+RH*LS** +F *+ ++   + GK** W+ +P +                         ++
```

Figure 1. Alignment of the Trident DNA binding domain with five other members of the fork head family. The conserved amino acids are indicated in the consensus sequence. * and + indicate conservation in five and four of the six proteins, respectively; % refers to sequence identity relative to the Trident sequence. References: HNF-3γ (22), fkh-1 (29), ILF (30), HTLF (31), FREAC-2 (14).

Northern blotting

Total RNA was isolated with RNAzol and 15 µg per lane was analyzed on a 1% agarose gel containing 2% formaldehyde. RNA was transferred to nitrocellulose, hybridized and washed under standard conditions (16). The cDNA coding for amino acids 197–757 was used as a probe.

Western blotting and phosphatase treatment

Western blotting was carried out as described (17). Approximately 3×10^5 cells per lane were used. For the phosphatase experiment, Nocodazole (250 ng/ml; Sigma) blocked Rat-1 cells were washed in PBS and split in three. One third was resuspended in SDS sample buffer, to the other samples calf intestinal phosphatase (1 U, Pharmacia) or phosphatase inhibitors (20 mM NaF and 25 µM phenylarsenic oxide) were added. After sonification for 15 s, the samples were incubated at 37°C for 15 min and subjected to SDS–PAGE for immunoblotting.

In vitro translation and production of recombinant protein

The *Trident* cDNA was subcloned into pcDNAI (Invitrogen) and subsequently translated *in vitro* using the TnT kit (Promega) according to the manufacturer's instructions.

Recombinant protein was made as a maltose binding protein fusion using the system developed by New England Biolabs. A cDNA fragment encoding the DNA binding domain (amino acids 207–348) was cloned into pIH902 for expression in *E.coli*.

Gel retardation assays and binding site selection

A typical gel shift was performed with 0.2 ng [γ-^{32}P]ATP end-labeled oligo and 10 ng recombinant protein in a buffer containing 10 mM Tris (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 5% glycerol and 0.1% Nonidet P-40. After an incubation at room temperature for 30 min the samples were subjected to electrophoresis on a 5% polyacrylamide gel run with 0.25× TBE.

The Trident consensus binding site was determined according to (18) with modifications according to (19). The random oligo contained two fixed A residues in the middle of the random stretch to enhance binding based on the consensus binding sequence of other fork head proteins (12,13).

The sequences of the oligos 2x and 0x are AGCTTGATTGTTTATAAACATGCCCGGG and AGCTTGATTGCCCATCCCCACCGGG, respectively.

[^{3}H]thymidine incorporation

Rat-1 cells were plated on 24-well plates (10 000 cells per well) in medium containing 10% FCS. The medium was removed after an overnight incubation and the cells were starved for 48 h on medium containing 0.5% FCS. On t=0 the medium was replaced by medium containing 10% FCS and on the indicated timepoints 1 µCi of [^{3}H]thymidine was added and the cells were incubated for an additional 1 h at 37°C. After three washes with PBS, the cells were fixed with 1 ml methanol for 30 min, washed again with PBS and resuspended in 0.75 ml of 0.2 N NaOH. An aliquot of 4 ml of scintillation liquid was added and the c.p.m. representing the amount of [^{3}H]thymidine was determined.

Transfections and CAT assays

An oligonucleotide containing a Trident binding site (AGCTTGATTGCCCTAAACATGCCCGG) was cloned into pBLCAT2 (20) to yield plasmid TK1. The Trident cDNA was cloned into pcDNAI (Invitrogen) to yield a Trident expression vector driven by the CMV promoter.

Transfections of AZU2 B cells and CAT assays were performed as described previously (21). 5×10^6 cells were transfected with 10 µg of the CAT reporter plasmid and 1 µg of the Trident expression plasmid. Cells were harvested and CAT activity was measured 48 h after transfection. COS cells were transfected and stained as described (17).

RESULTS

Cloning of *Trident* cDNA

In order to identify new members of the fork head family with a possible role in the differentiation of T cells, degenerate primers were designed, based on conserved amino acids in the fork head DNA binding domain. PCR reactions were carried out on mouse thymus cDNA. Subcloning and sequencing of PCR products revealed several clones encoding HNF-3γ (22). In addition, a novel sequence with low but significant homology to the fork head consensus domain was found. Using this 153 bp product as a probe, an EL-4 thymoma cDNA library was screened and a clone with a 2.5 kb insert was isolated, coding for a putative protein of 757 amino acids. The gene was termed *Trident*, and appeared to encode a distant member of the fork head family, with relatively low homology to other genes coding for winged-helix proteins. In Figure 1 the fork head DNA binding domain is aligned with the five most closely related family members, showing identities of ~45%. Database searches revealed a high match with a partial-length human cDNA described by Westendorf *et al.* (23) coding for a putative M phase phosphoprotein (MPP2; see Discussion).

Binding site selection

To identify the DNA binding specificity of Trident, binding site selection was performed using a recombinant protein consisting of

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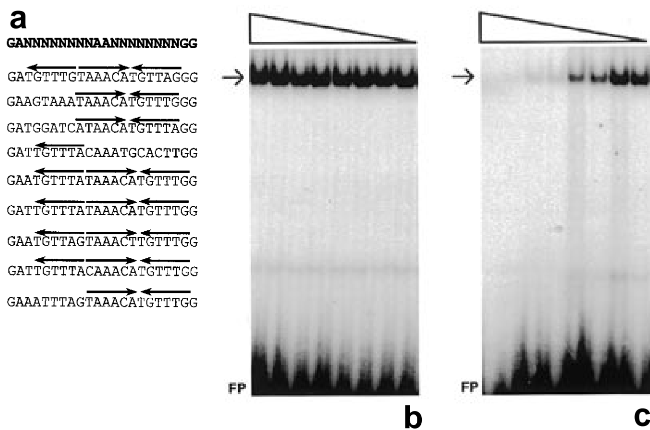

Figure 2. (**a**) Binding motifs of the Trident protein found by binding site selection. Only sequences containing more than one consensus site are shown. Arrows indicate the direction of these sites in alternating order. The sequence on top in bold represents the sequence of the random starting oligo. (**b** and **c**) Gel retardation analysis showing sequence specificity of the fusion protein containing the Trident DNA binding domain. The protein is incubated with 0.2 ng of labeled oligo (2x) and decreasing amounts (200 ng lane 1, 1 ng lane 8) of either the same oligo (c) or a mutant oligo (0x) as a competitor (b). The arrow indicates the position of the Trident retarded band. FP, free probe.

the fork head domain fused with the maltose binding protein. This fusion protein was incubated with a pool of random oligonucleotides; sequences binding to the fusion protein were isolated in a gel shift assay. Purified DNA from the shifted band was amplified using PCR and labeled for the next round of selection. After six rounds the oligonucleotides were subcloned and sequenced (Fig. 2). All of 36 selected sequences contained the consensus site TAAACA, in agreement with findings for other members of the family (12,13,24). These data confirmed that *Trident* encodes a *bona fide* fork head-type DNA binding domain. In 22 out of 36 sequenced motifs this site was found two or three times. The repeats of these motifs within individual selected oligos were always characterized by alternating orientation and fixed spacing between the motifs, as is indicated by the arrows in Figure 2a.

To confirm the specificity of the TAAACA binding site, recombinant fusion protein was incubated with a labeled oligonucleotide (2x) containing two of these sites in alternating order. The binding of the fusion protein to this oligonucleotide could be specifically competed by an excess of unlabeled oligo 2x, but not by an excess of an unlabeled oligo (0x) containing mutant binding sites (Fig. 2b and c).

Transactivation by Trident

We used the site found in the binding site selection experiment to determine whether the Trident protein is a transcriptional activator. AZU2 B cells were cotransfected with a Trident expression vector and a TK-CAT reporter plasmid with or without the TAAACA Trident binding site (designated TK1 and TK0). CAT activity from TK1 showed an induction of 4.7-fold by the Trident expression vector, while expression from TK0 was elevated slightly (1.4-fold) by a non-specific mechanism (Fig. 3). These results demonstrate that the Trident protein is able to activate transcription through the TAAACA site, as a weak transactivator.

Tissue-restricted expression of *Trident*

We analyzed the expression of the *Trident* gene in a range of adult mouse tissues (Fig. 4A). Total RNA from various tissues of a 6 week-old mouse was analyzed by Northern hybridization to determine expression of the *Trident* gene. Two transcripts of ~3.5 kb were identified uniquely in thymus. We have not resolved the size difference between the two transcripts, which is most likely due to alternative splicing or polyadenylation.

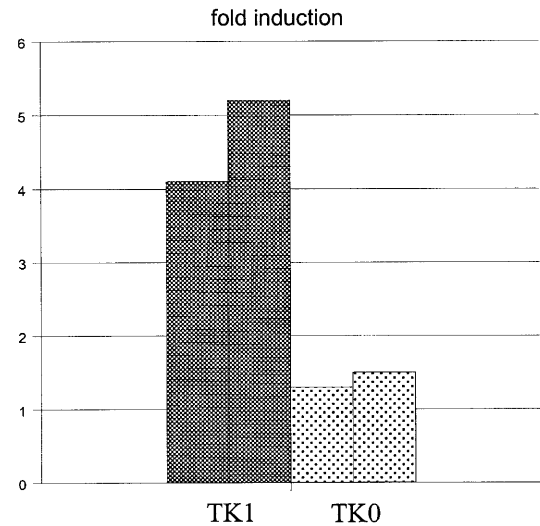

Figure 3. Transcriptional activation by Trident. The Trident expression plasmid (pcDNAI-Trident) was cotransfected with the indicated reporter plasmids in AZU2 B cells. Shown is the fold stimulation of the reporter plasmid by the Trident expression vector compared with that by the empty expression vector (pcDNAI).

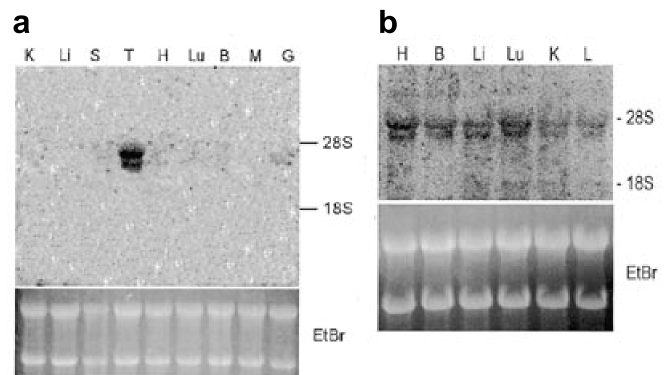

Figure 4. (**a**) Tissue-restricted expression of *Trident*. Northern blot analysis of total RNA prepared from various tissues of a 6 week-old mouse. Tissues: K, kidney; Li, liver; S, spleen; T, thymus; H, heart; Lu, lung; B, brain; M, muscle; G, gut. (**b**) Ubiquitous expression of *Trident* in embryonic tissues. Northern blot analysis of total RNA prepared from various tissues of a day E14 mouse embryo. L, limb.

Ubiquitous expression of *Trident* in embryonic tissues and cell lines

The expression of the *Trident* gene was tested in the developing mouse embryo. By analyzing RNA from six tissues of a day E14 mouse embryo, expression was found in all tissues including heart, brain, liver and lung (Fig. 4b), in contrast with the tissue-restricted expression in adult animals. Furthermore, expression was found in a large number of cell lines, ranging from B and T lymphoid, myeloid and erythroid to several carcinoma cell lines

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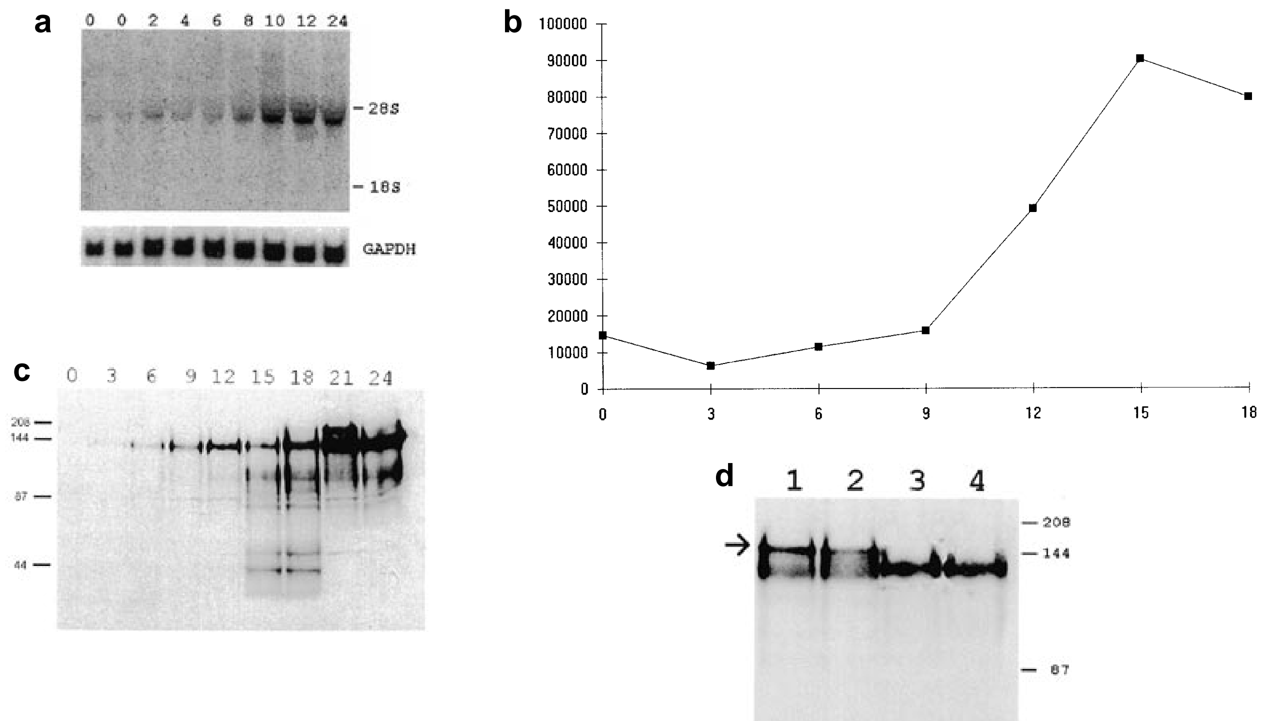

Figure 5. Expression of *Trident* is induced in stimulated Rat-1 fibroblasts. (**a**) Northern blot analysis of total RNA from cells harvested after 0, 2, 4, 6, 8, 10, 12 and 24 h after stimulation as indicated. (**b**) [^{3}H]Thymidine incorporation in Rat-1 cells after restimulation with FCS. (**c**) Western blot analysis of Trident protein in synchronized Rat-1 cells run on an 8% SDS–PAGE gel. Cells were harvested 0, 3, 6, 9, 12, 15, 18, 21 and 24 h after restimulation as indicated. The sizes of marker proteins are shown on the left in kiloDaltons. (**d**) Dephosphorylation of Trident. Extracts of nocodazole arrested RAT-1 cells were subjected to Western blotting. Lane 1, untreated sample; lane 2, sample incubated with phosphatase inhibitors; lane 3, phosphatase treated sample; lane 4, *in vitro* translated Trident protein.

(not shown). These findings prompted us to study *Trident* expression in the cell cycle.

Trident expression is induced in cells incited to cycling

In order to study Trident expression in cells entering the cell cycle, we used the rat fibroblast cell line Rat-1. Cells were grown to a confluent monolayer and synchronized in G_0 by serum starvation for 48 h. The cells were then allowed to reenter the cell cycle by replating in medium containing 10% fetal calf serum. Analysis of RNA isolated from samples taken every 2 h showed a sharp rise in *Trident* expression between 8 and 10 h after stimulation (Fig. 5a).

To correlate the timing of induction of *Trident* with cell cycle progression, a [^{3}H]thymidine incorporation assay in Rat-1 cells was performed. This experiment demonstrated that the rise in *Trident* mRNA levels coincided with the initiation of S phase (Fig. 5b).

Trident protein in cycling cells

To study Trident expression at the protein level we raised a monoclonal antibody against the recombinant TRIDENT protein (W.K. and H.C., unpublished). This anti-TRIDENT monoclonal antibody was found to be reactive in Western blotting and cell staining. It reacted with the human, mouse and rat Trident proteins. To confirm the specificity of the antibody, we used a transient transfection assay in COS cells as described (17, data not shown).

To confirm the observation of a rise in mRNA at the protein level we analyzed protein samples of synchronized Rat-1 cells by Western blotting. As shown in Figure 5c, a rise in the amount of protein followed the increase in mRNA levels ~9 h after reentering the cell cycle. Also, after 21 h a band with reduced mobility is visible, presumably representing the phosphorylated protein in M phase. Indeed, blocking of cells in mitosis with nocodazole resulted in an increase of phosphorylated Trident (see Discussion and Fig. 5d). Multiple bands representing smaller peptides are seen after 15 h of stimulation, presumably representing degradation products of Trident.

Phosphorylation of Trident in M phase

In order to confirm phosphorylation of Trident in M phase, Rat-1 cells were blocked in mitosis with nocodazole. Cells were sonicated in PBS alone or in the presence of either phosphatase inhibitors or phosphatase as described in the Materials and Methods section. The samples were subjected to SDS–PAGE and immunoblotted using the anti-TRIDENT antibody (Fig. 5d). The phosphorylated protein visible in lane 1 and 2 (arrow) is dephosphorylated after phosphatase treatment (lane 3).

The size of the Trident protein band (~130 kDa) did not correspond to the predicted size based on the amino acid sequence derived from the cDNA clone (~85 kDa). This is likely due to anomalous running behaviour on SDS–PAGE, since *in vitro* translated protein also runs as a 130 kDa band (Fig. 5d, lane 4).

DISCUSSION

We have cloned Trident, a novel member of the fork head family of transcription factors, studied its expression pattern and

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determined its DNA binding properties. Trident is expressed in all cycling, but not in resting cells. When cells are entering the cell cycle, Trident is expressed upon entry into S phase and phosphorylated during M phase. The Trident protein binds the motif TAAACA on DNA.

Trident expression was found in a large number of different cell types in cycle, but not in resting cells. It was shown that the expression is induced in S phase, implicating a role for the protein in the progression of cells through the cell cycle. Another observation that links Trident to the cell cycle was made by Westendorf *et al.* (23), who cloned MPP2, the 3′ end of the human *TRIDENT* cDNA, in a search for M phase phosphoproteins. In this study, MPM-2, a monoclonal antibody that binds an epitope containing a phospho amino acid was used to screen bacterially expressed proteins after phosphorylation with extracts from M phase enriched HeLa cells. These results suggest that Trident is phosphorylated upon entry into M phase, presumably by $p34^{cdc2}$. In accordance with this, we find a protein band of reduced mobility on a Western blot appearing 21 h after restimulation of synchronized Rat-1 cells, representing the phosphorylated protein, as we showed by phosphatase treatment. Moreover, the Trident sequence contains a cyclin–cdk2 recognition motif (amino acids 11–18) as defined by Adams *et al.* (25). This eight-residue sequence occurs in substrates and inhibitors of cyclin-dependent kinases, and is necessary and sufficient for binding to and phosphorylation by cyclin–cdk2 complexes. Together, this makes Trident a likely target for phosphorylation by cyclin-dependent kinases.

The consensus DNA binding site of the Trident protein as revealed by the binding site selection technique was similar to binding sites reported for other fork head proteins. The repeats of this motif in alternating orientation and fixed spacing, however, has not been reported before. This pattern reflects the organization of heat shock elements, which are found upstream of all heat shock genes [originally described by Pelham (26)]. Heat shock elements consist of up to seven repeats of a 5-nt module and are bound by heat shock factors, which activate the transcription of heat shock genes. Vuister *et al.* (27) reported NMR evidence for similarities between the DNA-binding domains of the *Drosophila* heat shock factor and HNF-3γ, prototype of the winged-helix family. The secondary structure of the heat shock factor and the pattern of residues involved in DNA binding are similar to those observed for the complexes between DNA and HNF-3γ (3). HNF-3γ binds DNA as a monomer, but the structure of the complex was elucidated as an asymmetric unit containing two oligonucleotides in a 5′–3′,3′–5′ orientation, reminiscent of the alternating binding sites found for Trident. This supports that winged-helix factors may multimerize on DNA.

Genes of the fork head family play important roles in developmental processes. The *Trident* gene, however, based on its expression, does not seem to function as a regulator of the differentiation pathway of specific cell types, but rather has a role in every cell in cycle. The majority of genes known to be expressed during the cell cycle are, like *Trident*, induced in, or shortly before, S phase (reviewed in 28). Transcriptional regulation of genes that control cell-cycle progression has been a subject of intensive study. The exact role in this process of many transcription factors, like MYC, JUN, BMYB and Trident, however, remains largely unknown. We were not able to show effects of ectopic overexpression of the Trident protein on cell-cycle kinetics in various transfected cells (W.K. and H.C., unpublished). Recently, a *Trident* knockout mouse was generated in our laboratory (Schilham *et al.*, unpublished results). So far, no $Trident^{-/-}$ mice were detected in litters from heterozygote matings, indicating that a null mutation in the *Trident* gene is lethal. Based on its expression pattern and its phosphorylation in M phase, the Trident protein is likely involved in cell cycle-specific gene regulation.

ACKNOWLEDGEMENTS

The authors thank Dr René Medema for comments on the manuscript and suggestions and the members of the Clevers laboratory for helpful discussions and support. This study was financially supported by a 'Pionier' grant from the Dutch scientific organization NWO.

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