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Characterization of the Testis-Specific Transcription Factors TTF-D and CREMtau, which Regulate the RT7 Gene

by

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Abstract

Spermatogenesis involves spatial and temporal changes in cell-specific gene expression. The co-ordination of gene expression is predominantly regulated at the level of transcription. Many promoter regions that direct testis-specific gene expression have been identified, but to date, only one testis-specific transcription factor (CREMτ) has been cloned and fully characterized. CREMτ has been shown to positively regulate the transcription of a number of testis-specific genes including RT7. A novel binding site was recently identified in the promoter of the RT7 gene for a second testis-specific protein, termed TTF-D. A homologous site was also identified in the testis *c-mos* promoter suggesting that TTF-D may play a role in the regulation of both pre-meiotic (*c-mos*) and post-meiotic (RT7) testis-specific genes.

The purpose of experiments described herein was to characterize TTF-D with respect to its binding site in the RT7 gene and its putative binding site in the *c-mos* gene, and to investigate the role of TTF-D in the regulation of RT7 transcription. The interaction of recombinant CREMτ protein with its RT7 binding site was also studied, and the role of CREMτ in the regulation of the mouse protamine 1 promoter investigated.

Three proteins, 36, 25, and 22 kDa in size, were shown to complex with the TTF-D binding site from both the RT7 and *c-mos* promoters. TTF-D activity was detected in mouse testis nuclear extracts as early as day 11 post partum, and in nuclear extracts from purified spermatocytes and spermatids. This

suggests that TTF-D is involved in the transcription regulation of both pre- and post-meiotic genes in the testis.

TTF-D was shown to be a positive regulator of RT7 gene transcription *in vitro*, and it interacts with its binding site in both double and single strand conformations. The central pyrimidine stretch of nucleotides CCTT was demonstrated to be essential for binding. A 40 kDa somatic nuclear protein was also shown to interact specifically with the 3' end of the TTF-D binding site, but the role of this protein in the regulation of RT7 is not known.

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" I Praise you because I am fearfully and wonderfully made;

your works are wonderful, I know that full well."

Psalm 139:14

To my Mom and Dad.

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List of Abbreviations

Units of Measure

cpm	counts per minute
°C	degrees centigrade
g	gram
hr	hour
Kb	kilobase
kDa	kilodalton
mAmp	milliampere
min	minute
ml	millilitre
M	molar
mM	millimolar
mm	millimetre
ng	nanogram
nm	nanometre
OD	optical density
r.p.m.	revolutions per minute
sec	second
Tm	melting temperature
µg	microgram
µl	microlitre
µM	micromolar
xg	times gravitational force

Chemical Compounds/Buffers/Solutions

3-AT	3-aminotriazole
amp	ampicillin
BLOTTO	Bovine Lacto Transfer Technique Optimizer

BSA	bovine serum albumin
CIP	calf intestinal phosphatase
dH_2O	deionized, distilled, sterile water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetatic acid
EGTA	ethylenebis(oxyethylenenitrilo)-tetraacetic acid
EtBr	ethidium bromide
FCS	fetal calf serum
GST	glutathione S transferase
GuHCl	guanidine hydrochloride
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
Klenow	DNA polymerase klenow fragment
LB	Luria-Bertani bacterial medium
NLB	nuclear lysis buffer
OA	okadaic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque forming unit
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
PNK	polynucleotide kinase
SD	synthetic dropout medium
SDS	sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)aminomethane
TBE	Tris-borate EDTA buffer
TBS	Tris buffered saline
TE	Tris EDTA buffer

Nucleic Acids

AdMLP	Adeno major late promoter
ATP	adenosine 5'-triphosphate
CTP	cytosine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dIdC	Poly (dI-dC)•Poly (dI-dC) double strand
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
O-Me-GTP	3'-O-Methylguanosine 5'-Triphosphate
NTP	nucleotide triphosphate
RNasin	RNase inhibitor
tRNA	transfer ribonucleic acid
UTP	uridine 5'-triphosphate

General

αs, as	anti-sense
Ab	antibody
BM	basement membrane
ds	double stranded
EMSA	electrophoretic mobility shift assay
GST-CT	GST-CREMτ
GST-ΔCT	GST-ΔCREMτ
L	liver nuclear extract
Lu	lumen
MW	molecular weight (markers)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RS	round spermatids
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
s	sense

SMS	somatostatin
Sp	spermatocytes
ss	single stranded
ST	seminiferous tubule nuclear extract
UV	ultraviolet

Chapter 1: Introduction

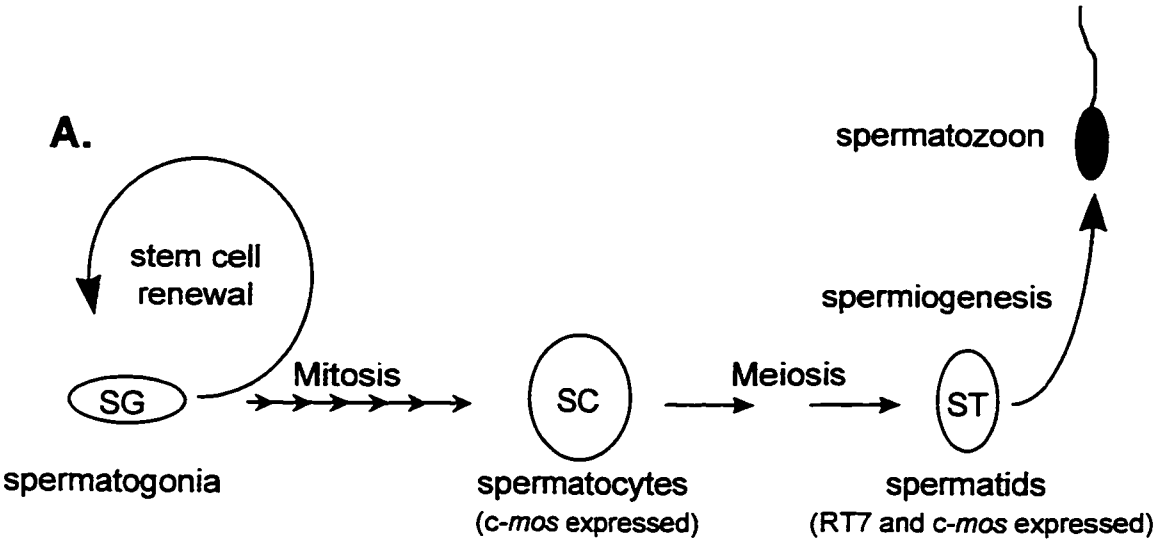
1.1 Spermatogenesis

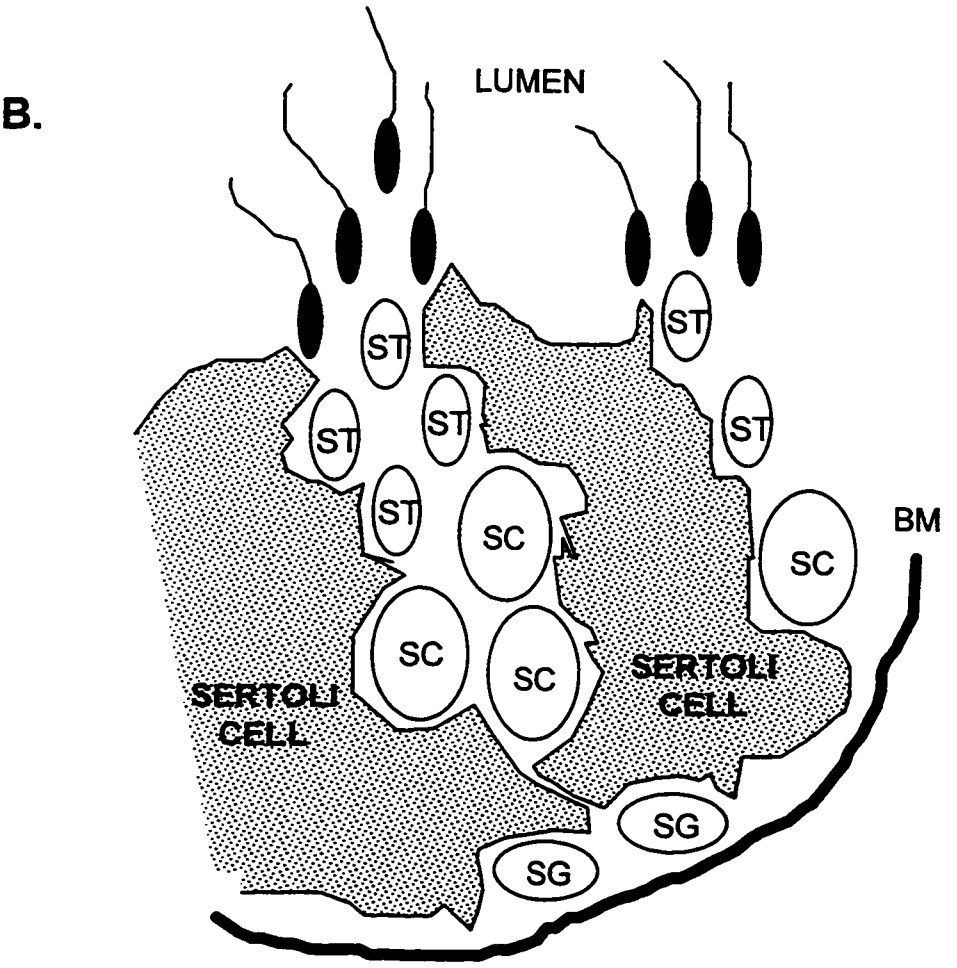
The process of male germ cell development involves a series of events termed spermatogenesis. In mammals, in particular the mouse and rat, the series of events which occur to produce mature spermatozoa has been studied in great detail at the morphological level. The physical characteristics of each cell type in the many stages of spermatogenesis have been analyzed and documented (Russell *et al.* 1990). Spermatogenesis can be divided into three phases, the proliferative phase (spermatogonia), meiotic phase (spermatocytes), and spermiogenic phase (spermatids). In the proliferative phase, Type A spermatogonia can either cycle and remain as stem cells, or they can, through six mitotic divisions, differentiate into Type B spermatogonia which are committed to the spermatogenetic pathway. Type B cells divide to form spermatocytes, which are the cell types defined to be in the meiotic phase. The first meiotic prophase is long (about three weeks), during which stepwise changes occur in the morphology of the spermatocytes. The sizes of the cells and their nuclei progressively increase during prophase, and the nuclear changes are the morphologic basis for the four stages of meiotic prophase. The four stages are termed leptotene, zygotene, pachytene and diplotene. During the pachytene spermatocyte stage, genetic recombination occurs between the paired chromosomes ensuring genetic diversity within the mature sperm. Two meiotic divisions follow to produce haploid spermatids. During the spermiogenic phase, spermatids undergo dramatic morphological changes culminating in the production of mature spermatozoa (Figure 1, Panel A).

Figure 1. Schematic of Spermatogenesis.

Panel A: Male germ cell differentiation program, spermatogonia can divide and remain as stem cells or undergo the differentiation pathway to produce mature spermatozoon.

Panel B: Schematic representation of a section of a seminiferous tubule including the basement membrane (BM), spermatogonia (SG), spermatocytes (SC), and spermatids (ST).


A.
spermatozoon
stem cell
renewal
spermiogenesis
Mitosis
Meiosis
SG
SC
ST
spermatogonia
spermatocytes
(c-mos expressed)
spermatids
(RT7 and c-mos expressed)


B.
LUMEN
ST
SC
BM
SERTOLI
CELL
SG

1.1.1 Spermiogenesis

The process of spermiogenesis is characterized by several events which occur simultaneously in a highly orchestrated and precise manner. Morphologically, in the rat, nineteen distinct stages have been assigned to the overall process which results in a species-specific sperm shape. These events include, development of flagellum, development of an acrosome, nuclear shaping and nuclear condensation, and elimination of cytoplasm. These events are dependent upon gene products that are expressed from the haploid genome. By the middle of spermiogenesis transcription is terminated as the histones are removed from the DNA and replaced with transition proteins (in some species) which are in turn replaced by protamines. The result is a highly condensed genetically inactive genome. Several extensive reviews have been devoted to the properties of protamines and the histone-to-protamine replacement reaction (Balhorn 1989; Hecht 1989; Olivia & Dixon 1991). Although much is known about the overall process of spermiogenesis at the morphological level, very little is known about the genes which play a role in the process at the molecular level.

1.1.2 Regulation of Spermatogenesis

In mice the entire process of spermatogenesis takes 35 days to complete, and is initiated every 12 days. This occurs in a precise coordinated manner in the seminiferous tubules, resulting in specific patterns of tubule organization and cellular associations. The signals that control the process of germ cell development at the cellular level are in part hormonal. Luteinizing hormone (LH)

and follicle-stimulating hormone (FSH) from the pituitary elicit responses from the testicular somatic Leydig cells and Sertoli cells respectively, in turn these cells influence the process of spermatogenesis (Maddocks *et al.* 1990). In the seminiferous tubules, the Sertoli cells extend from the base of the tubule to the lumen making contact with all of the germinal cells. Tight junction complexes between adjacent Sertoli cells separate the tubules into compartments (Figure 1, Panel B). Essentially only secretions from the Sertoli cells and germ cells contribute to the tubule and luminal compartments and disruption of these results in the disruption of spermatogenesis (Griswold 1995). Numerous paracrine (Spiteri-Grech & Nieschlag 1993) and endocrine (McLachlan *et al.* 1996) factors thought to influence the regulation of spermatogenesis have been studied, but the targets of these factors have yet to be fully elucidated. In recent years, the generation of knockout mice has given some broad insight into which genes may be playing an important role in spermatogenesis. The list of genes that, if disrupted, lead to complete or partial blocks at particular stages of spermatogenesis is growing, [reviewed in (Sassone-Corsi 1997)]. The role that these and other genes play at the molecular level however is largely still unknown. The study of genes involved in spermatogenesis is important to increase our knowledge of this process. As well, approaches to an effective male contraceptive, the dramatic rise in male infertility (Carlsen *et al.* 1992) and increases in the incidence of testicular cancer (Carlsen *et al.* 1995) require a basic understanding of molecular biology of the testis.

1.1.3 Spermatogenesis as an Experimental System

Spermatogenesis serves as a unique experimental system due to the functional differences between the male germ cell lineage and somatic cells during embryogenesis. Within this system, gene expression of eukaryotic cells with tetraploid, diploid, and haploid chromosome complements can be compared (Hecht 1990). Study of spermatogenesis at the molecular level has been delayed due to the lack of cell lines or a tissue culture system which can support male germ-cell development. Although progress has been made in this area, culture systems are not easy to manipulate and are not viable over the long term (Weiss *et al.* 1997). The precise spatial-temporal arrangement of cell types in functionally and morphologically distinct stages of development however is very well suited to molecular analysis. Centrifugal elutriation techniques allow for the purification of spermatids, spermatocytes, and spermatogonia in large enough quantities for biochemical and molecular analysis. As well, nuclear extracts from testes at various developmental stages or from mutant mice blocked at different stages of spermatogenesis (Willison & Ashworth 1987), can be utilized. Our lab has refined techniques first described by Gorski *et al.* (1986) to isolate nuclear extracts from rat seminiferous tubules that contain essentially only germ cells (>95%) (van der Hoorn & Tarnasky 1992). This allows us to study nuclear factors that are unique to male germ cells. Identification and cloning of testis-specific genes has been accomplished via subtractive hybridization techniques (Thomas *et al.* 1989) and more recently mRNA differential display (Catalano *et al.* 1997). Knockout mice have identified genes essential to spermatogenesis

(Sassone-Corsi 1997), and finally studies with transgenic mice allow for the delineation of promoter elements which are testis- and stage-specific. From identification of testis-specific promoters, transgenics can be constructed that aberrantly express testis-specific genes in the various germ cells. The consequences of these experiments on germ cell development and gene expression can then be evaluated.

1.2 Testis Specific Genes

Numerous genes have been identified that have unique patterns of expression during spermatogenesis (Wolgemuth & Watrin 1991). These genes can be divided into three major categories based on their expression pattern: 1) genes that are exclusively testis specific, expressed only during spermatogenesis, 2) genes that express a testis-specific variant of the somatic gene, and 3) somatic genes with expression levels that are many fold higher in the testis than in other tissues (Wolgemuth & Watrin 1991). Furthermore, within the stages of germ cell development, there are also different patterns of gene expression. Some genes are only expressed in pre-meiotic germ cells, others are first expressed at low levels in pre-meiotic spermatocytes followed by an increased level of expression in post-meiotic cells (Thomas *et al.* 1989). Finally there are a large number of testis-specific genes that are only expressed from the haploid genome of post-meiotic germ cells (Erickson 1990).

Transcriptional regulation plays a prominent role in the specificity of gene expression in the testis. The promoter regions that confer male germ cell-specific transcription onto reporter genes have been delineated for several testis-

specific genes. The testis-specificity of these genes appears to be regulated by *cis*-acting elements located within or close to their promoters. Control of spermatogenesis therefore intimately involves regulation of gene expression at the level of transcription. Our understanding of testis-specific transcription factors and how they function to regulate spermatogenesis however is limited. Several groups have cloned putative transcription factors which are exclusively expressed during spermatogenesis or are expressed at high levels in the testis compared to somatic tissue expression. The function of these putative transcription factors in spermatogenesis, and the promoters they interact with, remain to be determined. Putative transcription factors have also been identified based on specific protein-DNA interactions. Factors present in testis extracts have been shown to specifically interact with *cis*-acting elements in the promoters of several genes. These promoters and factors interacting with them will be discussed in detail.

Apart from tissue-specific factors regulating transcription, there is evidence that general transcription factors are differentially regulated in male germ cells. TATA-binding protein (TBP) has been shown to be highly over expressed in the testis, specifically in spermatids (Schmidt & Schibler 1995). TBP mRNA levels in early haploid germ cells are estimated to be more than 1000-fold greater than levels in somatic cell types. A molecular analysis of the 5' end of the *t bp* gene has shown that testis-specific *t bp* expression involves a modest up-regulation of the somatic *t bp* promoter and recruitment of at least two other major and three minor promoters (Schmidt *et al.* 1997). Several other non-

transcriptional mechanisms play a role in testis-specific gene expression (Hecht 1990). By mid-spermiogenesis, the haploid genome becomes transcriptionally silent and as a result, post-transcriptional control is especially important. Examples include addition of long poly(A) tracts to repress translation and specific deadenylation to activate translation. The timing of translation events can also be controlled by the 3' untranslated region of the mRNA as was shown for the *Prm 1* gene (Lee *et al.* 1995), and more recently for the *Tcp 10* gene (Ewulonu & Schimenti 1997). Only a few testis-specific genes have been studied in detail. The promoter elements which regulate testis-specific transcription and the factors that bind them are largely uncharacterized.

1.3 Testis Specific Promoters

Transgenic mice have provided a powerful tool for delineation of promoter sequences required to direct testis-specific transcription. In the past few years the promoter elements for several testis-specific genes have been examined using transgenics. These include: proacrosin (Nayernia *et al.* 1994), calmegin (Watanabe *et al.* 1995), Tcp-10bt (Ewulonu *et al.* 1996), c-*kit* (Albanesi *et al.* 1996), and *Hsp*70-2 (Dix *et al.* 1996). These transgenic mice give insight as to the precise temporal and spatial expression of these genes during spermatogenesis. The function of the genes or even the presence of a protein product, however is less clear. DNA binding studies, identification of the cis-acting elements and sequence comparisons to the promoter regions of well characterized testis-specific genes will lead to a greater understanding of the role of these genes in germ cell development. A very small number of testis-specific

gene promoters have been well characterized, both in transgenic work and by extensive promoter studies. These genes include Pdha-2, PGK-2, t-ACE, histone H1t and protamines.

1.3.1 Pdha-2

Pdha-2 is expressed during the meiotic prophase of germ cells, specifically in pachytene spermatocytes. The gene codes for the testis isoform of the mouse E1α subunit of the pyruvate dehydrogenase complex. Pdha-2 has similarities to another testis specific gene, PGK-2. Both genes are intronless, their somatic variants Pdha-1and PGK-1 are X-linked and both are expressed in pachytene spermatocytes (Iannello *et al.* 1994). The promoter for Pdha-2 was first studied by deletion analysis, and the region -187 to +22 was shown to direct transcription of a reporter gene in HeLa cells. DNase I footprinting of this region with testis, brain, and HeLa extracts revealed four regions of protein binding. Two of the sites were shown to be consensus sites for Sp1 and ATF/CREB. The remaining two were unique cis-elements named MEP-2 and MEP-3. A testis-specific factor first present in 2-week old mice was shown to bind MEP-2 and may be important for modulating Pdha-2 expression (Iannello *et al.* 1993). This same promoter region has been shown to direct testis-specific expression of a reporter gene in transgenic mice (Iannello *et al.* 1997). Expression of the Pdha-2 gene from its promoter is also regulated by methylation. A comparison of methylation status and activity of the transgenic or endogenous Pdha-2 promoter in testis, isolated germ cells, and somatic tissues, showed that the promoter was active only in the hypomethylated state. A hypomethylated promoter was seen

in 30 day old mouse testis and pachytene spermatocytes, but not in somatic tissue, 10 day old testis, spermatogonia, or spermatids (Iannello *et al.* 1997). The availability of promoter elements to testis-specific factors therefore may be regulated in part by methylation.

1.3.2 PGK-2

Transcription of PGK in the testis appears to switch from the somatic PGK-1 gene, expressed in spermatogonia until preleptotene spermatocytes, to the testis-specific PGK-2 gene, expressed in pachytene spermatocytes and haploid spermatids (Goto *et al.* 1990). Robinson *et al.* (1989) found that 323 bp 5' of the start site were necessary and sufficient to direct testis-specific expression of the human PGK-2 gene. Promoter elements common to many mammalian gene promoters were located in this region (CAAT box and GC box) but these alone were not sufficient to direct transcription, suggesting that other upstream motifs were essential (Robinson *et al.* 1989). A detailed analysis of the PGK-2 promoter by gel retardation assay identified several factors that may play a role in directing testis-specific transcription (Gebara & McCarrey 1992). Binding activity of four complexes was localized to a 40 bp region immediately upstream of the CAAT box. Two of the complexes were specifically associated with the expressed state of PGK-2 in adult testis nuclear extracts, and a third complex was generated with prepubertal testis nuclear extracts or HeLa cell nuclear extracts in conjunction with the non-expressed state of PGK-2. The fourth complex appeared to be present in all cases (Gebara & McCarrey 1992). The significance of these complexes towards transcriptional control of the PGK-2

gene remains to be determined, but the regulatory process is complex. A cis-element at -82/-64 shown to stimulate transcription in testis extracts binds a testis-specific protein called TAP-1. TAP-1 binds to GGAA which is the Ets-1 oncoprotein binding motif (Goto *et al.* 1993). A similar binding site is also located in the somatic PGK-1 gene. A testis-specific factor, TIN-1 has been shown to bind the PGK-1 site and inhibit transcription (Goto *et al.* 1991). The TIN-1 binding sequence can compete for the TAP-1 binding sequence in gel shift assay and vice versa (Goto *et al.* 1993). Thus a single *cis*-acting sequence appears to bind testis-specific factors that positively (TAP-1) and negatively (TIN-1) regulate the transcription of PGK-2 and PGK-1 genes, respectively. Putative TAP-1 binding sites (9 out of 11 nucleotides) have been reported in the mouse testis-specific cytochrome c gene at -1542 and -950, however protein interactions at these sites were not investigated (Yiu *et al.* 1997). The role of chromatin structure in expression of the PGK-2 gene has also been studied. DNase I-hypersensitive sites in the Pgk-2 gene appeared coincident with, or just prior to, transcriptional activation of this gene in spermatogenic cells (Kumari *et al.* 1996).

1.3.3 t-ACE

The testis specific isozyme of angiotensin-converting enzyme (t-ACE) initiates transcription from a testis-specific promoter within the 12th intron of the somatic ACE gene (Howard *et al.* 1990). The use of a cryptic promoter for tissue specific expression is also used by the c-kit gene which initiates testis-specific transcription from a promoter within the 16th intron of the somatic gene (Albanesi *et al.* 1996). The t-ACE enzyme was localized to elongating spermatids.

Transgenic studies demonstrate that transcription of t-ACE is controlled by a strong intragenic testis-specific promoter within 698 bp upstream from the start site (Langford *et al.* 1991). Further studies defined the minimal promoter region conferring germ cell-specific expression to -91 bp. A key motif identified in this region is a cAMP responsive element (CRE) consensus sequence located at -48/-55, a motif which is conserved within the t-ACE promoters of different species. DNase I footprinting revealed interaction of a testis-specific protein to this region and *in vitro* transcription of the t-ACE promoter could be effectively inhibited by specific competition for this protein with double stranded oligonucleotides (Howard *et al.* 1993). This protein was identified to be the testis-specific transcription factor CREMτ. CREMτ binds to the t-ACE CRE element and acts as a transcriptional activator of the t-ACE promoter (Zhou *et al.* 1996b). A second positive regulatory element at -32 in the t-ACE promoter was determined to be a nonconsensus TATA motif. The element is recognized by TBP, but is not responsible for the germ cell specific expression pattern of t-ACE (Zhou *et al.* 1996a).

1.3.4 Histone H1t

The role of linker histones in binding DNA and contributing to chromatin condensation has been well studied. A testis-specific variant of histone H1, H1t was isolated from rat testis extracts. The protein first appeared in 21 day old rat testis (Seyedin & Kistler 1980). The gene was cloned and found to be expressed only in mid to late pachytene spermatocytes (Cole *et al.* 1986), (Grimes *et al.* 1990; Kremer & Kistler 1991). Although the exact function of H1t is not known, it

binds DNA more weakly than other linker histones and is expressed in cells involved in crossing over events and mismatch repair suggesting a contributory role to these processes. Promoter elements in the H1t gene have been intensely studied via DNA binding and *in vitro* transcription assays. A promoter element, TE, containing the palindromic sequence CCTAGG has been reported to bind testis specific proteins (Grimes *et al.* 1992b). The proteins were present in extracts of adult testis, and extracts enriched for primary spermatocytes, but not in prepubertal testis (Grimes *et al.* 1992a). The size of TE binding proteins has been variously reported to be between 13 kDa and 50 kDa (Clare *et al.* 1997; Grimes *et al.* 1992b). Further study of this region of the H1t promoter expanded the TE element to include two imperfect inverted repeat sequences, TE1 and TE2, both of which bind specifically to nuclear proteins from primary spermatocytes. The TE1 element was shown to crosslink a complex of proteins with a molecular mass of roughly 180 kDa (van Wert *et al.* 1996; Wolfe *et al.* 1995). The discrepancy in the sizes of proteins binding to this region has not yet been resolved. The generation of transgenic mice expressing the rat H1t gene has served to establish the boundaries of the DNA region necessary for developmental and tissue-specific expression. The initial 6.8 Kb fragment of the rat H1t gene which conferred tissue-specific expression (vanWert *et al.* 1995), was truncated to a 1 Kb fragment (-948/+71) which expressed the rat H1t gene and a lacZ fusion construct specifically in spermatocytes and round spermatids (Bartell *et al.* 1996). The importance of transgenic mice to the study of testis specific gene promoters is highlighted by two recent results. An *in vitro*

transcription study showed that addition of the TE palindrome oligonucleotide as specific competitor did not significantly inhibit transcription from the H1t promoter construct (Clare *et al.* 1997). Transgenic mice generated with the rat H1t transgene containing a replacement of the TE sequence element with stuffer DNA however fail to express the rat H1t mRNA (Grimes *et al.* 1997).

1.3.5 Protamines

Protamines are small arginine rich proteins that replace histones and transition proteins on the DNA during later stages of spermiogenesis. The function and characteristics of protamines for several species have been extensively studied and it is one of the only testis-specific proteins which has a clearly assigned function [for review see (Olivia & Dixon 1991)]. There are two mouse protamine genes, mP1 and mP2, which are first expressed in round spermatids. The transcript is stored up to 8 days before the protein is translated in elongating spermatids (Johnson *et al.* 1988). Comparisons of the protamine promoters of various species show high levels of homology with a conserved CRE binding site invariably present at positions -48/-57 (Olivia & Dixon 1991). Three separate transgenic studies have narrowed the promoter region essential for tissue specific expression from 2.4 kB (Peschon *et al.* 1987), to 465 bp 5' of the start site (Peschon *et al.* 1989), to finally a region spanning -150/-37 (Zambrowicz *et al.* 1993). Promoter deletions studied in transgenics could be assigned broad functions with respect to spermatid-specific transcription. The region -224/-150 was required for high levels of transcription; in this region box C was identified that bound ubiquitous nuclear factors. The region -150/-110 was

essential for spermatid specific expression. Boxes B and O identified in this region also bound ubiquitous factors. Region -37/-77 was determined to be required for high levels of testis-specific expression as its deletion greatly reduced the level of transcription in round spermatids (Zambrowicz *et al.* 1993; Zambrowicz & Palmiter 1994). This region contains the CRE element and also binds a testis-specific factor Tet-1 (Tamura *et al.* 1992). Removal of all but the CRE sequences abolished transcription of the reporter gene in mice. This region, therefore, does not function efficiently by itself to activate testis-specific transcription, highlighting the need for co-operativity between several testis-specific and ubiquitous factors to direct transcription. A comparison of 10 mammalian protamine 1 gene promoters revealed 3 highly conserved elements. These were Prot1C (CRE) -64/-53, Box E -80/-86, and a SRE -113/-132 that was similar to the c-fos SRE (Queralt & Oliva 1993). No proteins were found to bind a fragment containing box E (Zambrowicz *et al.* 1993). DNase I footprinting of the rat protamine 1 promoter region with nuclear extracts from testis and other tissues, revealed protection of the SRE region and two flanking regions SAP (-153/-141) and SEP (-114/-100). Ubiquitous factors were also seen to bind these regions in gel shift assays (Queralt & Oliva 1995). The regulation of protamine 1 transcription involves a complex array of both tissue-specific and ubiquitous factors. Identification and cloning of these factors has yet to be accomplished.

Study of the mouse protamine 2 promoter has also given insight into the regulatory mechanisms of testis-specific transcription. Transgenic studies indicate that 859 bp immediately 5' to the transcription start site confers correct

spatial and temporal transcriptional activation (Stewart *et al.* 1988). An initial DNA binding study of the promoter region -419/-141 defined potential *cis*-acting elements in this region (Johnson *et al.* 1991). More detailed studies of the promoter region indicate 5 sites of protein binding. The region -64/-48 which harbors the CRE sequence interacted with both testis and liver proteins, and could bind CREMτ (Ha *et al.* 1997). Within this region, -59/-47 was shown to interact with a novel testis-specific protein PAF-1. Deletion of the PAF-1 binding region reduced *in vitro* transcription levels from the promoter 3 fold. PAF-1 was suggested to be different from CREMτ by virtue of differences in protein:DNA mobility in EMSA (Yiu & N.B 1997). Supershifts with CREMτ antibodies were not performed which would have provided stronger evidence that PAF-1 and CREMτ are indeed different proteins. Liver and testis proteins also bound the region -87/-67, which contains a CAAT box (Ha *et al.* 1997). Within this region, -83/-72 was shown to interact with the Y-box binding protein p48/p52 (Yiu & N.B 1997). p48/p52 is the germ cell-specific mouse homologue of the germ cell-specific Xenopus protein p54/p56, and is detected in pachytene spermatocytes and round spermatids (Oko *et al.* 1996). A putative Y-box sequence at -489/-478 in the mP2 promoter also binds p48/p52 (Nikolajczyk *et al.* 1995). The mouse testis-specific cytochrome c gene has also recently been shown to contain a putative Y-box at -13/-2 which binds p48/p52 as well (Yiu *et al.* 1997). The presence of Y-boxes in testis-specific gene promoters may be involved in directing tissue-specific expression. Y-box consensus sequences in other testis-specific promoters have not been extensively looked at. Finally the regions -

239/-210 and -328/-311 in the mP2 promoter were also seen to bind ubiquitous and testis specific proteins, respectively. Both of these regions share sequence homology to HREs, but the proteins binding them have not been further studied (Nikolajczyk *et al.* 1995).

Transcription of eukaryotic genes is complex, requiring multiple protein, tissue-specific factors and ubiquitous transcription factors. The delineation of promoters which confer testis-specific transcription onto genes is an important step in determining how this transcription is regulated. Comparison of sequences allows for identification of conserved or novel motifs which may serve as binding sites for specific transcription factors. Generation of transgenic mice gives enormous insight into which regions of the promoter are required for high levels and tissue specificity of transcript expression. Interaction of promoter regions with nuclear proteins from somatic or germ cells gives focus to which sequences may be involved in general or tissue-specific direction of transcription. The next step that is key for our understanding of the process is identifying, characterizing, and cloning these transcription factors.

1.4 Putative Transcription Factors

Genes have been cloned from testis cDNA libraries on the basis that they contain DNA binding motifs found in transcriptional regulatory proteins. These include high mobility group (HMG) proteins, homeodomain proteins, and zinc finger proteins (Zfp). As well several nuclear proteins with structures similar to nuclear receptors for hormones with no identified ligands have been cloned and

termed orphan receptors. The role of many of these genes in spermatogenesis and their target genes remain to be defined.

1.4.1 Zinc Finger Proteins

Zinc finger proteins (Zfp) make up a large family of proteins that exhibit specific nucleic acid binding properties. Members of this family include the transcription factors TFIIIA and SP1. Within the mouse genome there are a large number of zinc finger proteins (Ashworth & Denny 1991). Several of these have been shown to exhibit testis-specific expression or be upregulated during spermatogenesis. Although none of these genes have been proven to act as germ cell-specific transcription factors, it is of interest that their expression appears to be limited to one or two cell types in spermatogenesis. Zfp-35 expression is restricted to pachytene spermatocytes (Cunliffe *et al.* 1990). Zfp-38 expression is seen to accompany the progression from pachytene spermatocytes to haploid round spermatids. Fusion of the transactivation domain of Zfp-38 to the DNA binding domain of the yeast Gal 4 protein generated a protein that efficiently transactivates the expression of a reporter gene in transfection studies (Chowdhury *et al.* 1992). This suggests that Zfp-38 may be acting as a transcriptional activator, however the target genes are unknown. The Zfp-29 is expressed in round spermatids and also in developing embryos (Denny & Ashworth 1991). Zfp-37 mRNA is first detected in male germ cells at day 19. Two proteins are detected in testis extract, a larger protein in extracts from day 22 testis, and a smaller one in adult (day 34) testis. Localization studies showed the protein to be predominantly nuclear (Hosseini *et*

al. 1994). Localization to the nucleus was also seen with the protein product of Zfp-59, expression of which is restricted to postmeiotic germ cells (Passananti *et al.* 1995). The Y-chromosomal gene Zfy-1 is expressed in adult testis and thought to act as a transcription activator. Transgenic studies show that the gene is expressed in both somatic cells in the testis and in germ cells (Zambrowicz *et al.* 1994). The protein localizes to the nucleus and binds DNA in a sequence specific manner (Taylor-Harris *et al.* 1995). All of these zinc finger proteins have the potential to act as transcription factors. Specific expression patterns, protein localization and in some cases DNA binding properties for these proteins suggest they may play a role in spermatogenesis. The exact biological function or the gene targets of these proteins however remains unknown.

1.4.2 Homeodomain and High Mobility Group Proteins

The homeobox gene family of proteins are involved in the genetic control of development and differentiation. The homeobox encodes a 60 amino acid residue polypeptide, the homeodomain, that represents the DNA binding domain of the respective protein [reviewed in (Gehring *et al.* 1994)]. Homeobox domains have been identified in several mammalian tissue-specific transcription factors. *Hox*-1.4 has been identified as a testis-specific homeobox containing gene (Wolgemuth *et al.* 1987). *Hox*-1.4 is expressed in the adult testis in pachytene spermatocytes and round spermatids (Wolgemuth *et al.* 1991). No protein product of the *Hox*-1.4 gene nor its function in the testis have been reported.

The high mobility group box (HMG) motif has been found in several sequence specific DNA binding proteins where the HMG box was both

necessary and sufficient for DNA binding. A few HMG proteins have been cloned that are expressed at high levels in adult testis. Testis-specific HMG (tsHMG) is expressed in postpubertal testis and specifically binds to a 12 bp sequence. This sequence was also found in the promoters of human PGK-2, mouse PGK-2, and mouse Zpf-35 genes (Boissonneault & Lau 1993). The protein product of tsHMG however is restricted to elongating and condensing spermatids which are transcriptionally quiescent. It is thought that tsHMG functions as a DNA-packaging factor in these germ cells (Alami-Ouahabi *et al.* 1996). A testis-specific isoform of mitochondrial transcription factor A (mtFA) has been described that lacks the mitochondrial targeting sequence. The isoform, mtTFA, is present in the nucleus of spermatocytes. The carboxy-terminal of the protein is identical to mtFA suggesting that mtTFA may also function as a transcription factor (Larsson *et al.* 1996). Finally, three SRY related genes that have unique isoforms expressed in the testis, are HMG proteins and may function as transcription factors. Sox5 binds the consensus motif AACAAT. It is expressed at high levels in the testis and the 46 kDa Sox5 protein is restricted to round spermatids (Denny *et al.* 1991). Sox6 is a HMG protein highly homologous to Sox5 that is also testis-specific and binds to the AACAAT motif (Connor *et al.* 1995). *Sox*17 has two mRNA isoforms, Sox17 and t-Sox17 which has a partial deletion of the HMG box region. Both isoforms are expressed initially in pachytene spermatocytes and accumulate to high levels in round spermatids. Protein products are also seen for both forms. Recombinant Sox17 binds DNA in a sequence specific manner, while t-Sox17 has no apparent DNA

binding activity. Interestingly, Sox17 could stimulate transcription through its binding site in a co-transfection study using a luciferase reporter gene (Kanai *et al.* 1996). Sox17 may be a testis-specific transcription activator, however no target genes are known.

1.4.3 Orphan Receptors

Orphan receptors are classified based on sequence similarities with known steroid hormone receptors but have no identified ligands. Many nuclear receptors are ligand-activated transcription factors regulating target genes by binding to specific *cis*-acting elements in the promoter of the target genes. The receptors share a common modular structure with a conserved DNA-binding domain containing two zinc-finger motifs. Nuclear receptors are one of the largest known protein families and more than 75% of the known nuclear receptors are orphan receptors [reviewed in (Enmark & Gustafsson 1996)]. Orphan receptors have been cloned that are expressed at high levels in the testis. GCNF (Chen *et al.* 1994; Katz *et al.* 1997) and GCNF/RTR (Lei *et al.* 1997; Yan *et al.* 1997) bind to the consensus sequence TCAAGGTCA and are expressed in round spermatids. Finally the mouse Tr2-11 orphan receptor is also expressed in spermatids (Lee *et al.* 1996). Target genes for these orphan receptors expressed in the testis, have not yet been identified.

1.5 CREMτ

The proteins described above are classified as putative regulators of testis-specific gene expression. The promoters that they interact with and their role in the transcription of testis-specific genes, either positive or negative,

remain to be determined. It is evident from studies on promoters that conserved sequence motifs may act as targets for specific transcription factors. The cyclic-AMP response element (CRE) appears to play an important role as it is a conserved motif in many testis-specific promoters. Proteins interacting with this element have been identified as cyclic-AMP response element binding (CREB) and modulator (CREM) proteins [for review see (Lee & Masson 1993; Sassone-Corsi 1995)]. A testis-specific isoform of the CRE Mfamily, CREMτ, is to date the only well characterized testis-specific transcription factor (Delmas & Sassone-Corsi 1994).

At least eleven isoforms of CRE Mhave been identified [(Lalli *et al.* 1996) and references within]. Alternative splicing of the CRE Mgene generates both repressors (CRE Mα, β, γ) and activators (CRE Mτ, τ_1, τ_2, τ_α). An alternative intronic promoter within the CRE Mgene generates ICER (I, Iγ, IIγ, II) proteins which are cAMP - inducible repressors. Two CRE elements in the ICER promoter have been shown to bind ICER in an autoregulatory feedback loop (Monila *et al.* 1993). CRE Mproteins contain a leucine zipper region and can homodimerize or form heterodimers with other CRE Misoforms or with CREBs. CREMα, β, γ act as negative regulators of transcription by; 1) dimerization and binding the CRE site, thus preventing the positive regulator CREB from binding, or 2) by inactivating CREB or by forming heterodimers (Foulkes *et al.* 1991; Laoide *et al.* 1993). In the testis, alternative splicing events generate the CREMτ isoforms which act as positive regulators of transcription (Foulkes *et al.* 1992; Laoide *et al.* 1993).

A developmental switch occurs in the testis from expression of CRE Mrepressors to CRE Mactivators. CREMα, β, γ are expressed at low levels in pre-meiotic germ cells, Sertoli and Leydig cells. From the pachytene spermatocyte stage onward, alternative splicing only generates CREMτ activator isoforms (Foulkes *et al.* 1992; Foulkes & Sassone-Corsi 1992). This switch is regulated by FSH stimulation of signals to the germ cells via the Sertoli cells. Induction of CREMτ in round spermatids is due to the use of an alternative polyadenylation site which truncates the 3' untranslated region leading to a dramatic increase in transcript stability (Foulkes *et al.* 1993). CREMτ protein is synthesized in post-meiotic germ cells namely haploid round spermatids. The protein is phosphorylated at serine-117 by a nuclear cAMP-dependent protein kinase A (PKA) activity present in germ cells. Phosphorylation results in a powerful activation of CREMτ (De Groot *et al.* 1993; Delmas *et al.* 1993). Activated CREMτ can efficiently bind CREs present in promoters of a variety of genes specifically activated in haploid germ cells (Delmas *et al.* 1993). At least four genes have been shown to be targets of CREMτ-mediated transactivation in germ cells; RT7 (Delmas *et al.* 1993), transition protein-1 (Kistler *et al.* 1994), calsper min(Sun *et al.* 1995), and tACE (Zhou *et al.* 1996b). CREMτ knockout mice have been generated by two groups (Blendy *et al.* 1996; Nantel *et al.* 1996). The mice displayed a severe impairment in spermatogenesis with a post-meiotic arrest seen at the first step of spermiogenesis. No late spermatids or mature spermatozoa were seen in the homozygous mutants. The expression of post meiotic genes in the testis completely absent (RT7, mP1, mP2, transition

proteins 1 and 2) or severely reduced (calspermin). CREMτ clearly plays an important role in transcriptional activation of many essential testis-specific haploid expressed genes. It remains to date the only characterized testis-specific transcription factor.

1.6 RT7

RT7 is a haploid-specific gene that is abundantly expressed in round spermatids. The 1.1 kB transcript is as abundant as that of protamine-1 (van der Hoorn *et al.* 1990). The gene encodes the 27 kDa outer dense fiber protein, ODF27 that localizes to the sperm tail (Higgy *et al.* 1994). ODF27 can self interact via a leucine zipper region in the N-terminus, and weak self association is also seen between PCX repeats in the C-terminus of the protein (Shao & van der Hoorn 1996). Interaction between ODF27 and another sperm tail protein ODF84 has also been shown to involve the leucine zipper regions of both proteins (Shao *et al.* 1997). Transgenic studies show that the RT7 promoter directs transcription of a reporter gene exclusively in post-meiotic spermatids (Higgy *et al.* 1995).

A male germ cell derived *in vitro* transcription system was used to identify the promoter regions involved in the transcriptional regulation of RT7. Several testis-specific and ubiquitous nuclear proteins were seen to bind in the RT7 promoter region -447/+1. Deletion of -167/-90 resulted in a severe reduction in transcription with seminiferous tubule nuclear extract but not with liver nuclear extract. A testis specific protein, TTF-D was shown to bind specifically to this region (-124), suggesting that it acts as a testis-specific positive regulator of RT7

transcription. A CRE consensus element was also identified at -56 (van der Hoorn & Tarnasky 1992). The testis specific transcription factor CREMτ was shown to bind specifically to the RT7 CRE. Addition of α-CREMτ antibodies to *in vitro* transcription assays reduced RT7 promoter activity to 65% (Delmas *et al.* 1993). CREMτ plays an important regulatory role in the expression of RT7 as evidenced by the CREMτ knock out mice which did not express RT7 (see above). Thus two testis-specific transcription factors CREMτ and TTF-D, play an important role in the regulation of the RT7 gene. CREMτ has been speculated to play a role in regulation of several haploid specific genes besides RT7. A consensus binding site for TTF-D has also been identified in the pre-meiotic expressed testis-specific variant of the *c-mos* gene (van der Hoorn 1992). TTF-D may be an important novel testis-specific transcription factor involved in the regulation of both pre- and post-meiotic expressed genes.

1.7 Objectives

To date only one testis-specific transcription factor, CREMτ has been fully characterized and cloned. The identification of a binding site for a novel testis-specific factor within the promoter region of the RT7 gene that is required for high levels of testis-specific transcription *in vitro* suggested that this factor also may be a unique germ cell transcriptional regulator (van der Hoorn & Tarnasky 1992). The present study was initiated to characterize and clone this factor, termed TTF-D. A putative TTF-D binding site had also been identified in the testis promoter of the c-*mos* gene (van der Hoorn 1992). The interaction of TTF-D with the c-*mos* site was also investigated.

The regulation of RT7 gene expression also involves CREMτ (Delmas *et al.* 1993). RT7 promoter-protein interactions with recombinant CREMτ proteins were investigated and the *in vivo* activity of these proteins studied. The ability of CREMτ to activate transcription in a second testis-specific gene, mP1, was also studied.

Chapter 2: Materials and Methods

2.1 Protein Isolation from Cells and Bacteria

2.1.1 Seminiferous Tubule Nuclear Extract

Nuclear extracts from rat seminiferous tubules were prepared by a method adapted from Gorski (Gorski *et al.* 1986). Twenty to thirty Sprague Dawley rats were euthanized under CO_2 and the testes were immediately dissected. An incision was made in the outer testicular membrane and the testicular contents isolated and placed in a beaker on ice. The interstitial cells and seminiferous tubules were then covered with DME Mpyruvate-lactate buffer, 1.5 - 2 mg of collagenase A was added per ml followed by a 20 minincubation at 32°C, with frequent swirling to break up the tubules. Following collagenase treatment, the tubules were washed three times with DME Mbuffer. The tubules were then centrifuged briefly in 50 ml Falcon tubes and the remaining buffer aspirated away from the tubules which were then placed on ice. An approximate volume of 60 ml seminiferous tubules was obtained.

The following steps were performed at 4°C. In a pre-cooled modified Waring blendor (Lichtsteiner & Schibler 1989), the cells were added to 250 ml ice cold homogenization buffer, 1/20 volume of a 20% milk solution, protease inhibitors, and 141 µl cocktail per 10 ml homogenization buffer (see below for recipes). This mixture was blended for 3 X 10 seconds. The homogenate was then loaded on top of a 10 ml cushion (plus protease inhibitors), in SW28 tubes on ice. The tubes were centrifuged in a pre-cooled SW28 rotor and chamber at 24K r.p.m. for 60 minat 0°C.

With a spatula the solid disk of whole cells and debris was removed from the top of the tubes. The supernatant was carefully aspirated off with a 10 ml plastic pipette, but not aspirated completely to the pellet. The tubes were inverted and the wall of the tubes rinsed with distilled water using a 50 ml syringe with a bent Hamilton needle. Caution was taken to avoid getting water on the pellet. The sides of the inverted tube were then dried with a kimwipe. The tubes were placed on ice, each pellet was resuspended in 2 ml nuclear lysis buffer (NLB) plus protease inhibitors and cocktail, using a 1 ml pipette tip that had been cut to create a larger bore. The nuclei were transferred to a large glass homogenizer (40 ml), on ice. An additional 1 ml NLB was added to each tube to ensure that all nuclei were collected and transferred to the homogenizer. Clumps of nuclei were broken up by homogenizing 5-6X using an A pestle. The A_{260} of a 1:50 dilution in 0.5% SDS (blank against a 1:50 dilution of NLB) was measured.(DU-65 Spectrophotometer, Beckman, Fullerton, CA.) From this reading, the approximate concentration of DNA was calculated (1 mg/ml gives an A_{260} of 20). A final DNA concentration of 0.5 mg/ml was obtained by dilution with NLB.

To this was added 3 MKCl, to a final concentration of 0.55 M(note: the NLB already contains 0.1 MKCl, therefore the volume of 3 MKCl that is added is calculated as follows: (0.55 - 0.1) x volume of nuclear suspension/3 -0.45.) The tube was then incubated on ice for 30min, with occasional mixing, followed by centrifugation for 90 minat 28K r.p.m. at 0°C.

Immediately after the centrifuge stopped, the tubes were removed and the supernatant transferred to a clean tube. The volume was measured and 0.3 g solid $(NH_4)_2SO_4$ per ml of supernatant was added. This was gently mixed by inverting the tube, and left on ice until all crystals had dissolved, with occasional mixing. After all the salt crystals had dissolved, the tube was left on ice for 60 min. Precipitated proteins were collected by centrifugation for 90 minat 28K r.p.m., at 0°C. The supernatant was aspirated completely and discarded. At this point the pellet was stored on ice overnight or the procedure completed, depending on time constraints.

The pellet was resuspended in 2 ml cold dialysis buffer and kept on ice as much as possible. The proteins were dialyzed twice against 250 ml cold dialysis buffer for 90 minutes each in the cold room (4°C). Finally, the dialysate was transferred to a clean cold 1.5 ml eppendorf tube, and centrifuged for 5 minat 14 000 r.p.m.. The supernatant was then aliquoted and fractions stored at -70°C. The protein concentration was calculated as follows. Measure the A_{230} and A_{260} of a 1:100 dilution of extract, in water. A 1:100 dilution of dialysis buffer was used as a blank.

Calculation of approximate protein concentration: $187 \times A_{230} - 81.7 \times A_{260} = \mu g/ml$

Buffers and Solutions for Nuclear Extraction:

Homogenization Buffer: 10 mM HEPES (pH 7.6), 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.2 M sucrose (store at -20°C)

Cushion: 10 mM HEPES (pH 7.6), 15mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol (store at -20°C)

Protease Inhibitors + DDT: add just before use 0.5 mM DTT, 1 mM benzamidine (fresh), 0.5 mM PMSF(0.1 M in i-propanol)

Cocktail: 1% final aprotinin, 1μg/ml pepstatin, 1.14 μg/ml leupeptin

Milk: make 20% non-fat, BBL skim milk powder (Becton Dickinson, Cockeysville, MD.) in H_2O, centrifuge 20 minat 8000rpm, add 1/20 of supernatant to homogenization buffer just before use, make fresh each time

Nuclear Lysis Buffer: 10 mM HEPES (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 10% glycerol, just before use add: 1 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine (fresh), 141 μl cocktail per 10 ml

Dialysis Buffer: 25 mM HEPES (pH 7.6), 0.1 mM EDTA, 40 mM KCl, 10% glycerol, 1 mM DTT

2.1.2 Whole Testis Nuclear Extract

Crude nuclear extracts from mouse testes were made essentially according to Lilienbaum (Lilienbaum & Paulin 1993). Testes were dissected out from the mice, decapsulated into a petri dish and washed briefly with a small amount of cold 1X PBS. The tissue was then transferred to a 7 ml glass homogenizer, on ice. To this was added 3 ml of cold buffer [10 mM HEPES (pH 8), 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA (pH 8), 0.5 mM spermidine, 0.15 mM spermine], and Nonidet P-40 to a final concentration of 0.1%. The tissue was homogenized ten times with an A pestle to assist in breaking up the cells and releasing the nuclei. The homogenate was then transferred to a 15 ml polypropylene tube and centrifuged for 10 minat 3000 r.p.m. at 4°C to pellet the

nuclei. The pellet was resuspended in 200 μl extraction buffer (10 mM HEPES, 350 mM NaCl, 1 mM PMSF, 2 mM benzamidine, and 5 μg/ml of each: pepstatin, leupeptin, aprotinin) and transferred to a 1.5 ml eppendorf tube. Nuclear proteins were extracted by incubation at 4°C for 45 minon a Nutator rocking platform. Protein concentration of extracts was determined by Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA.) and extracts stored at -80°C.

2.1.3 Glutathione S Transferase Fusion Proteins

A single colony of bacteria TB1 transformed with pGEX-CREMτ full length or pGEX-ΔCREMτ was inoculated into 50 ml of Enriched Medium [2% tryptone, 1% yeast extract, 50 mM KPO_4 (pH 7.4), 0.2% glycerol, 100 mM NaCl] plus antibiotic. The culture was grown overnight at 37°C with shaking. The next day, the culture was diluted 1:10 into fresh Enriched Medium plus antibiotic and incubated at 37°C with shaking for one hour before induction with 0.1 mM IPTG for three hours. The bacteria were harvested by centrifugation and resuspended in 40 ml PBS containing 1 mM PMSF and 5 mM EDTA. The bacteria were then lysed by French press twice, diluted to 100 ml with PBS (1 mM PMSF, 5 mM EDTA) and incubated with glutathione-agarose (2 ml bed volume) overnight at 4°C with gentle shaking. The bead slurry was washed several times with cold PBS to remove any unbound or loosely bound proteins and transferred to a 10 ml column. The fusion protein was eluted by addition of 5 ml elution buffer (75 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM glutathione, 0.1% Triton-X) in ten

500 µl fractions. Protein concentration of each fraction was determined by Bio-Rad assay, and proteins were visualized by SDS-PAGE.

2.2 Protein Analyses

2.2.1 Silver Nitrate Staining

SDS-Page gels were fixed for 45 minin 50% methanol and 10% acetic acid, followed by 45 minin 5% methanol and 7% acetic acid. Fixation was followed by soaking the gel in 1% glutaraldehyde. The glutaraldehyde was removed by washing the gel in distilled water for 2-3 hours and the water changed every 20 min. Alternatively, the gel was washed in water overnight. The gel was then soaked in 5 µg/ml DTT for 45 min. The DTT solution was removed and a 0.1% silver nitrate solution added for 45 min. The gel was then rinsed in a small amount of distilled water and then rinsed twice more with a small amount of developer (0.0185% formaldehyde in 3% sodium carbonate). The gel was then soaked in developer until the desired intensity of the bands was reached. The staining reaction was stopped by addition of 2.3 M acetic acid. The gel was then washed overnight in distilled water.

2.2.2 Southwestern Blotting

For Southwestern blotting experiments, liver or ST extracts were loaded onto a 10% SDS polyacrylamide gel in an equal volume of loading buffer (5% SDS, 5 mM Tris-Cl (pH 6.8), 200 mM DTT, 20% glycerol, bromo phenol blue) without boiling. Following electrophoresis, proteins were transblotted onto

NitroPlus nitrocellulose transfer membrane (Micron Separations Inc., Fisher Scientific, Nepean, ON), using a semi-dry transblotter (Semi-phor, Hoefer Scientific Instruments, San Francisco, CA) and transblot buffer (25 mM Tris, 190 mM glycine), following the manufacturer's directions. Proteins were electro-blotted for 75 min at 105 mAmp. After transfer, the portion of the blot with the molecular weight markers was cut off and the markers visualized by ponceau S staining.

The remainder of the blot was blocked for 1 hour at 4°C in BLOTTO (50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% BBL skim milk powder (Becton Dickinson, Cockeysville, MD.)), with gentle shaking. The membrane was then incubated overnight at 4°C in binding buffer (25 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM EDTA). The following day, the blot was probed with the appropriate radiolabeled oligonucleotide sequence in fresh binding buffer to the following specifications: 100 µl buffer/cm^2, 100 000 cpm/ml. Blots were probed for 6 hours or overnight at 4°C, followed by 4x8 min washes in binding buffer also at 4°C. Blots were placed on Whatman paper protein side up to remove the majority of the moisture, they were then wrapped between a sheet of saran wrap and exposed to film at -80°C.

2.2.3 Elution of Protein from PAGE Gels

ST extracts were size separated on 10% or 12.5% SDS polyacrylamide gels. The molecular weight marker lanes were removed and the marker proteins visualized by cold KCl staining as reported by Hager (Hager & Burgess 1980).

Briefly, the gel section was rinsed in ice cold distilled H_2O, incubated for 5 min in cold 0.25 M KCl, 1 mM DTT, followed by rinsing in cold H_2O, 1 mM DTT until protein markers could be visualized. Markers were then realigned with the rest of the gel and gel slices in the desired molecular weight range of ST proteins were excised. Gel slices were crushed in a 15 ml falcon tube and 2 ml of elution buffer was added (50 mM Tris, 0.1 mM EDTA, 0.1% SDS, 5 mM DTT, 150 mM NaCl, 0.1 mg/ml BSA). Proteins were eluted overnight by gentle mixing on a Nutator. Tubes were then centrifuged for 5 min at 3000 r.p.m. to pellet the acrylamide, the supernatant was collected and transferred to a 15 ml siliconized glass falcon tube. Proteins were precipitated in 4 volumes of acetone (-20°C) plus 25 μg BSA, for 2 hours at -20°C, followed by centrifugation for 20 min at 10,000 r.p.m.. The supernatant was poured off and the protein pellet rinsed with 1 ml of 95% EtOH (-20°C). Pellets were allowed to air dry and resuspended in 25 μl of a saturated urea solution. The protein suspension was then diluted 50 fold by addition of dilution buffer (20 mM Tris, 10 mM KCl, 2 mM DTT, 0.1 mM PMSF) (McCaffrey *et al.* 1993). Proteins were allowed to renature by incubation overnight at 4°C.

2.2.4 PKA of GST-fusion proteins

GST-fusion proteins were radiolabeled in the presence of 10μl reaction buffer, (20 mM TRIS, 1 mM EGTA, 5 mM $MgCl_2$, 1 μg/ml PKA (gift of Dr. M. Walsh), 0.2 mM γ^{32}P-ATP) and 1 μl 20 nM okadaic acid. The reaction was complete after a 10 minute incubation on ice. An equal volume of loading buffer was then added,

samples were boiled for 3 minutes and analyzed by 10% SDS PAGE. The gels were stained by coomassie blue, followed by destaining to visualize the molecular weight markers. The gels were then dried under vacuum and analyzed by autoradioaugraphy.

2.3 Antibody Production and Analyses

2.3.1 Generation of Polyclonal Antibodies

Polyclonal antibodies to GST-CREMτ were generated following a six month protocol using New Zealand White Rabbits as the host. An 8 ml pre-bleed was taken from each rabbit one month prior to the first injection. The first injection consisted of 250 μl complete Freunds adjuvant and 250 μl protein (50-75 μg) in PBS. All following injections consisted of 250 μl incomplete Freunds adjuvant and 250 μl protein (50-75 μg) in PBS. A maximum of 500 μl injection volume per rabbit was allowed. The second and third injections followed one and two months after the first injection. Seven days after the third injection, the first bleed was taken from each animal. Eight millilitres of blood were collected into a serum separator test tube (Beckton and Dickinson, Bedford, MA) and placed on ice for ten minutes prior to centrifugation at 1000g for five minutes. The serum was collected and stored in 500μl aliquots at -80°C. The fourth injection was given one month after the third injection. Seven days following the fourth injection a second bleed was taken in the same manner as the first bleed. A fifth and final injection was given one month after the fourth injection. Seven days after the final injection a final bleed was taken by heart puncture with a volume of

100-130 ml blood collected into 50 ml centrifuge tubes on ice. The blood was allowed to coagulate overnight at 4°C and the serum was aliquoted and stored at -80°C. Following each bleed, the serum was analyzed by Western Blot to determine if antibodies to the fusion protein were being produced. The final bleed was only taken if the antibody production was at a suitable level as determined by Western blotting and immunoprecipitation assays.

2.3.2 In Vitro Transcription/Translation, Immunoprecipitation

In vitro transcription and translation was performed according to and using the Promega TNT Lysate Coupled Transcription/Translation kit. T7 RNA polymerase was used to transcribe pBCREMτ (gift of Dr. P. Sassone-Corsi) from the T7 promoter. Following the TNT reaction, samples were analyzed further by immunoprecipitation. In a 1.5 ml eppendorf tube 500 µl 1XTAG buffer [20 mM Tris (pH 8), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA], 4.5 µl antibody, 10 µl TNT reaction mix, and 20 µl 10% Protein A Sepharose fast flow beads (Pharmacia, Baie d'Urfe, Quebec) were mixed and incubated overnight at 4°C on a rotating platform. The following day the beads were washed 3 x 2 min with 1XTAG buffer, and centrifuged briefly at 3000 r.p.m. in a microfuge. All of the supernatant was pipetted off and the beads resuspended in 1X sample buffer [1% SDS, 1% glycerol, 80 mM Tris (pH 6.8), 10% β-mercaptoethanol]. Samples were boiled for 3 min and run on a 10% SDS-PAGE gel. The gel was then stained by Coomassie blue, destained in (5% MeOH:7% acetic acid) dried, and exposed to film.

2.4 Functional Protein Assays

2.4.1 In Vitro Transcription

Nuclear extract (3,6,9, or 12 μl) was incubated with 1 μl of template DNA (1μg) for ten minutes on ice with the volume not exceeding 13 μl. The remaining volume was made up with dialysis buffer. To this was added 7 μl of a master mix. The master mix was prepared as follows: per reaction: 4 μl 5X mix (see below), 0.7 μl α^{32}P-UTP(400 Ci/millimol), 1 μl RNasin (Pharmacia), 1 μl (0.3 μg) AdMLP, 0.3 μl H_2O. The reactions were then incubated at 28°C for 45 minutes. This was followed by addition of 284 μl STOP mix, [per reaction: 280 μl STOP buffer (see below), 2 μl 10 mg/ml tRNA, 2 μl 20 mg/ml Proteinase K] and a 30 minute incubation at 37°C. The reaction was then extracted once with phenol:chloroform (pH 7) and precipitated with 1 ml 95% EtOH (-20°C) for 45 minutes at -80°C. Samples were centrifuged for 20 minutes in a microfuge at 4°C and the pellet washed once with 500 μl 70% EtOH (-20°C). Pellets were dried under vacuum and resuspended in 3 μl formamide loading buffer. Samples were boiled for two minutes and then placed on ice. A 6% polyacrylamide/UREA gel was pre-run for 1 hour at 45W. Samples were loaded and run for 11/2 hours at 55W. The gel was fixed for 20 minutes in 20% EtOH, 10% acetic acid followed by drying under vacuum and exposure to autoradiographic film.

5X mix: (store in 200 µl aliquots at -20°C)

50 mM HEPES pH 7.6

15.2 % glycerol

127.5 mM KCl

30 mM $MgCl_2$

3.125 mM ATP

3.126 mM CTP

0.175 mM UTP

1 mM O-Me GTP (Pharmacia)

STOP buffer: 0.25 M NaCl

1.0% SDS

20 mM Tris, pH 7.5

5 mM EDTA

Make up and incubate at 37°C for at least 30 minutes prior to use

2.4.2 Native EMSA

The majority of gel shift assays were performed as follows: 1 or 2 ng of radiolabeled oligonucleotide was incubated with ST or liver nuclear extract, 1 µl of 10X footprinting buffer (250 mM HEPES pH 7.6, 50 mM $MgCl_2$, 340 mM KCl), 0,1, or 2 µl of poly dIdC(1 µg/µl), competitor oligo (specific and or non-specific), and dialysis buffer to bring the final volume of each reaction to 10 µl. Reactions were incubated on ice for 15 minutes. For some assays, extract was

preincubated with all components except the competitor oligo for 15 min on ice, a further 15 min incubation on ice followed addition of competitor oligo. 1 μl of loading buffer(30% glycerol in H_2O, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was then added to each sample and samples were loaded onto a pre-run (1 hour, 20 mAmp, 4°C) native PAGE gel (8% or 10%) and run at 4°C. Gels were then dried under vacuum and exposed.

2.4.3 UV/EMSA

Gel shifts which incorporated a UV crosslinking step were performed exactly as above. Prior to addition of the loading buffer, the samples were crosslinked on "auto-link" (1200 μJoulesX100, 5 cm from light source) in a UV-Stratalinker 1800 (Stratagene cloning systems, La Jolla, CA.).

2.4.4 UV/EMSA/SDS PAGE

EMSA assays were performed as above, and crosslinked as above. Following UV irradiation, an equal volume of SDS-PAGE loading buffer was added and samples were boiled for 3 min. Proteins crosslinked to the radiolabeled oligo nucleotides were then separated by 10% SDS PAGE. Gels were stained with coomassie blue followed by destaining to visualize the molecular weight markers. Gels were then dried and exposed to autoradiographic film to visualize crosslinked complexes.

2.4.5 EMSA/Alkaline Phosphatase

Nuclear extract, (liver or ST) was mixed with, 2 μl dIdC, 2 μl 10X footprint buffer, 4 μl protease inhibitors (0.5 mM DTT, 0.1 M PMSF, 0.1 mg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin), 2 μl 10X dephosphorylation buffer (Pharmacia), plus or minus 1 μl CIP (Pharmacia), and dialysis buffer for a final volume of 19 μl. Samples were incubated at 37°C for 1 hr. One nanogram of radiolabeled probe was then added and reactions were incubated for 10 min on ice. After addition of 2 μl loading buffer samples were loaded onto a pre-run (1 hr, 20 mAmp, 4°C) 8% native gel at 4°C and electrophoresed for 1 hr at 20 mAmp. The gel was then dried under vacuum and exposed to film.

2.4.6 Dynabead Assay

Columns for dynabead assays were prepared by washing 100μl DYNABEADS® M-280 streptavidin beads (Dynal Inc. New York, NY.) twice with PBS pH 7.4, 0.1% BSA. In all steps, the dynabeads were separated away from solution using the Dynal Magnetic Particle Concentrator according to manufacturer's directions. Beads were then mixed with biotinylated oligonucleotides harboring the TTF-D binding site for 45 min at room temperature with mixing. The beads were washed 3 times with PBS pH 7.4, 0.1% BSA. ST extract was mixed with dialysis buffer, dIdC and 1X footprinting buffer and incubated with the beads for 15 min on ice with occasional mixing. The flow-through was removed from the beads and the beads washed with 100 μl dialysis buffer for 5 min on ice. Protein bound to the dynabead-linked oligonucleotide was eluted with 1 or 2 M KCl, dialysis

buffer, followed by micro-dialysis for 30 min at 4°C on filter disks (MMF Millipore Membrane Filters, 0.025 μ MVSWP 002500, Bedford MA). Flow-through, wash, and eluted protein were all analyzed by EMSA following the dynabead assay.

2.4.7 Microinjections

Cells were plated on acid-washed (2 hr in concentrated HCl, followed by extensive rinsing in distilled H_2O and stored in 95% EtOH) and flamed glass coverslips, and allowed to spread for at least 24 hr prior to use. Solutions for injection were prepared in 1/2X PBS to the following concentrations: fusion protein: 0.3 μg/μl, plasmid DNA: 200 ng/μl, non-specific rabbit or mouse IgG: 2.0 μg/μl. Samples were centrifuged for 5 min at 13K at 4°C prior to use, and kept on ice at all times. Several fields of cells were injected per coverslip, typically 100-200 cells/duplicate or triplicate coverslips were injected for each solution tested. Following injection, forskolin was added to the media to a final concentration of 5 μM, and coverslips were incubated overnight. Microinjections were carried out using equipment gernerously provided by Dr. K. Riabowol.

2.4.8 Immunofluorescence

Coverslips with injected cells, or control testis sections were fixed one of two ways. 1) Ten minutes at room temperature in PBS containing 3.7% formaldehyde, followed by sequential 10 min washings in PBS containing 0.5% Triton X-100, and PBS with 0.05% Tween 20. 2) Five minutes in -20°C acetone followed by 10 min in PBS at room temperature. Primary Abs (30-50 μl needed

per coverslip) were diluted in PBS containing 1% BSA to a 1:100 concentration. Prior to use, diluted antibodies were centrifuged at 13K,4°C for 15 min to remove precipitates and aggregates.

Fixed cells on coverslips or tissue sections were placed on a layer of parafilm in a humidified chamber, 40 μl of primary Ab solution was added to each section or coverslip and cells were incubated for 30 min at 37°C. Coverslips were then washed for ten minutes at room temperature in PBS containing 0.05% Tween 20. Coverslips or slides were returned to the humidified chamber for incubation with a secondary antibody conjugated to fluor, 30 min at 37°C. Secondary antibodies were used at 1:200 dilution in PBS 1% BSA, of α-mouse fluorescein and/or Cy3-labeled-goat-anti-rabbit-IgG. Sections or cells were then washed as above. Following the final PBS/Tween wash, coverslips were rinsed briefly in dH_2O and dry mounted on microscope slides. Tissue sections were washed briefly in dH_2O and wet mounted (2.3% Daco, 20 mM Tris pH 8, 90% glycerol). Photographs made of tissue sections were taken using 160 ASA Kodak film with tungsten.

2.4.9 LacZ Staining

Coverslips injected for LacZ analysis, and control slides [RT7lacZ transgenic mice (Higgy *et al.* 1995), frozen sections] were fixed in PBS (1% formaldehyde, 0.02% NP-40) for ten minutes, followed by 3X 5 min washes in 1X PBS. Cells and slides were then incubated overnight in a humidified chamber at 37°C in X-gal staining solution [5 mM potassium ferricyanide, 5 mM potassium

ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal (added last, dropwise with vortexing to avoid crystal formation) in PBS]. If required coverslips were processed for immunofluorescence as described above.

2.5 Transcription Factor Screening Assays

2.5.1 λgt11 Expression Library Screening

A culture of *E. coli* Y1090 was grown overnight in LB-medium containing 0.2% maltose, 10 mM $MgCl_2$, and 50 μg/ml ampicillin at 37°C. 500 μl aliquots of the Y1090 were infected with 3-5 x 10^4 pfu of the λgt11 testis cDNA library (Shao *et al.* 1997) and incubated at 37°C for 15 min. To each aliquot of infected cells, 9 ml of top agarose (melted and equilibrated to 47°C) was added, the tube inverted twice and the mixture quickly spread on a prewarmed 150 mm LB/ampicillin plate. Plates were inverted and incubated at 42°C until pin-prick size plaques were visible (3-4 hr). In the meantime, NitroPlus filters were labeled appropriately with ink pen and soaked in 10 mM IPTG for 30 min and air dried. Each plate was carefully overlaid with a filter from above, and markings transferred to the bottom of the plate. Plates were incubated for 4-6 hr at 37°C. At the end of the first incubation, the primary set of filters were removed. Secondary IPTG soaked filters were overlaid on the plates, markings transferred, and the plates were incubated for an additional 5hr at 37°C. Both sets of filters were treated the same following removal from the plates. Filters were transferred to BLOTTO (50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% BBL skim milk powder (Becton Dickinson, Cockeysville, MD.) for 1 hr at

4°C with gentle agitation. The filters were then incubated overnight at 4°C in binding buffer [25 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT] with gentle agitation. Fresh binding buffer at a volume of 150 $\mu l/cm^2$/filter with 5 µg/ml dIdC (or an optimized concentration of non-specific ss-oligo) and probe (1 x 10^5 cpm/ml) was added to the filters. Following a 4 hr incubation at 4°C with gentle agitation, the filters were washed 4 x 8 min with binding buffer, air dried and exposed overnight at -80°C.

2.5.2 λgt11 Expression Library Screening using GuHCl

Plates were prepared and incubated with IPTG filters as above. Following removal from the plates, both primary and secondary filters were treated as follows. Filters were placed protein side up on Whatman paper and air dried for 15 min. The filters were then immersed in 6 M GuHCl in HEPES binding buffer (25 mM HEPES (pH 7.9), 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT) and incubated with gentle shaking for 10 min at 4°C. This step was repeated with fresh 6 M GuHCl-HEPES binding buffer. This solution was then decanted off, diluted 1:1 with HEPES binding buffer, to give 3 M GuHCl-HEPES binding buffer and the filters were incubated in this diluted solution for 5 min at 4°C. This step was repeated four times. Therefore the filters were incubated for 5 min at 4°C in each of the following serial dilutions of GuHCl-HEPES binding buffer: 3 M, 1.5 M, 0.75 M, 0.375 M, 190 mM . This was followed by 2 x 5 min 4°C incubations in HEPES binding buffer. The filters were then blocked in BLOTTO for 1 hr at 4°C, followed by an overnight incubation in HEPES-binding buffer at 4°C. As

described above, but using the HEPES-binding buffer, the filters were probed for 4 hr at 4°C, washed 4 x 8 min, dried and exposed to film.

2.5.3 Yeast-1-Hybrid Assay

The Yeast-1-Hybrid assay first described by Wang (Wang & Reed 1993) was available as a kit from CLONTECH Laboratories Inc. (Palo Alto, CA.). The protocols for the majority of the assay steps and preparation of solutions were as described in the kit (Protocol# PT1031-1, Version# PR58486). The yeast strain used to prepare all reporter strains was YM4271 (*ura*$^-$, *his*$^-$).

2.5.3.1 Competent Yeast Cells

To prepare competent yeast cells several colonies of the appropriate strain 2-3 mm in diameter, were inoculated into 1 ml YPD (20 g peptone, 10 g yeast extract per litre, 2% glucose, pH 5.8). This was vortexed well to break up and disperse the yeast colonies. The cell suspension was then transferred to 50 ml YPD and incubated at 30°C, with shaking at 250 r.p.m. for 16-18 hr to the stationary phase (OD_{600}>1.5). The overnight culture was then diluted to produce an OD_{600}=0.2-0.3 in 300 ml YPD. The diluted culture was incubated at 230 r.p.m. for 3 hr at 30°C. The culture was then centrifuged at 1,000 xg for 5 min at room temperature and the pellet resuspended 25 ml 1X TE (10X TE buffer: 0.1 M Tris, 10 mM EDTA, pH 7.5). The yeast cells were centrifuged a second time as indicated above and the cells resuspended in 1 ml 1X TE/LiAc (10X LiAc: 1 M lithium acetate pH 7.5) for a final volume of 1.5 ml.

2.5.3.2 Generating Reporter Yeast Strains

Reporter vectors, which contained the desired TTFD binding sites cloned into the reporter plasmids pHisi, pHisi-1 or pLacZi (described below), were linearized by restriction digestion. One microgram of the pHisi or pHisi-1 (digested with Xho I) or the pLacZi (digested with Nco I) constructs were incubated with the appropriate enzyme at 37°C for 2 hr in a 20 μl reaction volume. One hundred micrograms of carrier DNA (sheared herring sperm DNA, Pharmacia, Baie d'Urfe, Quebec) was added to the digested plasmid and mixed well. As a control, 100 μg carrier DNA was mixed with 1 μg of the same undigested reporter construct. To the DNA, 100 μl of competent YM4271 cells (see above) were added and mixed well. Next, 600 μl of PEG/LiAc (40% PEG, 1X TE, 1X LiAc) (PEG is from a 50% stock solution) was added and mixed well by vortexing. This solution was then incubated for 30 min at 30°C. Seventy microlitres of DMSO were added to each tube and mixed well by inverting the tube gently several times. The cells were then heat shocked at 42°C for 15 min followed by a 2 min incubation on ice. The cells were centrifuged at 14,000 r.p.m. for 5-10 sec in a microfuge. The supernatant was pipetted off and the pellet resuspended in 150 μl TE buffer. Reporter strains with pHisi or pHisi-1 constructs were plated on SD/-His and pLacZi transformants on SD/-Ura plates to select for colonies with an integrated reporter gene. [SD medium: 6.7 g Difco yeast nitrogen base without amino acids, 20g Agar (for plates) per litre, pH 5.8] Plates were incubated at 30°C for 4-6 days, colonies 2-3 mm in size were restreaked on the

same selection medium as above for master plates or grown up in SD/-His (-Ura) liquid culture and frozen at -80°C in 50% glycerol as stock.

2.5.3.3 Transforming Competent Reporter Strains

Competent reporter strains were prepared as described above. In a 50 ml tube 20 μg pGAD424-testis cDNA library (Shao *et al.* 1997) was mixed well with 2 mg of carrier DNA. To the DNA was added 1 ml of competent reporter cells with thorough mixing to ensure a good transformation efficiency. The protocol described above for transformation of competent cells was then followed exactly from the addition of PEG/LiAc to the two minute incubation on ice, with all volumes scaled up 10 fold. Following the two minute incubation on ice, the cells were centrifuged at 1,000xg for 5 min at room temperature. The pellet was resuspended in 2 x 25 ml SD/-His liquid medium in two 50 ml tubes and the cells incubated with shaking at 200 r.p.m. for 1 hr at 30°C. The cells were then centrifuged a second time as above and the pellets resuspended together in ~7 ml TE buffer to give a final volume of ~7.5 ml. To follow the transformation efficiency, 10 μl of the suspended cells were diluted in 1 ml TE buffer and 200 μl of this dilution was plated on a SD/-Leu plate. The remainder of the cell suspension was plated in 500 μl aliquots on 150 mm plates containing SD/-Leu/-His/plus the appropriate concentration of 3-AT (Sigma Chemical Co. St. Louis, MO). The concentration of 3-AT used was determined in a series of control experiments not described (see CLONTECH yeast-1-hybrid protocol). Plates were incubated at 30°C for 4-6 days. Positive colonies were identified,

restreaked on appropriate plates and liquid cultures grown for isolation and characterization of the plasmid generating a positive colony.

2.5.3.4 Isolation of Plasmid Vectors from Yeast

Isolation of plasmid vectors from yeast for subsequent transformation into *E. coli* and analysis were performed according to Hoffman (Hoffman & Winston 1987). Small (1.5-3 ml) cultures of yeast were grown overnight at 30°C in media that maintained the selective pressure for the plasmid of interest. Yeast cells were collected in 1.5 ml eppendorf tubes by centrifugation for 5 sec in a microfuge. The supernatant was decanted and the pellet resuspended in the residual liquid by briefly vortexing the tube. To this was added, 200 µl solution (0.2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA), 200 µl phenol-chloroform-isoamyl alcohol (25:24:1) and 0.3 g acid-washed glass beads (212-300 microns, Sigma Chemical Co.). The mixture was then vortexed for 2 min to break open the yeast and release the plasmid DNA. The sample was then centrifuged for 5 min in a microfuge and the aqueous layer transferred to a clean microfuge tube. Competent *E. coli* cells were then transformed with 1-5 µl of the aqueous layer which contained the plasmid DNA.

2.6 DNA Constructs

2.6.1 GST-fusion Protein Plasmid Constructs

A plasmid preparation (pBCREMτ) of the CREMτ gene cloned into the NotI site in pBKS$^-$ was used as starting material (gift of Dr. P. Sassone-Corsi). pBCREMτ

was restriction digested with NotI and the insert was gel isolated from an agarose gel as described below. The insert was then amplified by PCR using primers which introduced a BamHI site 5' and a XbaI site 3' to the tCREM-sense and tCREM-αsense, respectively. For the PCR reaction, 10ng CREMτ NotI insert, 10 μM tCREM-sense, 10 μM tCREM-αsense, 10 μM NTPs, 1X 10X PCR, and 1 unit Taq Polymerase were combined in a 20 μl reaction and a drop of PCR mineral oil added. The samples were PCR amplified as follows: 2 min at 95°C, 30 cycles at: 30 sec 95°C, 36 sec at a Tm of 52°C, 84 sec at 72°C, followed by 10 min at 72°C using a mini cycler PCR machine (MJ Research Inc. Waterton, MA). The samples were phenol:chloroform extracted once and then EtOH precipitated. The amplified CREMτ insert and the vector pGEX-KG were then restriction enzyme digested with BamHI and XbaI and the products gel isolated from agarose gels. The products were combined in a ligation reaction and the desired recombinant vector, pGEX-CREMτ (pGEX-CT) was identified and isolated.

To generate the GST-CREMτ mutant fusion protein vector (pGEX-ΔCT), pGEX-CT was digested with BamHI and Sal I. This restriction digest completely removed the DNA binding domain coding region from the CREMτ gene. The 5 Kb vector and the 0.9 Kb fragment (ΔCREMτ) from the digest were gel isolated and religated to generate the desired construct. Positive recombinants were identified and isolated.

2.6.2 Concatamers of TTFD Binding Sites

Concatamers of the TTFD binding site oligos were generated for cloning and screening purposes. Double stranded oligos were made blunt ended by a non-radioactive Klenow reaction (10 μl) for 1 hr at room temperature, the DNA molecules were then phosphorylated in a kinase reaction (15 μl) for 1 hr at room temperature. Two units of T4DNA ligase, 2 μl 10X ligation buffer were then added to the PNK reaction for a final volume of 20 μl. The ligation was incubated overnight at 16°C. The ligation reaction was desalted through a G-25 Sephadex spun column (Maniatis *et al.* 1982). The DNA molecules were separated on a 10% native PAGE and visualized by ethidium bromide staining of the gel. Concatamers in the desired size range were excised from the gel and the DNA molecules isolated as described below.

2.6.3 Yeast-1-Hybrid Plasmid Constructs

Concatamers of the TTFD binding sites were cloned into the reporter plasmids for the yeast-1-hybrid assay. pHIS-I, pHISi-1, or pLacZi vectors were digested with Sma I restriction enzyme, the linear plasmid gel isolated and combined in a ligation reaction with the desired concatamer as insert (described below).

2.7 Isolation of DNA from Agarose and Polyacrylamide Gels

2.7.1 Isolation of DNA from Agarose Gels

DNA bands of interest size were separated on agarose gels, visualized by a hand held UV (254 nm) light source and excised with a scalpel. The DNA was

extracted from the agarose slice as described by Heery (Heery *et al.* 1990). The gel slice was transferred to a punctured sterile 0.5 ml eppendorf tube that was plugged with 2-3 mm of siliconized sterile glass wool. This mini column was then placed in a 1.5 ml eppendorf tube which serves as a collection tube. The DNA was collected by centrifugation for 15 min at 6000 r.p.m., 4°C. The DNA was recovered from the eluate by EtOH precipitation.

2.7.2 Isolation of DNA from Native PAGE

DNA plasmid preparations containing multimers of the TTFD binding site were restriction enzyme digested with appropriate enzymes and the insert isolated. In general, the digest reaction was loaded onto a 10% native PAGE gel next to molecular weight markers. The DNA was visualized by ethidium bromide staining of the gel (0.5 μg/ml EtBr in 1x TBE, for 30 min at room temperature), the insert was excised and the gel slice transferred to an eppendorf tube. The DNA was eluted overnight in 200 μl elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) overnight on a rocking platform at 37°C. The sample was then centrifuged for 1 min at 12,000xg and the supernatant transferred to a fresh eppendorf tube. The acrylamide pellet was washed once with 100 μl elution buffer, vortexed briefly and centrifuged to collect the wash. The DNA was then EtOH precipitated for 45 min at -80°C in three volumes 95% EtOH, and recovered by centrifugation at 12,000xg for 20 min at 4°C. The pellet was air dried and resuspended in a small volume of TE.

2.8 Recombinant DNA Techniques

2.8.1 Restriction Enzyme Digestion

Restriction enzyme digestion of double stranded DNA was performed according to guidelines for buffer and temperature conditions supplied with the restriction enzyme. Reactions included restriction enzyme buffer, 0.1-10 μg of DNA, and one unit of restriction enzyme (Pharmacia; Gibco-BRL, Burlington, Ontario: Boehringer Mannheim, Laval, Quebec) per μg of DNA in a final volume of 10-20 μl. Reactions were incubated at the appropriate temperature for 1-2 hr. Digested DNA was analyzed by agarose gel electrophoresis.

2.8.2 Ligation of DNA Molecules

Insert or plasmid DNA was restriction digested and purified by agarose gel electrophoresis. Quantity and quality of the DNA was analyzed by agarose gel electrophoresis prior to ligation. For vector and insert ligations a 1:5 molar ratio of vector to insert was desired. Typically 100 ng of vector was combined with the appropriate amount of insert DNA, 1 μl of 10X ligase buffer (Pharmacia), 1unit T4 DNA ligase (Boehringer Mannheim) in a 10 μl final volume. Control ligations of restriction digested plasmid alone were always performed to determine the efficiency of generation of recombinants. Reactions were incubated at 15°C overnight.

2.9 Plasmid Vectors

2.9.1 Transformation of Competent E. coli bacteria with Plasmid DNA

For the majority of experiments, *E coli* XL1-Blue was the bacterial strain of choice for transformation. An overnight culture of XL1-Blue grown in LB broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per litre (pH 7)), 15 μg/ml tetracycline, was diluted 1:100 in 10 ml LB without antibiotics. This culture was then grown at 37°C to an OD_{600} =0.3-0.4. The bacteria were collected by centrifugation for 5 min at 3000 r.p.m., 4°C and the supernatant discarded. The bacteria were resuspended in 5 ml ice cold solution 1 [50 mM $CaCl_2$, 10 mM Tris (pH 7.6)] and incubated on ice for 15 min. The cells were centrifuged a second time as above and resuspended in 700 μl ice cold solution 1 and incubated on ice for 15 min. The appropriate amount of plasmid preparation or ligation reaction was then added to a 100 μl aliquot of the bacteria and placed on ice for 30 min. The cells were then heat shocked for 120 sec at 42°C, followed by 10 sec on ice. To the cells 400 μl of LB broth was added and the mixture incubated at 37°C for 1 hr to allow expression of the ampicillin resistance marker from the plasmid. The bacteria were plated on LB-Amp plates (dried for 3 hr at 37°C) and incubated overnight at 37°C.

2.9.2 Identification of Recombinant Plasmids

Several single bacterial colonies from a transfection experiment were selected and grown overnight in 3 ml LB-broth plus 50 μg/ml amp cultures. DNA from

small scale or mini-preparations were isolated by the alkaline lysis method (Maniatis *et al.* 1982). Five microlitres of each purified plasmid were analyzed by restriction enzyme digest and agarose gel electrophoresis.

2.9.3 Plasmid Preparations

Bacterial cultures containing the desired recombinant plasmid were grown in large scale cultures (500-1000 ml). Isolation of plasmid DNA followed cell lysis by alkali method and DNA purification by cesium chloride density gradient centrifugation according to Maniatis (Maniatis *et al.* 1982).

2.9.4 Sequence Analysis of Plasmid DNA

DNA sequences were determined manually using a Cycle Sequencing Kit (Pharmacia), using the dideoxy method. The labeling step is combined with the primer extension/chain termination reactions.

2.10 Preparation of DNA Radiolabeled Probes

2.10.1 Probes for Electrophoritic Mobility Shift Assays

All oligonucleotide sequences used were generated by the University Core DNA Services. Single stranded oligos were labeled by a PNK reaction with γ^{32}P-ATP. Double stranded oligos were prepared as follows. Equal concentrations of sense and anti-sense oligos were mixed and placed in a heating block at 100°C for five minutes. The temperature was then adjusted to Tm minus 5°C and the heating block allowed to slowly cool to the appropriate temperature. Oligos were

annealed overnight. Double stranded oligos were then labeled by either a Klenow reaction with one of the nucleotides radiolabeled (α^{32}P-dATP, 3000 Ci/millimol), or by a PNK reaction with γ^{32}P-ATP(3000 Ci/millimol) followed by a cold Klenow reaction. Unincorporated radioactive nucleotides were removed on a G-25 sephadex spun column according to (Maniatis *et al.* 1982).

2.10.2 Random Priming

Probes for screening λgt11 expression libraries or Southwestern blots were radiolabeled by random priming. In an eppendorf tube 1 μl dATP, dGTP, dTTP (5 mM), 2 μl 20 mM DTT, 2 μl 5X random prime buffer, 1 μl pdN_6 (30ng, Pharmacia, Baie d'Urfe, Quebec) and the insert DNA were mixed and adjusted to a final volume of 16 μl. This reaction was boiled for 3 min and after allowing to cool slightly, 3 μl α^{32}P-dCTP(3000 Ci/millimol) and 1 μl Klenow were added. The random prime reaction was incubated at 37°C for 1 hr and the radiolabeled probe separated from unincorporated nucleotides on a G-25 sephadex spun column according to (Maniatis *et al.* 1982). One microlitre of the reaction was then counted in a LS 5000 CE beta counter (Beckman) to determine the specific activity of the probe.

Chapter 3: Results

The molecular mechanisms that regulate testis-specific transcription are largely uncharacterized. Through the use of transgenic mice, the promoter elements which regulate testis-specificity of several testis-specific genes have been delineated (Langford *et al.* 1991; Peschon *et al.* 1989; Robinson *et al.* 1989). Transcription factors that interact with the promoter elements in testis-specific genes however, remain largely unknown or poorly characterized. In 1992, prior to my joining the lab, two reports were published which identified factors that interacted with the testis-specific gene promoters, RT7 and c-*mos* (Figure 2) (van der Hoorn 1992; van der Hoorn & Tarnasky 1992). The main focus of my doctoral thesis was to characterize and clone one of these factors, TTF-D. A secondary project involved further characterizing the interaction of the testis-specific transcription factor CREMτ with its binding site within the RT7 promoter.

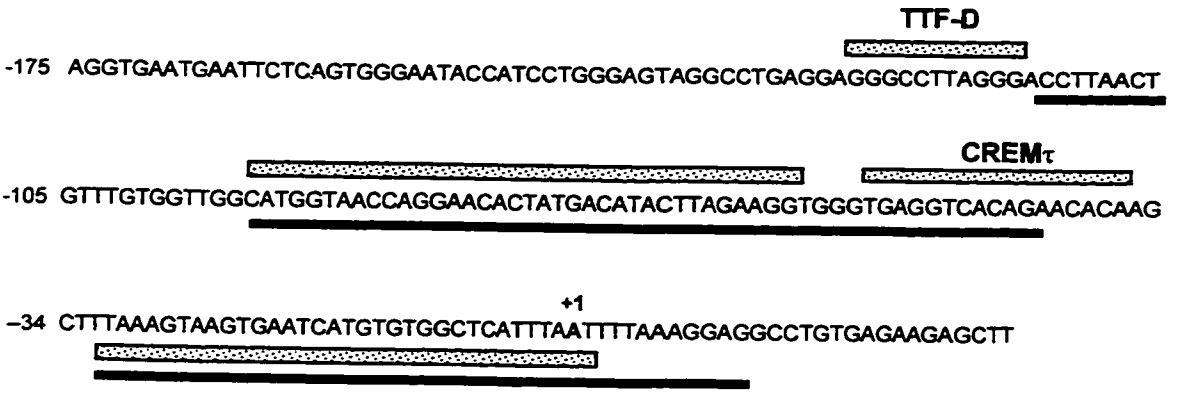
TTF-D was identified as a testis-specific transcription factor which positively regulates RT7. A region of the RT7 promoter (RT7D1) important for transcription, as assayed *in vitro*, was also shown via DNase I footprinting to bind a testis specific protein. The nucleotide sequence for this binding site was determined and gel retardation assays with seminiferous tubule (ST) nuclear extracts generated four specific complexes (van der Hoorn & Tarnasky 1992). A homologous binding site to RT7D1, was also found in the testis c-*mos* promoter (cmosD1). Gel retardation assays showed a relatedness between the testis-specific protein binding the c-*mos* promoter element and TTF-D (van der Hoorn 1992). It was hoped that further characterization and cloning of TTF-D would

Figure 2. DNA-Protein interactions in the RT7 and *cmos* Promoters

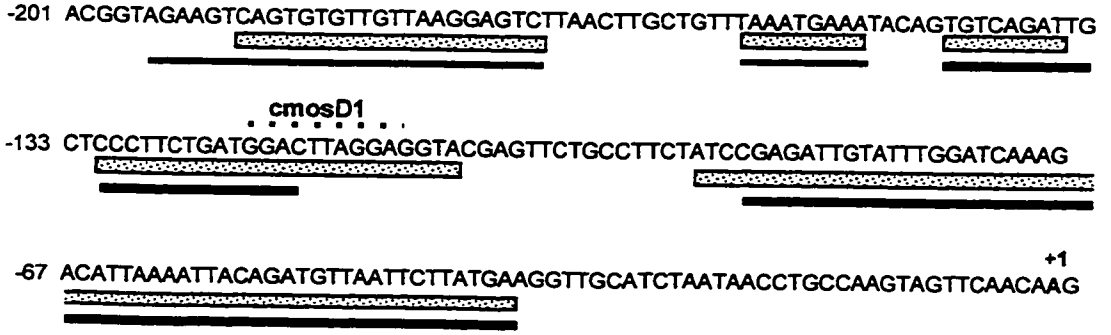
(A). Location of DNA-protein interactions relative to the RT7 promoter as deduced by DNase I footprinting experiments. Binding of testis nuclear proteins (stippled bars) includes TTF-D and CREMτ proteins. Footprints generated by liver nuclear proteins are also shown (solid bars), as is the transcription start site (**+1**) (van der Hoorn & Tarnasky 1992).

(B). Location of DNA-protein interactions relative to the *cmos* promoter as deduced by DNase I footprinting experiments. Testis nuclear protein (stippled bars) and liver nuclear protein (solid bars) footprints are indicated. The cmosD1 binding site, which binds TTF-D, is indicated by a dotted line, the transcription start site is shown as (**+1**) (van der Hoorn, 1992).

A. RT7 Promoter



B. *cmos* Promoter



identify a testis-specific transcription factor that in part regulated several testis-specific genes and therefore help explain the molecular basis of male germ cell-specific transcription.

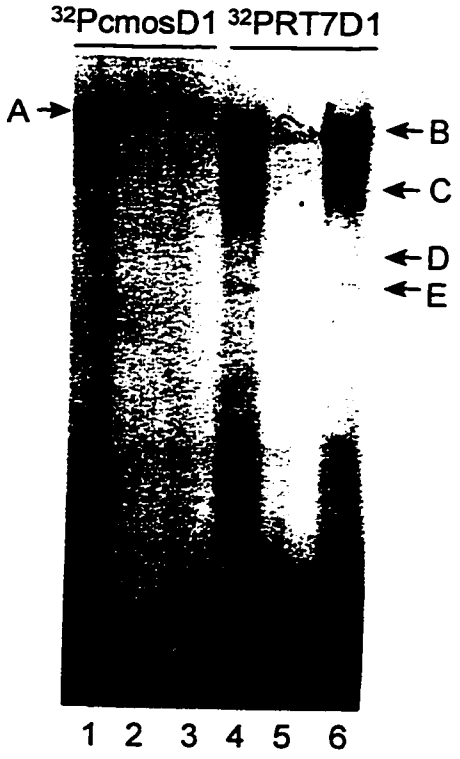
3.1 The TTF-D Consensus Sequence and Binding Characteristics

3.1.1 Interaction of TTF-D with the cmosD1 and RT7D1 Binding Sites.

The binding sites for a testis-specific nuclear factor within the RT7 promoter 5'-GGCCTTAGGG-3' and c-*mos* promoter 5'-GGACTTAGGA-3', are 80% identical and may bind a common testis-specific nuclear protein, TTF-D. Interaction of TTF-D with each binding site generates a specific banding pattern in a gel retardation assay (Figure 3). Incubation of ST nuclear extracts with the cmosD1 oligo gives a pattern of one major band (lane 1, complex A), whereas TTF-D binding RT7D1 generates four major bands (lane 4, complexes B-E) (note: complex A is also generated by TTF-D binding RT7D1 oligo; see below). The observed DNA-protein interactions can be efficiently competed by the self-oligo (lanes 2 and 5). Furthermore the RT7D1 oligo efficiently competes for formation of the TTF-D:cmosD1 complex (lane 3) and the cmosD1 oligo competes well for formation of the TTF-D:RT7D1 complex (lane 6) in cross-competition assays. Competition experiments using excess unrelated oligo indicated that the interaction of TTF-D with the cmosD1 binding site and RT7D1 binding site is specific (not shown) in agreement with our previous results (van der Hoorn 1992; van der Hoorn & Tarnasky 1992).

Figure. 3. TTF-D binds both c-mosD1 and RT7D1 oligos.

Gel retardation assays with indicated radiolabeled c-mosD1 oligo (lanes 1-3) and RT7D1 oligo (lanes 4-6) generate specific banding patterns (A-E). Competition with unlabeled excess (100 ng) cmosD1 oligo (lanes 2 and 6) or RT7D1 oligos (lanes 3 and 5) is specific.


32PcmosD1
32PRT7D1
A
B
C
D
E
1 2 3 4 5 6

3.1.2 Binding Pattern of TTF-D Varies with Different ST Extracts

Seminiferous tubule nuclear extracts prepared from rat testis were used as a source of nuclear proteins in characterizing TTF-D. Over the course of this thesis work, several extracts were prepared and used. The protein concentration of each extract varied, see Table 1. With this in mind, each extract prepared was tested for its binding properties in gel retardation assays, and also for its activity in *in vitro* transcription assays (not shown). Figure 4 shows a comparison of several ST extracts gel shifted with the RT7D1 oligo as probe. The TTF-D:DNA complexes are indicated A-E. Liver nuclear extract (LVI) controlled for somatic proteins binding to the probe. It was evident that the amount or even presence of each complex varied depending on the extract. Not all extracts prepared were active in *in vitro* transcription assay (summarized in Table 1), but a correlation between activity and TTF-D:DNA complexes present in EMSA could not be made. It appeared that the TTF-D protein(s) in ST nuclear extracts were not always present when assayed for by EMSA, possibly due to these proteins being highly unstable, or present in very low levels in the testis.

3.1.3 Phosphorylation State of TTF-D Does Not Affect the Binding Pattern

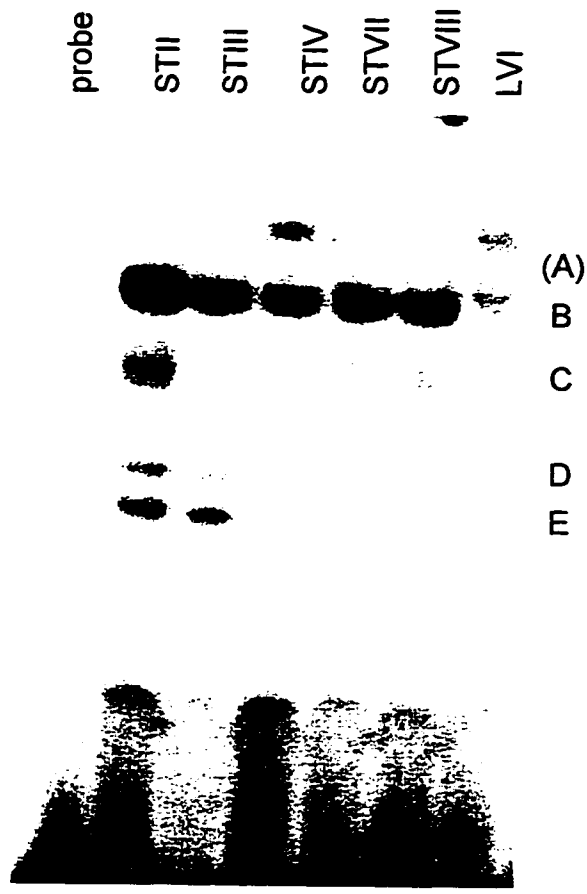
The possibility that phosphorylation of TTF-D might be playing a role in generating the multiple complexes seen in gel shift assays was examined. ST extracts were incubated in the absence or presence of calf intestinal phosphatase prior to EMSA analysis. Figure 5 shows that no significant change in the gel shift pattern of ST extracts (lanes 2 and 4) was seen upon treatment of the extracts with CIP (lanes 3 and 5). Binding of CREMτ to the RT7CRE binding

Table 1. Protein Concentration of Nuclear Extracts.

Nuclear Extracts	**μg/μl**	**active *in vitro***
LVI	9.6	YES
STII	22	YES
STIII	6.35	N/D
STIV	7.5	YES
STOA	6.9	YES
STVII	10.7	YES
STVIII	9.2	N/D
STIX	14.37	YES
STX	16.2	YES
STXI	9.7	NO
STXII	5.86	NO
STXIII	5.9	NO
STXV	6.41	YES
STXVI	4.05	YES
Crude Extracts		
STXIV	8.8	N/D
TAC	11.4	N/D
T11	4.02	N/D
T16	1.6	N/D
T21	4.54	N/D
T25	5.44	N/D

Figure. 4. EMSA of various ST nuclear extracts.

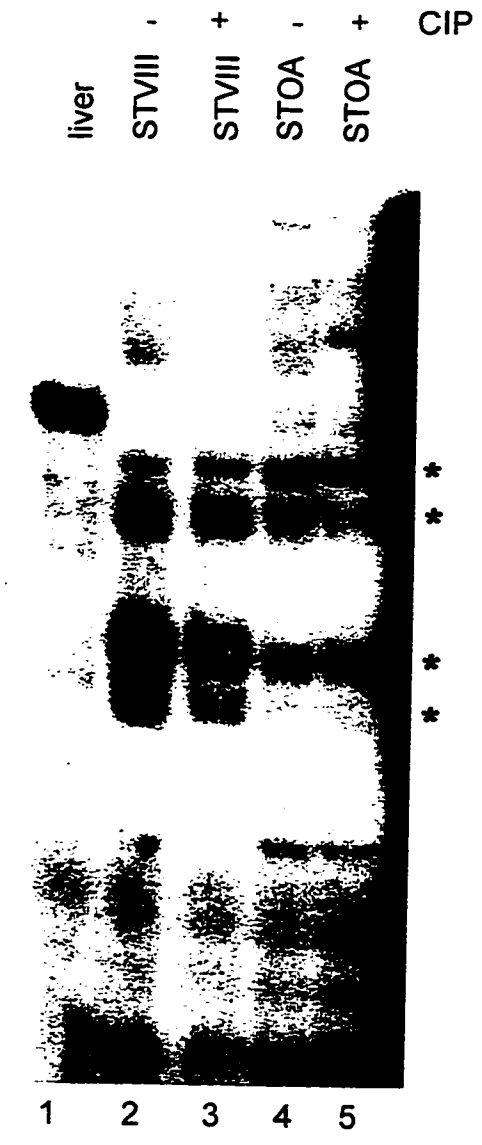
Ten micrograms of ST or liver (LVI) nuclear extracts were incubated with ^{32}PdsRT7D1 oligo. Five major protein complexes are generated which are testis specific (A-E) although the complexes A, D and E are not always present (extract dependent). None of the four complexes generated are present when liver nuclear extract is used.



ds^{32}PRT7D1

Figure. 5. EMSA of ST nuclear extracts treated with calf intestinal phosphatase.

Double stranded ^{32}PRT7D1 oligo probe(1ng) and 2 μg dIdC were incubated with liver (lane 1), or ST extracts (lanes 2-5). STVIII alone (lane 2), STVIII plus CIP (lane 3), STOA alone (lane 4), STOA plus CIP (lane 5). No changes in the TTFD protein complexes formed (*) were seen in the presence of CIP.



^{32}PdsRT7D1

site was seen to decrease in the presence of CIP as expected (not shown). STOA was an extract prepared in the presence of the phosphatase inhibitor okadaic acid. The gel shift pattern of this extract did not significantly differ from other ST extract preparations.

3.2 TTF-D is Expressed Early in Post-Partum Development

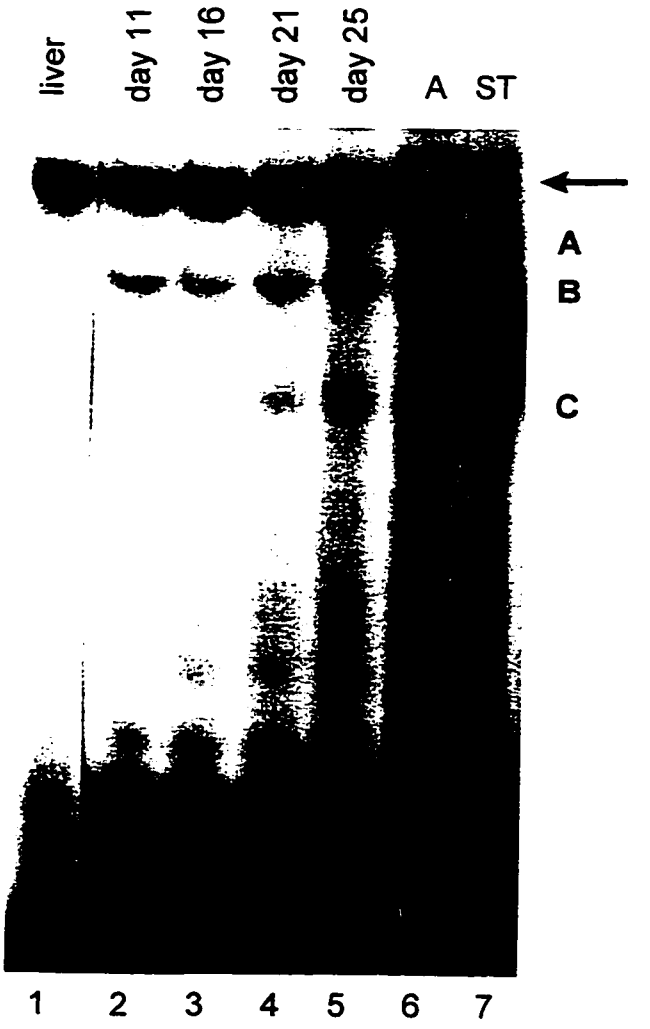
3.2.1 Developmental Expression of TTF-D in Mouse Testes

The c-*mos* and RT7 testis-specific promoters are active in pachytene spermatocytes and round spermatids, respectively. In the mouse, pachytene spermatocytes are produced for the first time at day 12-14 after birth, whereas round spermatids are not produced until days 21-22 after birth. To determine if TTF-D is present in the mouse testis at the time of expression of the c-*mos* and RT7 genes, nuclear extracts were prepared from total testes of mice 11, 16, 21, 25 days old, and adult mouse. These extracts were analyzed for TTF-D activity in gel retardation assays. The total testis proteins were compared to seminiferous tubule nuclear proteins (predominantly derived from germ cells) and liver nuclear proteins (somatic cells). The results shown in Figure 6 demonstrate that TTF-D activity can be detected as early as 11 days after birth (lane 2). TTF-D activity increases over time into adulthood (lanes 2-6). The uncharacterized somatic protein that also binds to the probe is present in all total testis extracts (lanes 2-6, arrow) and in liver nuclear extract (lane 1, arrow). Extracts derived from germ cells contain little of this somatic cell nuclear protein as expected (lane 7, arrow). These experiments show that TTF-D is present at

the time of expression of both the c-*mos* and RT7 genes.

Figure. 6. Developmental Expression of TTF-D.

Gel retardation assay of nuclear extracts from liver (lane 1), mouse testis days 11, 16, 21, 25, adult mouse testis (A) and seminiferous tubules (ST). Equal concentrations of proteins were incubated with ^{32}PdsRT7D1 oligo. A somatic cell protein is detected in liver and mouse testis (arrow) but not in ST extract. TTF-D DNA complexes are indicated by letters A-C.



^{32}PdsRT7D1

3.2.2 *TTF-D is Expressed in Rat Spermatocytes and Spermatids*

The use of centrifugal elutriation techniques allowed for the isolation of germ cell fractions from rat testes consisting of spermatids or spermatocytes. Nuclear extracts were then made from these germ cell fractions to be assayed for TTF-D. Figure 7 compares the gel shift pattern for ST nuclear extract with RT7D1 or cmosD1 oligos as probe (lanes 1, 2, 3, and 8 respectively), to the gel shift pattern of elutriated fractions with RT7D1 probe (lanes 4, 5, 6, and 7). The gel shift pattern generated by the spermatocyte (lanes 4 and 5) and spermatid (lanes 6 and 7) nuclear proteins appeared identical. These results show that the use of decreasing amounts of STII extract increased the amount of complex A relative to the amount of complexes B and C (lanes 1-3). Complex A co-migrates with the complexes generated by pachytene spermatocyte and spermatid nuclear extracts (lanes 5 and 7). Furthermore, this analysis suggested that the TTF-D:cmosD1 complex co-migrates with complex A (lane 8).

3.3 Size Determination of TTF-D Protein(s)

3.3.1 *Approximate Molecular Weight of TTF-D:DNA Complexes*

To further characterize TTF-D, preliminary experiments were performed to determine the approximate molecular weight of each of the TTF-D:DNA complexes generated in gel shift assays. ST extract was incubated with radiolabeled RT7D1 oligo under standard gel shift conditions. Prior to loading on a native polyacrylamide gel however, reactions were UV-irradiated. The wet gel was exposed to film and complexes A-E, (Figure 8, Panel 1) were excised. The

Figure. 7. EMSA of nuclear extracts from elutriated spermatocytes and spermatids.

STII nuclear extract (22 μg, lane 1; 6.6 μg, lane 2; 2.2 μg, lane 3; 22 μg, lane 8) or nuclear extract (4.5 and 9 μl) from elutriated pachytene spermatocytes (lanes 4 and 5) or spermatids (lanes 6 and 7) were incubated with ^{32}PdsRT7D1 (lanes 1-7) or ^{32}PdscmosD1 (lane 8).

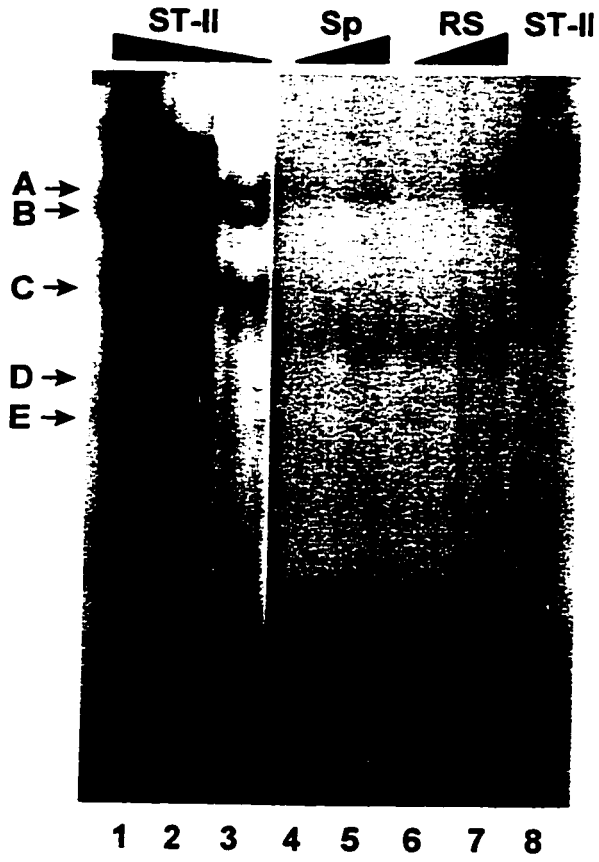
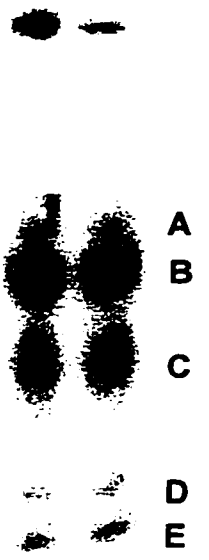

ST-II
Sp
RS
ST-II
A
B
C
D
E
1 2 3 4 5 6 7 8

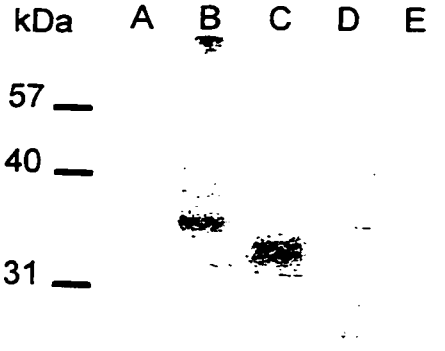
Figure. 8. UV-crosslinking analysis of TTF-D proteins.

Radiolabeled dsRT7D1 oligo was incubated with ST extract, UV-irradiated and complexes were resolved on a non-denaturing polyacrylamide gel. Complexes A-E (panel 1) were excised, eluted and analyzed by SDS-PAGE using a 12% SDS-PAGE (panel 2), or an 8% SDS-PAGE (panel 3).

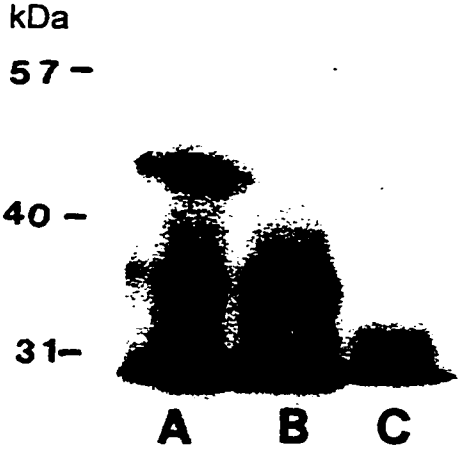
1.



2.



3.



radiolabeled protein:DNA complexes were then eluted according to the Materials and Methods and resolved by SDS PAGE, followed by autoradioaugraphy (Panels 2 and 3). Protein:DNA complex molecular weights were estimated as follows: A-45 kDa, B-35 kDa, C-32 kDa. After subtracting the molecular weight of the oligonucleotide (approximateiy 12 kDa), the molecular weight of the TTF-D proteins were estimated to be 33 kDa, 23 kDa, and 20 kDa. Complexes D and E, which are generated by proteins in the 30-40 kDa range (see below) were apparently not present in high enough concentrations to be visualized by this assay.

3.3.2 Size Fractionation of ST extract and EMSA of Eluted Fractions

Following estimation of TTF-D protein size, a larger quantity of protein was needed for analysis. Large quantities of ST nuclear extracts (800-1000μg) were loaded over 5-8 lanes on gels and separated by SDS-PAGE. Nuclear proteins in the size range 15-25 kDa, 25-30 kDa, and 30-40 kDa, were isolated as described in Materials and Methods. These molecular weight ranges were confirmed by silver staining (Figure 9, lanes 4-6). Some overlap in the size of fractionated proteins was seen, this could be due to overloading of protein in the lanes or the tendency of SDS gels to 'smile' when run. The isolated proteins were renatured and assayed for TTF-D activity by gel retardation using labeled RT7D1 as probe. Figure 10 shows the band shift pattern generated by total ST extract (lane 1) and the pattern generated by the size fractionated ST proteins (lanes 2-8). Proteins in the 30-40 kDa range bind to the RT7D1 oligo to generate three specific complexes A, D and E (lane 2). The proteins in the 25-30 kDa

Figure. 9. Silver staining of eluted protein fractions.

ST extracts were separated on SDS-PAGE and proteins ranging from 30-40 kD, 25-30 kD, and 15-25 kD were eluted and analyzed by SDS-PAGE and silver staining. BSA (*) was added during precipitation of eluted proteins (lanes 4-6). Molecular weight markers (lanes 1 and 2) indicate that the eluted fractions contain proteins in the following molecular weights. Lane 4: 31-40 kD, lane 5: 23-33 kD, and lane 6: 16-23 kD. Total ST extract is shown in lane 3.

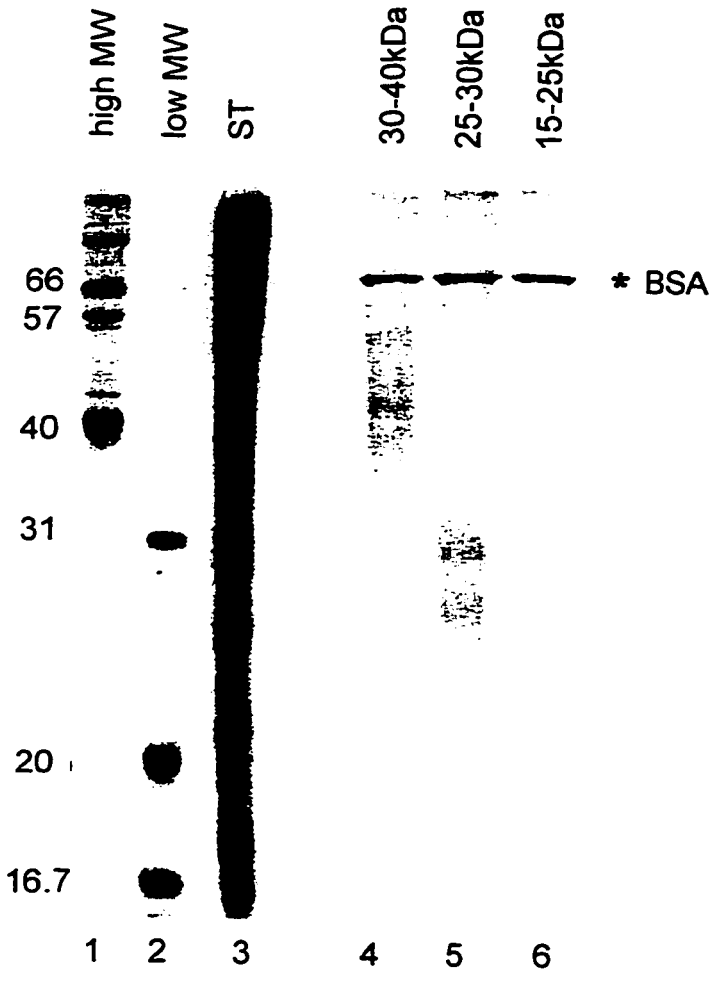
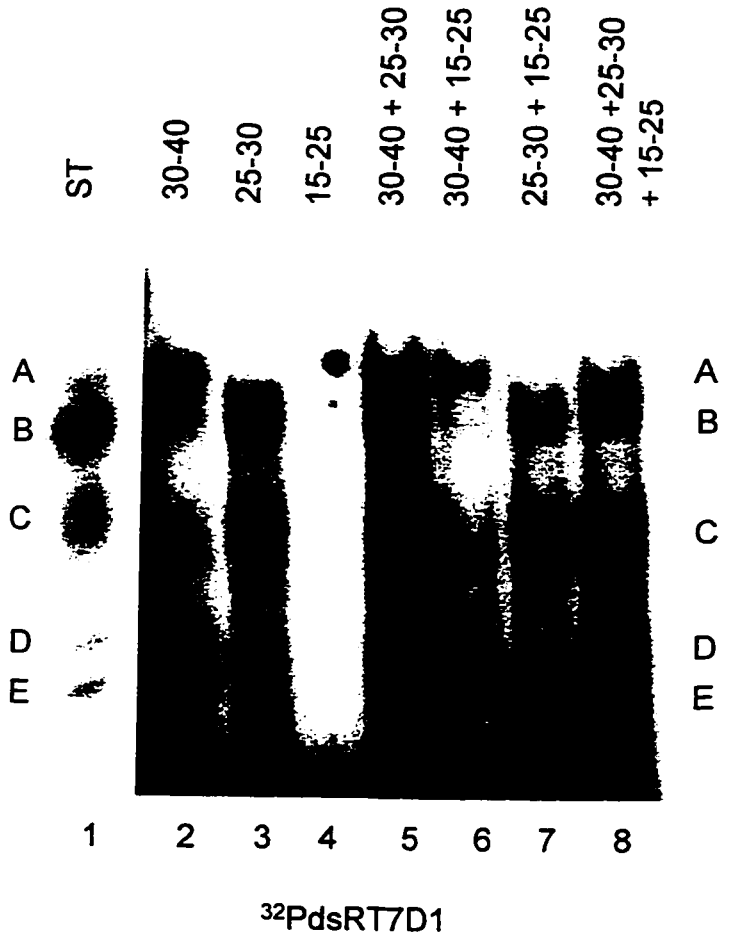

high MW
low MW
ST
30-40kDa
25-30kDa
15-25kDa
66
57
40
31
20
16.7
* BSA
1
2
3
4
5
6

Figure. 10. Proteins in the 25-40kD range regenerate the TTF-D gel shift pattern.

Gel retardation assay of control extracts and eluted ST proteins using radiolabeled dsRT7D1 as probe. TTF-D complexes generated by ST extract (lane 1) are indicated by the letters A-E. Eluted protein fractions were 30-40 kD (lane 2), 25-30 kD (lane 3), and 15-25 kD (lane 4) in size. The 30-40 kD fraction was mixed with the 25-30 kD fraction (lane 5) and the 15-25 kD fraction (lane 6). Proteins from the 25-30 kD fraction were mixed with those from the 15-25 kD fraction (lane 7). Finally, all three fractions were combined (lane 8).


ST
30-40
25-30
15-25
30-40 + 25-30
30-40 + 15-25
25-30 + 15-25
30-40 +25-30
+ 15-25
A
B
C
D
E
A
B
C
D
E
1
2
3
4
5
6
7
8

^{32}PdsRT7D1

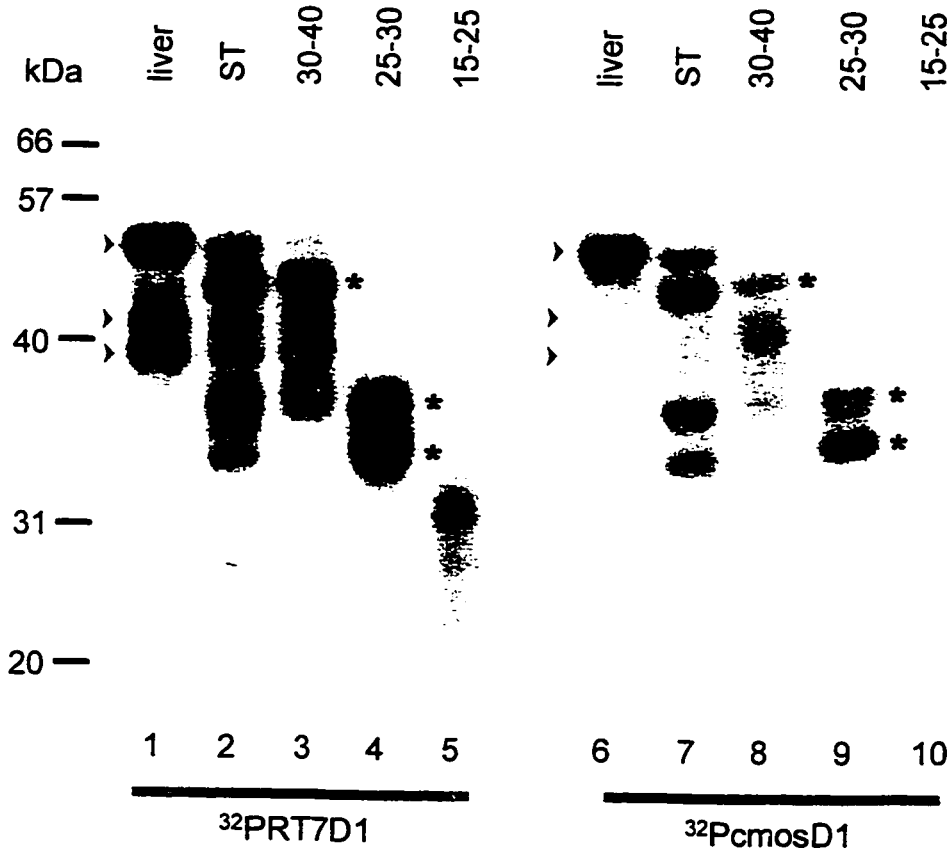
fraction generate the complexes B and C, but not A, D or E (lane 3). No complexes were formed using proteins ranging from 15-25 kDa (lane 4).This was surprising as the estimated sizes of TTF-D proteins were 33, 23, and 20 kDa. The overlap seen in the silver stain gel (Figure. 9) may account for this size discrepancy. No complexes other than the complexes generated by total ST extracts were observed when the fractions were mixed in pairs (lanes 5-7), or when all three fractions were combined (lane 8). Thus the complete TTF-D gel retardation pattern generated by ST nuclear extracts can be reproduced by proteins present in the 25-30 kDa and 30-40 kDa fractions.

3.3.3 Molecular Weight Determination of TTF-D Protein:DNA Complexes

Fractionated proteins, RT7D1 oligo, and cmosD1 oligo were next used in UV-crosslinking assays to determine the molecular weights of protein:DNA complexes. We had previously shown that these two oligos, which specifically interact with TTF-D in germ cell-derived nuclear extracts, can bind a nuclear protein present in somatic cells (van der Hoorn 1992; van der Hoorn & Tarnasky 1992). Therefore, control experiments were performed with liver nuclear extracts in all UV-crosslinking assays to distinguish interactions of the two oligos with TTF-D constituents from interactions with somatic protein(s). The results are presented in Figure 11. Liver or ST nuclear extracts were incubated with radiolabeled RT7D1 (lanes 1-5) or cmosD1 (lanes 6-10) and the UV crosslinked protein:DNA complexes resolved by SDS-PAGE. Three somatic proteins (arrowheads) were crosslinked in both liver (lanes 1 and 6) and ST fractions (lanes 2, 3, 7, and 8). The amount of somatic protein in ST fractions was, as

Figure. 11. Molecular weight determination of TTF-D protein:DNA complexes.

Liver, ST nuclear extracts and eluted ST proteins were UV crosslinked to ^{32}PdsRT7D1 (lanes 1-5) or ^{32}P dscmosD1 (lanes 6-10) oligos. The protein:DNA complexes were then resolved by SDS-PAGE. Liver extract, (lanes 1 and 6), ST extract, (lanes 2 and 7). Three major protein:DNA complexes of approximately 48, 37 and 34 kD (*) specific to testis are formed in the 30-40 kD and 25-30 kD fractions (lanes 3,4, and 8,9 respectively). Complexes seen in the 15-25 kD fraction (lanes 5 and 10) do not contribute to TTFD binding seen in gel retardation experiments. Arrowheads indicate complexes containing somatic cell derived protein(s).


kDa
liver
ST
30-40
25-30
15-25
liver
ST
30-40
25-30
15-25
66
57
40
31
20
1 2 3 4 5
6 7 8 9 10
32PRT7D1
32PcmosD1

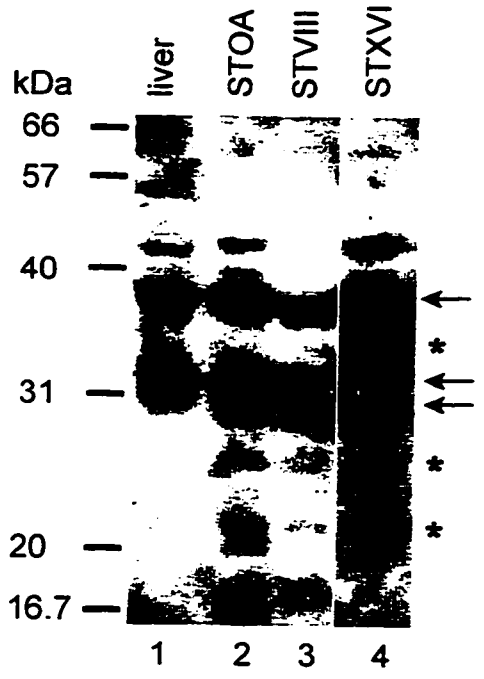
expected, less, as seminiferous tubules contain fewer than 5% somatic cells. Total ST extract generated three testis specific bands with each of the probes used (lanes 2 and 7). Eluted fractions in the 30-40 kDa range generated the slowest migrating TTF-D complex (star, lanes 3 and 8). The remaining two TTF-D complexes were generated by proteins in the 25-30 kDa range (stars, lanes 4 and 9). The protein complexes seen in the 15-25 kDa range (lanes 5 and 10) can crosslink to the probe but do not contribute to the total ST extract pattern seen in this assay or in gel retardation assays (see Figure 10, lane 4). These proteins could be degradation products of TTF-D proteins that do not gel shift but could be crosslinked to the DNA probe. The molecular weights of the three TTF-D complexes binding to RT7D1 and cmosD1 were calculated to be 36 kDa, 25 kDa and 22 kDa (after subtraction of the molecular weight of each respective probe). These molecular weights correlate well with the preliminary results of 33, 23, and 20 kDa seen above. The fact that a protein of apparent 22 kDa molecular weight was consistently isolated in the 25-30 kDa gel slice may be due to the isolation protocol in which the molecular weight marker lanes of the gel are removed for cold KCl staining and then realigned to the gel. This gel section was seen to swell slightly during the procedure and may account for inappropriate assignment of molecular weights to the isolated protein fractions. It was clear however, from the above experiments, that the cmosD1 and RT7D1 oligos bind three testis-specific nuclear proteins that have the same molecular weights.

In light of the discrepancies in the above experiments with the molecular weights of the TTF-D proteins, it was important to confirm the molecular weight of TTF-D binding proteins under non-crosslinking conditions. This was accomplished using Southwestern assays. Figure 12 shows various ST extracts (lanes 2-4) and a control liver extract (lane 1) probed with a concatamerized probe of the mutated RT7D1 oligo, MUT3 (Table 2). In gel shift assays (see below) MUT3 oligo was shown to bind ST proteins but not liver proteins and the same was expected in Southwestern assays. Somatic proteins however were bound by this probe and are indicated by arrows. The testis-specific proteins in ST extracts are indicated by stars. The difference in presence or concentration of TTF-D protein(s) seen in gel shift experiments (see above) was also reflected in the Southwestern assay, compare lanes 2, 3, and 4. The molecular weights of the TTF-D proteins were 36 kDa, 25 kDa and 22 kDa. The Southwestern assay was repeated using several TTF-D binding site oligos (cmosD1-multimer, dsRT7D1, RT7D1-sense, and TTFD-multimer), each of which generated similar results as shown in Figure 12 (data not shown).

3.4 Analysis of the TTF-D Binding Site Consensus Sequence

As was shown above, the cmosD1 and RT7D1 nucleotide sequences can be crosslinked to testis-specific proteins of the same molecular weights. The two nucleotide changes in the cmosD1 site therefore are not considered essential for TTF-D binding. These changes may however affect the stability of binding as in EMSA under non-crosslinking conditions, the cmosD1:TTF-D binding pattern

Figure. 12. Southwestern analysis of liver and ST nuclear proteins. Liver (lane 1) and ST extracts (lanes 2-4) processed for Southwestern analysis were probed with a concatamer of the MUT3 binding site, ^{32}PMUT3-concatamer. TTFD proteins of 36, 25, and 21 kDa are seen in the ST lanes only (*). The presence of the TTFD bands however varies with the ST extract used. Somatic proteins are indicated with arrows.



^{32}PMUT3-concatamer

Table 2. Oligonucleotides.

RT7D1	a a t t G G C C T T A G G G
cmosD1	t t G G A C T T A G G A
MUT1	a a t t C T C C T T A G G G
MUT2	a a t t G G C A C G A G G G
MUT3	a a t t G G C C T T C T A G
TTFD	T A G G C C T G A G G A G G G C C T T A G G G A C
D2	a a t t A C C T T A A C T G
D1screen	A G G A G G G C C T T A G G G A C C
RT7CREMτ	a a t t G G G T G A G G T C A C
SMS	G A T C C T T G G C T G A C G T C A G A G A G A G
FOOTIII	G A T C A A T A T G G C A T C C T T A T G
B	a a t t G T G G C T C C T G C C C
ΔT	a a t t A C A G A A C A C A A
Primers:	
T7primer	A A T A C G A C T C A C T A T A G
tCREM sense	C A A G G A T C C A T G A C C A T G G A A A C
tCREM α-sense	G A A T C T A G A C T A A T C T G T T T T G G G

was observed to be significantly weaker and more difficult to generate than the RT7D1:TTF-D binding pattern (not shown). The two nucleotides that differ in the cmosD1 binding site could be playing a role in the binding of somatic proteins. Figure 13 shows a significant difference in binding of liver nuclear proteins crosslinked to either RT7D1 oligo or cmosD1 oligo probe (lanes 1 and 3 respectively, arrows; see also Figure 11, lanes 1 and 6, and Figure 14, lanes 1 and 7). This difference was not always seen however, (see Figure 17 lanes 1 and 5). The significance of this difference was not pursued.

3.4.1 Proteins of the Same Molecular Weight Crosslink To, and are Specific for the RT7D1 and cmosD1 Binding Sites.

In gel shift assays it was shown that the RT7D1 and c*mos*D1 promoter elements bind highly related proteins based on cross competition with these two binding site oligos (Figure 3). Under crosslinking conditions, the proteins binding to each of these oligos were determined to have the same molecular weight both in size separated fractions (Figure 11), and ST extract (Figure 13). Competition assays were then used to identify which of the 36, 25, and 22 kDa TTF-D proteins could be specifically competed for by the RT7D1 and cmosD1 binding sites. ST nuclear extracts were incubated with either RT7D1 or cmosD1 probe in the presence or absence of competitor oligo. Following UV crosslinking the complexes were resolved by SDS PAGE. Figure 14 shows the three TTF-D complexes generated with either RT7D1 oligo (lane2, stars) or cmosD1 oligo (lane 8, stars). TTF-D protein(s) binding to RT7D1 probe were specifically competed for by addition of increasing amounts of cold dsRT7D1 oligo (lanes 3

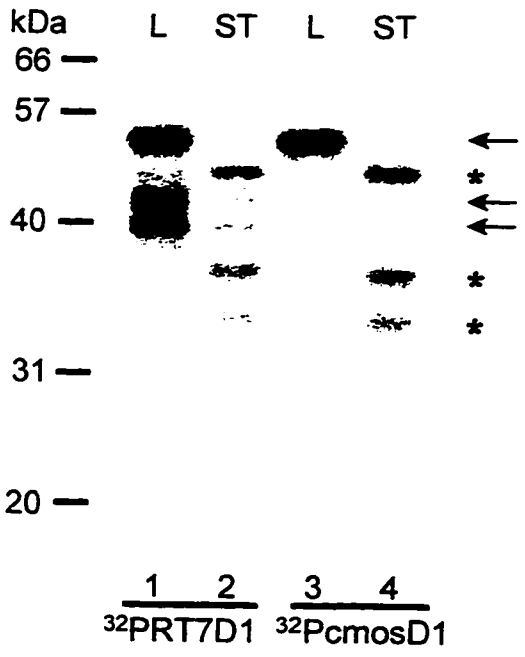
and 4) or cold dscmosD1 oligo (lanes 5 and 6). Similarly TTF-D protein(s) binding to cmosD1 probe were specifically competed for by addition of cold dscmosD1 oligo (lanes 9 and 10) and cold dsRT7D1 oligo (lanes 11 and 12). In each instance, self competition of the oligo was more considerably more efficient than cross competition. These results reflect the EMSA competition results shown in Figure 3, and confirm that proteins of the same molecular weight specifically bind both the RT7D1 and cmosD1 binding sites.

The difference in binding of somatic proteins, as shown previously, to the RT7D1 and cmosD1 probes (lanes 1 and 7 respectively) was seen here again. The faster migrating doublet of somatic proteins was not competed for by addition of cold cmosD1 oligo (lanes 5 and 6, arrowheads) but were efficiently competed for by cold RT7D1 (lanes 3 and 4).

Figure. 13. RT7D1 and cmosD1 oligos crosslink proteins of the same molecular weight.

Liver (L) or ST nuclear extracts were incubated with 2 μl dIdC, 50 ng RT7crem, and radiolabeled RT7D1 or cmosD1 and the reactions UV irradiated prior to resolving the complexes by SDS-PAGE. Three major testis specific protein:DNA complexes (*) of approximately 48, 37, and 34 kD are generated with both oligos.

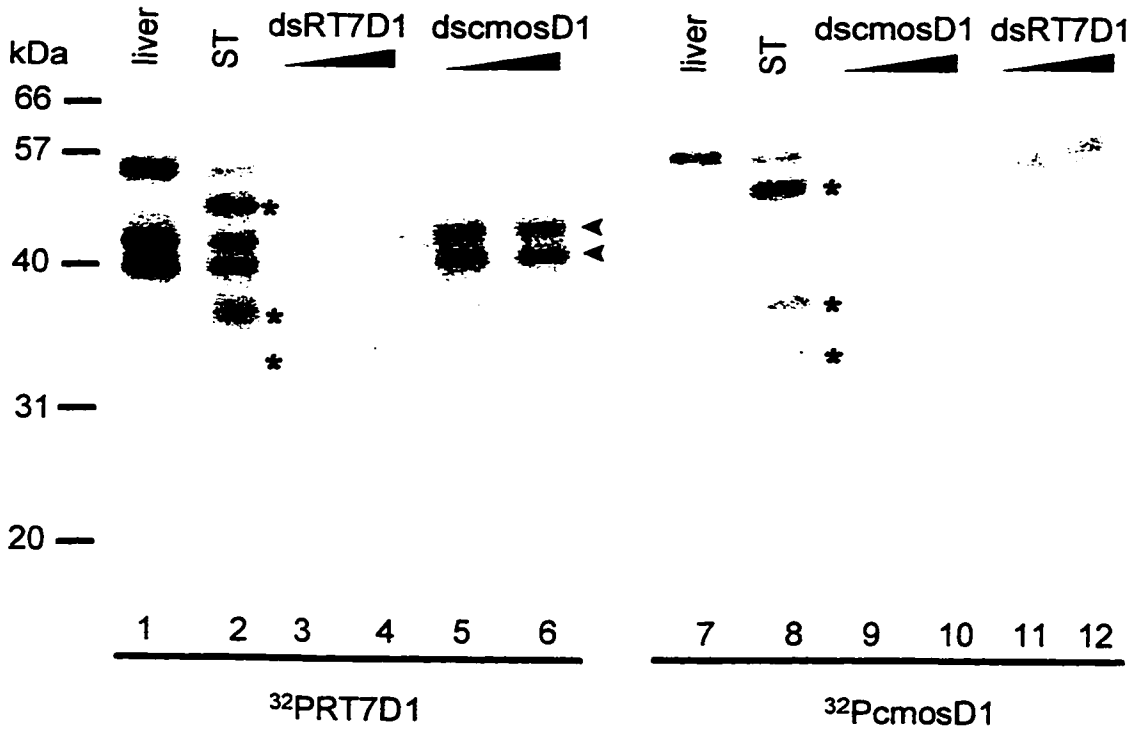
Arrows indicate complexes containing somatic cell derived protein(s).



RT7D1: aattGGCCTTAGGG
cmosD1: ttGGACTTAGGA

Figure. 14. dsRT7D1:TTF-D and dscmosD1:TTF-D complexes are self and cross competed.

Liver (lanes 1 and 7) or ST (lanes 2-6 and 8-12) nuclear extracts were incubated with ^{32}PdsRT7D1 or ^{32}P dscmosD1, 2 μg dIdC, and 50 or 100 ng competitor oligo, followed by UV crosslinking. Complexes were resolved by SDS-PAGE. TTF-D complexes (*) binding to dsRT7D1 could be competed by addition of 50 and 100 ng dsRT7D1 (lanes 3 and 4), and 50 and 100 ng dscmosD1 (lanes 5 and 6). Similarly, TTF-D complexes (*) binding to dscmosD1 could be competed by 50 and 100 ng cmosD1 (lanes 8 and 9) and 50 and 100 ng dsRT7D1 (lanes 11 and 12).


kDa
liver
ST
dsRT7D1
dscmosD1
liver
ST
dscmosD1
dsRT7D1
66
57
40
31
20
1 2 3 4 5 6
7 8 9 10 11 12
32PRT7D1
32PcmosD1

3.4.2 *TTF-D Also Binds Single Strand Binding Sites*

During the course of this thesis work, due to available materials, the method of radiolabeling oligonucleotides for probes was changed from Klenow labeling to end labeling using PNK followed by a cold Klenow reaction to fill in the ends of the double stranded oligo. Whereas Klenow labeling generates radiolabeled oligos that are double stranded, labeling with PNK results in both single and double stranded radiolabeled oligos. After annealing and prior to labeling, double stranded oligos were not gel purified away from the remaining population of single stranded oligos. The use of a mixed population of both single and double stranded probes, suggested that TTF-D may be binding a single stranded binding site in addition to its regular double stranded binding site. Interestingly, a comparison between a double strand probe and one that was denatured by boiling in EMSA with ST extracts revealed that the TTF-D complexes were generated equally well by either probe (not shown). A study of the single stranded binding properties of TTF-D was then initiated.

EMSA assays using either the sense or anti-sense strand of RT7D1 as probes are shown in Figures 15 and 16. Binding of TTF-D to the RT7D1-sense site in the absence of competitor oligos generated TTF-D complexes whose pattern was very similar to that generated by double stranded probes (Figure 15, lane 1). Competition with ds, sense, or αsense RT7D1 oligos (lanes 2, 3, 4 respectively) revealed that all three configurations of oligo could compete well for TTF-D binding. RT7D1-αsense however, appeared to have the highest affinity for TTF-D proteins. CmosD1 sense and αsense oligos could also compete for

TTF-D complex formation (lanes 5 and 6), with the sense sequence being a stronger competitor than the αsense. A non-specific oligo B (see Table 2) was also used in competition assays to verify that the TTF-D complexes were specific and not aspecific single stranded binding proteins. As well, B-oligo was designed with the same AATT end sequence for labeling purposes and to ensure that the protein complexes seen as TTF-D were not just binding to the common sequence at the ends of the oligonucleotides used as probes or competitors. No competition was seen by either B-sense or B-αsense oligos (lanes 7 and 8). In some experiments addition of B-oligo was seen to enhance binding of TTF-D (see below).

TTF-D complexes were also seen when RT7D1-αsense was used as probe in gel shift assays, Figure 16 (lane 1). These complexes could be specifically competed for by addition of RT7D1 ds, s, and αs oligos (lanes 2, 3, 4, respectively). As seen above, αsense RT7D1 oligo was the best competitor for TTF-D followed by RT7D1-sense and double stranded oligo. CmosD1 was also seen to compete for TTF-D either as sense (lane 5) or αsense (lane 6). Although cmosD1-sense appeared to compete better than αsense for the RT7D1-αsense binding site, these oligos were not as efficient as when competing for the RT7D1-sense binding site, (compare Figure 16 lanes 5 and 6 to Figure 15 lanes 5 and 6). As expected, B-oligos did not compete for TTF-D complex formation (lanes 7 and 8).

Figure. 15. Binding of TTF-D to the RT7D1-sense site is specific.

ST nuclear extract was incubated with 1 ng ^{32}PRT7D1-sense and 2 μg dIdC in the absence (lane 1) or presence (lanes 2-8) of 200 ng competitor oligo as indicated, under UV/EMSA conditions followed by 8% native PAGE.


RT7D1
cmosD1
B-oligo
ds
s
αs
s
αs
s
αs
200ng competition
1 2 3 4 5 6 7 8
32PRT7D1-sense

Figure. 16. Binding of TTF-D to the RT7D1-αsense site is specific.

ST nuclear extract was incubated with 1 ng ^{32}PRT7D1-αsense and 2 μg dIdC in the absence (lane 1) or presence (lanes 2-8) of 200 ng competitor oligo as indicated, under UV/EMSA conditions followed by 8% native PAGE.


RT7D1
cmosD1
B-oligo
200ng competition
ds
s
αs
s
αs
s
αs
1 2 3 4 5 6 7 8
32PRT7D1-αsense

To further analyze which of the 36, 25 and 22 kDa TTF-D proteins were involved in binding to the single stranded binding site, complexes were crosslinked to a single or double stranded probe followed by SDS PAGE. Figure 17 demonstrates that TTF-D:DNA complexes are formed with both double and single stranded RT7D1 and cmosD1binding sites (lanes 2-4 and 6-8). The three TTF-D complexes, indicated by stars are seen with each probe used and were calculated to have the expected molecular weights of 36, 25, and 22 kDa. A weaker signal with the double stranded RT7D1 was due to the age of the probe used.

Competition assays for either the RT7D1-sense or RT7D1-αsense binding site showed that TTF-D protein(s) binding to a single stranded binding site was specific (Figure 18). The background bands appeared to be more intense with either the RT7D1-sense probe (lanes 1-5) or the RT7D1-αsense probe (lanes 6-10) compared to double strand probe so that resolution of TTF-D complexes was only seen for the lower two complexes (arrowheads). These complexes could be barely competed from the sense binding site by addition of 200 ng dsRT7D1 oligo (lane 3), better competition was seen with the RT7D1-sense oligo (lane 4), however RT7D1-αsense oligo (lane 5) competed the best for the TTF-D complexes. Double stranded RT7D1 oligos could compete well for TTF-D: RT7D1-αsense complexes (lane 8), with complete competition for the lower complexes by both sense (lane 9) and αsense (lane 10) oligos.

Figure. 17. TTF-D UV-crosslinks to both double strand and single strand oligo binding sites.

Liver and ST extracts were incubated with and UV crosslinked to double stranded (ds), sense (s) and anti-sense (αs) radiolabeled RT7D1 or cmosD1 oligos as indicated, followed by 10% SDS-PAGE. The three TTFD-DNA complexes specific to ST extract were seen with each probe used (*).

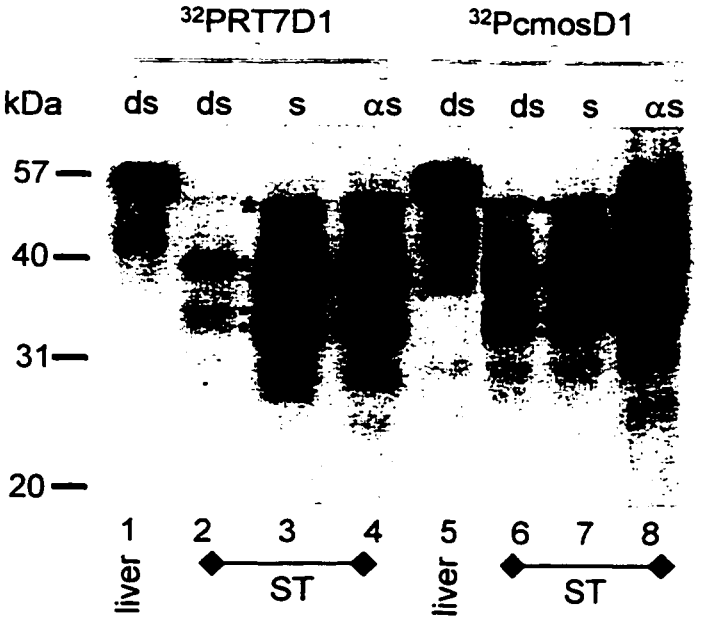
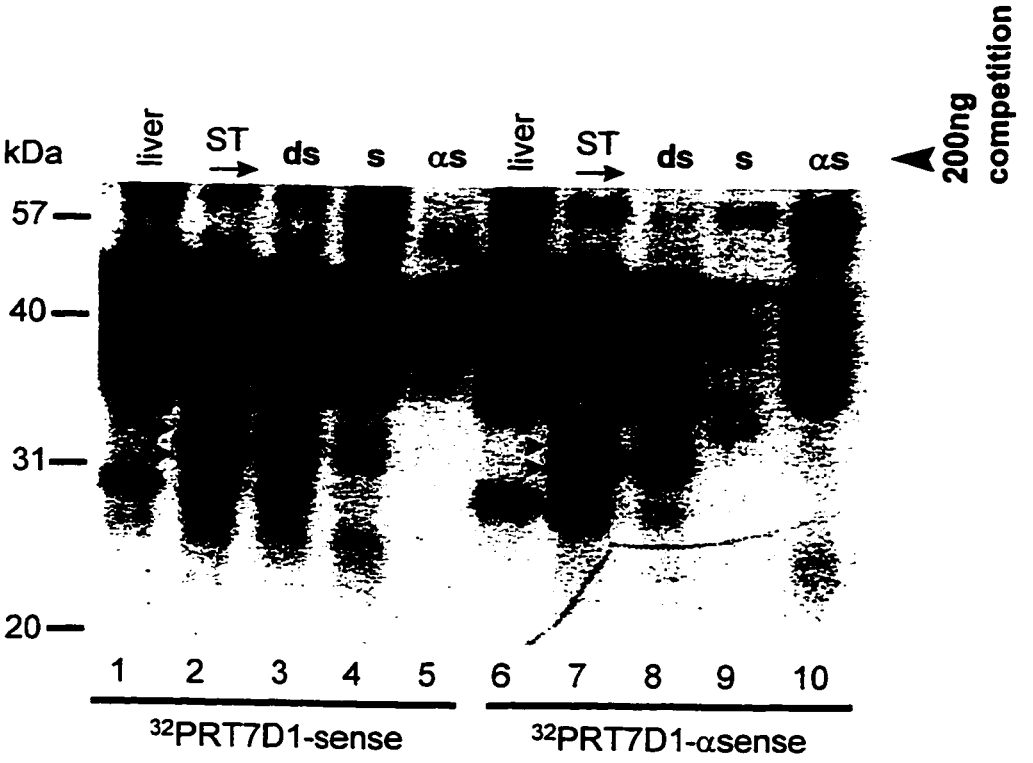

32PRT7D1
32PcmosD1
kDa
ds
ds
s
αs
ds
ds
s
αs
57
40
31
20
1
2
3
4
5
6
7
8
liver
ST
liver
ST

Figure. 18. Competition of TTF-D complexes with double strand and single strand oligos.

Liver (lanes 1 and 6) or ST extract (lanes 2-5 and 7-10) were incubated with ^{32}PRT7D1sense (lanes 1-5), or ^{32}PRT7D1-αsense (lanes 6-10) oligos, with or without competitor oligos, complexes UV irradiated and resolved on SDS-PAGE. Competition with 200 ng dsRT7D1 (lanes 5 and 10), 200 ng sense (lanes 6 and 11) or 200 ng α-sense (lanes 7 and 12) varied depending on the oligo used as probe. TTF-D complexes are indicated by arrowheads, the upper complex, not indicated, cannot be resolved above background. In general all three oligos compete but the best competition was seen with the oligo complementary to the oligo used as probe.


kDa
liver
ST
ds
s
αs
liver
ST
ds
s
αs
200ng competition
57
40
31
20
1 2 3 4 5 6 7 8 9 10
32PRT7D1-sense
32PRT7D1-αsense

3.4.3 TTF-D Binding to the minimal or larger RT7D1 Binding Site

Examination of the nucleotide sequence immediately upstream of the RT7D1 site revealed a second potential binding site. The eleven nucleotides 5' to RT7D1 had seven bases identical to the RT7D1 sequence. The region containing the known and the potential TTF-D binding sites was generated as a 25mer oligonucleotide termed TTFD (see Table 2). TTFD oligos (ds, sense, and αsense) were then used in mobility shift assays as competitor for TTF-D:RT7D1(ds, sense, and α-sense) complex formation.

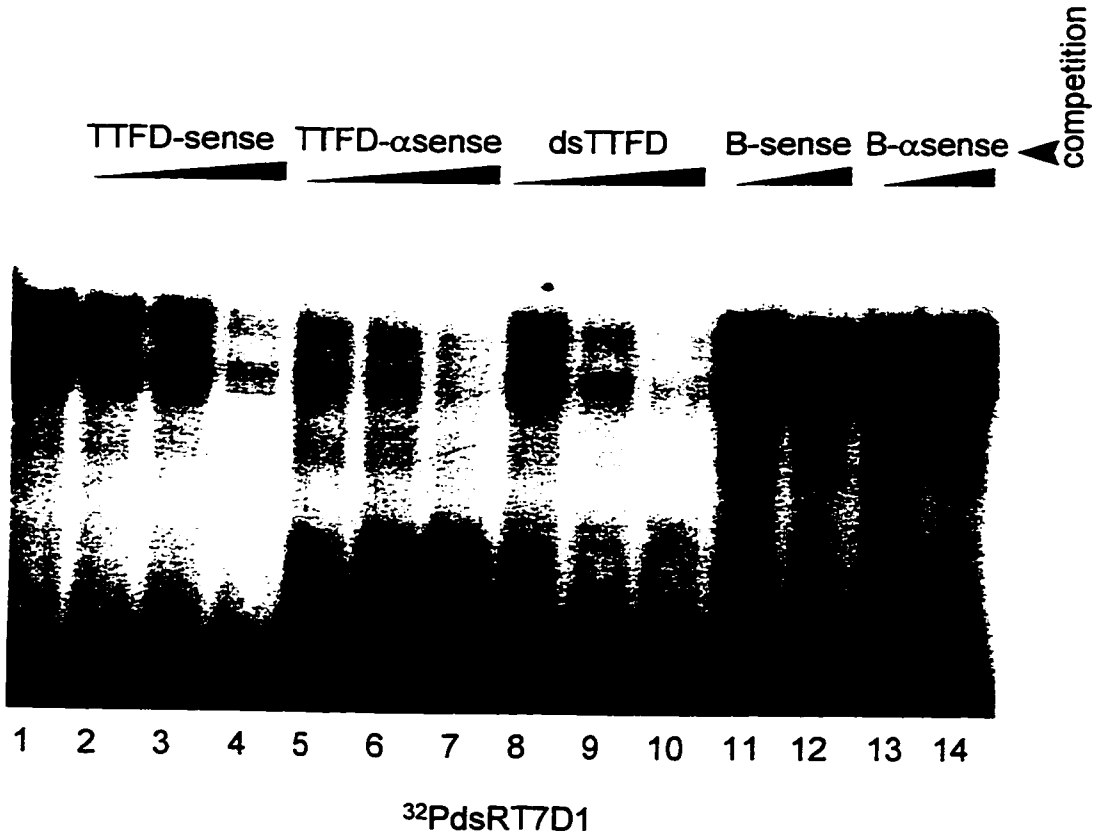
As shown in Figure 19, increasing amounts of TTFD-αsense (lanes 5-7) was the most efficient competitor for the ds binding site, followed by dsTTFD (lanes 8-10), TTFD-sense oligo (lanes 2-4) was the poorest competitor. B-oligo was used as a competitor which is non-related to the RT7D1 binding site except for the AATT end of the oligo (see Table 2). No competition was seen for the TTF-D complexes when B-oligo was added to the reaction. As described earlier, the addition of B-oligo appears to enhance binding of TTF-D at the level of 100 ng B-sense (lane 11) and both 100 ng and 200ng B-αsense (lanes 13 and 14). The reduced signal seen when 200 ng B-sense was added (lane 12) was not seen as competition as the signal does not significantly differ from the starting condition (lane 1).

The larger TTFD oligo was also used in gel shift assays to compete for TTF-D binding to the single stranded RT7D1 binding site. When RT7D1-sense was used as probe, shown in Figure 20, TTFD-αsense oligos (lanes 4, 5) appeared to compete most efficiently followed by the TTFD-sense oligo (lanes 2,

3). Addition of dsTTFD oligo did compete for complex formation (lanes 6, 7) but only at the higher concentration of oligo. Figure 21 shows competition by the TTFD oligo for the RT7D1-αsense binding site. Double stranded TTFD was still seen to be a poor competitor for the single stranded binding site (lanes 6, 7). The TTFD-sense oligo (lanes 2, 3) however competes better than the TTFD-αsense oligo (lanes 4, 5).

Figure. 19. TTFD oligonucleotides compete for binding of the TTF-D protein(s) to the dsRT7D1 binding site.

ST nuclear extract was incubated with 1ng ^{32}PdsRT7D1and 1μg dIdC, without (lane 1) or with (lanes 2-14) increasing amounts of competitor oligos under EMSA conditions. Complexes were resolved by 6% native PAGE. Addition of 50 ng, 100 ng, and 200 ng: TTFD-sense (lanes 2,3,4), TTFD-αsense (lanes 5, 6, 7) and dsTTFD (lanes 8, 9, 10) showed varied levels of competition. TTFD-αsense competed well at all concentrations, dsTTFD showed increasing competition with increasing oligo concentration, and significant competition with the TTFD-sense oligo was only seen at the 200 ng level. Addition of 100 ng and 200 ng of: B-sense (lanes 11, 12) or B-αsense (lanes 13,14) did not compete for the TTF-D complexes.



^{32}PdsRT7D1

RT7D1: aattGGCCTTAGGG
TTFD: taGGCCTGAGGAGGGCCTTAGGGAC

Figure. 20. TTFD oligonucleotides compete for binding of the TTF-D protein(s) to the RT7D1-sense binding site.

ST nuclear extract was incubated with 1 ng ^{32}PRT7D1-sense, 1 μg dIdC under EMSA conditions in the absence (lane 1) or presence (lanes 2-7) of competitor oligos. Reactions were UV crosslinked prior to separation on 10% native PAGE. Varied degrees of competition for TTF-D complexes was seen by addition of 100 ng and 200 ng: TTFD-sense (lanes 2,3), TTFD-αsense (lanes 4,5) and dsTTFD (lanes 6,7). TTFD-αsense oligos competed better than the ds or sense oligos.

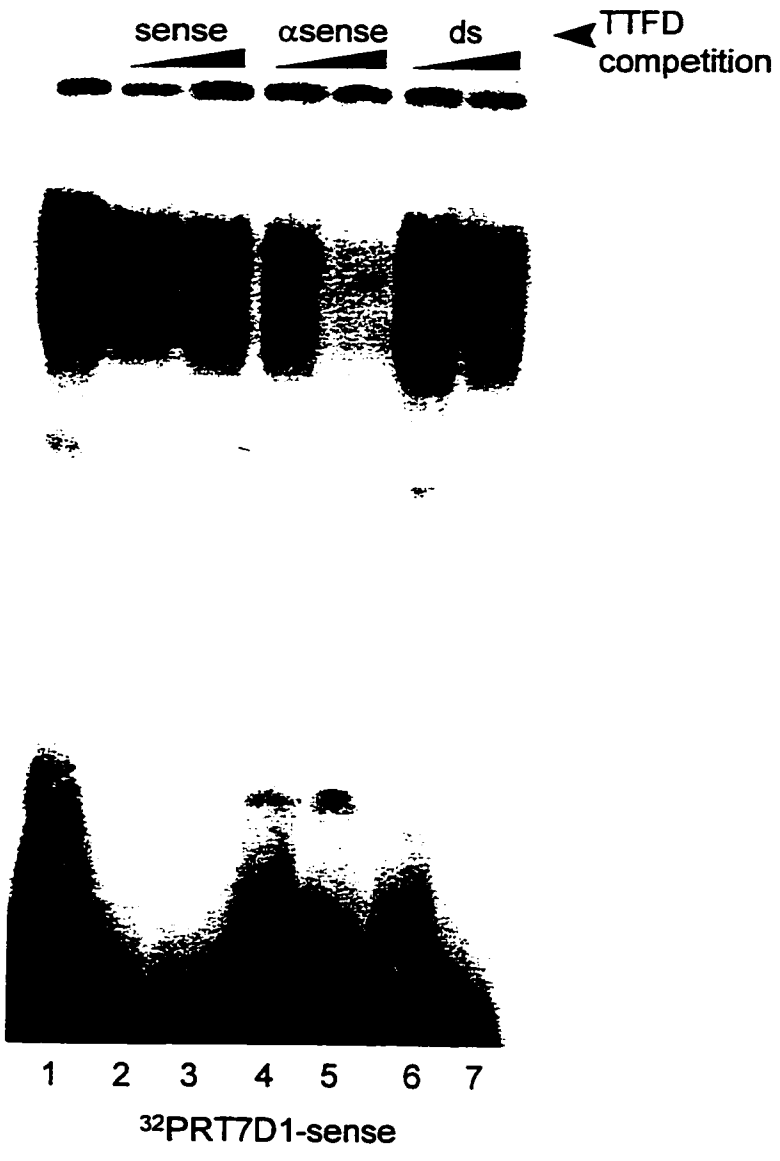
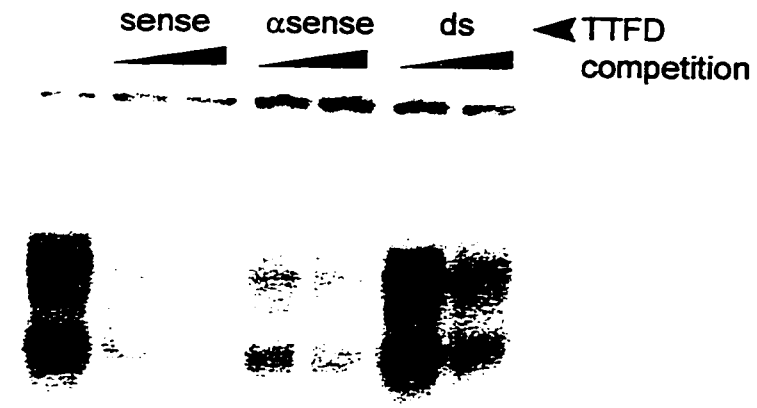

sense
αsense
ds
TTFD
competition
1 2 3 4 5 6 7
32PRT7D1-sense

Figure. 21. TTFD oligonucleotides compete for binding of the TTF-D protein(s) to the RT7D1-αsense binding site.

ST nuclear extract was incubated with 1 ng ^{32}PRT7D1-αsense, 1 μg dIdC under EMSA conditions in the absence (lane 1) or presence (lanes 2-7) of competitor oligos. Reactions were UV crosslinked prior to separation on 10% native PAGE. Varied degrees of competition for TTF-D complexes was seen by addition of 100 ng and 200 ng: TTFD-sense (lanes 2,3), TTFD-αsense (lanes 4,5) and dsTTFD (lanes 6,7). TTFD-sense oligos competed better than the ds or αsense oligos.



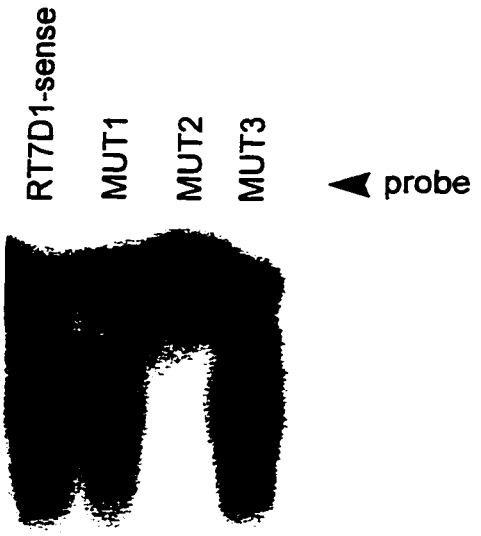
^{32}PRT7D1-αsense

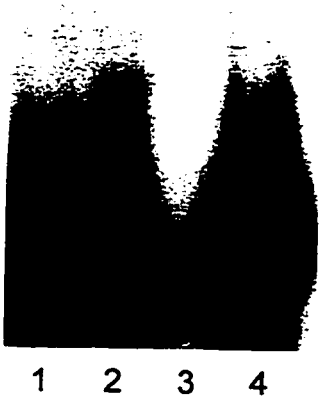
3.4.4 Mutation of the TTF-D Binding Site

Having studied the nucleotide sequence immediately upstream of the RT7D1 site and not finding any significant changes in TTF-D binding properties the next step was to focus on the RT7D1 nucleotide sequence. In order to analyze which region in the TTF-D binding site that was important for TTF-D binding, several base changes along the RT7D1 binding site were made generating MUT1, MUT2, and MUT3 oligos (see Table 2). The oligos reflect changes in the 5', central, and 3' regions of the binding site, respectively. Mutated oligos were then used as single strand probes in gel retardation assays to evaluate the effects of base changes on TTF-D complex formation (Figure 22). The expected TTF-D gel shift pattern was seen when RT7D1-sense was used as probe (lane 1). MUT1 probe (lane 2) generates the same TTF-D pattern. Changes in the five prime end (MUT1) of the binding site appeared to slightly affect binding as seen by the lighter intensity of the gel shift pattern to that of the starting condition (compare lanes 1 and 2). Changes in the 3'end (MUT3) does not appear to affect TTF-D binding (lane 4). TTF-D binding was not seen however with the MUT2 probe (lane3). It appeared that the majority of the probe was bound up and retarded in the gel slot, however competition gel shift assays and UV crosslinking studies (see below) confirmed that changes in MUT2 severely reduced binding of TTF-D proteins. Therefore, the central nucleotides CTT, in the RT7D1 binding site were seen to be essential for TTF-D binding.

Figure. 22. Mutation of the TTF-D binding site severely affects protein binding.

STXVI extract (6 μg) was incubated with 2 μg dIdC and 1 ng radiolabeled RT7D1-sense, MUT1, MUT2 or MUT3 oligos. Probes were counted before use and had approximately equal cpm. Complexes were UV crosslinked and resolved by native PAGE. Changes in nucleotide sequence in the MUT1and MUT3 oligos have almost no effect on TTF-D binding affinity, however MUT2 oligo severely diminishes TTF-D binding.





RT7D1: AATTGGCCTTAGGG
MUT1: AATT c t CCTTAGGG
MUT2: AATTGGC acg AGGG
MUT3: AATTGGCCTT c t a G

Differences in the ability of the native ds, sense, and αsense RT7D1 binding site to compete for TTF-D complexes was shown (see above). It was of interest to determine if the mutant oligos could compete for TTF-D binding to the RT7D1-sense or RT7D1-αsense binding site in gel shift assays. TTF-D complexes formed with RT7D1-sense probe in the presence of non-specific B-sense oligo served as the starting condition (Figure 23, lane 1). Addition of RT7D1-sense or -αsense oligos competed for TTF-D as expected (lanes 2 and 3). MUT1 oligo could compete for TTF-D but not as efficiently as the wild type binding site (lane 4). MUT3 was also seen to compete for TTF-D but to a lesser degree than MUT1 (lane 6). Surprisingly, MUT2 appeared to compete somewhat albeit very poorly for TTF-D complex formation (lane 5). Similar results were seen when RT7D1-αsense was used as probe in MUT oligo competition EMSA (Figure 24). It appeared that overall, [with the exception of RT7D1-αsense oligo (lane 3)], the RT7D1-sense (lane 2), MUT1 (lane 4) and MUT3 (lane 6) oligos were less efficient competitors for the RT7D1-αsense site than for the sense site. Finally, MUT2 (lane 5) did not compete for the RT7D1-αsense binding site.

The mutant binding sites were then used as probes under EMSA/UV/SDS conditions to determine if the binding 36 kDa, 25 kDa, and 22 kDa TTF-D complexes were affected by the base changes. Figure 25 shows a comparison of the native binding site in double and single strand conformations and the three mutated binding sites. Liver extract (lane 1) was used as a control to differentiate somatic and testis-specific proteins. As expected in ST extract, the

Figure. 23. EMSA competition of native and mutant oligos for the RT7D1-sense binding site.

ST nuclear extract was incubated with 1 ng ^{32}PRT7D1-sense, 2 μg dIdC, and 200 ng competitor oligo under UV/EMSA conditions followed by 8% native PAGE. Addition of the non-specific oligo B-sense (lane 1) served as the starting condition. RT7D1-sense and -αsense oligos (lanes 2 and 3) compete very well for TTF-D complexes, with αsense oligos competing better than sense. Mutant oligos, (lanes 4-6) compete very poorly for complex formation with MUT2 serving as the poorest competitor.

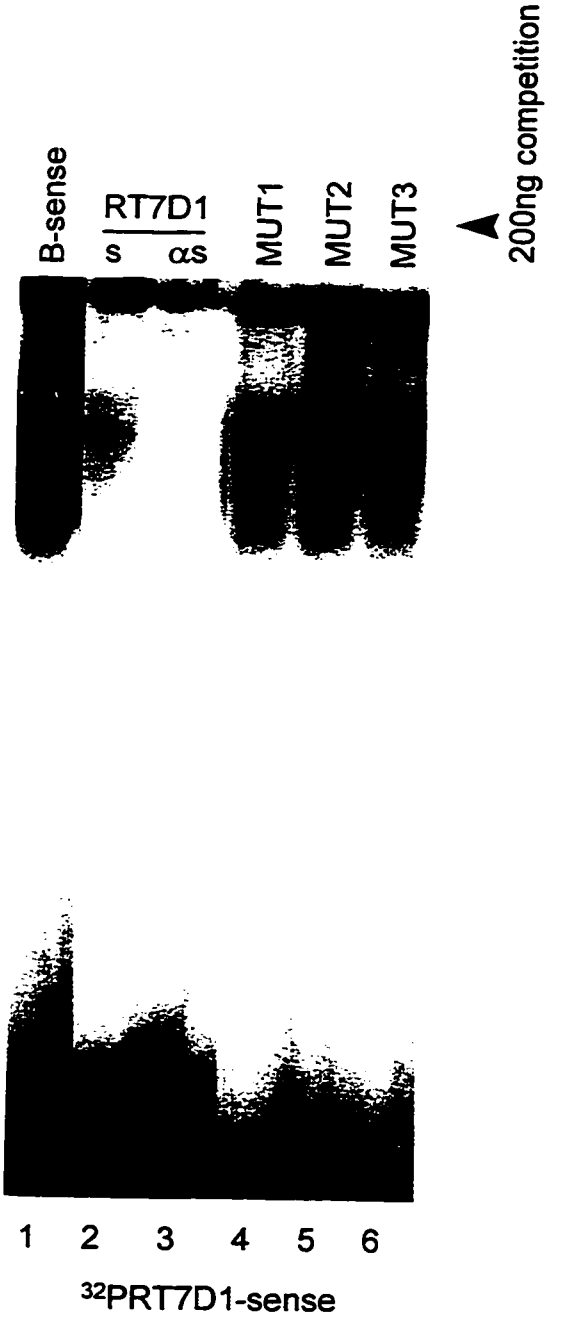

B-sense
RT7D1
s
αs
MUT1
MUT2
MUT3
200ng competition
1 2 3 4 5 6
32PRT7D1-sense

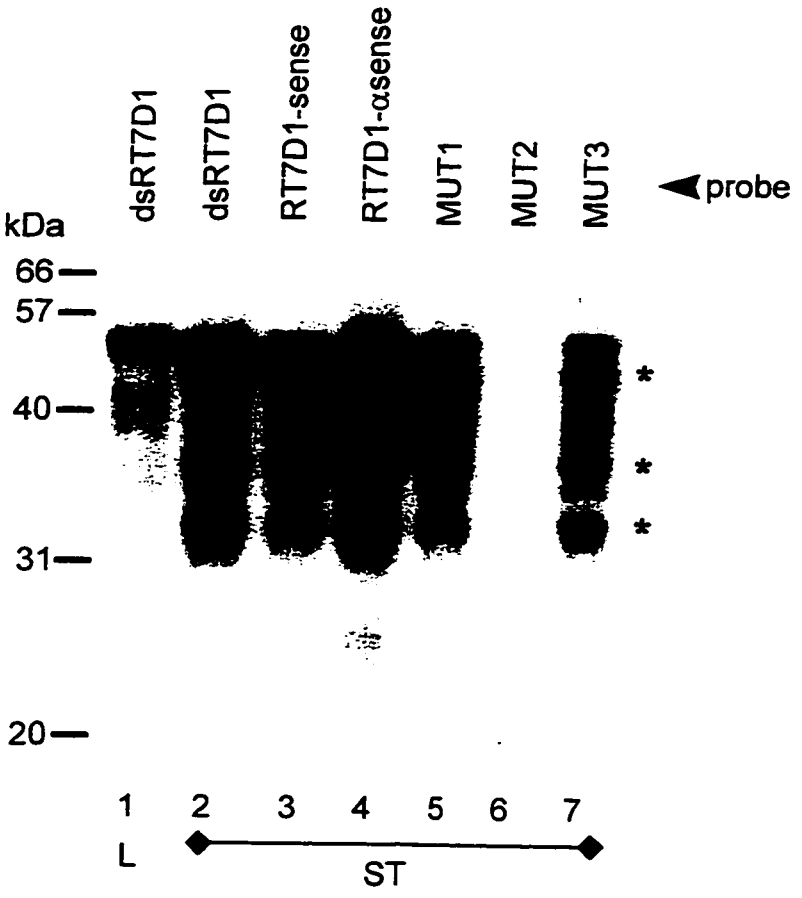
Figure. 24. EMSA competition of native and mutant oligos for the RT7D1-αsense binding site.

ST nuclear extract was incubated with 1ng ^{32}PRT7D1-αsense, 2 μg dIdC, and 200 ng competitor oligo under UV/EMSA conditions followed by 8% native PAGE. Addition of the non-specific oligo B-sense (lane 1) served as the starting condition. RT7D1-sense oligo (lane 2) can compete for TTF-D complexes but not as well as RT7D1-αsense oligos (lane 3). Mutant oligos (lanes 4-6) compete very poorly for complex formation with MUT2 serving as the poorest competitor.


B-sense
RT7D1
s
αs
MUT1
MUT2
MUT3
200ng competition
1
2
3
4
5
6
32PRT7D1-αsense

Figure. 25. TTF-D complexes are not crosslinked to MUT2 oligo.

Under EMSA/UV/SDS conditions, liver (lane 1) or ST extract (lanes 2-7) were incubated with 2 μg dIdC and ^{32}PdsRT7D1 (lanes 1 and 2), ^{32}PRT7D1-sense (lane 3), ^{32}PRT7D1-αsense (lane 4), ^{32}PMUT1 (lane 5), ^{32}PMUT2 (lane 6) or ^{32}PMUT3 (lane 7) oligos. TTF-D:DNA complexes are seen in ST lanes (*) except when MUT2 was used as probe.


dsRT7D1
dsRT7D1
RT7D1-sense
RT7D1-αsense
MUT1
MUT2
MUT3
probe
kDa
66
57
40
31
20
*
*
*
1
2
3
4
5
6
7
L
ST

three TTF-D:DNA complexes (stars) are generated when ds-, sense-, and αsense- RT7D1 oligos were used as probes (lanes 2, 3, and 4 respectively). MUT1 and MUT3 oligos form the testis-specific complexes (lanes 5 and 7), however MUT2 oligo does not form any of the TTF-D complexes (lane 6). Equal cpm of probe were added to each reaction and all lanes had free probe which ran at the gel front (not shown). The base changes made in MUT2 are therefore essential for TTF-D binding, and the MUT1 and MUT3 oligos bind the same peptides as RT7D1-sense oligo.

3.4.5 EMSA Competition for TTF-D Binding by D2 Oligonucleotide

The D2 oligo (see Table 2) was designed to study the nucleotide sequence directly 3-prime to the RT7D1 binding site which generated a DNase I footprint using liver nuclear extract (see Figure 2)(van der Hoorn & Tarnasky 1992). Initially D2 oligos were used in TTF-D EMSA studies as a negative control for oligo competition. It was discovered that D2 oligo could however, compete weakly for TTF-D binding. Closer inspection of the D2 nucleotide sequence revealed the CTT triplet of nucleotides that had been shown to be important in mutation studies for TTF-D binding. Figure 26 shows competition of D2 single stranded oligos for RT7D1-sense (lanes 1-3) or -αsense (lanes 4-6) binding in EMSA. Addition of 200ng B-sense oligo was used as the starting condition for both probes. D2-αsense competes better than D2-sense in each case. D2 oligos could not compete as efficiently for the RT7D1-αsense probe compared to the RT7D1-sense probe (compare lanes 5 and 6 to lanes 2 and 3).

Figure. 26. D2 oligonucleotides can compete for the single strand TTF-D binding site.

ST nuclear extract was incubated with 1 ng RT7D1-sense (lanes 1-3) or RT7D1-αsense (lanes 4-6) probe, 2 μg dIdC, and 200 ng B or D2 oligo. EMSA complexes were UV crosslinked followed by 8% native PAGE. TTF-D:DNA complexes in the presence of the non-specific B-sense oligo served as starting conditions (lanes 1 and 4). D2 oligos could weakly compete for both probes. D2-αsense appeared to be a slightly stronger competitor than D2-sense for TTF-D complexes.

RT7D1: aattGGCCTTAGGG
D2: aattACCTTAACTG

3.5 *In Vitro* Transcription

The analysis of testis-specific promoter elements has been limited, because no male germ cell lines exist. The isolation of transcriptionally active testis-specific nuclear factors however allows for limited study using the *in vitro* transcription assay. Previous *in vitro* transcription studies had shown that the region from -167 to -90 in the RT7 promoter acts as a testis-specific cis-acting element (van der Hoorn & Tarnasky 1992). It was also demonstrated that this promoter region only contains the TTF-D binding site located at -124: no other protein-DNA interactions were detected in the -167 to -90 region (van der Hoorn & Tarnasky 1992). To further study the effect of TTF-D binding on RT7 promoter activity *in vitro*, transcription assays with TTF-D binding site oligos added as competitors were performed.

3.5.1 Repression of RT7 Promoter Activity with Double Stranded Binding Site Oligonucleotides

Figure 27 demonstrates that TTF-D acts as a positive transcription factor. ST nuclear extract, the RT7 promoter linked to a G-free cassette reporter gene and the internal AdMLP control were incubated in the absence (lane 1) or presence (lanes 2-4) of competitor oligos. Addition of RT7D1 oligos and cmosD1 oligos, which compete for binding of TTF-D to the RT7 promoter, results in an approximately 30 % reduction of the activity of the RT7 promoter (lanes 2 and 4). Addition of the D2 oligo, resulted in a reduction of promoter activity of approximately 40% (lane 3). The D2 oligo, which contains the nucleotide sequence CTT shown to be essential for TTF-D binding, was also seen to

compete in EMSA (see above). The competitor oligos had no effect on the activity of the Adenovirus major late promoter used as internal control in all reactions (lanes 1-4).

3.5.2 Repression of RT7 Promoter Activity with Single Stranded Binding Site Oligonucleotides

Gel shift assays have shown that TTF-D can bind to a single stranded binding site. It was important to determine if single stranded oligos could specifically compete for TTF-D binding under *in vitro* transcription conditions. Figure 28 compares transcription levels from an RT7 promoter reporter construct with or without addition of single stranded competitor oligos. The AdMLP was used as an internal control. Addition of RT7D1-sense, MUT1, MUT3, and D2-sense (lanes 2, 3 ,5 and 6 respectively) resulted in approximately fifty percent drop in the level of promoter activity. MUT2 oligo (lane 4) which essentially does not bind TTF-D in EMSA, reduced transcription by fifteen percent. The non-specific B oligo (lane 7) also appeared to reduce transcription but not significantly. It must be noted that these reductions were not consistently observed in all single stranded oligo *in vitro* transcription experiments carried out. In some, no significant reduction in RT7 promoter activity was seen. However, an increase in transcription levels was never observed upon the addition of single stranded oligos and the competition for TTF-D in *in vitro* transcription assays by single stranded binding sites was therefore not an artifact. The reason for this is not known, but a decreased change in promoter activity was noticed when using a large excess of single strand oligo. Taking all results

Figure. 27. Repression of RT7 promoter activity by double stranded oligonucleotides.

In vitro transcription assay of ST nuclear extract with pRT7CAP0.2 and AdMLP (internal control) promoter constructs. Addition of 100 ng: dsRT7D1 (lane2), D2 (lane 3) or dscmosD1 (lane 4) oligonucleotides, resulted in a repression of promoter activity compared to standard conditions (lane1).

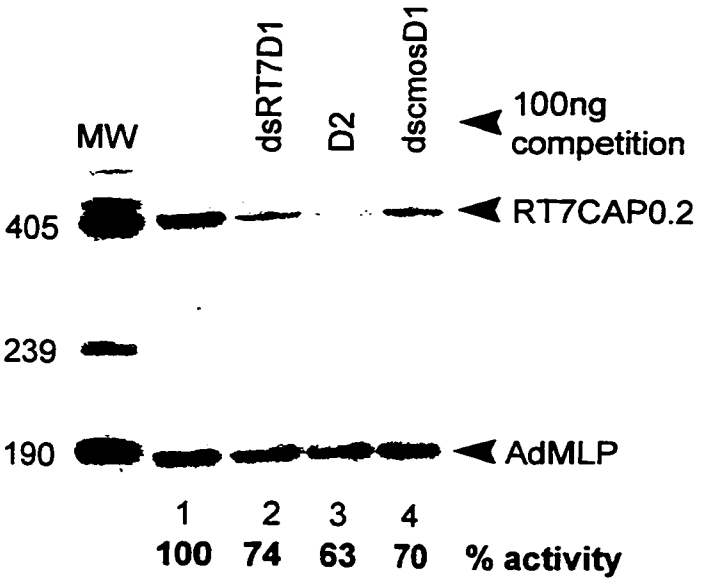
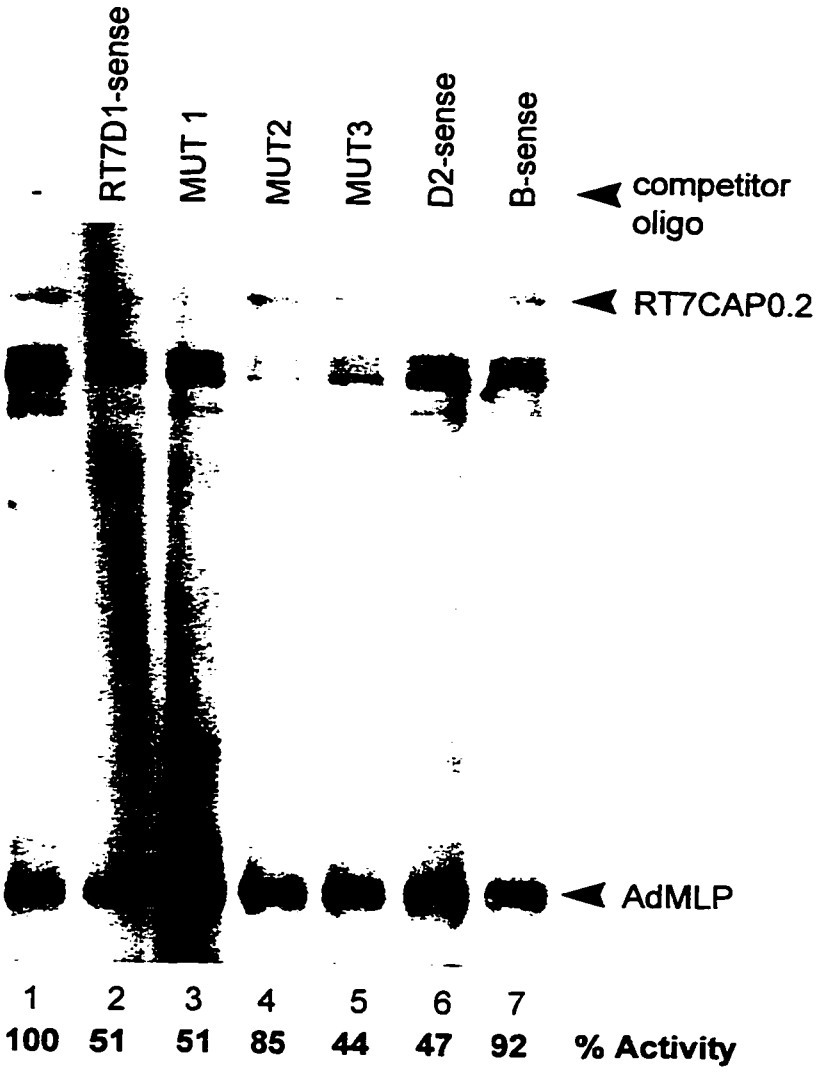

MW
dsRT7D1
D2
dscmosD1
100ng competition
405
RT7CAP0.2
239
190
AdMLP
1 2 3 4
100 74 63 70 % activity

Figure. 28. Repression of RT7 promoter activity by single stranded oligonucleotides.

In vitro transcription assay of ST nuclear extract with pRT7CAP0.2 and AdMLP (internal control) promoter constructs. Addition of 200 ng single stranded competitor oligos (lanes2-7) resulted in varied degrees of transcription repression from the RT7 promoter construct.


RT7D1-sense
MUT 1
MUT2
MUT3
D2-sense
B-sense
competitor oligo
RT7CAP0.2
AdMLP
1 2 3 4 5 6 7
100 51 51 85 44 47 92
% Activity

together we can conclude that competition with single strand oligos (wt, MUT1, and MUT3) can significantly reduce RT7 promoter activity. In contrast MUT2 and the unrelated B oligo never produced a significant drop in RT7 promoter activity.

3.6 Cloning Trials

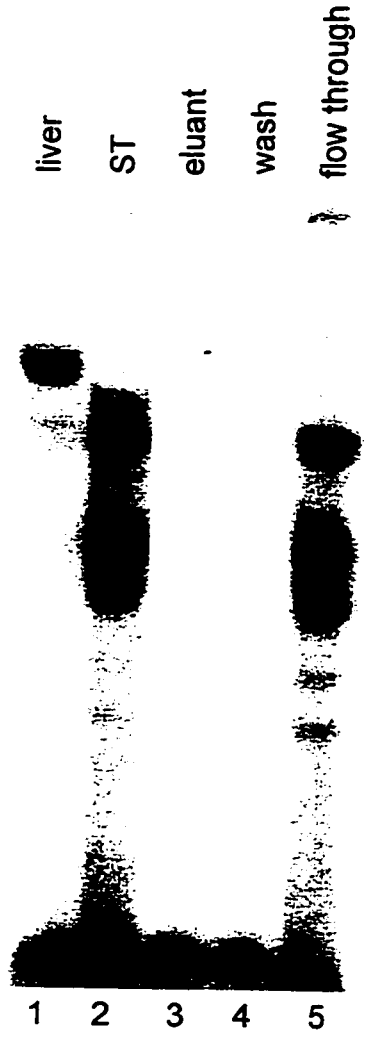
3.6.1 Dyna-Bead Purification of TTF-D Protein(s)

Following characterization of TTF-D binding properties, the next specific aim in my thesis work was to clone TTF-D. The initial method of choice was using DNA affinity columns to purify enough TTF-D protein for micro-sequencing analysis. Magnetic DNA affinity columns were chosen as the method was rapid (under 1 hr), and reactions could easily be kept on ice. Both of these features were important to reduce TTF-D degradation in the ST extracts. Biotinylated oligos with the RT7D1 site were generated and combined with Dyna-bead M-280 streptavidin beads to prepare DNA affinity columns. ST extracts were incubated on the Dyna-bead columns according to the Materials and Methods, and column fractions assayed for TTF-D activity by EMSA. Figures 29, 30, and 31 were typical of the inconsistent results generated by this assay. Gel retardation assays were used to analyze the proteins eluted from the Dyna-bead column as well as wash and flow-through fractions. The EMSA shown in Figure 29 compares Dyna-bead fractions to liver (lane 1) and ST extract (lane2) binding to dsRT7D1 as probe. No TTF-D proteins were seen in the eluant (lane 3) or wash (lane 4) fractions. It appeared that the majority of the TTF-D proteins

remained in the flow-through fraction (lane 5). In some experiments, gel shift analysis of Dyna-bead fractions revealed the presence of TTF-D proteins in the assay eluant. However, this was only detectable using large volumes of the eluted fractions. As well, only complexes B and C, but not A, D and E were recovered (Figure 30). Conditions used to elute proteins bound to the Dyna-bead columns, were also varied. Figure 31 shows the EMSA analysis of one such experiment. Proteins were eluted from the column using either 1 M KCl-dialysis buffer (lane 2) or 2 M KCl-dialysis buffer (lane 4). No significant TTF-D binding activity was isolated in either fraction, and the majority of the protein remained in the flow through fractions (lanes 3,5). Eluted fractions were also run on SDS-polyacrylamide gels followed by silver staining in attempts to identify column isolated proteins. No specific protein bands of the appropriate sizes could be distinguished above background bands (not shown). Several different columns using the various oligos containing TTF-D binding sites were also generated and tried (cmosD1, TTFD, RT7D1-multimers, TTFD-multimers, RT7D1-sense) without success. Insufficient amounts of TTF-D proteins could be isolated by the Dyna-bead assay approach and fractions isolated were highly contaminated with other ST proteins. As a result this part of my thesis work was not pursued further (see Discussion).

Figure. 29. EMSA of Dyna-bead assay fractions I.

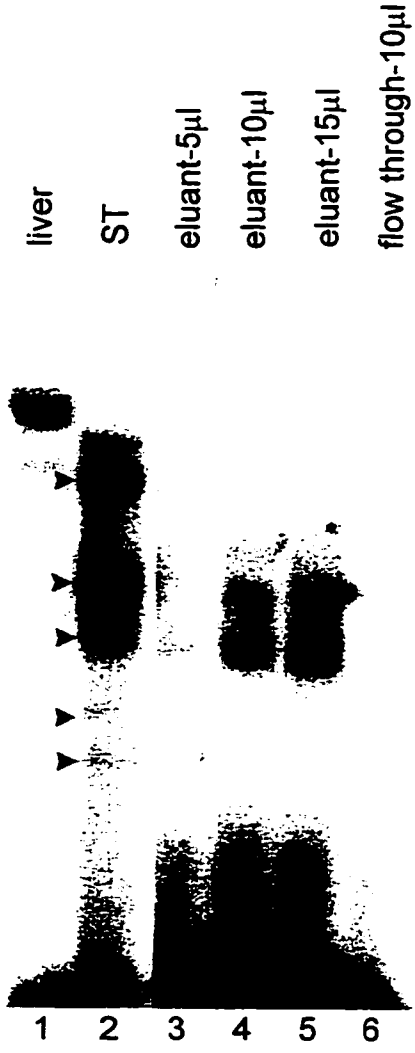
Nuclear extracts or fractions from a dsRT7D1 Dyna-bead column were incubated with radiolabeled dsRT7D1 under EMSA conditions and complexes resolved by 10% native PAGE. Amounts assayed are as follows: liver nuclear extract; 9 μg (lane 1), ST nuclear extract; 9 μg (lane 2), column eluant; 5 μl (lane 3), column wash; 5 μl (lane 4), and column flow through; 10 μl (lane 5).



^{32}PdsRT7D1

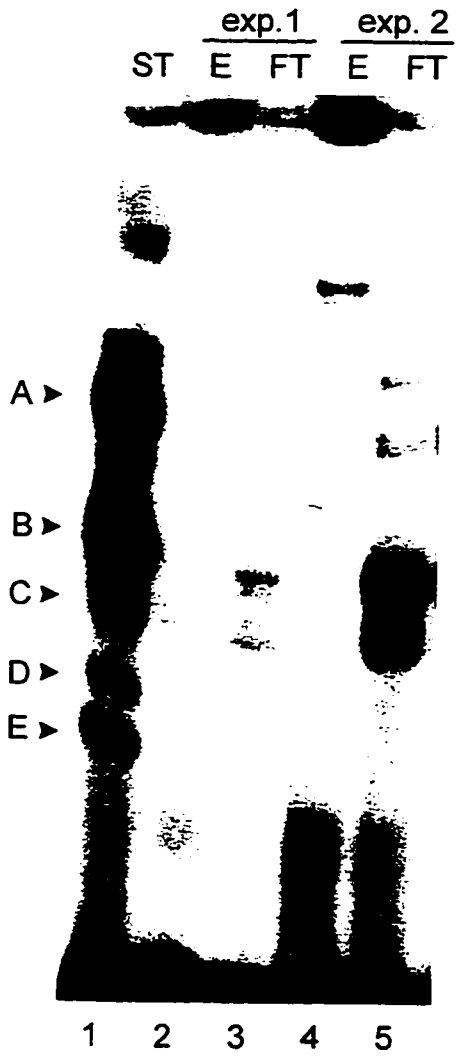
Figure. 30. EMSA of Dyna-bead assay fractions II.

Liver (lane 1) nuclear extract, ST (lane 2) nuclear extract, or fractions from a dsRT7D1 Dyna-bead column (lanes 3-6) were incubated with ^{32}PdsRT7D1 under EMSA conditions and complexes resolved by 10% native PAGE. TTF-D complexes are indicated by arrowheads in the ST control lane (2).


liver
ST
eluant-5µl
eluant-10µl
eluant-15µl
flow through-10µl
1
2
3
4
5
6

^{32}PdsRT7D1

Figure. 31. EMSA of Dyna-bead column fractions, 1 MKCl vs 2 MKCl. ST nuclear extract, column eluant (E) or flow through (FT) fractions were incubated with ^{32}PdsRT7D1 under EMSA conditions followed by 10% native PAGE. TTF-D complexes in the ST control lane are indicated A-E.



^{32}PdsRT7D1

3.6.2 λgt11 Screen

As TTF-D could not be purified in an amount sufficient for micro-sequencing, more direct cloning techniques were tried. Southwestern assays (see above) were encouraging in identifying TTF-D binding and optimized conditions (described in Materials and Methods) from those experiments were used for the library screening. A testis λgt11 expression library was screened for TTF-D with the following probes: RT7D1 (monomers, and concatamers), cmosD1(monomers, and concatamers), TTFD-2mer, cmosD1-6mer +/- GuHCl. No positive clones were identified beyond a secondary screening with any of the above probes. Screening under GuHCl conditions as described in Materials and Methods did improve the screen. The testis library used in the protocol had been successfully used for expression screening and cloning by other members of the laboratory (Higgy *et al.* 1997). It was possible that TTF-D binding to the D1 site involved a complex of peptides which would not be successfully identified in the screening of an expression library. Alternately, it was possible that the bacterially expressed proteins were not in an active conformation for DNA binding (see Discussion).

3.6.3 Yeast-One-Hybrid Assay

The yeast-one-hybrid system, an *in vivo* genetic assay used for isolating novel genes encoding proteins that bind to a DNA target sequence, was the final tool used in attempts to clone TTF-D. Successful cloning of transcription factors, OLF-1 (Wang & Reed 1993), REST (Gstaiger *et al.* 1995), and ORC-6 (Li & Herskowitz 1993), had been reported, and the components necessary for the

assay were commercially available in a kit. As well, a testis cDNA library of activation domain fusion proteins in the yeast vector pGAD424 (see Figure 32, panel A) was available in the lab (Shao *et al.* 1997). Concatamers of the TTF-D binding site were cloned into the three reporter plasmids (Figure 32. Panels B, C, D). Following several screenings of the library in an RTD1-6X-HISi yeast reporter strain, nine potential positives were isolated. Plasmid DNA was isolated from each of the nine yeast colonies. The plasmid constructs were then introduced into the RT7D1-6X-LacZi reporter strain and colonies assayed for β-galactosidase activity. None of the nine potential positives were positive in the LacZ screen, and therefore considered to be false positives. A second reporter construct with the TTFD-2X binding site cloned into the pHISi-1 vector was screened with the library. Two putative positives were isolated from this screen. One colony contained an empty pGAD424 plasmid (see below). The second plasmid contained a 0.7 Kb insert which was subcloned and sequenced. Sequence analysis identified the insert as rat ribosomal protein L17 (Suzuki & Wool 1991).

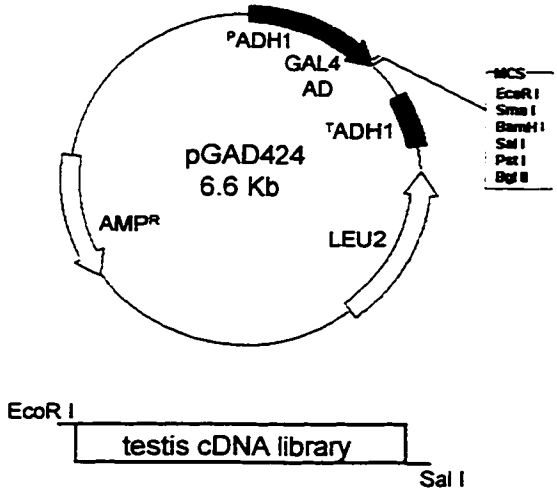
The yeast-one-hybrid system, despite its apparent simplicity, was fraught with problems. Histidine reporter genes were integrated into the reporter strains resulting in a requirement for leaky expression of the reporter gene to establish colony growth on media lacking histidine. This led to high numbers of background colonies. To curb this problem, 3-aminotriazole (3-AT), a competitive inhibitor of the yeast HIS3 protein was added to the medium in the concentrations recommended; 0, 15, 30, 45 and 60 mM . High levels of

background colonies were still being observed at the 60 mM 3-AT concentration. Concentrations of 3-AT as high as 90 mM were tested in an attempt to limit background colony formation. A major problem with higher 3-AT concentrations however was the subsequent slow growth of the yeast colonies. The yeast strains used in this assay were slow growing (4-6 days). At higher concentrations of 3-AT the appearance of 'positive' colonies could take up to ten days and there was a concern that these colonies might represent artifacts. Surprisingly, pGAD vector alone was isolated from a putative positive colony. As a result, reporter strains were tested as follows to establish screening conditions: the reporter strain was transfected plus or minus the empty pGAD242 vector and plated on increasing concentrations of 3-AT. The conditions where no colony growth was seen was chosen. Even with these stringent conditions in use, one of the two potential positive colonies generated by the library screen still harbored an apparently empty pGAD424 vector. Problems were also encountered in generating the yeast reporter strains. Strain generation was dependent on integration of the linear reporter plasmid into the yeast genome with the leaky expression of the reporter gene allowing for strain growth and selection on minimal media. Repeated attempts were made to integrate a cmosD16X-HISi-1 reporter construct into the yeast genome. No viable yeast strain could be generated (see Discussion).

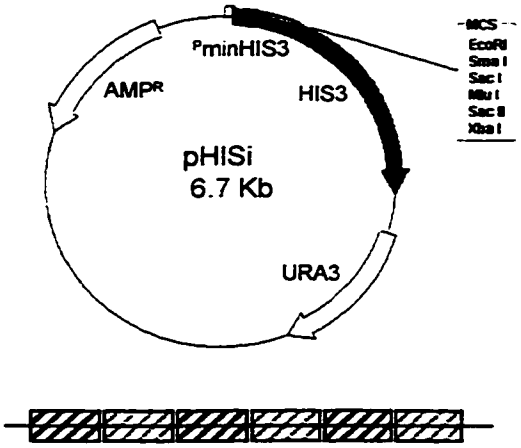
Figure. 32. Plasmid constructs generated for the yeast-one-hybrid screen.

Schematics of the plasmid constructs used in the yeast-one-hybrid screen. Panel A: a testis specific cDNA library cloned into the pGAD424 vector had previously been generated in the lab by Xueping Shao for screening in yeast systems. Multimers of the RT7D1 binding site were cloned into the three reporter plasmids, (Panels B, C, and D) supplied in the yeast one hybrid kit, to generate TTF-D target reporter strains for library screening.

A.

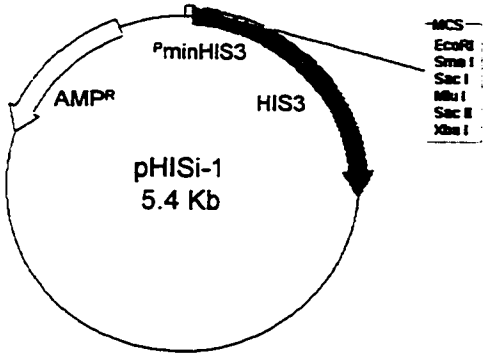


B.



RT7D1-6x cloned into Sma I

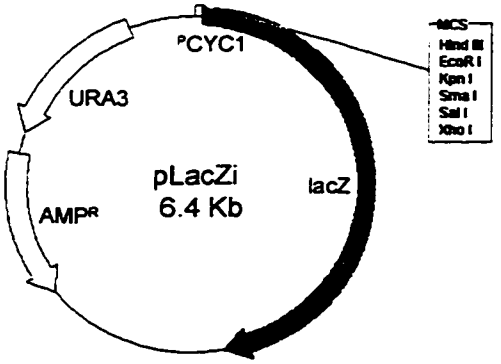
C.



multimerized binding site

RT7D1-6x, TTFD-2x, cmosD1-6x cloned into Sma I site

D.



RT7D1-6x cloned into Sma I

3.7 Interaction of the Testis-Specific Transcription Factor CREMτ with its Binding Site in the RT7 Promoter

3.7.1 Generation of CREMτ GST-Fusion Proteins

To study the *in vitro* binding properties of CREMτ, GST-fusion proteins were generated as described in Materials and Methods using the plasmids outlined in Figure 33. A full length wild type GST-CREMτ (GST-CT) and a mutant GST-CREMτ lacking the DNA binding domain (GST-ΔCT) were each bacterially expressed and column isolated. Figure 34 shows the size of each of the purified proteins on an SDS polyacrylamide gel stained with Coomassie blue. GST-CT (★) and GST-ΔCT (Δ) ran as expected at 67 kDa and 65 kDa respectively. The lower molecular weight bands present in each preparation were always seen in column purified fractions and may be due to either protein degradation or the result of potential internal translation start or stop sequences.

3.7.2 Properties of GST-CREMτ and GST-ΔCREMτ

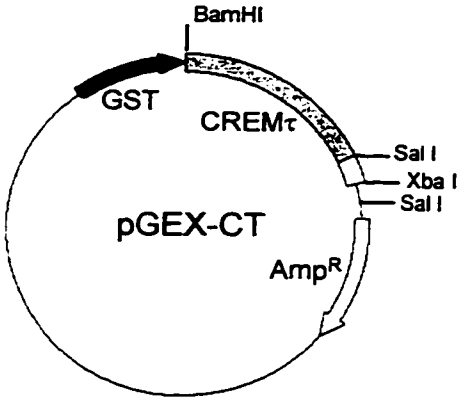
The ability of the fusion proteins to bind the RT7CRE site present in the oligo probe RT7CREMτ (see Table 2) was tested using EMSA. GST-CT but not GST-ΔCT could specifically bind the RT7CREMτ probe as shown in Figure 35. Liver extract (lane 2) which contain CRE-binding CREBs, and ST extract (lane 3) which contain both CREBs and CREMs were used as positive controls. Bacterially produced GST alone which did not bind (lane 4) was the negative control. GST-CT (lane 5) binding to RT7CREMτ was specific as addition of 200ng cold RT7CREMτ oligo (lane 6) competed for complex formation. GST-

ΔCT, which lacks the DNA binding domain, was unable to bind as expected (lanes 7 and 8).

Next, PKA was used to phosphorylate the GST-fusion proteins; previous results had demonstrated that CREMτ is a target for nuclear PKA in spermatids (De Groot *et al.* 1993). GST, GST-CT and GST-ΔCT were incubated in the absence (Figure 36, lanes 1, 2, 3 respectively) and the presence of PKA (Figure 36, lanes 4, 5, 6 respectively). Only GST-CT and GST-ΔCT were phosphorylated by PKA. The effect of phosphorylation on binding activity of GST-CT was assayed by EMSA. Figure 37 shows that binding of GST-CT to the CRE is enhanced by phosphorylation, as evidenced by increased amounts of retarded complex (compare lanes 3 and 5). Phosphorylation of GST has no effect on binding (lanes 2 and 4). The specificity of GST-CT binding was assayed by EMSA using the RT7CREMτ CRE sequence (Figure 38) or the somatostatin CRE (SMS) sequence (Figure 39) as probes and competitor oligos. In Figure 38, binding of the RT7CREMτ probe by GST-CT (lane 3) can be efficiently competed for by addition of SMS oligo (lane 4) but not by the ΔT oligo (lane 5) which contains a CRE-half site. In Figure 39, GST-CT binds to the SMS probe (lane 1) and the generated complex can be competed by addition of RT7CREMτ oligo (lanes 2 and 3) or SMS oligo (lanes 4 and 5) but not by the ΔT oligo (lane 6).

Figure. 33. GST-fusion protein plasmids.

pGEX-CT plasmid which generates the full length GST-CREMτ fusion protein was digested with BamHI and Sal I, both the digested plasmid and the truncated CREMτ gene were gel isolated and religated to generate the pGEX-ΔCT plasmid.



BamHI and Sal I restriction enzyme digest

Gel isolate 5 Kb and 0.9 Kb fragments and religate

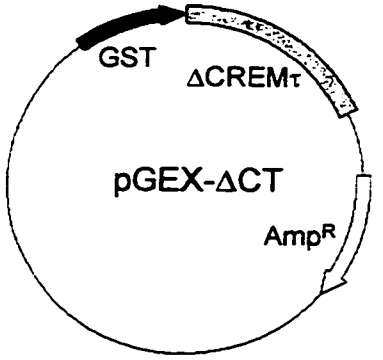


Figure. 34. Column purified GST-CREMτ and GST-ΔCREMτ.

Column purified GST-CT and GST-ΔCT were resolved by 10% SDS-PAGE followed by coomassie blue staining and destaining. GST-CT (★) runs at 67 kDa, GST-ΔCT (Δ) runs at 65 kDa.

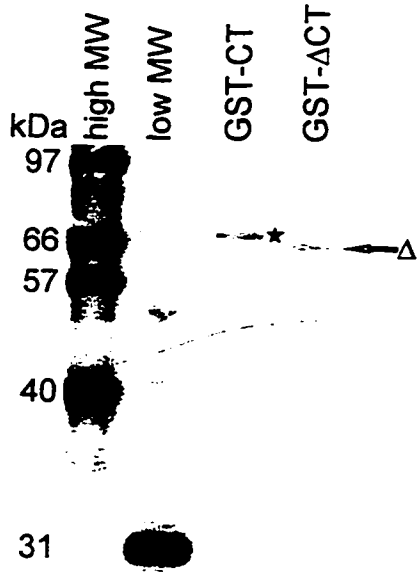

high MW
low MW
GST-CT
GST-ΔCT
kDa
97
66
57
40
31
Δ

Figure. 35. GST-CREMτ but not GST-ΔCREMτ interacts with its binding site.

EMSA of liver (lane 1), or ST (lane 2) nuclear extracts, GST (lane 4) GST-CT (lanes 5,6) and GST-ΔCT (lanes 7,8). Extracts or proteins were incubated with 1 ng ^{32}PRT7cremτ, 1 μg dIdC, and 200 ng cold RT7cremτ (lanes 6 and 8 only). Complexes were UV crosslinked and resolved by 10% native PAGE. GST alone is unable to bind. GST-CT binding is specific for the RT7cremτ site and removal of the DNA binding domain from GST-ΔCT disrupts binding completely. Probe alone, lane 1.

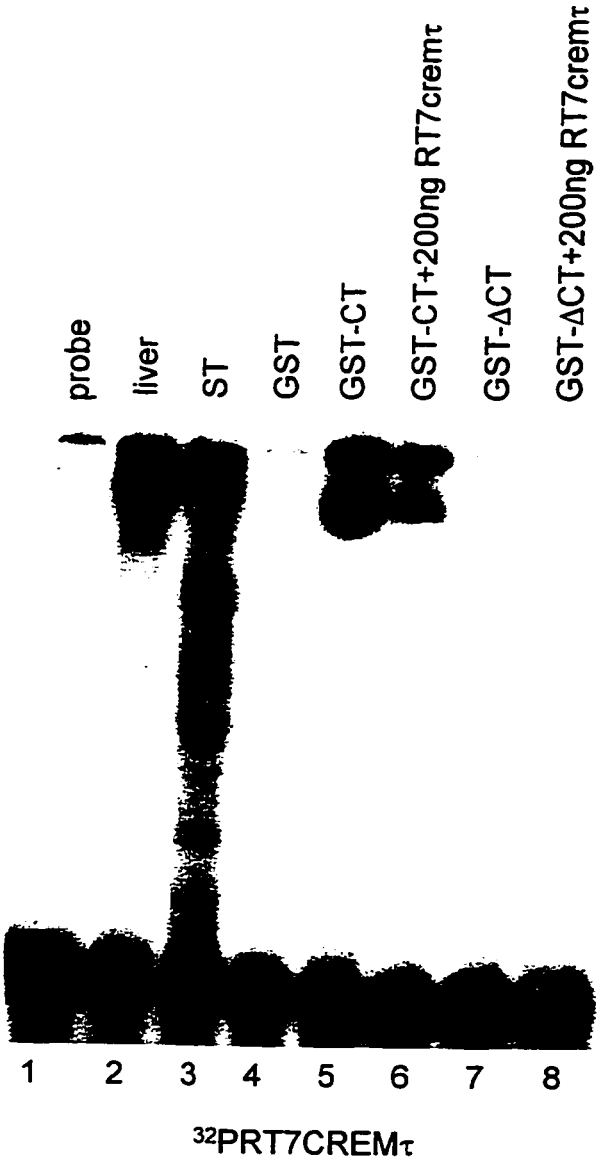

probe
liver
ST
GST
GST-CT
GST-CT+200ng RT7cremτ
GST-ΔCT
GST-ΔCT+200ng RT7cremτ
1 2 3 4 5 6 7 8
32PRT7CREMτ

Figure. 36. GST-CREMτ and GST-ΔCREMτ are phosphorylated by PKA. GST alone, GST-CT and GST-ΔCT were incubated under identical reaction conditions without (lanes 1-3) or with the addition of PKA (lanes 4-6). Reactions were then resolved by 10% SDS PAGE along with molecular weight markers. The gel was then stained, fixed and exposed to film. Only in the presence of PKA, are the GST-CT and GST-ΔCT phosphorylated.

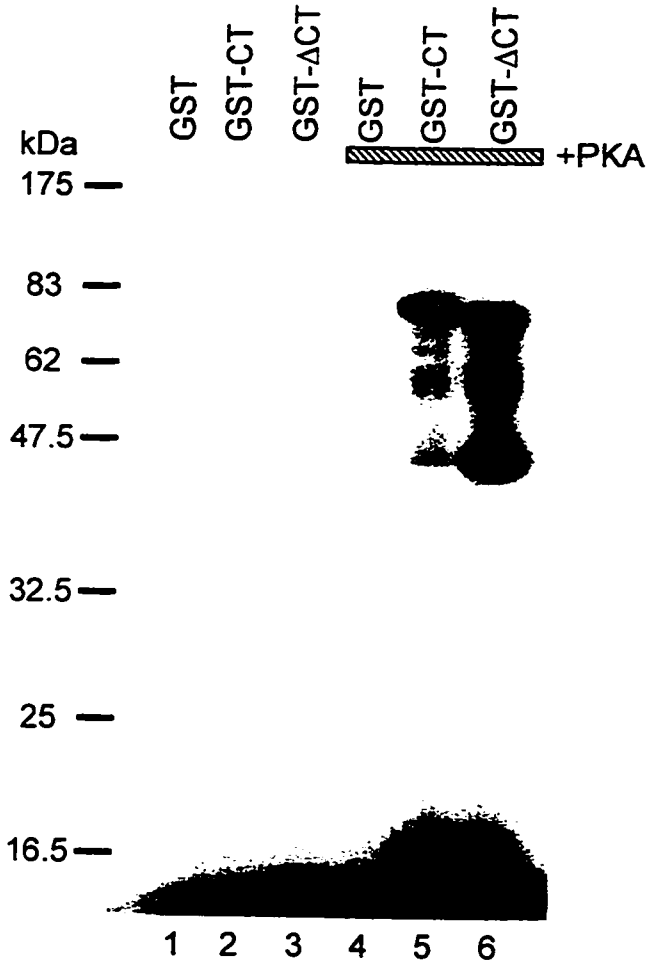
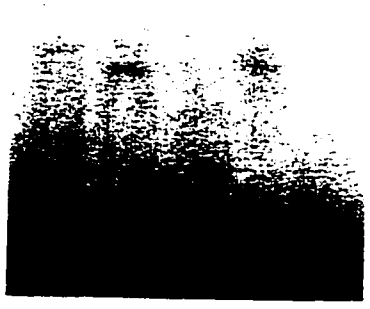

GST
GST-CT
GST-ΔCT
GST
GST-CT
GST-ΔCT
+PKA
kDa
175
83
62
47.5
32.5
25
16.5
1 2 3 4 5 6

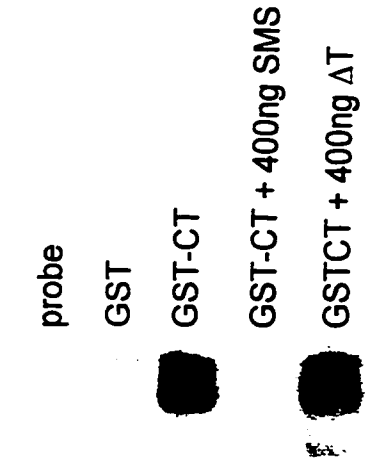
Figure. 37. Phosphorylation enhances DNA binding of GST-CREMτ.

GST and GST-CT were incubated with ^{32}PRT7cremτ without (lanes 2,3) or with PKA (lanes 4,5) under gel retardation conditions. Complexes were resolved by 10% native PAGE. Phosphorylation of GST-CT by PKA greatly enhances DNA binding. Probe alone, lane 1.



^{32}PRT7cremτ

Figure. 38. GST-CREMτ binding can be competed for by somatostatin CRE. EMSA of GST (lane 2), or GST-CT (lanes 3-5) proteins incubated with radiolabeled RT7cremτ without or with addition of competitor oligo. Addition of 400ng somatostatin CRE (SMS) specifically competes for the GST-CT (lane 4) where as 400 ng of the CRE half site ΔT oligo (lane 5) does not compete. Probe alone, lane 1.


probe
GST
GST-CT
GST-CT + 400ng SMS
GSTCT + 400ng ΔT

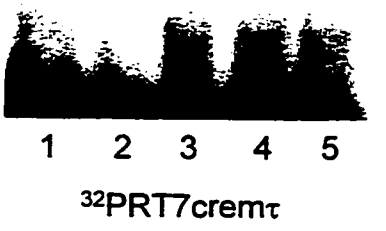
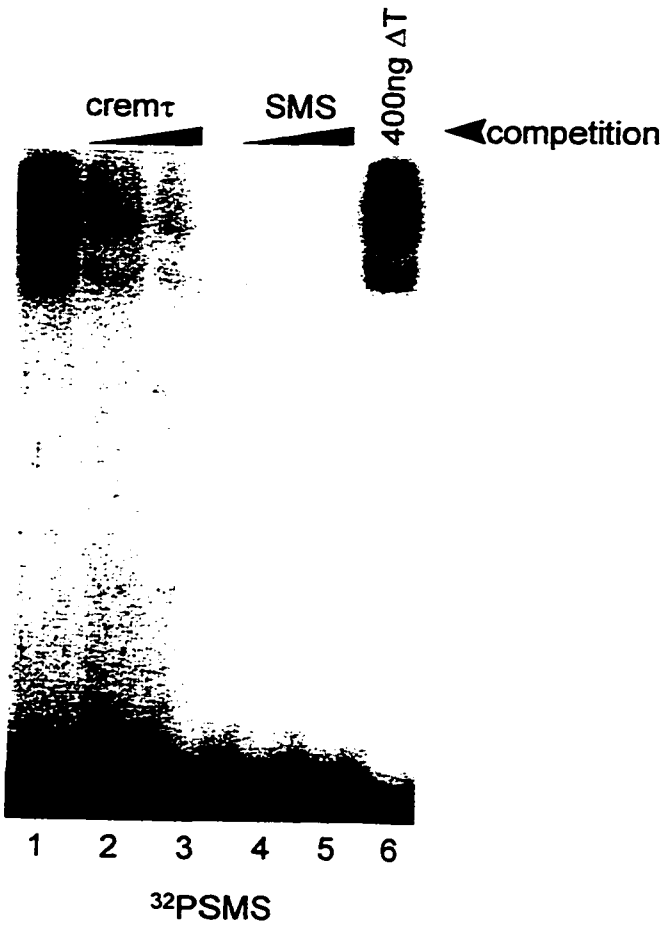

1
2
3
4
5
32PRT7cremτ

Figure. 39. GST-CREMτ binds the somatostatin CRE.

GST-CT was incubated with ^{32}PSMS under EMSA conditions without (lane 1) or with (lanes 2-6) addition of competitor oligos. Competitions included an excess of 200 and 400 ng RT7cremτ (lanes 2,3), 200 and 400 ng SMS CRE (lanes 4,5) and 400 ng ΔT (lane 6).


cremτ
SMS
400ng ΔT
competition
1 2 3 4 5 6
32PSMS

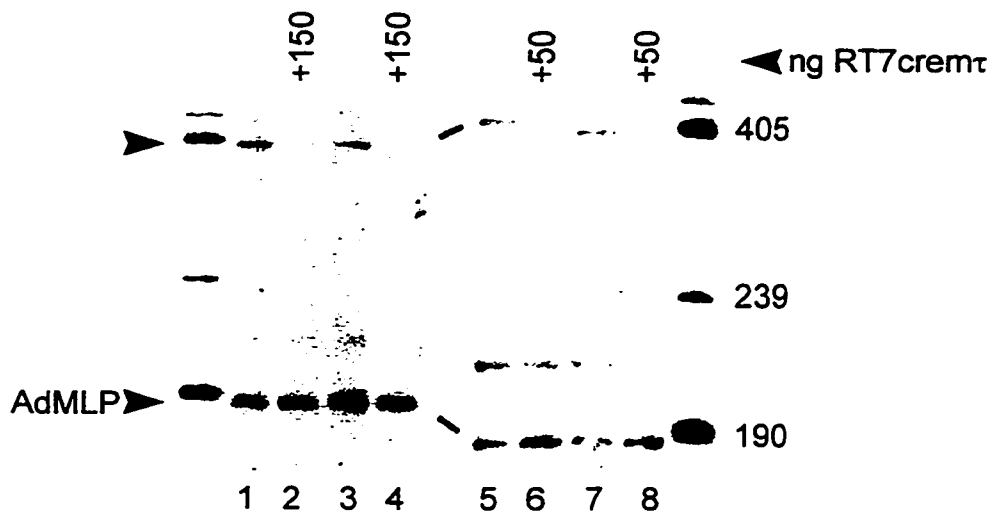
3.7.3 Repression of RT7 and mP1 Promoter in vitro Activity by RT7CREMτ oligo.

The potential of CREMτ protein to activate not only the RT7 but also the mouse protamine (mP1) promoter *in vitro* was also investigated. The mP1 promoter contains a CRE that binds bacterially expressed CREMτ protein (Delmas *et al.* 1993). The RT7 and mP1 promoters, each linked to a G-free cassette reporter gene were incubated in ST extracts with or without addition of different amounts of RT7CREMτ oligonucleotide, which acts as competitor for the binding of endogenous CREMτ to these promoters. Figure 40 shows that the addition of 150ng of this oligo reduces RT7 promoter activity (lanes 1 and 2) and mP1 promoter activity (lanes 3 and 4) to undetectable levels. The addition of 50ng RT7CREMτ oligo indicates that this decrease depends on the excess of RT7 CRE added (lanes 5-8). The oligo had no effect on the activity of internal control promoter AdMLP (lanes 1-8) which lacks a CRE.

It was of interest to test the activity of the GST-CT and GST-ΔCT fusion proteins in an *in vitro* transcription assay system. Recombinant CREB protein had been shown to stimulate transcription from a 3xCRE-MLP in an *in vitro* transcription assay with purified basal transcription factors (Nakajima *et al.* 1997). Addition of PKA-phosphorylated GST-CT to ST extract in an *in vitro* transcription assay did not stimulate transcription from the RT7 promoter (not shown).

Figure. 40. Repression of RT7 and mouse protamine 1 promoter activity by RT7cremτ oligo.

In vitro transcription assay with ST extract and the RT7 promoter constructs pRT7-C_2AT (lanes 1,2) and pRT7-CAP0.2 (lanes 5,6) or the mouse protamine 1 (mP1) promoter construct pmP1-C_2AT (lanes 3,4,7,8) without (lanes 1,3,5,7) or with (lanes 2,4,6,8) addition of RT7cremτ oligo. All reactions contained pAdMLP as an internal control. Addition of RT7cremτ oligo represses transcription of the test promoter driven transcript (arrow head) in all cases.


+150
+150
+50
+50
ng RT7cremτ
405
239
190
AdMLP
1 2 3 4 5 6 7 8

3.7.4 *Polyclonal Antibodies to GST-CREMτ*

Polyclonal antibodies to the bacterially expressed GST-CREMτ were generated according to the Materials and Methods. The specificity of the antibodies to CREMτ was analyzed in immunoprecipitation and immunofluorescence assays. Figure 41 shows an immunoprecipitation of *in vitro* transcribed and translated CREMτ protein with a control antibody against CREMτ (lane 1) and the two rabbit polyclonal antibodies generated against GST-CT (lanes 2 and 3). The antibodies specifically recognized the CREMτ protein. This specificity was also seen in immunofluorescence analysis of rat testis frozen sections. The primary antibody, rabbit-αCREMτ-polyclonal, was detected by the secondary antibody α-rabbit-Cy3 which fluoresces red. The results, shown in Figure 42 indicated that the antibody was specific to CREMτ. Panel A shows a phase contrast cross section of a rat seminiferous tubule, including the basement membrane (BM) and the lumen (Lu). Immunofluorescence of the same section, (Panel B) shows that the only cells which express CREMτ protein are round spermatids (RS; arrow). Spermatocytes do not express CREMτ (Sp; arrowhead). Panel C is a computer generated overlay of Panels A and B (Panel B was made transparent and superimposed over Panel A) to highlight which cells react to the antibody. Panel D is a 100X magnification of round spermatids which show the fluorescent staining to be exclusively nuclear, the punctate pattern within the nuclei indicates that CREMτ is excluded from the nucleoli. The results, shown in Figure 42 indicated that the antibody was specific to CREMτ.

Figure. 41. Polyclonal αCREMτ antibodies immunoprecipitate *in vitro* translated CREMτ protein.

In vitro translated CREMτ protein (42 kDa) was immunopreciptated with a control antibody (gift of Dr. P. Sassone-Corsi) (lane 1) and two rabbit polyclonal antibodies generated against GST-CT (lanes 2 and 3).

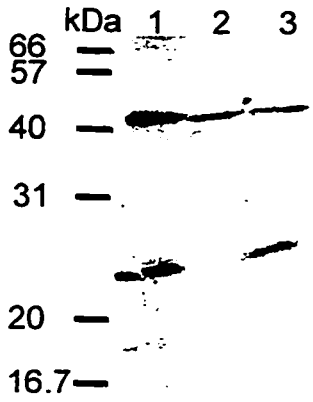
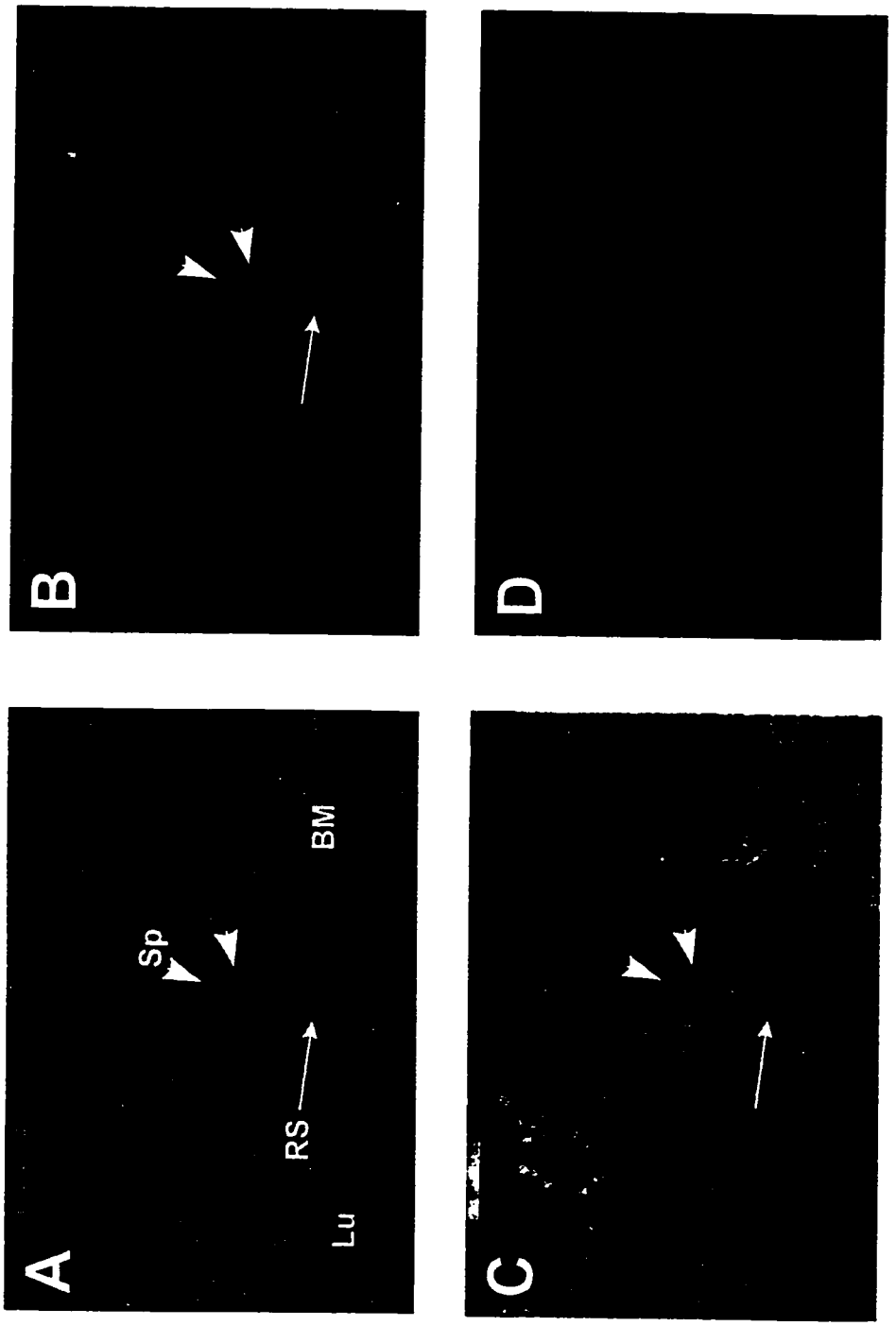

kDa
1
2
3
66
57
40
31
20
16.7

Figure. 42. Polyclonal αCREMτ antibody is specific for endogenous CREMτ.

Rat testis frozen sections were processed for immunofluorescence with polyclonal anti-CREMτ antibodies. A cross section of a seminiferous tubule at 40X magnification is shown in phase contrast (Panel A) and under immunofluorescence (Panel B). The basement membrane (BM), examples of spermatocytes (Sp) and round spermatids (RS), and lumen (Lu) are indicated (Panel A). Panel C is an overlay of Panels A and B to highlight that only the round spermatids contain CREMτ. Panel D is a 100X magnification of round spermatids showing that the antibody staining is exclusively nuclear.


A
Sp
RS
BM
Lu
B
C
D

3.7.5 Microinjection Studies with GST-CREMτ and GST-ΔCREMτ

The ability of GST-CT protein to activate gene expression from the pRT7lacZ reporter construct was tested *in vivo* by microinjection studies. Co-injections of pSVCREMτ and pRT7lacZ, or pRT7lacZ DNA alone into Hs68 cells (gift of Dr. K. Riabowol) were used as controls. GST-CT or GST-ΔCT were co-injected with pRT7lacZ into Hs68 cells. After an overnight incubation in the presence of forskolin, cells were fixed and assayed for lacZ activity. Results, shown in Table 3, indicate that GST-CT did not significantly transactivate the RT7 promoter in this assay.

3.8 A 40 kDa Somatic Protein Binds the TTF-D Binding Site

Throughout the thesis work on TTF-D, liver nuclear extract was used as a control extract to differentiate between somatic and testis-specific proteins. DNase I footprinting experiments had shown a liver protein footprint partially overlapping with both the RT7D1 and cmosD1 binding sites (van der Hoorn 1992; van der Hoorn & Tarnasky 1992). One major protein complex was consistently seen in EMSA using liver extract and RT7D1 or related oligo probe. The developmental study of TTF-D expression also indicated that this liver protein was expressed throughout post-partum development (Figure 6, arrow). UV crosslinking experiments followed by SDS PAGE showed that the major liver protein crosslinked to RT7D1 or cmosD1 oligo was approximately 40 kDa in size (see Figure 11. lanes 1 and 6). Figure 43 shows EMSA of liver nuclear extract with oligo competition for the 40 kDa protein. Addition of 100 ng of dsRT7D1 oligo competed well for the 40 kDa liver protein (lane 3). CmosD1 (lane 4) and

D2 (lane 5) oligos competed to a lesser degree and FOOTIII oligo (see Table 2) did not compete (lane 6). Liver nuclear extract was also analyzed by gel shift assay using the three mutant oligos as probe (Figure 44). MUT1 oligo (lane 2) bound the 40 kDa protein as well as the dsRT7D1 oligo (lane 1). Binding affinity for MUT2 oligo appeared to be reduced (lane 3) and MUT3 oligo did not bind the 40 kDa liver protein under EMSA conditions (lane 4).

Table 3. Microinjection of Hs68 cells with pSVCREMτ or GST-CREMτ fusion proteins and pRT7lacZ DNA.

INJECTION	CELLS INJECTED	X-GAL POSITIVE CELLS	PERCENT POSITIVE
pSVCREMτ + pRT7lacZ	450	54	12
pRT7lacZ	450	21	5
GST-CT + pRT7lacZ	900	18	2
GST-ΔCT + pRT7lacZ	450	5	1

Figure. 43. A liver nuclear protein binds to and is specific for the RT7D1 binding site.

ST nuclear extract (lane 1), or liver nuclear extract (lanes 2-8), were incubated with 1 ng ^{32}PdsRT7D1 and 1 μg dIdC. The specificity of the major protein in the liver extract for the RT7D1 binding site was tested by competition with 100 ng of double stranded oligonucleotides. Lanes 3-8 represent the oligos RT7D1, cmosD1, D1screen, RT7crem, D2, and FOOTIII respectively.

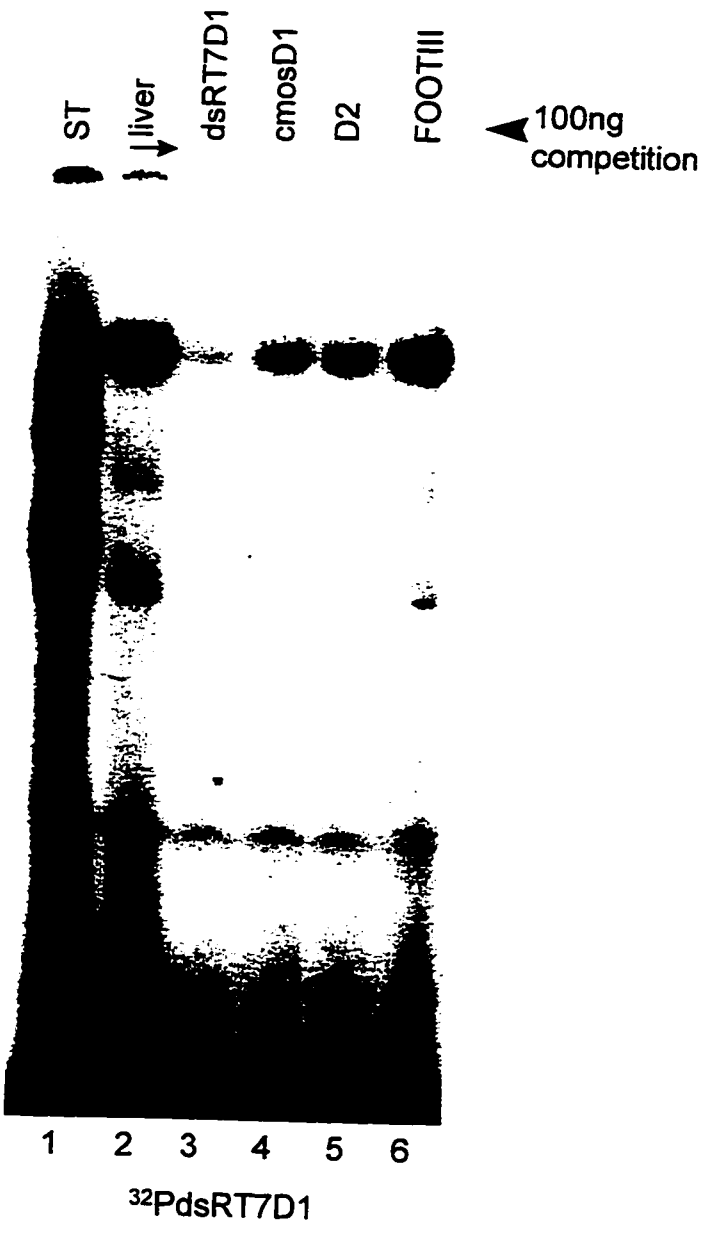
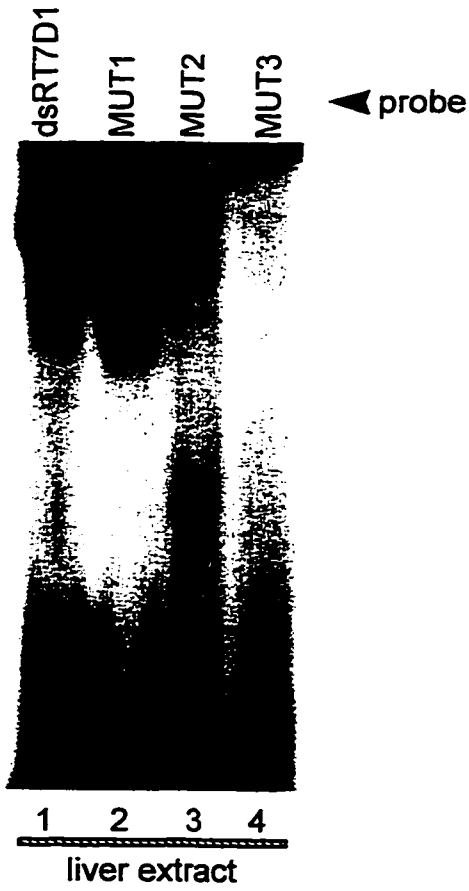

ST
liver
dsRT7D1
cmosD1
D2
FOOTIII
100ng
competition
1
2
3
4
5
6
32PdsRT7D1

Figure. 44. EMSA of liver nuclear extract and mutant RT7D1 binding sites. Liver nuclear extract was incubated with radiolabeled dsRT7D1, MUT1, MUT2, or MUT3 oligos and complexes resolved by native PAGE. MUT1 oligo has no effect on complex formation, however the affinity of the protein for the MUT2 site is reduced and no binding was seen when MUT3 oligo was used as probe.



RT7D1: AATTGGCCTTAGGG
MUT1: AATT c t CCTTAGGG
MUT2: AATTGGC acg AGGG
MUT3: AATTGGCCTT c t a G

Chapter 4: Discussion

Transcriptional regulation of genes plays a key role in their tissue- and cell-specific expression. In the testis, little is known regarding the transcription factors that play a role in the positive or negative regulation of testis-specific genes. The binding of testis-specific proteins within the promoters of several testis-specific genes has been reported. Further characterization of these proteins however, with the exception of CREMτ, has not occurred. TTF-D was identified as a testis-specific protein binding to the promoter region of the RT7 gene that was important for directing testis-specific transcription *in vitro*. The sequence of the TTF-D binding site (RT7D1) was deduced from DNAse I footprinting assays and gel retardation experiments (van der Hoorn & Tarnasky 1992). A binding site for a testis-specific protein, that was 80% homologous to RT7D1 was also found in the *c-mos* promoter (cmosD1) (van der Hoorn 1992). To gain a better understanding of the transcriptional regulation of RT7 and perhaps other testis-specific genes, we sought to characterize and clone TTF-D. Through the course of this work we have shown that TTF-D is a positive, testis-specific transcription factor which specifically binds both the RT7D1 element and the cmosD1 element. TTF-D activity is detectable in mice as early as day 11 after birth. TTF-D is composed of three germ cell-specific proteins 36, 25, and 22 kDa in size that specifically bind to both the RT7D1 and cmosD1 elements, and to the single stranded conformation of the RT7D1. Despite numerous and varied attempts, cloning of TTF-D was not successful. We also demonstrated that a 40 kDa somatic protein, possibly a transcriptional repressor, interacts specifically with the RT7D1 binding site.

It was recently shown that CREMτ is a transcriptional regulator of the RT7 gene (Delmas *et al.* 1993). In this thesis work, RT7 promoter-protein interaction was studied using bacterially expressed full length and truncated GSTCREMτ fusion proteins. The ability of these proteins to activate RT7 transcription *in vivo* was also tested. Importantly, the potential of endogenous CREMτ protein to activate the mouse protamine 1 promoter *in vitro* was demonstrated.

TTF-D binds both RT7D1 and cmosD1

TTF-D was shown to specifically bind both the RT7D1 and cmosD1 binding sites. Specific gel retardation patterns were generated by each of these two binding sites with ST extracts. In general, complex A, and not B-E, was seen to bind to cmosD1 and each of the complexes A-E were seen to bind to the RT7D1 site. The formation of the complexes gel shifted by RT7D1, particularly A, D and E, was extract dependent (see Figure 4). The basis of the different gel retardation pattern between cmosD1 and RT7D1 is not known, but may reflect differences in the structure of the DNA:TTF-D complexes, or perhaps cmosD1 is only interacting with one of TTF-D factors. The variability of the gel shift pattern seen with different extracts could reflect that these proteins are labile, or present in very low amounts in the testis.

The proteins generating the cmosD1 and RT7D1 gel shift patterns are highly related, if not identical, as each of these oligos could successfully self and cross compete for complex binding. The cmosD1 binding site was however, a weaker competitor, for TTF-D binding to RT7D1 oligo. The two nucleotides that differ in the cmosD1 site from the RT7D1 site may affect the stability of the

protein:DNA complex. Evidence in favor of this possibility came from our observation that the amount of TTF-D:cmosD1 complexes could be dramatically increased by UV irradiation prior to gel shift analysis (personal observation)

TTF-D activity is present early in germ cell development

The expression of RT7 is restricted to haploid spermatids (van der Hoorn *et al.* 1990), whereas expression of the *c-mos* gene in the testis is restricted to pachytene spermatocytes (van der Hoorn *et al.* 1991). Spermatocytes are produced for the first time 12-14 days post partum. We demonstrate that TTF-D activity is present early in testis development, from day 11 onward. Importantly, TTF-D activity was also demonstrated in nuclear extracts generated from elutriated germ cell fractions containing essentially only spermatocytes, or essentially only spermatids. A single TTF-D:RT7D1 complex was generated by pachytene spermatocyte and spermatid nuclear extracts (Figure 7). Significantly, this complex co-migrates with the TTF-D:RT7D1 complex A, and with the TTF-D:cmosD1 complex generated by ST extracts. Nuclear extracts from elutriated cells were difficult to generate, and they have a very low concentration of proteins. The low levels of TTF-D complex formation by these cell specific extracts is reflective of this. The presence of TTF-D activity early in testis development and in both pre- and post-meiotic germ cells, suggests a role for TTF-D in the transcriptional regulation of both pre-meiotic (*c-mos*) and post-meiotic (RT7) genes.

TTF-D proteins are 36, 25, and 22 kDa in size

Preliminary crosslinking experiments indicated that three testis-specific proteins of approximately 33, 23, and 20 kDa bind to the RT7D1 oligo. Based on these estimates, we were able to reconstitute the complete TTF-D gel retardation pattern with gel-purified, renatured ST proteins 25-40 kDa in size (Figure 10). Importantly, no new bands were generated by combining fractionated proteins of different molecular weight ranges. The TTF-D gel retardation patterns can be interpreted as follows. *A)* The 30-40 kDa fraction of ST nuclear protein, contains a 36 kDa protein which crosslinks to both cmosD1 and RT7D1 oligos (Figure 11, lanes 3 and 8). This fraction generates three retarded complexes A, D, and E (Figure 10, lane 2). Complex A is not always clearly detectable in gel retardation assays using different ST extracts. In our experience with crude ST nuclear extracts we see either complexes D/E or complex A (not shown). Figure 7 (lanes 1-3) demonstrates that the use of decreasing amounts of ST extract increased the amount of complex A, whereas complexes D and E decreased. These results demonstrate that complexes A, D and E contain the 36 kDa protein. The slower migration of complex A could result from the presence of either a homodimer of the 36 kDa protein, or a heterodimer of the 36 kDa protein and an uncharacterized second protein(whose molecular weight must be between 30-40 kDa) that does not crosslink to DNA. *B)* The TTF-D complexes B and C are generated by protein(s) with an apparent molecular weight of 25-30 kDa (Figure 10, lane 3). UV-crosslinking shows that proteins generating bands B and C are the 25 and 22 kDa TTF-D components that bind both the RT7D1 and cmosD1

sites (Figure 11, lanes 4 and 9 respectively). We do not know how or if these two proteins are related. The molecular weight of the TTF-D complexes was confirmed under non-crosslinking conditions by Southwestern assays (Figure 12). Testis-specific proteins 36, 25, and 22 kDa were detected in Southwesterns using several TTF-D binding site containing oligos, including the RT7D1 and cmosD1 sites. The somatic doublet of protein at 30/32 kDa detected in both liver and ST nuclear extracts is believed to be histone H1. Histone H1 is loosely bound to chromatin and is present at low levels in these extracts. Histone H1 binds DNA, and runs as a doublet at this molecular weight under these conditions (Campoy *et al.* 1995; DiMario *et al.* 1989). This suggests that the somatic doublet RT7D1 crosslinks to, seen at 40/42 kDa (Figure 13, arrows), may also be histone H1.

Crosslinking analysis of total ST extract confirmed that testis-specific proteins of the same molecular weights (36, 25 and 22 kDa) bound to both the RT7D1 and cmosD1 sites (Figure 13). The specificity of these proteins for this binding site, as shown in gel retardation assays (see above), was also seen under crosslinking conditions. All three protein complexes could be efficiently self- and cross-competed for with RT7D1 and cmosD1 oligos (Figure 14). These results demonstrate that testis-specific DNA binding nuclear proteins of 36, 25, and 22 kDa are present in TTF-D, these proteins specifically bind to both the RT7D1 and cmosD1 binding sites, and TTF-D activity is present in germ cell development at the time of expression of both the RT7 and *c-mos* genes.

TTF-D binds a single stranded RT7D1 site

In studying the nature of TTF-D interacting with its binding site, novel properties were discovered. TTF-D was demonstrated to bind both the double and single stranded RT7D1 binding site. TTF-D could bind equally well to either the RT7D1-sense or the RT7D1-αsense oligonucleotides (compare lanes 1 in Figures 15 and 16). RT7D1-αsense oligo however appeared to compete for TTF-D:ss-RT7D1 complexes more efficiently than either the sense or double strand RT7D1 oligos. This is perhaps indicative that TTF-D preferentially interacts with the α-sense binding site, or that the kinetics of binding favor the αsense binding site. Interestingly, the cmosD1-sense strand appeared to compete more efficiently than cmosD1-αsense for TTF-D binding to the ss-RT7D1 site. The reason for this is not known, but may be reflective of the differences seen in the TTF-D gel shift patterns for TTF-D:RT7D1 and TTF-D:cmosD1. It is also possible that the various DNA binding domains of the peptides which generate TTF-D are interacting with different key nucleotides on either strand of the binding site, resulting in the differences seen in the EMSA competition with the single stranded oligos. Crosslinking studies show that the 36, 25, and 22 kDa TTF-D complexes which bind to the dsRT7D1 and dscmosD1 sites, also specifically bind the RT7D1 and cmosD1 sense and αsense oligos. This demonstrates novel single strand specific binding properties of TTF-D. The implications of this finding are not known, but these may contribute to TTF-D acting as a positive regulator of RT7 transcription (see below).

Numerous regulators of transcription have been identified and cloned on the basis of their double stranded DNA binding properties. Only a relatively small number of sequence-specific, single stranded-DNA-binding proteins have been described. Roles for these single stranded binding proteins have been implicated in the regulation of replication, for example in the c-*myc* gene (Negishi *et al.* 1994; Takai *et al.* 1994), as well as both positive and negative regulation of transcription. Negative regulation of transcription by proteins binding single stranded elements in the proximal promoter or enhancer regions of genes has been reported for genes such as, the myelin basic protein (Haas *et al.* 1995), the androgen receptor (Grossmann & Tindall 1995), nicotinic acetylcholine receptor delta-subunit (Sapru *et al.* 1996), and the growth hormone receptor (Menon *et al.* 1997). The Y-box protein YB-1 binds a single stranded DNA binding site and suppresses major histocompatibility class II gene expression (MacDonald *et al.* 1995). Other Y-box proteins highly homologous to YB-1 have also been shown to bind single stranded elements in the thyrotropin receptor gene (Ohmori *et al.* 1996), and in the chicken alpha 2(I) collagen gene (Bayarsaihan *et al.* 1996)

Two *Xenopus* Y-box proteins have been identified that stimulate transcription *in vitro*, namely FRG Y1 and FRG Y2 (Tafuri & Wolffe 1992). The mouse homologue of FRG Y2 is a testis-specific protein p54/p56. p54/p56 has recently been shown to interact with the promoters of the testis-specific cytochrome c gene (Yiu *et al.* 1997), and the mouse mP2 gene (Nikolajczyk *et al.* 1995). Binding activity of p54/p56 to its site in the mP2 promoter was seen to be preferentially single stranded (Nikolajczyk *et al.* 1995). The regulatory effects of

this Y-box protein have not yet been elucidated, but it is suggested to play a role in transcription of mP2 and possibly other testis-specific genes. Consensus binding sequences are found in other testis-specific gene promoters including: protamine 1, transition protein 2, PGK-2, and the testis-specific H2B promoter [references within, (Nikolajczyk *et al.* 1995)].

Single stranded binding proteins which have been shown to positively regulate transcription include Pur alpha (binds in the neuron-specific FE65 gene promoter) (Zambrano *et al.* 1997), Brn-3a (alpha-internexin gene promoter) (Budhram-Mahadeo *et al.* 1996), PYBP (human transferrin gene promoter) (Brunel *et al.* 1991), and the far upstream element-binding proteins (FBP) (Davis-Smyth *et al.* 1996). FBP was originally identified binding to the FUSE element in a single-stranded manner, in the c-*myc* gene where it acts to stimulate transcription (Duncan *et al.* 1994).

Mutation analysis of the RT7D1 site

A putative TTF-D binding site (70% identical) was identified in the sequence immediately upstream of the RT7D1 binding site. This larger sequence, termed TTFD oligo, was used as competitor in EMSA for the single TTF-D binding site (Figures 19, 20, and 21). Overall, the larger TTFD oligo was seen to compete more efficiently for protein binding than the smaller RT7D1 binding site, either single or double stranded. The efficiency of competition however, was not high enough to conclude that the extra nucleotides containing the RT7D1-like sequences play a significant role in TTF-D complex formation. Mutational analysis of the 5' (MUT1), central (MUT2), and 3' (MUT3) nucleotides

of RT7D1 served to demonstrate which nucleotides were playing a role in TTF-D binding. Gel retardation assays with the mutant oligos used as probes demonstrated varying effects on TTF-D complex formation (Figure 22). MUT3 oligo was as efficient as RT7D1-sense oligo in binding TTF-D, MUT1 oligo was slightly less efficient, and MUT2 oligo did not generate TTF-D complexes. The MUT oligos were also less efficient competitors in EMSA for TTF-D:RT7D1-sense or -αsense complexes. Surprisingly MUT2 oligo could compete somewhat (albeit very poorly) for the RT7D1-sense probe. This was unexpected as the MUT2 oligo did not gel shift or crosslink to (see below) any of the TTF-D complexes. In gel shift assays however MUT2 probe consistently generated a large complex that was unable to migrate out of the gel slot, perhaps masking any weak TTF-D:MUT2 interactions. MUT2 did not compete at all for TTF-D:RT7D1-αsense complexes. Crosslinking studies demonstrated that TTF-D complexes were generated by the 36, 25, and 22 kDa proteins and the MUT1 and MUT3 oligos, but not with the MUT2 oligo (Figure 25).

The dramatic loss of TTF-D binding activity with the MUT2 oligo demonstrated that the nucleotides CTT were essential for binding. The putative TTF-D binding site in the larger TTFD oligo, which did not significantly change binding activity (see above), does not contain this CTT sequence. The importance of the central CTT nucleotides for binding TTF-D was also seen using the D2 oligo. The D2 oligo which also has the nucleotides CTT could compete for TTF-D complex formation (Figure 26). D2 competition was weak compared to wild type RT7D1 oligo however, suggesting that the nucleotide

sequence surrounding the CTT core also plays a role in TTF-D binding. The inability of MUT1 and MUT3 oligos to compete as efficiently as wild type oligos for TTF-D complexes supports this. The ability of MUT1 oligo to bind and compete for TTF-D complexes was compromised. The nucleotides in the 5' end of the binding site may be important for stability of TTF-D:DNA complexes. The cmosD1 site has two nucleotides that differ from the RT7D1 site, one of which changes the CCTT to ACTT. TTF-D:cmosD1 complexes were seen to be less stable than TTF-D:RT7D1 complexes, supporting the possibility that the nucleotides 5' to the binding site play a role in the stability of the protein DNA interaction.

TTF-D is a positive regulator of RT7 transcription

Promoter deletion studies had previously suggested that the TTF-D *cis*-acting element played a role in positive regulation of gene expression *in vitro*. Work shown here demonstrates that TTF-D is a positive transcriptional regulator of the RT7 gene. Addition of double stranded competitor oligos for TTF-D resulted in a moderate decrease of RT7 transcription levels *in vitro* (Figure 27). A more dramatic repression of RT7 promoter activity was seen by addition of single stranded RT7D1 binding sites (Figure 28). This suggests that TTF-D, preferentially interacts with a single stranded binding site, a result that was also reflected by single stranded competition in gel shift assays. This leaves the possibility that the moderate competition seen with double stranded oligos is actually competition from remaining single stranded oligos. Importantly, the RT7 promoter activity was not repressed by addition of the MUT2 oligo, which does

not bind TTF-D, or the unrelated B-sense oligo. This confirms the key role that the central CTT nucleotides play in the TTF-D binding site.

Cloning assays

Through the course of this thesis work, several approaches were taken to try to clone TTF-D. Initial attempts were made using a magnetic bead DNA affinity column. This method was chosen since it was rapid and reactions could easily be kept on ice. The presence of TTF-D activity in eluted column fractions was inconsistent and if seen, it was in very low concentrations. In silver stain gels of eluted protein, protein of the expected size range for TTF-D could not be detected above background. The failure of the Dyna bead columns could be due to several factors. The nature of the TTF-D binding site on the beads may be compromising binding activity. The RT7D1 oligonucleotide is short, a 14mer, and attachment of a monomer of this sequence directly to the beads may be excluding the ability of TTF-D to bind. However, columns were generated with concatamers of the binding site, but binding of TTF-D did not significantly improve. Insertion of linker DNA between the bead and the binding site, or generation of longer concatamers, were not tested but could potentially improve TTF-D binding. Another possibility appears more likely: TTF-D proteins may be highly labile or present at very low levels in the testis thus making column purification impossible. In conjunction with this, a limited amount of starting material could reasonably be produced, an extract from 30 rats gives about 8-10 mg of total testis nuclear proteins.

A testis cDNA λgt11 expression library was screened for TTF-D with various RT7D1 binding site oligonucleotide probes. No positive clones were identified beyond a secondary screening. It was possible that the bacterially expressed proteins were not generated in an active conformation with respect to the DNA binding domain. Denaturation/renaturation screening conditions using GuHCl did not improve the screening results. Bacterially expressed proteins are also not processed the same as eukaryotic proteins. In particular glycosylation of protein, which may be essential for a functional protein conformation, does not occur in bacteria. Perhaps such post-translational modifications of TTF-D are required for a DNA binding activity. Finally, TTF-D has been shown to be made up of three peptides. It is possible that DNA binding activity is dependent on the interaction of two possibly all three of these peptides. By definition the λgt11 library only allows for a single protein to be expressed per plaque which would prohibit successful cloning of TTF-D. However, this possibility seems less likely given our Southwestern results

The yeast-one-hybrid screen was also utilized in an effort to clone TTF-D. Several potential positives isolated were discarded after a second screening using a LacZ reporter strain. The only putative positive isolated clone was identified as rat ribosomal protein L17 (Suzuki & Wool 1991). This is a ubiquitous, protein that is not involved in transcriptional regulation and therefore was not considered as a candidate TTF-D. Problems inherent to the yeast-one-hybrid system were encountered, including high numbers of background colonies, and very slow growing yeast strains. As well it was not possible to

generate a yeast reporter strain with a cmosD1-6mer binding site for the target sequence. It was possible that the cmosD1-6mer sequence was being bound by a yeast repressor protein thus inhibiting the leaky HISi-1 reporter gene expression necessary for strain establishment. The multi-peptide nature of TTF-D, may make it impossible to clone if TTF-D binding to its target sequence is dependent on more than one peptide. As well the post-translational modifications of a foreign protein in yeast cells may not be carried out correctly to produce a functional protein. Overall, considering the problems outlined above and detailed in the Results, the use of the yeast-one-hybrid assay for cloning purposes should be approached with caution.

A 40 kDa somatic protein binds RT7D1

Previously, DNase I footprinting experiments had shown a liver protein footprint partially overlapping with the 3' end of both the RT7D1 and cmosD1 binding sites (van der Hoorn 1992; van der Hoorn & Tarnasky 1992). Gel shift assays with liver nuclear extract revealed one major protein that specifically interacted with the RT7D1 binding site (Figure 43), and crosslinking studies demonstrated the protein to be 40 kDa in size. This somatic protein was also demonstrated to be present as early as day 11 in total testis nuclear extracts, as expected. EMSA conditions demonstrated that mutations in the 3' end (MUT3) and the central region (MUT2) of the RT7D1 binding site were important for binding of the 40 kDa protein (Figure 44). The role of this protein is not known, however an intriguing possibility is that it plays a role in the repression of RT7 expression in somatic tissues.

Role of TTF-D

Work presented here has characterized TTF-D as a novel positive testis-specific transcription factor that plays a role in regulating RT7 gene expression. The presence of TTF-D activity early in germ cell development and the fact that it specifically binds the cmosD1 site suggests that it plays a regulatory role in both pre-meiotic (c-*mos*) and post-meiotic (RT7) genes. Sequence analysis of other testis-specific gene promoters revealed homologous binding sites in mP1 and mP2 (Table 4). TTF-D binding in these promoters however, has not been studied.

Proteins 36, 25, and 22 kDa in size generate the complete TTF-D:RT7D1 gel shift pattern and specifically bind to both double and single stranded RT7D1 and cmosD1 binding sites. TTF-D appeared to bind preferentially to the αsense RT7D1 binding site. The binding of TTF-D to a single stranded binding site has functional implications for TTF-D acting as a transcription factor. Altered DNA conformations are important in the regulation of gene expression. S1 nuclease sensitive regions occur in the 5' flanking regions of genes, which in some cases is cell type-specific and developmentally regulated (Santoro *et al.* 1991). TTF-D binding could result in a change in chromatin conformation of the promoter, thus facilitating binding of other RT7 regulators like CREMτ. Binding of TTF-D may act to prime the promoter region for CREMτ binding. Upon binding DNA, CREMτ has been shown to bend DNA (de Groot *et al.* 1994), which may result in TTF-D:CREMτ interactions working together to positively activate RT7 transcription (Figure 45). Alternatively, the introduction of a single-stranded loop or area

could introduce flexibility to that region of the promoter to facilitate interaction between otherwise topologically constrained proteins.

Figure. 45. Model of TTF-D binding.

TTF-D binds intially to its double stranded binding site in the RT7 promoter resulting in DNA melting and stabilization of a single stranded bubble. This single stranded region may act to introduce flexibility to the topologically constrained DNA, or facilitate binding of a second testis-specific transcription factor CREMτ. Binding of CREMτ may introduce DNA bending which in turn could facilitate protein interactions which act to positively activate RT7 transcription.

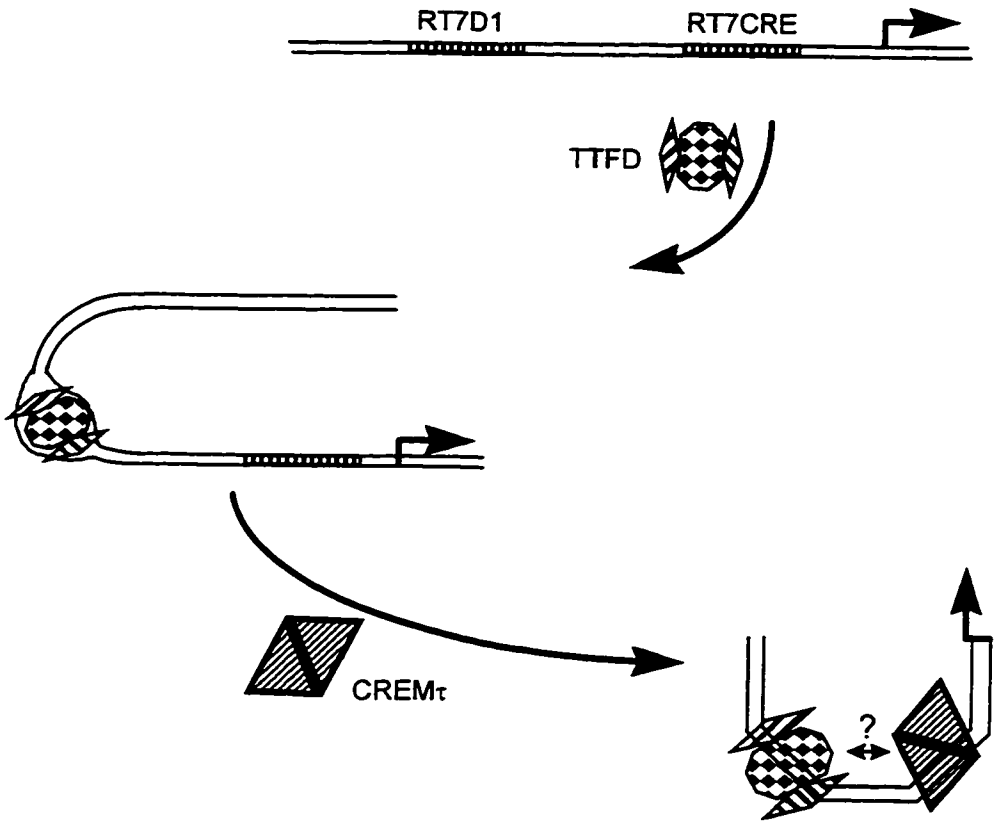

RT7D1
RT7CRE
TTFD
CREMτ
?

TABLE 4. Putative TTF-D Binding Site Homologs in Promoters of Testis-Specific Genes.

Listed are sequences which show homology to the RT7 and c-*mos* TTF-D binding sites. These sequences are present at the indicated positions in the testis-specific promoters of the mouse protamine 1 (mP1) and mouse protamine 2 (mP2) genes.

gene	location[1]	sequence	reference
PRE-MEIOTIC			
***mos*,r**	**-121**	**GGACTTAGGA**	(van der Hoorn 1992)
POST-MEIOTIC			
RT7,r	**-124**	**GGCCTTAGGG**	(van der Hoorn & Tarnasky 1992)
mP1	-287	GGCCTTGGGT	(Johnson *et al.* 1988)
mP2	-142	GGCTTAGGGG	(Johnson *et al.* 1988)

1) The location is according to the numbering of sequences published in the references and does not indicate the position with respect to transcription start sites.

The interaction of TTF-D with its binding site did not appear to be significantly affected by phosphorylation. This was only however briefly investigated and more work would need to be done using other phosphatases before such a role in TTF-D binding and transactivating activity can be established. The role of specific nucleotides in the RT7D1 binding site was investigated using mutant binding sites. The central triplet of pyrimidines CTT are essential for TTF-D binding with the 5' end of the binding site playing a role in the stability of complex formation. The 3' end of the RT7D1 binding site was shown to be involved in binding of the 40 kDa somatic protein. The TTFD sequence (RT7 binding site plus 12 upstream nucleotides) was entered for computer analysis in a transcription factor binding site database [http://agave.humgen.upenn.edu/]. None of the possible binding factors returned in the search, had binding sites which covered or even included the central CTT sequence shown to be necessary for TTF-D binding, or were of similar molecular weights (Table 5). TTF-D is a novel testis-specific transcription factor.

Table 5. Putative Transcription factor binding sites on the TTFD oligo as determined by comparison to entries in the transcription factor data base located at *http://agave.hu mgen.upenn.edu/.*

TTF-D binding site: 5' TAGGCCTGAGGAGGGCCTTAGGGAC 3'

Factor	Sequence	Molecular Weight[1]	Reference
TBF1*	TTAGGG	A= 63	(Brigati *et al.* 1993)
AP-2	CGCCTGCGGA GACCTGGGGA	A= 50-52 D= 48	(Mitchell & Tjian 1989)
Sp1	GAGGGCGT TGGGGAGGGGC GAGGCGGGGC	A= 95-105 D= 80	(Mitchell & Tjian 1989)
C/EBPα	GAGGAGGG	A= 42 D= 42	(Mitchell & Tjian 1989)
OCT	GAGGCGGGGC	*see note*	

[1]Size is approximate molecular mass: deduced from DNA sequence (D) or apparent from SDS PAGE (A)
*TBP: TATA binding factor
note: Octamer-binding factor was indicated but not which member of the family, molecular weight of OCT-1= 76 kDa, OCT-2= 51 kDa (Mitchell & Tjian 1989)

GST-CREMτ fusion protein binds to the RT7 promoter

The RT7 promoter contains a CRE and binds the testis-specific nuclear factor CREMτ. CREMτ has been shown to activate the RT7 promoter in both transient (Delmas *et al.* 1993), and stable (Oosterhuis & van der Hoorn 1996) transfection assays. RT7 promoter-protein interaction was studied using full length and truncated recombinant GST-CREMτ fusion proteins. Both the full length and the truncated CREMτ proteins were efficiently phosphorylated by PKA *in vitro*. The truncated protein, which lacks the DNA binding domain in the C-terminus of the protein, did not bind the RT7 or SMS CRE containing oligonucleotides. Specific binding of the full length GST-CREMτ was demonstrated, and binding activity was shown to be enhanced by phosphorylation of the GST-CREMτ protein. Recombinant CREMτ protein however was not transcriptionally active in *in vitro* assays (not shown), or *in vivo* as studied by microinjection. Bacterially expressed proteins do not have post-translational modifications that may be required for a protein to be transcriptionally active. Alternatively the GST moiety may interfere with the transactivating potential of the recombinant protein. It is also possible that the human cell line HS68, used in these experiments, may be expressing repressor proteins which upon binding to the RT7 promoter, inhibits expression of the reporter gene. Polyclonal antibodies were generated against GST-CREMτ and these were demonstrated to be specific for endogenous CREMτ protein (Figure 42). Immunofluorescence studies of rat testis sections with the α-CREMτ antibody only detected CREMτ in round spermatids, CREM antagonists and

CREB proteins which are expressed in pre-meiotic germs cells were undetectable. Staining for CREMτ in the round spermatids was demonstrated to be exclusively nuclear, and the punctate staining pattern within the nucleus suggested that nucleoli exclude CREMτ.

CREMτ regulates RT7 and mP1

The potential of endogenous CREMτ protein to activate the RT7 and mouse protamine 1 (mP1) promoters *in vitro* was demonstrated (Figure 40). CREMτ was shown to be a major player in the transcription of both the mP1 and RT7 promoters, since an excess of CRE oligo essentially abolished transcription from these promoters. The mP1 promoter had previously been shown to bind a recombinant CREMτ protein (Delmas *et al.* 1993), but this is the first evidence that CREMτ is playing a direct role in regulating mP1 transcription.

CHAPTER 5: Significance and Future Directions

The characterization of TTF-D as a positive testis-specific transcription factor, is significant when presented in the context of the field of spermatogenesis. To date only one testis-specific transcription factor, CREMτ, has been cloned and fully characterized. The factors involved in the precisely coordinated spatial and temporal expression of testis-specific genes remain largely unknown or only poorly characterized. Work presented here has shed light on the regulation of the RT7 gene by TTF-D. The specific interaction of TTF-D with a binding site present in the c-*mos* testis promoter also suggests that TTF-D plays a role regulation of c-*mos* as well. Significantly TTF-D activity is present in germ cell development at the time of expression for both of these genes. TTF-D may participate as a general testis-specific transcription factor for both pre- and post-meiotic genes. Novel single stranded binding properties of TTF-D were demonstrated, which may point to a functional role for TTF-D in generating conformational changes in promoter chromatin structure, thereby augmenting transcriptional activation of the gene. A 40 kDa somatic protein was also identified which specifically interacted with the RT7D1 binding site. The role of this protein is not known but its presence in somatic cells and specificity of binding to the RT7D1 site suggest that it may play a role as a repressor of RT7 expression. The fact that TTF-D proved to be unclonable was a source of both disappointment and frustration. A variety of approaches were taken and each of them failed to clone this factor. As a result, work on TTF-D and further efforts to clone it will not be pursued by the lab.

The regulation of RT7 by CREMτ has been well documented. Results presented here led to the publication of some of this work, (Oosterhuis & van der Hoorn 1996). Antibodies generated against CREMτ will be of further use in the lab to detect production of CREMτ in an inducible cell system. Importantly we have shown CREMτ to be a major player in transcription of the mP1 promoter. This discovery will further aide the understanding of the regulation of this testis-specific gene.

References

Alami-Ouahabi, N., Veilleux, S., Meistrich, M. L. & Boissonneault, G. 1996 The testis-specific high-mobility-group protein, a phosphorylation- dependent DNA-packaging factor of elongating and condensing spermatids. *Mol Cell Biol* **16**, 3720-9.

Albanesi, C., Geremia, R., Giorgio, M., Doici, S., Sette, C. & Rossi, P. 1996 A cell- and developmentl stage-specific promoter drives the expression of a truncated c-kit protein during mouse spermatid elongation. *Development* **122**, 1291-1302.

Ashworth, A. & Denny, P. 1991 Zinc finger protein genes in the mouse genome. *Mamm Genome* **1**, 196-200.

Balhorn, R. 1989 *Mammalian Protamines: Structure and Molecular Interactions*. Molecualr Biology of Chromosome Function. New York: Springer Verlag.

Bartell, J. G., Davis, T., Kremer, E. J., Dewey, M. J. & Kistler, W. S. 1996 Expression of the rat testis-specific histone H1t gene in transgenic mice. One kilobase of 5'-flanking sequence mediates correct expression of a lacZ fusion gene. *J Biol Chem* **271**, 4046-4054.

Bayarsaihan, D., Enkhmandakh, B. & Lukens, L. N. 1996 Y-box proteins interact with the S1 nuclease-sensitive site in the chicken alpha 2(I) collagen gene promoter. *Biochem J* **319**, 203-7.

Blendy, J. A., Kaestner, K. H., Weinbauer, G. F., Nieschlag, E. & Schutz, G. 1996 Severe impairment of spermatogenesis in mice lacking the CREM gene. *Nature* **380**, 162-5.

Boissonneault, G. & Lau, Y.-F. C. 1993 A testis-specific gene encoding a nuclear high-mobility-group box protein located in elongating spermatids. *Mol Cell Biol* **13**, 4323-4330.

Brigati, C., Kurtz, S., Balderes, D., Vidali, G. & Shore, D. 1993 An essential yeast gene encoding a TTAGGG repeat-binding protein. *Mol Cell Biol* **13**, 1306-14.

Brunel, F., Alzari, P. M., Ferrara, P. & Zakin, M. M. 1991 Cloning and sequencing of PYBP, a pyrimidine-rich specific single strand DNA-binding protein. *Nucleic Acids Res* **19**, 5237-5245.

Budhram-Mahadeo, V., Morris, P. J., Lakin, N. D., Dawson, S. J. & Latchman, D. S. 1996 The different activities of the two activation domains of the Brn-3a transcription factor are dependent on the context of the binding site. *J Biol Chem* **271**, 9108-13.

Campoy, F. J., Meehan, R. R., McKay, S., Nixon, J. & Bird, A. 1995 Binind of histone H1 to DNA is indifferent to methylation at CpG sequences. *J Biol Chem* **270**, 26473-26481.

Carlsen, E., Giwercman, A., Keiding, N. & Skakkebaek, N. 1992 Evidence for decreasing quality of semen during past 50 years. *Br Med J* **305**, 609-613.

Carlsen, E., Giwercman, A., Keiding, N. & Skakkebaek, N. E. 1995 Declining semen quality and increasing incidence of testicular cancer: is there a common cause? *Environ Health Perspect* **7**, 137-9.

Catalano, R. D., Vlad, M. & Kennedy, R. C. 1997 Differential display to identify and isolate novel genes expressed during spermatogenesis. *Mol Biol Reprod* **3**, 215-221.

Chen, F., Cooney, A. J., Wang, Y., Law, S. W. & O'Malley, B. W. 1994 Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. *Mol Endocrinol* **8**, 1434-44.

Chowdhury, K., Goulding, M., Walther, C., Imai, K. & Fickenscher, H. 1992 The ubiquitous transactivator Zfp-38 is upregulated during spermatogenesis with differential transcription. *Mech Dev* **39**, 129-142.

Clare, S. E., Hatfield, W. R., Fantz, D. A., Kistler, W. S. & Kistler, M. K. 1997 Characterization of the promoter region of the rat testis-specific histone H1t gene. *Biol Reprod* **56**, 73-82.

Cole, K. D., Kandala, J. C. & Kistler, W. S. 1986 Isolation of the gene for the testis-specific H1 histone variant H1t. *J Biol Chem* **261**, 7178-7183.

Connor, F., Wright, E., Denny, P., Koopman, P. & Ashworth, A. 1995 The Sry-related HMG box-containing gene Sox6 is expressed in the adult testis and developing nervous system of the mouse. *Nucleic Acids Res* **23**, 3365-72.

Cunliffe, V., Koopman, P., McLaren, A. & Trowsdale, J. 1990 A mouse zinc finger gene which is transiently expressed during spermatogenesis. *Embo J* **9**, 197-205.

Davis-Smyth, T., Duncan, R. C., Zheng, T., Michelotti, G. & Levens, D. 1996 The far upstream element-binding proteins comprise an ancient family of single-strand DNA-binding transactivators. *J Biol Chem* **271**, 31679-87.

De Groot, R.P., Delmas, V. & Sassone-Corsi, P. 1994 DNA bending by transcription factors CREM and CREB. *Oncogene* **9**, 463-468.

De Groot, R. P., den Hertog, J., Vandenheede, J. R., Goris, J. & Sassone-Corsi, P. 1993 Multiple and cooperative phosphorylation events regulate the CREM activator function. *EMBO J* **12**, 3903-3911.

Delmas, V. & Sassone-Corsi, P. 1994 The key role of CREM in the cAMP signaling pathway in the testis. *Mol Cell Endocrinol* **100**, 121-4.

Delmas, V., van der Hoorn, F. A., Mellstrom, B. J. & Sassone-Corsi, P. 1993 Induction of CREM activator proteins in spermatids: down-stream targets and implications for haploid germ cell differentiation. *Mol Endo* **7**, 1502-1514.

Denny, P. & Ashworth, A. 1991 A zinc finger protein-encoding gene expressed in the post-meiotic phase of spermatogenesis. *Gene* **106**, 221-227.

Denny, P., Swift, S., Conner, F. & Ashworth, A. 1991 An SRY-related gene expressed during spermatogenesis in the mouse encodes a sequence-specific DNA-binding protein. *EMBO J* **11**, 3705-3712.

DiMario, P. J., Bromley, S. E. & Gall, J. G. 1989 DNA-binding proteins on lampbruch chromosome loops. *Chromosoma* **97**, 413-420.

Dix, D. J., Roasrio-Herrle, M., Gotoh, H., Mori, C., Goulding, E. H., Barrett, C. V. & Eddy, E. M. 1996 Developmentally regulated expression of Hsp 70-2 and a Hsp 70-2/lacZ transgene during spermatogenesis. *Develop Biol* **174**, 310-321.

Duncan, R., Bazar, L., Michelotti, G., Tomonaga, T., Krutzsch, H., Avigan, M. & Levens, D. 1994 A sequence-specific, single-strand binding portein activates the far upstream element of c-*myc* and defines a new DNA-binding motif. *Genes Devel* **8**, 465-480.

Enmark, E. & Gustafsson, J.-A. 1996 Orphan nuclear receptors - the first eight years. *Mol Endo* **10**, 1293-1307.

Erickson, R. P. 1990 Post-meiotic gene expression. *TIG* **6**, 264-269.

Ewulonu, U. K. & Schimenti, J. C. 1997 Function of untranslated regions in the mouse spermatogenesis-specific gene Tcp10 evaluated in transgenic mice. *DNA Cell Biol* **16**, 645-51.

Ewulonu, U. K., Snyder, L., Silver, L. M. & Schimenti, J. C. 1996 Promoter mapping of the mouse Tcp-10bt gene in transgenic mice identifies essential male germ cell regulatory sequences. *Mol Reprod Dev* **43**, 290-297.

Foulkes, N. S., Borrelli, E. & Sassone-Corsi, P. 1991 CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**, 739-49.

Foulkes, N. S., Mellstrom, B., Benusiglio, E. & Sassone-Corsi, P. 1992 Developmental switch of CREM function during spermatogenesis: from antagonist to activator. *Nature* **355**, 80-4.

Foulkes, N. S. & Sassone-Corsi, P. 1992 More is better: activators and repressors from the same gene. *Cell* **68**, 411-4.

Foulkes, N. S., Schlotter, F., Pevet, P. & Sassone-Corsi, P. 1993 Pituitary hormone FSH directs the CREM functional switch during spermatogenesis. *Nature* **362**, 264-7.

Gebara, M. M. & McCarrey, J. R. 1992 Protein-DNA interactions associated with the onset of testis-specific expression of the mammalian Pgk-2 gene. *Mol Cell Biol* **12**, 1422-1431.

Gehring, W. J., Qiu Qian, Y., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G. & Wuthrich, K. 1994 Homeodomain-DNA Recognition. *Cell* **78**, 211-223.

Gorski, K., Carneiro, M. & Schibler, U. 1986 Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47**, 767-776.

Goto, M., Koji, T., Mizuno, K., Tamaru, M., Koikeda, S., Nakane, P. K., Mori, N., Masamune, Y. & Nakanishi, Y. 1990 Transcription switch of two phosphoglycerate kinase genes during spermatogenesis as determined with mouse testis sections in situ. *Exp Cell Res* **186**, 273-8.

Goto, M., Masamune, Y. & Nakanishi, Y. 1993 A factor stimulating transcription of the testis-specific Pgk-2 gene recognizes a sequence similar to the binding site for a transcription inhibitor of the somatic-type Pgk-1 gene. *Nucleic Acids Res* **21**, 209-214.

Goto, M., Tamura, T., Mikoshiba, K., Masamune, Y. & Nakanishi, Y. 1991 Transcription inhibition of the somatic-type phosphoglycerate kinase 1 gene in vitro by a testis-specific factor that recognizes a sequence similar to the binding site for Ets oncoproteins. *Nucleic Acids Res* **19**, 3959-63.

Grimes, S. R., van Wert, J. & Wolfe, S. A. 1997 Regulation of transcription of the testis-specific histone H1t gene by multiple promoter elements. *Mol Biol Rep* **24**, 175-184.

Grimes, S. R., Wolfe, S. A., Anderson, J. V., Stein, G. S. & Stein, J. L. 1990 Structural and functional analysis of the rat testis-specific histone H1t gene. *J Cell Biochem* **44**, 1-17.

Grimes, S. R., Wolfe, S. A. & Koppel, D. A. 1992a Temporal correlation between the appearance of testis-specific DNA- binding proteins and the onset of transcription of the testis-specific histone H1t gene. *Exp Cell Res* **201**, 216-224.

Grimes, S. R., Wolfe, S. A. & Koppel, D. A. 1992b Tissue-specific binding of testis nuclear proteins to a sequence element within the promoter of the testis-specific histone H1t gene. *Arch Biochem Biophys* **296**, 402-409.

Griswold, M. D. 1995 Interactions between Germ Cells and Sertoli Cells in the Testis. *Biol Repro* **52**, 2111-216.

Grossmann, M. E. & Tindall, D. J. 1995 The androgen receptor is transcriptionally suppressed by proteins that bind single-stranded DNA. *J Biol Chem* **270**, 10968-10975.

Gstaiger, M., Knoepfel, L., Georglev, O., Schaffner, W. & Hovens, C. M. 1995 A B-cell coactivator of octomer-binding transcription factors. *Nature* **373**, 360-362.

Ha, H., van Wijnen, A. J. & Hecht, N. B. 1997 Tissue-specific protein-DNA interactions of the mouse protamine 2 gene promoter. *J Cell Biochem* **64**, 94-105.

Haas, S., Steplewski, A., Siracusa, L. D., Amini, S. & Khalili, K. 1995 Identification of a sequence-specific single-stranded DNA binding protein that suppresses transcription of the mouse myelin basic protein gene. *J Biol Chem* **270**, 12503-10.

Hager, D. A. & Burgess, R. R. 1980 Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of

Escherichia coli RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Anal Biochem* **109**, 76-86.

Hecht, N. B. 1989 Molecular biology of structural chromosomal proteins of the testis. In *Molecular Biology of Chromosome Function* (ed. K. W. Adolph), pp. 396-420. New York: Springer Verlag.

Hecht, N. B. 1990 Regulation of 'haploid expressed genes' in male germ cells. *J Reprod Fert* **88**, 679-693.

Heery, D. M., Gannon, F. & Powell, R. 1990 A simple method for subcloning DNA fragments from gel slices. *TIG* **6**, 173.

Higgy, N. A., Pastoor, T., Renz, C., Tarnasky, H. A. & van der Hoorn, F. A. 1994 Testis-specific RT7 protein localizes to the sperm tail and associates with itself. *Biol Reprod* **50**, 1357-1366.

Higgy, N. A., Tarnasky, H. A., Valarche, I., Nepveu, A. & van der Hoorn, F. A. 1997 Cux/CDP homeodomain protein binds to an enhancer in the rat c-mos locus and represses its activity. *Biochim Biophys Acta* **1351**, 313-24.

Higgy, N. A., Zackson, S. L. & van der Hoorn, F. A. 1995 Cell Interactions in Testis Development: Over expression of c-mos in Spermatocytes Leads to Increased Germ Cell Proliferation. *Dev Genet* **16**, 190-200.

Hoffman, C. S. & Winston, F. 1987 A ten-minute DNA preparation from yeast effciantly releases autonomous plasmids for transformation of Escherichia coli. *Gene* , 267-272.

Hosseini, R., Marsh, P., Pizzey, J., Leonard, L., Ruddy, S., Bains, S. & Dudley, K. 1994 Restricted expression of a zinc finger portein in male germ cells. *J. Mol. Endocrinol* **13**, 157-165.

Howard, T., Balogh, R., Overbeek, P. & Bernstein, K. E. 1993 Sperm-specific expression of angiotensin-converting enzyme (ACE) is mediated by a 91-base-pair promoter containing a CRE-like element. *Mol Cell Biol* **13**, 18-27.

Howard, T. E., Shai, S.-Y., Langford, K. G., Martin, B. M. & Bernstein, K. E. 1990 Transcription of testicular angiotensin-converting enzyme (ACE) is initiated within the 12th intron of the somatic ACE gene. *Mol Cell Biol* **10**, 4294-4302.

Iannello, R. C., Kola, I. & Dahl, H. H. 1993 Temporal and tissue-specific interactions involving novel transcription factors and the proximal promoter of the mouse Pdha-2 gene. *J Biol Chem* **268**, 22581-22590.

Iannello, R. C., Young, J., Sumarsono, S., Tymms, M. J., Dahl, H.-H. M., Gould, J., Hedger, M. & Kola, I. 1997 regulation of *Pdha*-2 expression is mediated by proximal promoter sequences and CpG Methylation. *Mol Cell Biol* **17**, 612-619.

Iannello, R. C., Young, J. C. & Kola, I. 1994 Pdha-2: a model for studying transcriptional regulation in early spermatocytes. *Mol Reprod Dev* **39**, 194-199.

Johnson, P. A., Bunick, D. & Hecht, N. B. 1991 Protein binding regions in the mouse and rat protamine-2 genes. *Biol Reprod* **44**, 127-134.

Johnson, P. A., Peschon, J. J., Yelick, P. C., Palmiter, R. D. & Hecht, N. B. 1988 Sequence homologies in the mouse protamine 1 and 2 genes. *Biochim Biophys Acta* **950**, 45-53.

Kanai, Y., Kanai-Azuma, M., Noce, T., Saido, T. C., Shiroishi, T., Hayashi, Y. & Yazaki, K. 1996 Identification of two Sox17 messenger RNA isoforms, with and without the high mobility group box region, and their differential expression in mouse spermatogenesis. *J Cell Biol* **133**, 667-81.

Katz, D., Niederberger, C., Slaughter, G. R. & Cooney, A. J. 1997 Characterization of germ cell-specific expression of the orphan nuclear receptor, germ cell nuclear factor. *Endocrinology* **138**, 4364-72.

Kistler, M. K., Sassone-Corsi, P. & Kistler, W. S. 1994 Identification of a functional cyclic adenosine 3',5'-monophosphate response element in the 5'-flanking region of the gene for transition protein 1 (TP1), a basic chromosomal protein of mammalian spermatids. *Biol Reprod* **51**, 1322-9.

Kremer, E. J. & Kistler, W. S. 1991 Localization of mRNA for testis-specific histone H1t by *in situ* hybridization. *Exp Cell Res* **197**, 330-332.

Kumari, M., Stroud, J. C., Anji, A. & McCarrey, J. R. 1996 Differential appearance of DNase I-hypersensitive sites correlates with differential transcription of Pgk genes during spermatogenesis in the mouse. *J Biol Chem* **271**, 14390-14397.

Lalli, E., Lee, J. S., Lamas, M., Tamai, K., Zazopoulos, E., Nantel, F., Penna, L., Foulkes, N. S. & Sassone-Corsi, P. 1996 The nuclear response to cAMP: role of transcription factor CREM. *Philos Trans R Soc Lond B Biol Sci* **351**, 201-9.

Langford, K. G., Shai, S. Y., Howard, T. E., Kovac, M. J., Overbeek, P. A. & Bernstein, K. E. 1991 Transgenic mice demonstrate a testis-specific promoter for angiotensin-converting enzyme. *J Biol Chem* **266**, 15559-62.

Laoide, B. M., Foulkes, N. S., Schlotter, F. & Sassone, C. P. 1993 The functional versatility of CREM is determined by its modular structure. *Embo J* **12**, 1179-91.

Larsson, N. G., Garman, J. D., Oldfors, A., Barsh, G. S. & Clayton, D. A. 1996 A single mouse gene encodes the mitochondrial transcription factor A and a testis-specific nuclear HMG-box protein. *Nat Genet* **13**, 296-302.

Lee, C.-H., Chang, L. & Wei, L.-N. 1996 Molecular cloning and characterization of a mouse nuclear orphan receptor expressed in embryos and testes. *Mol Reprod Devel* **44**, 305-314.

Lee, K., Haugen, H. S., Clegg, C. H. & Braun, R. E. 1995 Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. *Proc Natl Acad Sci U S A* **92**, 12451-5.

Lee, K. A. & Masson, N. 1993 Transcriptional regulation by CREB and its relatives. *Biochim Biophys Acta* **1174**, 221-33.

Lei, W., Hirose, T., Zhang, L. X., Adachi, H., Spinella, M. J., Dmitrovsky, E. & Jetten, A. M. 1997 Cloning of the human orphan receptor germ cell nuclear factor/retinoid receptor-related testis-associated receptor and its differential regulation during embryonal carcinoma cell differentiation. *J Mol Endocrinol* **18**, 167-76.

Li, J. J. & Herskowitz, I. 1993 Isolation of ORC6, a component of the yeast origin of recognition complex by a one-hybrid system. *Science* **262**, 1870-1873.

Lichtsteiner, S. & Schibler, U. 1989 A glycosylated liver-specific transcription factor stimulates transcription of the albumin gene. *Cell* **57**, 1179-87.

Lilienbaum, A. & Paulin, D. 1993 Activation of the human vimentin gene by the Tax human T-cell leukemia virus. I. Mechanisms of regulation by the NF-kappa B transcription factor. *J Biol Chem* **268**, 2180-8.

MacDonald, G. H., Itoh-Lindstrom, Y. & Ting, J. P. 1995 The transcriptional regulatory protein, YB-1, promotes single-stranded regions in the DRA promoter. *J Biol Chem* **270**, 3527-33.

Maddocks, S., Parvinen, M., Soder, O., Punnonen, J. & Pollanen, P. 1990 Regulation of the testis. *J Reprod Immunol* **18**, 33-50.

Maniatis, T., Fritsch, E. F. & Sambrook, J. 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor NY: Cold Spring Harbor Laboratory.

McCaffrey, P. G., Perrino, B. A., Soderling, T. R. & Rao, A. 1993 NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *J Biol Chem* **268**, 3747-52.

McLachlan, R. I., Wreford, N. G., O'Donnell, L., de Kretser, D. M. & Robertson, D. M. 1996 The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. *J Endo* **148**, 1-9.

Menon, R. K., Cheng, H. & Singh, M. 1997 Identification and characterization of single strand DNA-binding protein that represses growth hormone receptor gene expression. *Mol Endocrinol* **11**, 1291-304.

Mitchell, P. J. & Tjian, R. 1989 Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-378.

Monila, C. A., Foulkes, N. S., Lalli, E. & Sassone-Corsi, P. 1993 Inducibility and negative autoregulation of CREM: An alternative promoter directs the expression of ICER, an early response repressor. *Cell* **75**, 875-886.

Nakajima, T., Uchida, C., Anderson, S. F., Parvin, J. D. & Montminy, M. 1997 Analysis of a cAMP-responsive activator reveals a two-component mechanism for transcriptional induction via signal-dependent factors. *Gen Dev* **11**.

Nantel, F., Monaco, L., Foulkes, N. S., Masquilier, D., LeMeur, M., Henriksen, K., Dierich, A., Parvinen, M. & Sassone-Corsi, P. 1996 Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature* **380**, 159-62.

Nayernia, K., Nieter, S., Kremling, H., Oberwinkler, H. & Engel, W. 1994 Functional and molecular characterization of the transcriptional regulatory region of the proacrosin gene. *J Biol Chem* **269**, 32181-32186.

Negishi, Y., Nishita, Y., Saegusa, Y., Kakizaki, I., Galli, I., Kihara, F., Tamai, K., Miyajima, N., Iguchi-Ariga, S. M. M. & Ariga, H. 1994 Identification and cDNA cloning of single-stranded DNA binding proteins that interact with the region upstream of the human c-*myc* gene. *Oncogene* **9**, 1133-1143.

Nikolajczyk, B. S., Murray, M. T. & Hecht, N. B. 1995 A mouse homologue of the Xenopus germ cell-specific ribonucleic acid/deoxyribonucleic acid-binding proteins p54/p56 interacts with the protamine 2 promoter. *Biol Repro* **52**, 524-30.

Ohmori, M., Ohta, M., Shimura, H., Shimurat, Y., Suzuki, K. & Kohn, L. D. 1996 Cloning of the single strand DNA-binding protein important for maximal expression and thyrotropin (TSH)-induced negative regulation of the TSH receptor. *Mol Endocrinol* **10**, 1407-24.

Oko, R., Korley, R., Murray, M. T., Hecht, N. B. & Hermo, L. 1996 Germ cell-specific DNA and RNA binding proteins p48/52 are expressed at specific stages of male germ cell development and are present in the chromatoid body. *Mol Reprod Dev* **44**, 1-13.

Olivia, R. & Dixon, G. H. 1991 Vertebrate Protamine Genes and the Histone-to-Protamine Replacement Reaction. *Prog Nucleic Acids Res* **40**, 25-94.

Oosterhuis, J. H. & van der Hoorn, F. A. 1996 CREMtau activates the spermatid-specific RT7 and mouse protamine 1 promoters. In *Cellular and Molecular Regulation of Testicular Cells* (ed. C. Desjardins), pp. 73-82. Norwell, MA: Springer.

Passananti, C., Corbi, N., Paggi, M. G., Russo, M. A., Perez, M., Cotelli, F., Stefanini, M. & Amati, P. 1995 The product of Zfp59 (Mfg2), a mouse gene expressed at the spermatid stage of spermatogenesis, accumulates in spermatozoa nuclei. *Cell Growth Differ* **6**, 1037-1044.

Peschon, J. J., Behringer, R. R., Brinsster, R. L. & Palmiter, R. D. 1987 Spermatid-specific expression of protamine 1 in transgenic mice. *Proc Natl Acad Sci USA* **84**, 5316-5319.

Peschon, J. J., Behringer, R. R., Palmiter, R. D. & Brinster, R. L. 1989 Expression of mouse protamine 1 genes in transgenic mice. *Ann NY Acad Sci* **564**, 186-197.

Queralt, R. & Oliva, R. 1993 Identification of conserved potential regulatory sequences of the protamine-encoding P1 genes from ten different mammals. *Gene* **133**, 197-204.

Queralt, R. & Oliva, R. 1995 Demonstration of trans-acting factors binding to the promoter region of the testis-specific rat protamine P1 gene. *Biochem Biophys Res Commun* **208**, 802-812.

Robinson, M. O., McCarrey, J. R. & Simon, M. I. 1989 Transcriptional regulatory regions of testis-specific PGK-2 defined in transgenic mice. *Proc Natl Acad Sci USA* **86**, 8537-8441.

Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P. & Clegg, E. D. 1990 *Mammalian Spermatogenesis. In: Histological and Histopathological Evaluation of the Testis.* Clearwater Florida: Cache River Press.

Santoro, I. M., Yi, T. M. & Walsh, K. 1991 Identification of single-stranded-DNA-binding proteins that interact with muscle gene elements. *Mol Cell Biol* **11**, 1944-53.

Sapru, M. K., Gao, J. P., Walke, W., Burmeister, M. & Goldman, D. 1996 Cloning and characterization of a novel transcriptional repressor of the nicotinic acetylcholine receptor delta-subunit gene. *J Biol Chem* **271**, 7203-11.

Sassone-Corsi, P. 1995 Transcription factors responsive to cAMP. *Ann Rev Cell Dev Biol* **11**, 355-77.

Sassone-Corsi, P. 1997 Transcriptional Checkpoints Determining the Fate of Male Germ Cells. *Cell* **88**, 163-166.

Schmidt, E. E., Ohbayashi, T., Makino, Y., Tamura, T. & Shibler, U. 1997 Spermatid-specific over expression of the TATA-binding protein gene involves recruitment of two potent testis-specific promoters. *J Biol Chem* **272**, 5326-5334.

Schmidt, E. E. & Schibler, U. 1995 High accumulation of components of the RNA polymerase II transcription machinery in rodent spermatids. *Development* **121**, 2373-83.

Seyedin, S. N. & Kistler, W. S. 1980 Isolation and characterization of rat testis H1t. *J Biol Chem* **255**, 5949-5954.

Shao, X., Tarnasky, H. A., Schalles, U., Oko, R. & van der Hoorn, F. A. 1997 Interactional cloning of the 84-kDa major outer dense fiber protein Odf84. Leucine zippers mediate associations of Odf84 and Odf27. *J Biol Chem* **272**, 6105-13.

Shao, X. & van der Hoorn, F. A. 1996 Self-interaction of the major 27-kilodalton outer dense fiber protein is in part mediated by a leucine zipper domain in the rat. *Biol Reprod* **55**, 1343-50.

Spiteri-Grech, J. & Nieschlag, E. 1993 Paracrine factors relevant to the regulation of spermatogenesis- a review. *J Reprod Fert* **98**, 1-14.

Stewart, T. A., Hecht, N. B., Hollingshead, P. G., Johnson, P. A., Leong, J. C. & Pitts, S. L. 1988 Haploid-specific transcription of protamine-myc and protamine-T-antigen fusion genes in transgenic mice. *Mol Cell Biol* **8**, 1748-1755.

Sun, Z., Sassone-Corsi, P. & Means, A. R. 1995 Calspermin gene transcription is regulated by two cyclic AMP response elements contained in an alternative promoter in the calmodulin kinase IV gene. *Mol Cell Biol* **15**, 561-71.

Suzuki, K. & Wool, I. G. 1991 The primary structure of rat ribosomal protein L17. *Biochem Biophys Res Commun* **178**, 322-328.

Tafuri, S. R. & Wolffe, A. P. 1992 DNA binding, multimerization, and transcription stimulation by the *Xenopus* Y box proteins *in vitro*. *New Biol* **4**, 349-359.

Takai, T., Nishita, Y., Iguchi-Ariga, S. M. M. & Ariga, H. 1994 Molecular cloning of MSSP-2, a c-myc gene single-strand binding protein: characterization of binding specificity and DNA replication activity. *Nucleic Acids Res* **22**, 5576-5581.

Tamura, T., Makino, Y., Mikoshiba, K. & Muramatsu, M. 1992 Demonstration of a testis-specific trans-acting factor Tet-1 in vitro that binds to the promoter of the mouse protamine 1 gene. *J Biol Chem* **267**, 4327-4332.

Taylor-Harris, P., Swift, S. & Ashworth, A. 1995 Zfy1 encodes a nuclear sequence-specific DNA binding protein. *FEBS Lett* **360**, 315-319.

Thomas, K. H., Wilkie, T. M., Tomashefsky, P., Bellve, A. R. & M.I., S. 1989 Differential gene expression during mouse spermatogenesis. *Biol Reprod* **41**, 729-739.

van der Hoorn, F. A. 1992 Identification of the testis c-*mos* promoter: specific activity in a seminiferous tubule-derived extract and binding of a testis-specific nuclear factor. *Oncogene* **7**, 1093-1099.

van der Hoorn, F. A., Spiegel, J. E., Maylie, P. M. & Nordeen, S. K. 1991 A 43 kD c-mos protein is only expressed before meiosis during rat spermatogenesis. *Oncogene* **6**, 929-32.

van der Hoorn, F. A. & Tarnasky, H. A. 1992 Factors involved in regulation of the RT7 promoter in a male germ cell-derived *in vitro* transcription system. *Proc Natl Acad Sci USA* **89**, 703-707.

van der Hoorn, F. A., Tarnasky, H. A. & Nordeen, S. K. 1990 A new rat gene RT7 is specifically expressed during spermatogenesis. *Dev Biol* **142**, 147-154.

van Wert, J. M., Wolfe, S. A. & Grimes, S. R. 1996 Binding of nuclear proteins to a conserved histone H1t promoter element suggests an important role in testis-specific transcription. *J Cell Biochem* **60**, 348-362.

vanWert, J. M., Wolfe, S. A. & Grimes, S. R. 1995 Testis-specific expression of the rat histone Hlt gene in transgenic mice. *Biochemistry* **34**, 8733-8743.

Wang, M. M. & Reed, R. R. 1993 Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. *Nature* **364**, 121-126.

Watanabe, D., Okabe, M., Hamajima, N., Morita, T., Nishina, Y. & Nishimune, Y. 1995 Characterization of the testis-specific gene 'calmegin' promoter sequence and its activity defined by transgenic mouse experiments. *FEBS Lett* **368**, 509-512.

Weiss, M., Vigier, M., Hue, D., Perrard-Sapori, M. H., Marret, C., Avallet, O. & Durand, P. 1997 Pre- and postmeiotic expression of male germ cell-specific genes throughout 2-week cocultures of rat germinal and Sertoli cells. *Biol Reprod* **57**, 68-76.

Willison, K. & Ashworth, A. 1987 Mammalian spermatogenic gene expression. *TIG* **3**, 351-355.

Wolfe, S. A., van Wert, J. M. & Grimes, S. R. 1995 Expression of the testis-specific histone H1t gene: evidence for involvement of multiple cis-acting promoter elements. *Biochemistry* **34**, 12461-12469.

Wolgemuth, D. J., Viviano, C. M., Gizang-Ginsberg, E., Frohman, M. A., Joyner, A. L. & Martin, G. R. 1987 Differential expression of the mouse homeobox-containing gene *Hox-1.4* during male germ cell differentiation and embryonic development. *Proc Natl Acad Sci USA* **84**, 5813-5817.

Wolgemuth, D. J., Viviano, C. M. & Watrin, F. 1991 Expression of homeobox genes during spermatogenesis. *Ann NY Acad Sci* **637**, 300-312.

Wolgemuth, D. J. & Watrin, F. 1991 List of cloned mouse genes with unique expression patterns during spermatogenesis. *Mamm Genome* **1**, 283-288.

Yan, Z. H., Medvedev, A., Hirose, T., Gotoh, H. & Jetten, A. M. 1997 Characterization of the response element and DNA binding properties of the nuclear orphan receptor germ cell nuclear factor/retinoid receptor-related testis-associated receptor. *J Biol Chem* **272**, 10565-72.

Yiu, G. K., Murray, M. T. & Hecht, N. B. 1997 Deoxyribonucleic acid-protein interactions associated with transcriptional initiation of the mouse testis-specific cytochrome c gene. *Biol Reprod* **56**, 1439-1449.

Yiu, G. K. & N.B, H. 1997 Novel testis-specific protein-DNA interactions activate transcription of the mouse protamine 2 gene during spermatogenesis. *J Biol Chem* **272**, 26926-26933.

Zambrano, N., De Renzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F. & Russo, T. 1997 DNA-binding protein Pur alpha and transcription factor YY1 function as transcription activators of the neuron-specific FE65 gene promoter. *Biochem J* **328**, 293-300.

Zambrowicz, B. P., Harendza, C. J., Zimmermann, J. W., Brinster, R. L. & Palmiter, R. D. 1993 Analysis of the mouse protamine 1 promoter in transgenic mice. *Proc Natl Acad Sci U S A* **90**, 5071-5075.

Zambrowicz, B. P. & Palmiter, R. D. 1994 Testis-specific and ubiquitous proteins bind to functionally important regions of the mouse protamine-1 promoter. *Biol Reprod* **50**, 65-72.

Zambrowicz, B. P., Zimmermann, J. W., Harendza, C. J., Simpson, E. M., Page, D., C., Brinster, R. L. & Palmiter, R. D. 1994 Expression of a mouse Zfy-1/lacZ transgene in the somatic cells of the embryonic gonad and germ cells of the adult testis. *Development* **120**, 1549-1559.

Zhou, Y., Overbeek, P. A. & Bernstein, K. E. 1996a Tissue specific expression of testis angiotensin converting enzyme is not determined by the -32 nonconsensus TATA motif. *Biochem Biophys Res Commun* **223**, 48-53.

Zhou, Y., Sun, Z., Means, A. R., Sassone-Corsi, P. & Bernstein, K. E. 1996b cAmp-response element modulator tau is a positive regulator of testis angiotensin converting enzyme transcription. *Proc Natl Acad Sci USA* **93**, 12262-12266.

IMAGE EVALUATION
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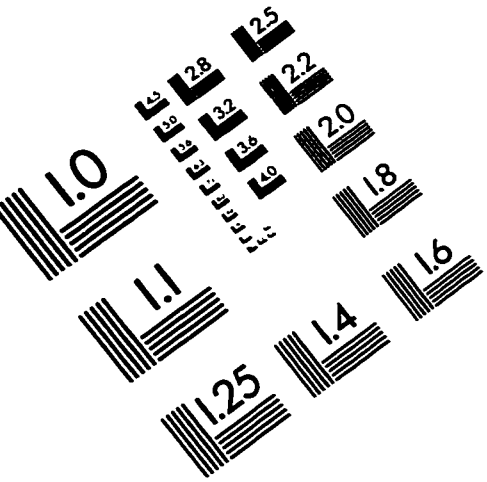

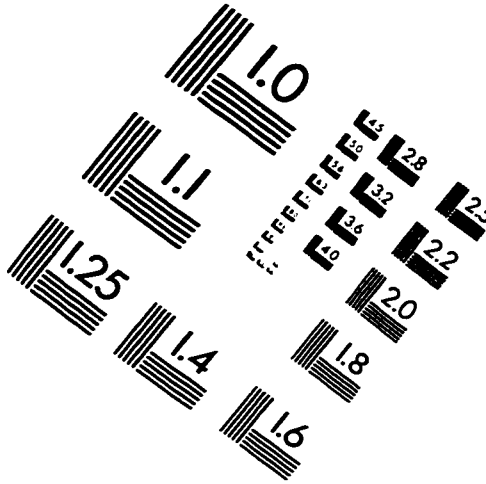

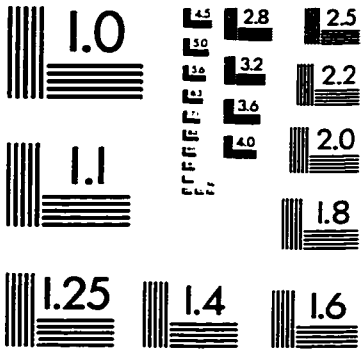

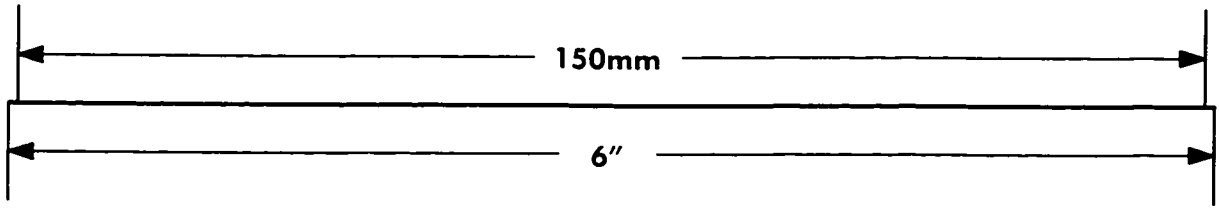

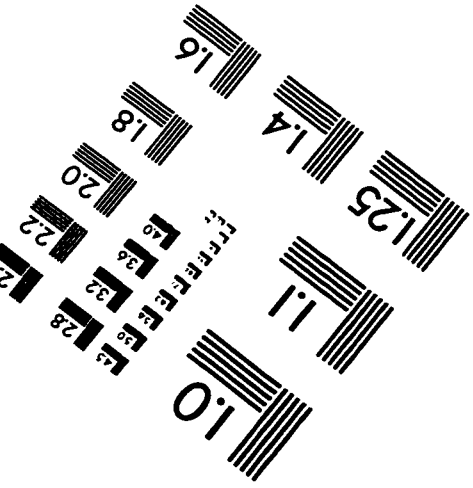

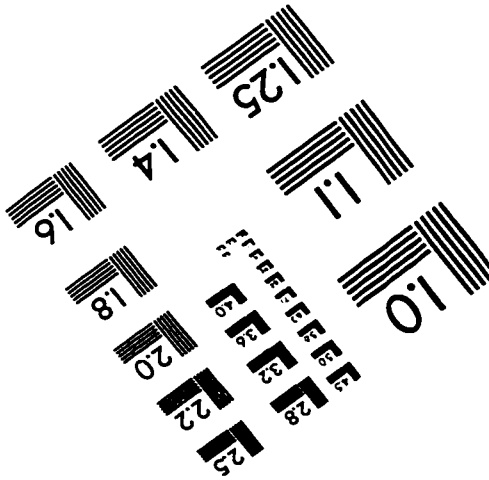

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