We recently identified testis-specific nuclear factor binding sites in the testis-specific promoters of the c-mos gene and the Odf1 gene, which are 80% identical. Here we characterize a testis-specific nuclear factor, TTF-D, which is able to complex with both binding sites and stimulates Odf1 promoter activity. TTF-D is detectable in mouse testis as early as day 11 postpartum and contains three peptides of 22, 25, and 35 kDa in size. Surprisingly, TTF-D binds specifically to its cognate double-stranded DNA binding site as well as to its single-stranded DNA binding site. Both double-stranded and single-stranded binding site oligonucleotide DNA can specifically repress Odf1 promoter activity. Our results suggest that TTF-D is involved in positive transcription regulation of a pre-meiotic and a post-meiotic gene in the testis.

Spermatogenesis represents a model system for mammalian development. Within a tightly controlled environment, spermatogenesis undergoes rapid successive divisions to form spermatocytes, which, upon passage through a meiotic phase, enter a differentiation or spermiogenic phase in which spermatids undergo morphological changes to form mature sperm. The processes resulting in the formation of spermatozoa are well defined at the morphological level. However, the molecular signals responsible for tissue- and stage-specific gene expression throughout germ cell development are less well defined. Several genes have been identified that are only expressed in the testis or express a testis-specific variant of a somatic gene (1–5). Using transgenic mice, promoter regions in mouse protamine mP1 (4), mP2 (5), human phosphoglycerate kinase 2 (PGK-2) (6), testis angiotensin-converting enzyme (7), and rat Odf1 (8, 9) genes that confer germ cell-specific transcription onto reporter genes have been delineated. These studies identified transcription factor binding sites crucial for their expression. Although the transcription factors that bind to such sites have not been positively identified for most cases, a small number have been characterized (reviewed in Refs. 10 and 11). The best characterized testis-specific transcription factor is perhaps CREM. Full-length CREM+ acts as a positive regulator of transcription in spermatids (12, 13). Under the control of follicle-stimulating hormone, a switch in the regulation of expression of CREM occurs during spermatogenesis. Negative regulators CREMα, CREMβ, and CREMγ are shut off, and the positive regulator CREMγ is switched on (12, 14). An unstable form of CREM RNA is first expressed in spermatocytes. In spermatids, CREM RNA stability is greatly increased due to the use of an alternative polyadenylation site, and the CREM protein is only produced in these cells (15). Phosphorylation sites on CREM regulate its activity (16). Finally, the appearance of CREM activator protein in spermatids correlates with the transcription activation of Odf1, which contains a CREM binding site (17). Strong positive regulation of Odf1 by CREM was further shown in vitro transcription assays and transfection assays, implicating an important regulatory role for CREM in Odf1 transcription during spermatogenesis (17). The generation of CREM knockout mice (18, 19) further showed that in addition to Odf1, mP1, testis angiotensin-converting enzyme, and TP1 are also regulated by CREM in spermatids. In addition to CREM, several genes have been cloned from testis cDNA libraries that contain motifs found in transcriptional regulatory proteins. Two zinc finger-containing genes have been identified: Zfp-35, which is pachytene spermatocyte-specific (20), and Zfp-29, which is expressed in round spermatids (21). Testis-specific genes encoding high mobility group box motifs have also been cloned. Boissonneault and Lau (22) isolated the testis-specific high mobility group box motif that is predominantly located in elongating spermatids and may be involved in the regulation of gene expression of the haploid male genome. An SHY-related gene that also encodes a high mobility group box designated Sox-5 was isolated (23). Sox-5 is most highly expressed in round spermatids (reviewed in Ref. 24). The function of these putative transcription factors in spermatogenesis and the promoters that they interact with remain to be determined. Putative transcription factors were also identified based on specific protein-DNA interactions. Factors present in testis extracts have been shown to interact specifically with cis-acting elements in the promoters for several genes including mP1 (25), testis-specific histone H1t (26), and PGK-2 (27, 28). The appearance of a 13-kDa protein and a 30-kDa protein, which bind the H1t promoter, coincides with the onset of transcription of the H1t gene (Ref. 29; reviewed in Ref. 30). Testis-specific and promoter-specific regulation of PGK-2 transcription involves the binding of TAP-1, resulting in stimulation of PGK-2 transcription (28). We previously demonstrated that an uncharacterized testis-specific transcription factor, which we called TTF-D, binds to the Odf1 promoter (31). A related binding site was identified in the c-mos promoter (32). Using testis-specific nuclear extracts, we analyzed TTF-D and its pattern of expression in the developing testis, and we investigated the role of TTF-D in Odf1 promoter activity.
TTF-D Regulates Odf1 Transcription

EXPERIMENTAL PROCEDURES

Nuclear Extract Isolation

Nuclei from rat liver and seminiferous tubules (STs) were prepared as described by van der Hoorn and Tarnasky (31). The protein concentrations in the dialyzed nuclear extracts ranged from 9 to 16 mg/ml. Nuclear extracts from mouse testes were prepared as described by Lilienbaum and Paulin (33). Protein concentrations were determined by colorimetric assay (Bio-Rad).

DNA-Protein Interactions

Gel Retardation Assay—Gel retardation assays were performed as described previously (34) using double-stranded (ds) oligonucleotides Odf1D1 (5′-AATTGGCTTTAGG-3′) and c-mosD1 (5′-ATGGACT-3′) labeled with Klenow polymerase. In the indicated experiments, ss oligonucleotides were labeled with polynucleotide kinase. Autoradiography. La Jolla, CA) before separation on native polyacrylamide gels or on linked by UV irradiation using a UV Stratalinker 1800 (Stratagene, Canada), and incubated in blocking buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% skim milk powder) for 1 h at 4 °C. Filters were incubated overnight at 4 °C in binding buffer (25 mM NaCl, 10 mM Na3Citrate, 1 mM EDTA, 1 mM dithiothreitol, and 5% skim milk powder). Reaction mixtures contained 1 ng of labeled oligonucleotide DNA, 1.0 μg of poly(dI-DC)/poly(dI-DC), and extract. The reaction products were separated on 8% nondenaturing polyacrylamide gels and visualized by autoradiography. In the indicated experiments, an excess of unlabelled ds or ss oligonucleotide DNA was used as competitor DNA. Oligonucleotides used in such competition experiments were D2 (5′-AATTACCTTAAACTG-3′), B (5′-AATTGGTGGCCTTGCCA3′-C′), MUT1 (5′-AATTCTCTCAGG-3′), MUT2 (5′-AATTGGACAGGAGG-3′), and MUT3 (5′-AATTGGCCCTTCTAG-3′).

UV Cross-linking Assays—To determine the molecular weight of proteins capable of forming complexes in the gel retardation assays, the radiolabeled oligonucleotide DNA-protein complexes were cross-linked by UV irradiation using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) before separation on native polyacrylamide gels or on complexes were separated by size using SDS-PAGE. Reactions in lanes c and f contained a 50-fold molar excess of unlabeled c-mosD1 and Odf1D1 oligonucleotide DNA, respectively, in addition to the probe and extract. Molecular mass markers and testis-specific complexes (arrows) are indicated.

Southwestern Blotting Assays—Liver and ST nuclear proteins (50 μg) were separated by SDS-PAGE, transferred to NitroPlus nitrocellulose filters (Micron Separations Inc., Fisher Scientific, Nepean, Ontario, Canada), and incubated in blocking buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM diethiothreitol, and 5% skim milk powder) (Becton Dickinson, Cockeysville, MD) for 1 h at 4 °C. Filters were incubated overnight at 4 °C in binding buffer (25 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM EDTA, and 100 μg/ml ss hybridizing sperm DNA). The filters were then probed using 105 cpm/ml Odf1D1 DNA in binding buffer for 6 h at 4 °C. After four 8-min washes in binding buffer at 4 °C, filters were exposed to Kodak XAR film.

In vitro Transcriptions

In vitro transcriptions were performed as described previously by van der Hoorn and Tarnasky (31, 32), using pRT7–0.2C2AT construct obtained with the c-mosD1 and Odf1D1 oligonucleotides are identical (the Odf1D1 results are shown in Fig. 2). Somatic cells contain 38-, 30-, and 32-kDa nuclear factors capable of binding the TTF-D oligonucleotides (Fig. 2, lanes a and b). Male germ cells specifically express 35-, 25-, and 22-kDa-binding peptides (lanes c and d, arrows), in close agreement with our UV cross-linking results.

Developmental Expression of TTF-D—We predicted that if TTF-D binding to the c-mos and Odf1 promoters has physiological significance, then TTF-D must be present in cells that express c-mos and Odf1. The c-mos testis-specific promoter is active in pachytene spermatocytes and round spermatids, and the Odf1 promoter is active in spermatids. In the mouse, pachytene spermatocytes are produced for the first time at days 12–14 after birth, whereas round spermatids are not produced until days 21–22 after birth. To determine whether TTF-D is present in mouse testis at the time of expression of the c-mos and Odf1 genes, nuclear extracts were prepared from total testes of 11-, 16-, 21-, and 25-day-old mice and an adult mouse. These extracts were analyzed for TTF-D activity in gel retardation assays. The results shown in Fig. 3A demonstrate that TTF-D activity (indicated by the asterisks) can be detected as early as 11 days after birth (lane a). TTF-D activity is detectible into adulthood (lane e) when a full complement of germ cells is established. The 38-kDa somatic protein that binds the oligonucleotide is present in all total testis extracts (lanes a–e), as expected. These experiments show that TTF-D is present at the time of first expression of both the c-mos and Odf1 genes. The increased TTF-D signal likely results from the increased percentage of male germ cells during postnatal development from day 11 onward: control gel retardation assays using SP1 oligonucleotides demonstrated no change in the...
FIG. 2. Size determination of TTF-D DNA binding components. Different isolates of liver nuclear proteins (lanes a and b) and ST proteins (lanes c and d) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with radiolabeled Odf1D1 oligonucleotide DNA. Arrows indicate the peptides present in ST nuclear extracts but not in liver nuclear extracts.

FIG. 3. TTF-D activity during testis development. A, gel retardation assays of total mouse testis nuclear extracts isolated 11, 16, 21, and 25 days after birth (lanes a–d) and adult mouse testis nuclear extract (lane e) using radiolabeled Odf1D2 oligonucleotide DNA. TTF-D DNA complexes are indicated by asterisks. s, the retarded complex generated by the somatic nuclear protein. B, nuclear proteins were isolated from pachytene spermatocytes (lanes f and g) and round spermatids (lanes h and i) purified by centrifugal elutriation and from total male germ cells (lane j) and analyzed by gel retardation using the c-mosD1 oligonucleotide probe. The reactions shown in lanes f and h contained 0.5 μg of protein, the reactions shown in lanes g and i contained 5 μg of protein, and 25 μg of protein were used in lane j. The TTF-D complex is indicated by an arrow.

amount of complex formed (data not shown). To further document the expression of TTF-D in male germ cells before and after meiosis, we performed gel retardation assays using c-mosD1 oligonucleotide as a probe and nuclear proteins isolated from elutriated pachytene spermatocytes and round spermatids. The results (Fig. 3B) show that TTF-D-binding activity is detectable in these germ cell types (arrow, lanes g and i, respectively) and that the pattern is similar to that seen using ST extract (lane j).

TTF-D Acts as a Weak Positive Transcription Factor—Our previous in vitro transcription studies have shown that the Odf1 promoter region harboring the TTF-D and CREM sites acts as a testis-specific cis-acting element (31). Transfection experiments and analysis of CREM
transient knockout mice have established CREM as the major regulator of the Odf1 promoter (13, 19). The testis-specific c-mos promoter lacks a CREM binding site but shares a TTF-D site with the Odf1 promoter. It is weak compared with the Odf1 promoter. To demonstrate a role for TTF-D in promoter activation, we analyzed promoter activity in in vitro transcription assays, using ST nuclear extracts with and without an excess of Odf1D1, c-mosD1, and other indicated oligonucleotides as competitors for TTF-D binding. pAdMLP was included in all assays as an internal control. The addition of excess ds Odf1D1 oligonucleotide and ds c-mosD1 oligonucleotide resulted in a reduction of the activity of the Odf1 promoter by approximately 30% (Fig. 4, lanes b and d, respectively) in comparison with a competitor oligonucleotide (B oligonucleotide) that had no effect on Odf1 promoter activity (lane e). Interestingly, a related oligonucleotide (D2) also repressed the activity of the Odf1 promoter in vitro (lane e). None of the competitor oligonucleotide DNAs used affected the activity of the adenovirus promoter (compare lane a and lanes b–e). We conclude from these assays that TTF-D acts as a testis-specific, positive transcription factor.

TTF-D Specifically Interacts with ss Binding Site Oligonucleotides—In the course of the above-described gel retardation experiments, we observed in preliminary experiments that TTF-D might bind to ss DNA representing its binding site. Therefore, we performed the following studies to determine the specificity of such an interaction, and we asked whether the same TTF-D peptides could bind to ss DNA binding sites. We used ST nuclear proteins and sense and antisense Odf1D1 binding site oligonucleotides in gel retardation assays. In indicated experiments, we included an excess of ss competitor oligonucleotide. The results for the sense and antisense oligonucleotide were essentially identical: those for sense Odf1D1 ss DNA are shown in Fig. 5A. The results demonstrate that TTF-D binds efficiently to ss oligonucleotide (lane a, asterisks) and that this binding cannot be competed by a large excess of unrelated ss oligonucleotide (B-sense oligonucleotide and B-antisense oligonucleotide, lanes g and h, respectively). Binding to sense oligonucleotide is efficiently competed by ds Odf1D1 oligonucleotide and by sense and antisense self-oligonucleotide (lanes b–d, respectively). Competition was slightly less efficient with ss c-mosD1 oligonucleotides (lanes e and f).

UV cross-linking was used to ascertain whether the same or different TTF-D peptides are involved in the binding to ds and ss DNA. The results are shown in Fig. 5B and indicate that Odf1D1 (lanes i–k) and c-mosD1 (lanes l–n) sense and antisense oligonucleotides bind the same peptides compared with each other and with ds oligonucleotide. In this experiment, the 25-kDa TTF-D component appears resolved in two bands, a result that was not always evident. The increased intensities of the ssDNA-TTF-D complexes (lanes j, k, and n) could result from a greater affinity of TTF-D for those particular ss DNA cognate sites compared with the corresponding ds DNA binding sites. To further define the specificity of binding, we designed mutant Odf1D1 oligonucleotides called MUT1, MUT2, and MUT3 in which the first two, the middle three, or the last three nucleotides of the binding site were changed, respectively (see “Experimental Procedures”). Gel retardation analysis using these mutant oligonucleotides as competitors for DNA (Fig. 6A) shows that MUT1 and MUT3 oligonucleotides competed for binding of TTF-D to ss Odf1D1 oligonucleotide (lanes d and f, asterisks), although with reduced efficiency compared with self-oligonucleotide (lane c). MUT2 oligonucleotide did not compete (lane c), nor did the unrelated B oligonucleotide (lane a). Preliminary UV cross-linking experiments showed that the same peptides bind to Odf1D1, MUT1, and MUT3 DNA, but not to MUT2 DNA (data not shown). Thus, mutation of the central
Somatic 38-kDa Protein—The assays described above demonstrate that a 38-kDa somatic, nuclear protein can interact with the TTF-D binding site. We used ds wild type and mutant DNAs to gather further information on the nucleotides involved. Oligonucleotide DNA was incubated with liver nuclear extract, and the complexes were analyzed in the gel retardation assays shown in Fig. 7. The complex between the 38-kDa somatic protein and Odf1D1 ds DNA (s, lane a) can be competed by self-oligonucleotide (lane b) and c-mosD1 oligonucleotide (lane c) but cannot be significantly competed by D2 oligonucleotide (lane d). Because D2 and Odf1D1 only share sequences in the middle region, this shows that either the 5′ or 3′ end of the Odf1D1 oligonucleotide binds the somatic protein. We therefore analyzed whether the mutant oligonucleotides can complex with the somatic protein. The results (Fig. 7) indicate that the 3′ nucleotides in the Odf1D1 sequence are essential for binding, which is abolished by their mutation (lane g). Reduced binding was observed for MUT2 (lane f), suggesting that nucleotides in the middle of the Odf1D1 sequence contribute to the affinity. These results suggest that the binding site for TTF-D and the somatic 38-kDa protein overlap.

**DISCUSSION**

Transcriptional regulation of genes plays a key role in their tissue- and cell-specific expression. In the testis, with the exception of CREM, little is known regarding transcription factors that play a role in the positive or negative regulation of testis-specific genes (10, 11). We have recently shown that CREM is a regulator of transcription of the post-meiotic testis-specific gene Odf1 (17). We had previously identified a second putative regulator of Odf1 transcription that we called TTF-D (31). The sequence of the TTF-D binding site was deduced from DNase I footprinting assays and gel retardation experiments and is called Odf1D1 (31). In the c-mos promoter, an element that is 80% identical to the Odf1D1 sequence was identified that binds a testis-specific factor that we postulated could be related to or identical to TTF-D (32). Here we show that TTF-D is a testis-specific transcription factor, which binds to both the Odf1D1 oligonucleotide and the c-mosD1 oligonucleotide. Mutational analysis indicates that the central CTT nucleotides in the 5′-GGGCTTAGGG-3′ TTF-D binding site are essential for binding. The flanking GG and AGG nucleotides, however, increase the affinity of TTF-D for its cognate site. TTF-D contains three testis-specific peptides of 22, 25, and 35 kDa that surprisingly bind to both

The 3′ Half of the TTF-D Binding Site Interacts with the
ds and ss Odf1 and c-mos TTF-D binding site oligonucleotides. We
do not know the relationship between any of these peptides: it is
possible that one is a post-translationally modified version of an-
other one, for instance, by phosphorylation. Importantly, the same
peptides bind to both ds and ss versions of the Odf1 and the c-mos
TTF-D binding sites. TTF-D activation of Odf1 promoter activity in
vitro is reduced by the addition of TTF-D binding site oligonucleo-
tides (both as ds and ss DNA), but not by unrelated or mutant
oligonucleotides.

Our functional data and binding results with TTF-D strongly
suggest that TTF-D plays a role in the transcription of the c-mos and
Odf1 testis-specific promoters. The fact that TTF-D can be
detected as early as day 11 in germ cell development strengthens
such a role for TTF-D in this transcriptional regulation,
because c-mos is first detectable in spermatocytes that
develop 12–14 days after birth in the mouse. The in vitro
transcription assays that we performed previously (31, 32)
suggest that the c-mos promoter is weak in comparison to the
Odf1 promoter. This finding can be explained by the facts that (a)
compared with CREMt, TTF-D is a weak transacting factor, and (b)
it is the only testis-specific factor that binds to the c-mos
promoter detectable by DNase I footprint assays (32). It is more
difficult to interpret a role for TTF-D in the regulation of the
post-meiotic Odf1 promoter. Clearly, TTF-D is present in pre-
meiotic cells that do not express Odf1. One possibility is that
the binding of TTF-D to the Odf1 promoter does happen in
spermatocytes and that this event changes the Odf1 chromatin
structure to prepare the transcriptional activation, because c-
mos can be detected as early as day 11 in germ cell development
in vitro (19, 20).

We performed a chromatin immunoprecipitation experiment
in which the binding of the 38-kDa protein to its site blocks the
expression of Odf1 and/or c-mos in cells that accidentally
topically express TTF-D. Such a model, although unproven in
this case, is attractive because it has been shown to occur in
other systems: one of the mechanisms of action of the Cut
homeodomain repressor protein is its ability to block access of
the transcriptional activator CTF/NF1 protein to its binding
site (42). For this reason, Cut protein had also been named
CDP (CCAT-box displacement factor). The identity of the 38-kDa
somatic protein and its putative role in gene regulation
remain to be determined.

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