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Tyrosine phosphorylation of the TATA element modulatory factor by the FER nuclear tyrosine kinases

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Abstract The FER locus in the mouse encodes two tyrosine kinases, p94^{fer} and p51^{ferT}. While p94^{fer} accumulates in the cytoplasm and nucleus of most mammalian cells the expression of p51^{ferT} is restricted to the nucleus of meiotic primary spermatocytes. The cellular function of the FER kinases is not understood, nor has a substrate for these enzymes been characterized. To identify putative substrates of p94^{fer} and p51^{ferT}, the two enzymes were used as 'baits' in the yeast two-hybrid screening system. cDNAs encoding the mouse TATA element modulatory factor (TMF) were repeatedly isolated in this assay. TMF was previously shown to bind the TATA element in RNA polymerase II promoters and impaired their functioning in a cell free transcription system. Both p94^{fer} and p51^{ferT} phosphorylated the TMF protein in in vitro and in vivo kinase assays. Sequential deletions showed that the carboxy-terminal region of TMF was essential for phosphorylation. In situ hybridization analysis revealed the preferential accumulation of TMF transcripts in meiotic spermatogenic and oogenic cells. p94^{fer} and p51^{ferT} may thus modulate the suppressive activity of TMF during cellular growth and in defined differentiation processes.

Key words: Tyrosine kinase; TATA element; FER locus

1. Introduction

In mammalian cells signal transduction pathways induced by growth factors are usually initiated by tyrosine phosphorylation events which occur on membranal receptors [36] or on the tyrosine kinases associated with them [26]. As the signaling cascade propagates toward the cell nucleus these phosphorylation events lead to serine and threonine phosphorylation activities [9,22]. In the nucleus, tyrosine phosphorylation events are relatively rare, yet several nuclear tyrosine kinases have been identified and characterized. These can be divided into two main groups of src-related and non-related nuclear tyrosine kinases [30,35]. The role of most of these tyrosine kinases is however not well understood.

p94^{fer} is an evolutionarily conserved tyrosine kinase [29] encoded by the FER locus in mouse [11], rat [27] and human [19]. p94^{fer} accumulates in both cytoplasm and nucleus of cells [18]. Its nuclear accumulation becomes most prominent, however, concomitantly with the progression of cells toward the G1/S transition state (Ben-Dor and Nir, submitted). The presence of p94^{fer} has been documented in most mammalian cell lines analyzed [10,15,19,27]. It could not be detected, however, in pre-B, pre-T and T cells [15].

A truncated form of p94^{fer}, termed p51^{ferT}, is encoded by a testis-specific FER transcript [11,24]. This kinase accumulates in the nucleus of meiotic primary spermatocytes [11,20].

p51^{ferT} and p94^{fer} differ in their N-termini but they do share common SH2 and kinase domains [11,19]. The FER kinase domain [19,27] is 70% and 50% homologous to the kinase domains of two other known nuclear and cytoplasmic tyrosine kinases, c-Fes [14,31,38,39] and c-Abl [34], respectively. These four kinases encompass a group of the known SH2-containing mammalian nuclear tyrosine kinases, which are not src-related [34,38,39]. While the cellular role of the nuclear c-Fes is not known, the cytoplasmic fraction of that enzyme was shown to promote proliferation of vascular endothelial cells [13] and has been implicated in the signal transduction elicited by cytokines in hematopoietic cells [17,21]. The nuclear function of c-Abl has been linked to cellular response to genotoxic stress [40,41], and has been implicated in potentiating the activity of RNA polymerase II in S phase [37].

The cytoplasmic fraction of p94^{fer} interacts with the catenin-like substrate pp120 in fibroblastic cells [25] and its nuclear fraction was found to associate with the cell chromatin [18]. The kinase activity of p94^{fer} is elevated in growth factor-stimulated fibroblastic cell lines [25]. However, no direct link of p94^{fer} to regulation of cell growth has been shown, nor has a substrate for p94^{fer} or p51^{ferT} been characterized, thus leaving the cellular role of p94^{fer} and p51^{ferT} elusive.

Ectopic expression of p94^{fer} and p51^{ferT} led to tyrosine phosphorylation of 66 kDa and 120 kDa proteins in CHO cells [6]. These proteins may mediate the cellular functions of the FER tyrosine kinases [6].

In order to further the understanding of the cellular functions of the FER proteins, the yeast two-hybrid system has been applied for identification of putative substrates of these enzymes. By using that approach and complementary phosphorylation assays, the TATA element modulatory factor (TMF) was found to serve as a phosphorylation target for both p94^{fer} and p51^{ferT}.

2. Materials and methods

2.1. Point mutagenesis of the FER proteins

Mutations were introduced in the FER proteins using the oligonucleotide-directed mutagenesis without phenotypic selection procedure [2], with modifications as follows.

Mutagenized fragments were cloned in pBluescript (KS$^+$) and the R408 M13 strain (Promega) was used as a helper phage for preparation of single strand DNA stocks.

Gly-571 was changed to arginine in p94^{fer}, by using the oligonucleotide -gaattactgCgcaaggga- in which the marked cytosine replaced the original guanine in the fer cDNA, thus creating a new arginine encoding triplet -cgc [11]. The modified oligonucleotide was extended with T7 DNA polymerase and HB101 cells were then transformed with the double strand DNA.

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2.2. Construction of plasmids

2.2.1. Two-hybrid plasmids. Native and mutated fer or ferT cDNAs were flanked by *Bam*HI linkers and were then inserted in the *Bam*HI site of the pBTM116 vector [3]. In these plasmids, which served as 'bait' expression vectors in the two-hybrid screen, the FER proteins were produced as LexA fusion proteins.

The mouse testes cDNA library, screened in this work, was cloned in the pGAD10 plasmid from which the cDNAs were expressed as GAL4 activating domain fusion proteins (Clontech).

2.2.2. Maltose binding protein (MBP) expression plasmids. Fer and ferT cDNAs flanked by *Bam*HI linkers were inserted into a *Bam*HI site located at the 3′ end of the *malE* gene, in the pMAL-c2 vector. Inserting the FER cDNAs in the same translation frame as *malE* allowed the production of MBP-FER fusion proteins from the pMAL-c2 vector (New England BioLabs Inc.)

2.2.3. Glutathione S-transferase (GST) expression plasmids. D_1TMF encoding a mouse TMF fragment that extends from Asp-381 to Ile-1089 (Fig. 2A) was cut out of the pGAD10 expression vector with *Bam*HI and *Eco*RV. This fragment was then inserted into a *Bam*HI-*Eco*RI cut pGEX2 plasmid [33] in which the *Eco*RI end was filled in with the Klenow DNA polymerase fragment.

D_2TMF which encodes Asp-381 to Asp-864 in the mouse TMF (Fig. 2A) was obtained by cutting the D_1TMF fragment with *Asp*718 and filling in with Klenow fragment. This fragment was then inserted into a linear pGEX2 plasmid which harbored a cohesive *Bam*HI site and a filled in *Eco*RI site, at its ends.

The D_3TMF fragment encoding Lys-637 to Ile-1089 in the mouse TMF was obtained by cutting the D_1TMF fragment with *Bgl*II and *Sph*I. The *Sph*I end was turned blunt with T4 DNA polymerase and the obtained fragment was inserted into a pGEX2 vector harboring *Bam*HI and filled in *Eco*RI site at its ends.

2.2.4. pcDNA3 expression plasmids. The TMF cDNA fused to an influenza virus hemagglutinin epitope (HA) at its 5′ end (HA-TMF) was inserted between the *Bam*HI and *Not*I sites in the pcDNA3 expression vector (Invitrogen). In that vector, which carries the SV40 origin of replication, the TMF cDNA is transcribed under the control of the human cytomegalovirus promoter. This plasmid was termed pcDNA3TMF.

2.2.5. pECE expression plasmids. The native and mutated $p94^{fer}$ cDNAs were inserted between the *Hin*dIII and *Eco*RI sites in the pECE expression vector, [8]. In that vector, which also carries the SV40 origin of replication, the $p94^{fer}$ cDNA is transcribed under the control of the SV40 early promoter [8]. The plasmid that carries the native $p94^{fer}$ cDNA was termed pECEfer and the one carrying the mutated fer cDNA (Gly-571 to Arg) was termed $pECEfer^{571}$.

2.3. Yeast two-hybrid screening

$p94^{fer}$ and $p51^{ferT}$ pBTM116 expression plasmids were introduced into CTY10-5d yeast strain which harbors two LexA operators in a UAS-less promoter upstream of a *lacZ* reporter gene [3,4]. Western analysis using α-FER antibodies [20] confirmed high levels of expression of the LexA-FER fusion proteins (data not shown). 0.5×10^6 cDNA clones from a mouse testes library (Clontech), fused to the GAL4 activating domain in the pGAD10 vector [3,4], were then transfected to CTY10-5d yeast cells that expressed a LexA-FER fusion protein. Seven positive clones drove LexA-FER-dependent expression of the *lacZ* gene. These were isolated and further characterized. Inserts were cut out of the pBTM116 vector with *Sal*I and were recloned into the *Sal*I site of the pBluescript plasmid. DNA sequencing of the cloned inserts was performed by the Biological Services of the Weizmann Institute of Science using an Applied BioSystem 373 DNA Sequencer and dideoxy Taq terminators. The obtained sequences were compared against the GenBank/EMBL sequences databases.

2.4. Production of MBP fusion proteins in Escherichia coli cells

E. coli BL21 cells transformed with MBP expression plasmids were exposed to 0.1 mM IPTG for 20 min and were then sonicated in MBP buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 4 mM EDTA and 10 mM β-mercaptoethanol) to which protease inhibitor mixture (20 mg/ml Pefabloc, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 20 μg/ml benzamidine) was added.

The protein extracts were cleared by centrifugation for 30 min at $13000\times g$. Supernatants were loaded on an amylose column [5] that was then washed with MBP buffer. MBP fusion proteins were eluted and stored in MBP buffer containing 1 mM EDTA, 10 mM maltose and 10% glycerol at 4°C.

2.5. Production of GST fusion proteins in E. coli cells

E. coli BL21 cells transformed with GST expression plasmid were treated with 0.1 mM IPTG for 20 min and sonicated in GST buffer (Tris-HCl pH 7.5, 4 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol) and mixture of protease inhibitors. Cleared lysates were loaded on glutathione agarose beads [33] which were then washed with cold GST buffer containing 1% Triton X-100 and then with cold GST buffer alone. GST fusion proteins were stored on the glutathione beads in GST buffer containing 1 mM EDTA, at 4°C.

2.6. In vitro kinase assay

30 μl glutathione agarose beads carrying 1 μg substrate fused to GST were added to 1 μg MBP-FER fusion protein, in 60 μl kinase reaction containing HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM $MgCl_2$, 10 mM $MnCl_2$) and 6 μl [γ-^{32}P]ATP (3000 ci/mmol). The reaction was incubated for 15 min at room temperature. Glutathione agarose beads carrying the phosphorylated substrates were then washed with 150 mM NaCl, 50 mM Tris pH 7.5, 10 mM EDTA and 1% Triton. Washed beads were resuspended in Laemmli buffer, heated for 3 min at 95°C and loaded onto a SDS 8–16% polyacrylamide gradient gel. Proteins were electroblotted onto an Immobilon-P membrane (Millipore), which was dried and exposed to Fuji X-ray film.

2.7. Phosphoamino analysis

A PVDF piece carrying a phosphorylated protein was incubated in 300 μl 6 M HCl for 60 min at 110°C. The supernatant was transferred to an Eppendorf tube, dried and hydrolysis products were redissolved in 20 ml H_2O. 5 μl were loaded on a TLC plate together with 0.5 μg phosphotyrosine, phosphoserine and phosphothreonine standards. First-dimension electrophoresis was carried out for 20 min in 1500 V, with pH 1.9 buffer: 0.58 M formic acid and 1.36 M glacial acetic acid. The second dimension was run for 30 min in 1300 V with pH 3.5 buffer: 0.5 mM EDTA, 0.5% (v/v) pyridine and 0.87 mM glacial acetic acid. The TLC plate was dried for 20 min at 50°C, stained with ninhydrin dye and exposed to Kodak XAR-5 film.

2.8. In vivo kinase assays

The in vivo kinase assay was carried out in transiently transfected COS1 cells. 1.2×10^6 cells were cotransfected with 4.5 μg pECEfer or $pECEfer^{571}$ and 1.5 μg pcDNA3TMF, in the presence of 36 μl lipofectamine (Gibco-BRL). Cells were lysed 40 h later, using lysis buffer containing 3 mM sodium orthovanadate [6]. HA-TMF was immuno-

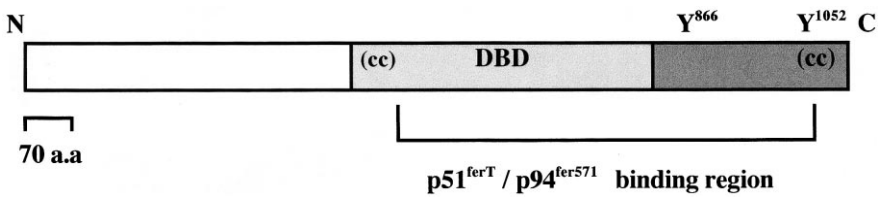

Fig. 1. Structure of TMF. Empty box represents the protein N-terminal portion. Light gray denotes the DNA binding domain (DBD) which contains at its N-terminal portion leucine zippers that can potentially form coiled coil (CC) structures. Dark gray defines the TMF carboxy-terminal tail which includes a potential coiled coil region at its end (cc). The region in TMF which harbors the $p51^{ferT}$/$p94^{fer571}$ (see text) binding sequences and potential tyrosine phosphorylation site in TMF is also shown.

precipitated with anti-hemagglutinin antibodies (Boehringer Mannheim), Western blotted [6] and reacted with monoclonal anti-phosphotyrosine antibodies (pT66, Sigma Immunochemicals).

2.9. Northern blot analysis

Whole cell RNA was extracted using the LiCl/urea precipitation methods, as described ([1], procedure C). RNA was resolved in a 1.2% formaldehyde agarose gel and transferred to a Magna nylon membrane. The filter was hybridized with a randomly primed mouse TMF cDNA (encoding the D_1TMF fragment, Fig. 2A) according to standard procedures [32] and washed in 0.1×SSC and 0.1% SDS at 60°C. The filter was exposed to Kodak XAR-5 film with intensifying screen.

2.10. In situ hybridization

8–10 μm cryosections were hybridized essentially as described before [28]. The sections were mounted on SuperFrost/Plus slides (Menzel Glaser), and refixed with 4% paraformaldehyde. Following prehybridization, the slides were hybridized at 50°C with antisense or sense TMF RNAs. A 1659 bp long TMF fragment encoding the last 553 aa of this protein was used as a probe. Posthybridization washes included treatment with RNase A (50 μg/ml), and stringent washes with 0.1×SSC at 60°C. The slides were dipped into NTB-2 emulsion (Kodak), exposed for 4–7 days (gonads and embryos, respectively), developed, and stained with toluidine blue.

3. Results

3.1. TATA element modulatory factor (TMF) is a putative substrate of the FER tyrosine kinases

To isolate cDNAs encoding potential substrates of $p94^{fer}$ and $p51^{ferT}$, the yeast two-hybrid system has been applied [3,4]. Usage of an enzyme as a 'bait' for detection of enzyme-substrate interactions in that assay may necessitate formation of relatively stable enzyme-substrate complexes. In order to impair the turnover of the FER protein enzymatic activity, mutations were introduced in the ATP binding site of these kinases [7]. These mutations should favor the formation of stable complexes of p94fer or p51ferT with their putative substrates [16]. The native and mutated FER cDNAs were cloned in the yeast pBTM116 expression vector, and the FER kinases were expressed as LexA fused 'baits' in the yeast CTY10-5d cells [3,4]. Of the expressed constructs, the native enzymes and the Gly^{571}-$p94^{fer}$ mutant, in which the first glycine of the GxGxxG ATP consensus binding site [7,11] is mutated to arginine, were most efficiently produced in the transformed yeast cells (data not shown). These proteins were further used as 'baits' in the two hybrid screen. 0.5×10^6 cDNA clones from a mouse testes library, fused to the GAL4 activating domain in the pGAD10 vector, were then transfected to CTY10-5d yeast cells that expressed each one of the LexA-FER enzymes. Seven clones encoding proteins that interact with the FER kinases were isolated and further characterized. Sequencing of these clones revealed that one clone was independently isolated with both native $p51^{ferT}$ and Gly^{571}-$p94^{fer}$ expressing cells. That clone encodes the TMF [12]. TMF is a 123 kDa protein ([12] and Fig. 1) that binds to the TATA element of some RNA polymerase II promoters and represses their activity by competing with the

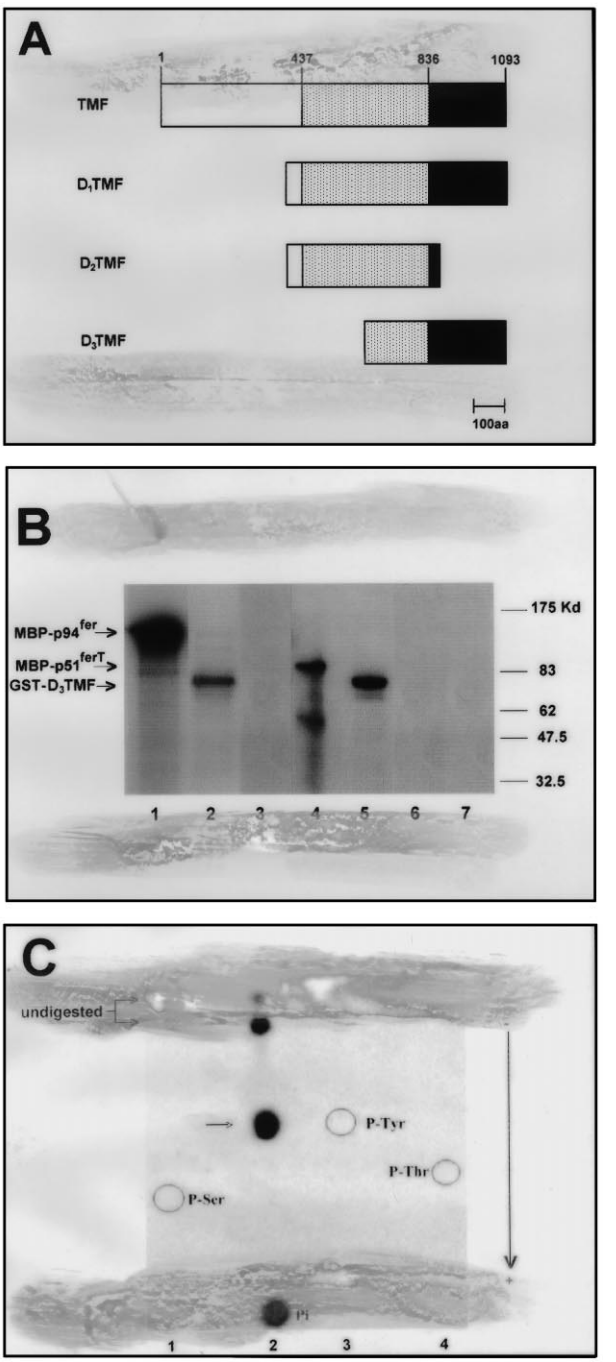

Fig. 2. In vitro phosphorylation of TMF by the FER tyrosine kinases. A: Murine TMF fragments exposed to in vitro phosphorylation assays. Empty box represents the protein N-terminal portion, stippled represents the TMF DNA binding region and black denotes the TMF carboxy-terminal domain. The numbers given in the TMF scheme correspond to the aa numbers in the human TMF [12]. B: MBP-$p94^{fer}$ (lane 1), MBP-$p94^{fer}$ with GST-D_3TMF (lane 2), $p94^{fer}$ with GST (lane 3), MBP-$p51^{ferT}$ (lane 4), MBP-$p51^{ferT}$ with GST-D_3TMF (lane 5), MBP- MBP-$p51^{ferT}$ with GST (lane 6) and GST-D_3TMF alone (lane 7) were incubated in in vitro kinase assays. The enzymes were washed away from the glutathione agarose beads at the end of the reactions presented in lanes 2, 3, 5 and 6. Phosphorylated proteins were resolved in PAGE and transferred to PVDF membrane which was exposed to Kodak XAR-5 film. Arrows on the left indicate migration distances of enzymes and substrates. Migration distances of known molecular weight markers are shown on the right. C: Phosphoamino analysis of in vitro phosphorylation products. Products from lane 5 in B were exposed to phosphoamino analysis. Locations of unlabeled, known phosphorylated amino acids are shown, phosphoserine (lane 1), phosphotyrosine (lane 3), phosphothreonine (lane 4). Degradation products of in vitro phosphorylated protein were loaded in lane 2. Arrow indicates labeled phosphotyrosine.

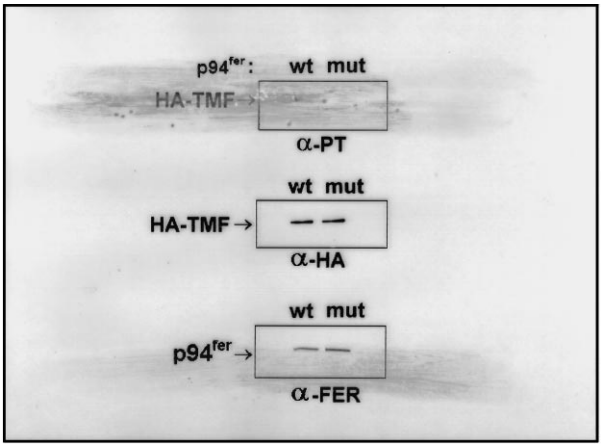

Fig. 3. In vivo phosphorylation of TMF by p94^{fer}. Hemagglutinin-tagged TMF (HA-TMF) was transiently coexpressed in COS1 cells together with native (wt) or Gly-571 mutated p94^{fer} (mut). HA-TMF was immunoprecipitated with anti-hemagglutinin antibodies, Western blotted and reacted with anti-phosphotyrosine (upper panel) or anti-hemagglutinin antibodies (middle panel). p94^{fer} was detected with anti-FER antibodies (lower panel).

binding of TATA binding protein (TBP), to the TATA element of these promoters [12]. TMF binds most efficiently to nucleotides −46 to −10 of the HIV TATA element [12]. Deletion mapping analysis revealed that Gly^{571}-p94^{fer} and the native p51^{ferT} bind to sequences within a 542 amino acids long region in the murine TMF (Fig. 1). This fragment, which extends from Asp-540 to Ile-1089 in the mouse TMF, contains the DNA binding and carboxy-terminal domains of that factor (Fig. 1). Wild type p94^{fer} did not bind to TMF in that two-hybrid assay (data not shown). This may reflect the transient interaction between p94^{fer} and TMF which most probably results from the autophosphorylation and self-activation of p94^{fer} but not p51^{ferT} in the expressing yeast cells (data not shown).

To test whether TMF can serve as a substrate for the p94^{fer} and p51^{ferT} kinases, a TMF fragment extending from Lys-637 to Ile-1089 (D_3TMF in Fig. 2A) was produced as a GST fusion protein [33] in bacterial cells. The GST-D_3TMF fusion protein (Fig. 2A) was incubated in the presence of bacterially produced MBP-p94^{fer} and MBP-p51^{ferT} fusion proteins [5], in an in vitro kinase assay [19]. Bacterially produced MBP-p51^{ferT} and MBP-p94^{fer} fusion proteins retained their enzymatic activities and exerted tyrosine autophosphorylation activity in that assay (Fig. 2B, lanes 1 and 4, respectively). MBP-p94^{fer} and p51^{ferT} also phosphorylated the GST-D_3TMF fusion protein (Fig. 2B, lanes 2 and 5, respectively) but they did not phosphorylate the GST protein itself (Fig. 2B, lanes 3 and 6). As expected, the GST-D_3TMF fusion protein did not exhibit any autophosphorylation activity (Fig. 2B, lane 7), thus attributing its phosphorylation in the in vitro kinase reaction to the kinase activities of p94^{fer} and p51^{ferT}.

Phosphoamino analysis confirmed that the phosphorylation activity exerted by the FER kinases in these in vitro assays led to the accumulation of tyrosine rather than serine or threonine phosphorylation products (Fig. 2C).

To test the tyrosine phosphorylation of TMF by p94^{fer} in vivo, a transient cotransfection expression kinase assay was established. An intact TMF cDNA linked to an influenza virus HA epitope at its 5′ end (HA-TMF) was inserted into the pcDNA3 expression vector. This vector (pcDNA3TMF) was cotransfected into COS1 cells together with a pECE vector which carries the native (pECEfer) or mutated (pECEfer^{571}) p94^{fer} cDNA.

Ectopically expressed TMF was immunoprecipitated from COS1 extracts 40 h after transfection, Western blotted and reacted with anti-phosphotyrosine monoclonal antibodies. Ectopic coexpression of native TMF and p94^{fer} led to tyrosine phosphorylation of TMF (Fig. 3). However, TMF was not phosphorylated when coexpressed with an inactive p94^{fer} (pECEfer^{517}) (Fig. 3), thus linking the tyrosine phosphorylation of TMF in that assay to the exogenous p94^{fer} kinase activity.

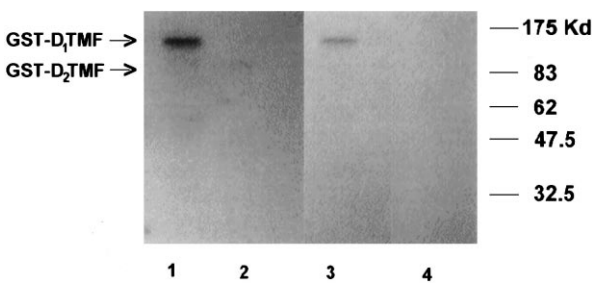

Fig. 4. In vitro phosphorylation of the carboxy-terminal domain of TMF. MBP-p94^{fer} with GST-D_1TMF (see Fig. 2A) (lane 1), MBP-p94^{fer} with GST-D_2TMF (see Fig. 2A) (lane 2), MBP-p51^{ferT} with GST-D_1TMF (lane 3) and MBP-p51^{ferT} with GST-D_2TMF (lane 4) were incubated in in vitro kinase assays. Phosphorylation products were resolved in PAGE, electroblotted onto PVDF membrane that was exposed to Kodak XAR-5 film. Arrows on the left indicate migration distances of putative substrates. Migration distances of known molecular weight markers are shown on the right side.

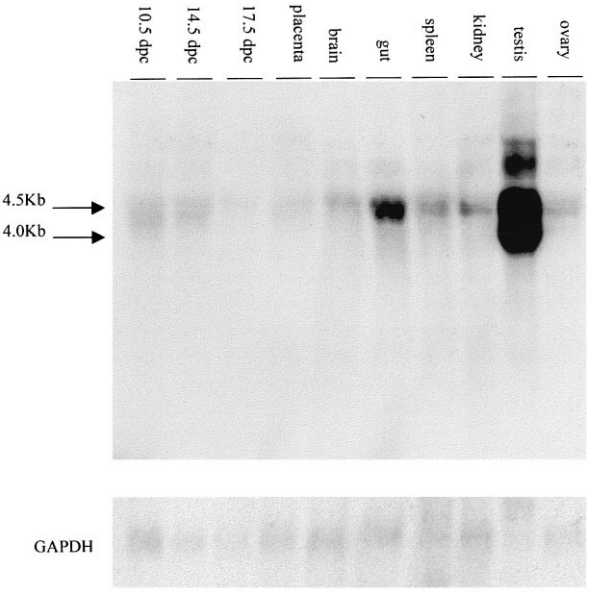

Fig. 5. Northern blot analysis of TMF expression. Whole cell RNA was extracted from 10.5 day post coitus (dpc) mouse embryos, 14.5 dpc, 17.5 dpc mouse placenta, brain of 45 day old mice, gut, spleen, kidney, testis and ovary. Samples were fractionated in a 1.2% formaldehyde agarose gel, transferred to Magna nylon membrane (MSI) which was then hybridized with a mouse TMF probe and was exposed to Kodak XAR-5 film. Arrows on the left indicate migration distances of the TMF transcripts. Longer exposure revealed the presence of the 4 and 4.5 kb transcripts in all tissues analyzed (data not shown).

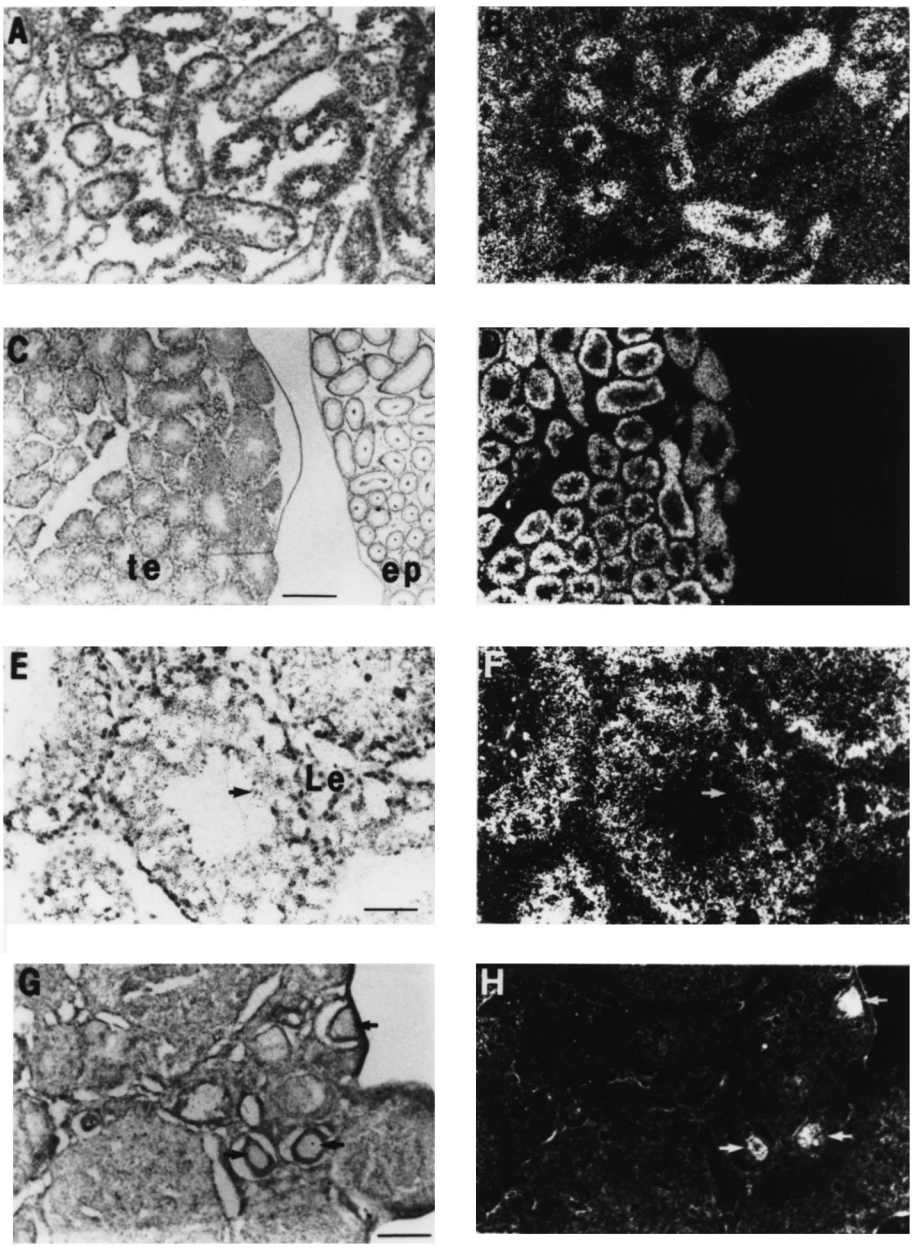

Fig. 6. TMF localization in the adult gonads. Bright (A) and dark (B) field view of a section of 17 day postnatal testis hybridized to TMF antisense probe. About half of the tubules express high levels of TMF, while the others express much lower levels. Bright (C) and dark (D) field view of adult testis (te) and epididymis (ep). All seminiferous tubules express very high levels of TMF, while no significant signal was observed in the spermatozoa stored in the epididymis. Bright (E) and dark (F) field enlargements of the adult testis. Hybridization signal is seen over the developing spermatocytes, but not over elongated spermatids (arrow), or interstitial Leydig cells (Le). Bright (G) and dark (H) field view of adult ovary. TMF RNA is localized in growing oocytes (marked by arrows). Bar = 100 μm (A, B, G and H), 250 μm (C and D), 50 μm (E and F).

3.2. The C-terminal region of TMF is essential for phosphorylation by the FER proteins

The FER tyrosine kinases bind to a 542 aa long fragment in TMF that contains the DNA binding domain and the carboxy-terminal tail of that protein (Fig. 1). This suggests the phosphorylation of one of these subregions by the FER tyrosine kinases. In order to find which part of TMF is phosphorylated by the FER enzymes, two TMF subfragments were exposed to the phosphorylation activity of these enzymes. One fragment contained the DNA binding and carboxy-terminal domains of TMF (D_1TMF, Fig. 2A), while the other included only the DNA binding domain (D_2TMF, Fig. 2A). These TMF segments were produced in *E. coli* as GST fusion proteins and equal amounts of them were incubated with MBP-$p94^{fer}$, in an in vitro kinase assay. Incubation of the GST-TMF-DNA binding domain (D_2TMF, Fig. 2A) with MBP-$p94^{fer}$ fusion protein did not lead to phosphorylation of this TMF subfragment (Fig. 4, lanes 2 and 4). The same result was obtained when the N-terminal portion of TMF (Fig. 1) was attached to the DNA binding domain (data not shown). Linking the TMF carboxy-terminal tail to the DNA binding domain of that factor (D_1TMF, Fig. 2A) restored the phosphorylation of TMF by $p94^{fer}$ and $p51^{ferT}$ (Fig. 4B, lanes 1 and 3, respectively). The C-terminal region of

TMF is thus essential for the phosphorylation of that protein by $p94^{fer}$.

3.3. Preferential expression of TMF in germ cells

The data presented above suggested a functional link between the FER proteins and TMF. Since the expression of $p51^{ferT}$ is restricted to spermatogenic cells [11,20] and $p94^{fer}$ is expressed in most mammalian cells [10,15,19,27] we turned to check whether TMF patterns of expression overlap with those of the FER RNAs. RNAs were extracted from various mouse tissues and analyzed by Northern blot hybridization using a TMF labeled probe. Two discrete TMF transcripts of 4.5 and 4 kb [12] were detected in all tissues analyzed (Fig. 5). These transcripts accumulated, however, to remarkably high levels in mouse testis (Fig. 5). The identity of the cells expressing TMF within the gonads was assessed by in situ RNA hybridization. Sections of developing prepuberal testis and of adult testis were examined. At 7 and 12 days postnatally, germ cells expressed moderate levels of TMF (not shown). By 17 days, about half of the tubules expressed high levels in the germ cell compartment, while the other half retained the moderate levels observed in earlier stages (Fig. 6A,B). 22-day (not shown) and adult testes (Fig. 6C,D) exhibited high levels in all tubules. Higher magnification indicated that germ cells from around mid-pachytene to round spermatid stage [23] expressed high levels of TMF (Fig. 6E,F, and not shown). Elongated spermatids (Fig. 6E,F) and spermatozoa stored in the epididymis (Fig. 6C,D) expressed only low levels. No expression could be seen in Sertoli or interstitial Leydig cells (Fig. 6E,F). Analogously, in the adult ovary, growing secondary oocytes expressed much higher levels than the surrounding granulosa or interstitial cells (Fig. 6G,H).

4. Discussion

$p94^{fer}$ and $p51^{ferT}$ belong to a group of nuclear tyrosine kinases whose cellular functions are not well understood. Identifying the substrates of these kinases should further the understanding of their cellular roles. In the present work, the transcription modulator, TMF, was identified as a putative substrate of the FER tyrosine kinases. This protein was shown to bind to the TATA element of RNA polymerase II promoters and consequently repressed their activity by competing the binding of TBP [12]. As was shown for various transcription factors, phosphorylation of TMF by the FER tyrosine kinases might modulate its activity. The induction of the $p94^{fer}$ phosphorylation activity upon stimulation of cells with growth factors [25] linked the functioning of $p94^{fer}$ to cell growth processes. $p94^{fer}$ may thus modulate the suppressive activity of TMF in somatic cell growth and differentiation processes while $p51^{ferT}$ may exert this function during meiosis. TMF is thus the first mammalian suppressor of transcription whose function might be affected by its tyrosine phosphorylation.

How do the FER tyrosine kinases modulate the suppressive activity of TMF?

Deletion mapping analysis indicated that the FER phosphorylation targets, reside within the carboxy-terminus of TMF (Figs. 5 and 1). Computer analysis using the PROSITE application revealed the existence of two potential tyrosine phosphorylation sites in TMF. One site, $KLEDENNRY^{866}$, extends from Lys-858 to Tyr-866, and the other, $RDLDQRY^{1052}$, lies between Arg-1046 and Tyr-1052. These two sites, which are highly conserved in the mouse and human TMF proteins (Schwartz and Nir, unpublished data), lie within the carboxy-terminal region of TMF (Fig. 1). The putative Tyr-1052 phosphorylation site resides within a potential coiled coil region which composes the carboxy-terminal end of TMF [12]. $p94^{fer}$ and $p51^{ferT}$ could thus modulate the functioning of TMF by affecting its oligomerization either with itself or with other cellular protein(s). This may affect the DNA binding activity or the subcellular distribution profile of TMF during cell growth and differentiation processes. Interestingly, another nuclear tyrosine kinase, c-Abl, was shown to potentiate the activity of RNA polymerase II during S phase progression in mammalian cells [37]. Nuclear tyrosine kinases may thus exert at least part of their regulatory functions by modulating the transcription machinery in mammalian cells.

TMF is a ubiquitously expressed gene. As was shown for the human TMF gene [12], two transcripts of 4 and 4.5 kb were found in all tissues examined (Fig. 5). These transcripts accumulate, however, to high levels in murine testis (Fig. 5). In situ localization revealed that, within the gonads, TMF transcripts are preferentially expressed in meiotic cells (Fig. 6), In the testis from around mid-pachytene stage to round spermatids, and in the ovary from secondary oocyte stage until, at least, ovulation. Further characterization of the two TMF transcripts should reveal whether they encode different proteins, and whether they accumulate at different levels in the spermatogenic and oogenic cells.

The relatively high expression levels of TMF RNA in both spermatogenic and oogenic cells suggest a functional role of TMF protein during meiosis. Interestingly, the testis-specific form of the FER kinases, $p51^{ferT}$, is highly expressed in pachytene spermatocytes [20,24] which also harbor high levels of the TMF transcripts (Fig. 6). It is thus plausible that $p51^{ferT}$ serves as a modulator of TMF phosphorylation and activity, at defined spermatogenic stages.

Further studies on the modulation of TMF activity by the FER proteins should reveal what roles are played by nuclear tyrosine kinases in control of nuclear suppressive activities during the progression of the mammalian cell cycle and defined differentiation pathways.

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