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Regulation of Gene Expression by Germ Cell Nuclear Factor

Angela Caldwell-Hudson

REGULATION OF GENE EXPRESSION BY GERM CELL NUCLEAR FACATOR

THESIS

BY

ANGELA CALDWELL-HUDSON

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THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of Texas Southern University

By

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1997

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REGULATION OF GENE EXPRESSION BY GERM CELL NUCLEAR FACTOR

By

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Texas Southern University, 1997

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Orphan receptors form an important subfamily of the nuclear receptor superfamily. They are identified on the basis of homology in the DNA-binding domain to previously cloned members. These novel members of the superfamily are named orphan receptors because of their unknown biological functions and putative ligands. A novel member of the nuclear receptor superfamily termed Germ Cell Nuclear Factor (GCNF) has been recently cloned. It is expressed predominantly in the male and female germ cells of the adult mouse. Analysis was undertaken to shed light on GCNF's transactivation properties and to identify target responsive genes. It has been show that GCNF binds to response elements which are configured as direct repeats of the consensus half site AGGTCA with a 0 bp spacing and that it binds to this element as a homodimer. A computer search of Genbank identified a number of potential responsive genes containing DR0 sequences, one of which was the Lactoferrin gene. Transcription of the Lactoferrin gene

is stimulated by estrogen in the mouse uterus. Analysis of the promoter revealed an estrogen responsive element (ERE) overlapping with a chicken ovalbumin up-stream promoter (COUP) element located at position -349 to -329 from the transcription initiation site. The ERE element differed from the consensus ERE sequences by one nucleotide at the second position of the 3' half of the element (G to A); the COUP element differed by one nucleotide from the consensus COUP element. The ERE/COUP element confers estrogen action to both homologous and heterologous promoters. Competition experiments with ERE and COUP oligonucleotides have been performed to confirm interaction between Lactoferrin ERE/COUP element with the COUP transcription factor and the estrogen receptor, respectively; and this sequence is a complex steroid response element and was renamed mERM for mouse lactoferrin estrogen response module. COUP-TF represses the promoter through this element. In knockout mice of the Lactoferrin gene, pre-implantation embryonic lethality is observed. The Lactoferrin gene is not expressed maternally in the oocyte; however, the Lactoferrin gene is turned on at the 2 cell and 4 cell stage of embryogenesis. Lactoferrin expression is maintained to the 32 cell stage. In the Lactoferrin ERM, four half sites are juxtaposed to generate a number of repeats; an IR3, which is the ERE, DR0 to which GCNF may bind, and DR1 to which COUP-TF binds. All of these half sites are 1 nucleotide away from a perfect AGGTCA core sequence except the central half site which is perfect. In this study, experiments were performed to determine whether GCNF binds to the promoter region in the Lactoferrin (targeting the ERM). Other experiments performed were dose-dependent binding of GCNF to the WT mERM,

competition with the WT mERM and PRE to establish specificity of binding, and binding of GCNF to mDR1, mDR0, mERE and mERM mutant sequences.

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LIST OF SYMBOLS AND ABBREVIATIONS

Steroid Response Elements	SREs
DNA-Binding Domain	DBD
Germ Cell Nuclear Factor	GCNF
Lactoferrin	LF or LTF
Polymorphonuclear leukocyte	PMN
Estrogen-Response Element	ERE or IR3
Chicken Ovalbumin Upstream Promoter	COUP
Estrogen Response Module	ERM
Direct Repeat with 0 base pair spacing	DR0
Direct Repeat with 1 base pair spacing	DR1
Complementary DNA	DNA
human Glucocorticoid Receptor	hGR
human Mineralcorticoid Receptor	hMR
human Androgen Receptor	hAR
human Progesterone Receptor	hPR
human Estrogen Receptor	hER
human Estrogen Receptor-Related 1 or 2	hERR1 or hERR2
human Retinoic acid Receptor	hRA
human Thyroid Hormone Receptor Beta	hTRß
human Vitamin D3 Receptor	hVDR

human Chicken Ovalbumin Upstream Promoter	hCOUP
Retinoic X Receptor	RXR
mouse Germ Cell Nuclear Factor	mGCNF
mouse Retinoid X Receptor alpha	mRXR alpha
mouse Retinoid Acid Receptor alpha	mRAR alpha
mouse peroxisome proliferator-activated receptor	mPPAR
mouse Steroidogenic Factor-1	mSF-1
mouse Progesterone Receptor	mPR
Diethylstilbesterol	DES
wildtype estrogen response module	WT ERM or WT
mutant direct repeat with 1 bp spacing	mDR1
mutant direct repeat with 0 bp spacing	mDR0
mutant estrogen response element	mERE
mutant estrogen response module	mERM
Progesterone Response Element	PRE
Polymerase Chain Reaction	PCR
Diothiothreitol	DTT
Disodium ethylene diamine tetraacetate	EDTA
Potassium Manganese DTT	KMD
United States Biochemical	USB

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CHAPTER 1
INTRODUCTION

A great deal of evidence has accumulated that proves the hypothesis that steroid hormones act at the level of nuclear DNA to regulate gene expression. Earlier qualitative studies, involved experiments showing that steroid hormones cause accumulation of new species of hybridizable RNAs, stimulation of synthesis of new specific proteins, a corresponding increase in the cellular levels of specific mRNAs, and stimulate the rate of transcription of specific nuclear genes. During, the early 1970s, the primary pathway for steroid hormone action was defined as follows: steroid -> (steroid-receptor) -> (steroid-receptor-DNA) -> mRNA -> functional response. Isolation of specific target genes for steroid hormones defined their structure and proved that cis-acting regulator sequences were located near such genes, usually in the 5'-flanking sequences. When these sequences (termed steroid response elements or SREs) are occupied by receptors, these genes come under control of the respective hormones. These receptors for steroid hormones were the most intensively studied and highly purified transcription factors for control of eukaryotic gene expression. Biochemical studies in the late 1970s suggested that steroid receptors, thyroid receptors, and receptors for Vitamin D and Vitamin A belonged to a family of gene regulatory proteins of ligand activated transcription factors. These proteins were organized into domains that contained the functions of (1) specific and high-affinity ligand binding,

(2) specific DNA binding, and (3) transcriptional modulation (O'Malley, 1992). These receptor genes belong to the structurally and genetically-related nuclear receptor superfamily. The common structural feature of this superfamily is a tripartite domain structure consisting of a hypervariable N-terminus that contributes mainly to the transactivation function; a highly conserved DNA-binding domain (DBD), which is responsible for DNA recognition and dimerization and the conserved C-terminus, which contains the subdomains II and III and is involved in nuclear localization, ligand binding, receptor dimerization, silencing, and transactivation (Chen, 1994). The DBD, which is the most conserved feature of this superfamily, contains 65-68 amino acid residues. Eight of the nine nonvarient cysteines form two type II zinc modules. The sequence identity in the DBD of any member to the rest of the family ranges between 40-98%, thus leading to the discovery of many more structurally related receptors which are termed orphan receptors because the identity of their ligands and physiological functions are unknown.

Orphan receptor is a name used to designate related proteins that may or may not be receptors in the endocrinological sense. They, nevertheless, comprise the vast majority of the steroid/thyroid/vitamin superfamily. They are proteins which contain distinctive homologies in their amino acid sequences (type II zinc fingers), which clearly mark them as members of this superfamily of gene regulators (O'Malley, 1992). Orphan receptors form an important subfamily of the nuclear receptor superfamily. The majority of them were identified on the basis of homology in the DBD to previously cloned members of the superfamily. The supposition that orphan receptors indeed have important cellular functions is based on the following: (1) they

are expressed as proteins in cells, sometimes with cell-type and developmental specificity; (2) they are authentic members of a highly evolved and powerful family of eukaryotic transcription factors; (3) certain of the orphan receptors already have been shown to function by regulating known specific genes and developmental processes; (4) selected orphans have been implicated in the mediation of cellular response to neurotransmitters, retinoic acid, and peroxisome proliferators; and (5) at least one orphan receptor has been reported to act as an immediate early mediator of growth factor and electrical response in the central nervous system (O'Malley, 1992).

Cloning and characterization of orphan receptor genes have played a significant role in the discovery of new signaling pathways and transactivation mechanisms. A novel member of the nuclear receptor superfamily termed Germ Cell Nuclear Factor (GCNF) has been recently cloned in our laboratory. This clone meets all of the criteria of the nuclear receptor superfamily, but it is distinct in that it is not closely related to any previously known receptor. GCNF is expressed predominantly in the male and female germ cells in the adult mouse. Further characterization of GCNF expression in the seminiferous tubules shows that it is expressed most highly in round spermatids. Computer-assisted analysis of the cellular distribution of the message and quantitation of the levels by Dr. Niederberger (University of Chicago, Department of Virology) showed that the specific timing of highest expression is in the stage VII round spermatids. This is one of the last stages before spermatids begin morphogenesis to become mature gametes.

Analysis of the amino acid sequence of the DBD of GCNF shows that it

shares the same P-box as the receptors for Vitamin D, thyroid hormone and retinoic acid, which preferentially bind to response elements containing the core sequence AGGTCA arranged in direct repeats with a 0 bp spacing (DR0) and that it binds to this element as a homodimer (Chen et al., 1994).

A computer search of the Genbank identified a number of potential responsive genes containing DR0 sequences in their promoter region, one of which was the lactoferrin gene. Lactoferrin (LF or LTF) is a nonheme iron binding glycoprotein first discovered in human milk. It is produced by immature neutrophilic leukocytes and various secretory epithelia including the uterine epithelium of the mouse. LF is a member of the transferrin family of iron-binding proteins and binds two ferric (Fe^{3+}) ions and two bicarbonate ions (McMaster et al., 1991). Functions of lactoferrin include epithelial cell proliferation, hematopoiesis, inhibition of bacterial growth, immunoregulation, and stimulation of growth and iron transport.

It has been shown that lactoferrin gene expression is regulated by estrogens in the reproductive tract of the mouse (Walmer et al., 1992). As an estrogen-regulated protein in the genital tract and a specific component of polymorphonuclear leukocyte (PMN), LTF is an interesting probe for studying the reproductive tract of the female mouse. Studies of expression of the lactoferrin gene indicate that it is regulated through upstream binding sites (Liu et al., 1993). Transcription of the lactoferrin gene is stimulated by estrogen in the mouse uterus. Analysis of the promoter revealed an estrogen-responsive element (ERE) overlapping a chicken ovalbumin upstream promoter (COUP) element located at -349 to -329 base pairs from the transcription initiation site (Liu et al., 1993). The ERE element differed from

the consensus ERE sequence by one nucleotide. Experiments with ERE and COUP oligonucleotides have been performed to confirm interaction between lactoferrin ERE/COUP element with the estrogen receptor (ER) and the COUP transcription factor (Liu et al., 1993). This sequence is a complex steroid response element and has been renamed the mERM for mouse lactoferrin estrogen response module. In the lactoferrin ERM, four half sites are juxtaposed to generate a number of repeats, an IR3 (also termed ERE), DR1 to which COUP-TF binds, and DR0 to which GCNF may bind. All of these half sites are one nucleotide away from a perfect AGGTCA core sequence except the central half site which is perfect and is shared by all of the response elements. The lactoferrin gene seems to be a good model system for determining the regulation of gene expression by GCNF because it has important biological functions, is known to be regulated by steroid receptors, and because it possesses a site to which GCNF may bind.

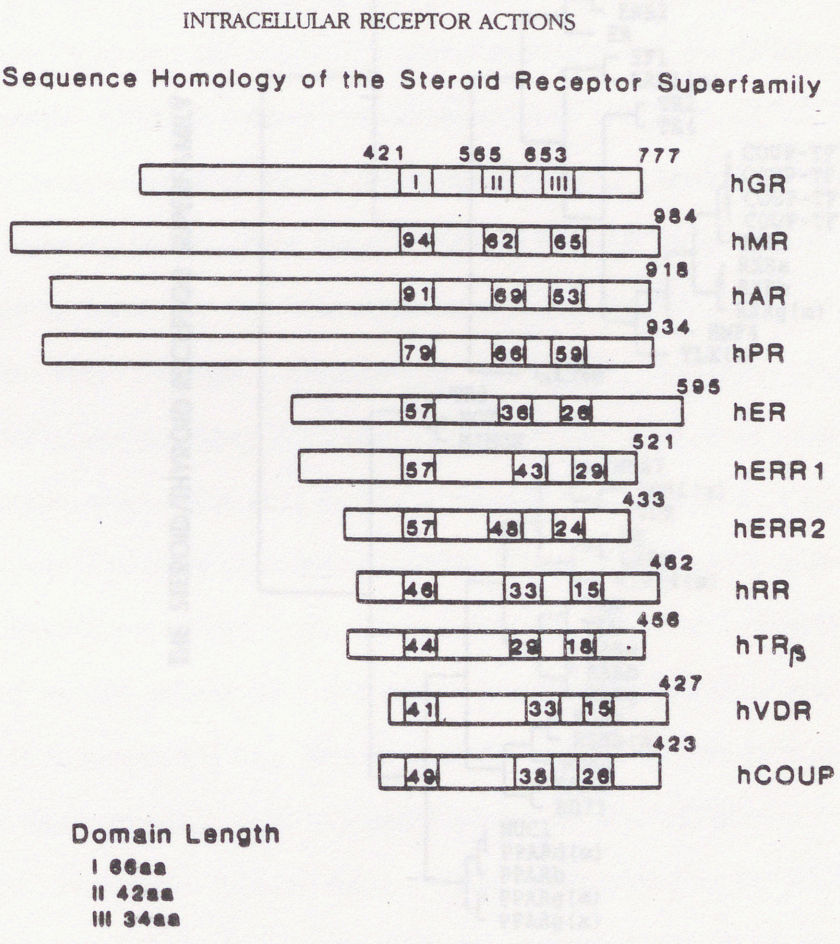
CHAPTER 2
LITERARY REVIEW

One of the most fascinating observations to have evolved from the cloning of cDNAs for steroid receptors is the fact that they are all genetically related, they form a steroid receptor superfamily of related genes; and they probably evolved from a primordial steroid receptor gene (O'Malley et al., 1992). Figure 1 shows a schematic representation of some of the selected members of this super family (O'Malley, et al., 1992). A large number of cloned "receptoroids" have been discovered after identification of the receptors for the more traditional members of this family (glucocorticoids, mineralocorticorids, sex steroids, thyroid hormone, Vitamin D3, and retinoic acid). Figure 2 is a phylogenetic tree of the steroid/thyroid hormone (orphan) nuclear receptor superfamily and illustrates the different groups and their relationship to each other. These molecules are considered to be "orphan receptors" in search of a function and a ligand.

In recent years, several groups have made progress in analyzing the physiological functions of orphan receptors. O'Malley and Tsai were involved in the purification and cloning of a transcription factor called COUP-TF (chicken ovalbumin upstream promoter-transcription factor) (O'Malley et al., 1992). COUP-TF is a high-affinity and specific DNA binding protein, which interacts as a dimer with the distal promoter sequence of the ovalbumin gene and promotes initiation of transcription of this gene by RNA

FIGURE 1

Sequence Homology of the Steroid Receptor Superfamily

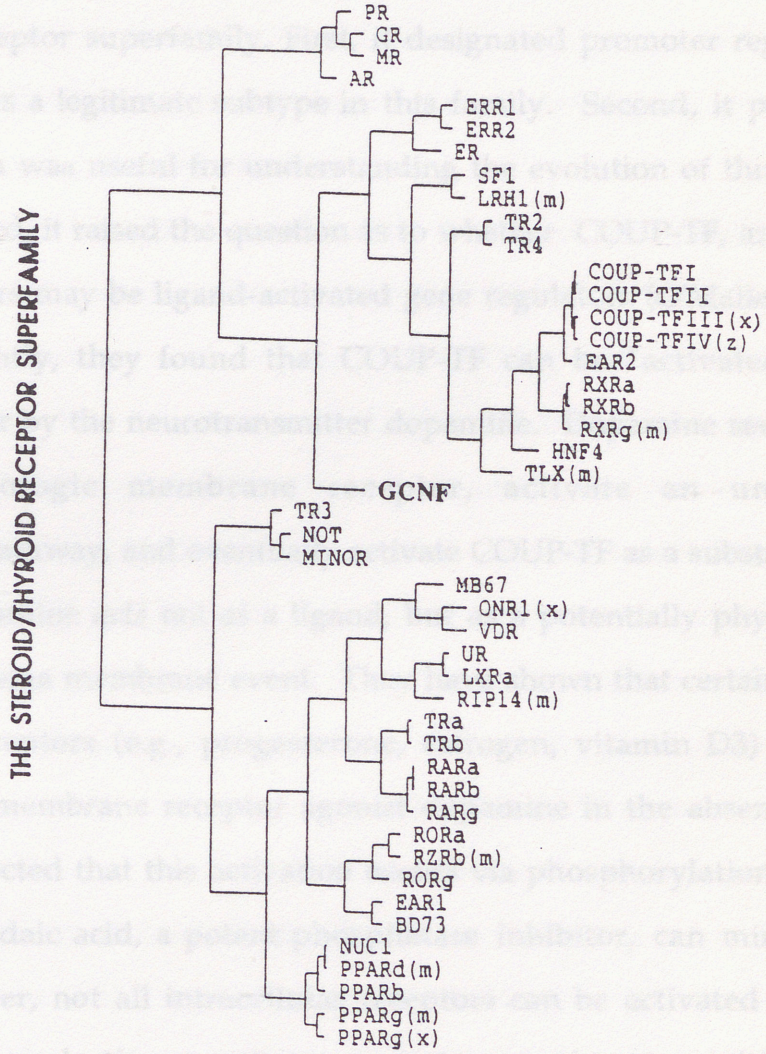


Biology of Reproduction Vol.46: 163-165. (1992)

This figure shows schematic representation of some of the selected members of this super family.

FIGURE 2

Phylogenetic Tree of The Steroid/Thyroid Receptor Superfamily



This illustrates the different groups and their relationship to each other. Using the DNA binding domain of each nuclear receptor, a multiple alignment of the progressive alignment method of Feng and Doolittle was created. PileUp makes multiple sequence alignments using progressive pairwise alignments. Then n the distance program was ran with the Kimura protein correction method, to create a table of the pairwise distances within the group of aligned sequences. Using the multiple alignment, the Growtree program was used to create a phylogenetic tree from the distance matrix using the UPGMA method.

polymerase (Wang et al., 1987). After having cloned COUP-TF and having derived its amino acid sequence, O'Malley and Tsai found that it was an authentic member of the steroid receptor superfamily (O'Malley et al., 1992). A number of apparent implications arose after the assignment of COUP-TF to the steroid receptor superfamily. First, it designated promoter regulatory binding proteins as a legitimate subtype in this family. Second, it provided information which was useful for understanding the evolution of this family of regulators. Third, it raised the question as to whether COUP-TF, and other promoter activators may be ligand-activated gene regulators (O'Malley et al., 1992). Subsequently, they found that COUP-TF can be "activated" as a transcription factor by the neurotransmitter dopamine. Dopamine seemed to bind to its biologic membrane receptor, activate an unknown phosphorylation pathway, and eventually activate COUP-TF as a substrate. In this instance, dopamine acts not as a ligand, but as a potentially physiologic activator via a plasma membrane event. They have shown that certain of the classic steroid receptors (e.g., progesterone, estrogen, vitamin D3) can be regulated by the membrane receptor agonist dopamine in the absence of a ligand. It is suspected that this activation occurs via phosphorylation of the receptor since okadaic acid, a potent phosphatase inhibitor, can mimic the response. However, not all intracellular receptors can be activated by this pathway and only selective membrane receptor agonists can initiate the response. O'Malley and Tsai's results demonstrated convergence and cross-talk between two major regulatory pathways of eukaryotic cells. Two other groups have shown that Steroidogenic factor-1 (SF-1) was an orphan receptor that regulated the expression of steroidogenic enzymes in the testis, ovaries,

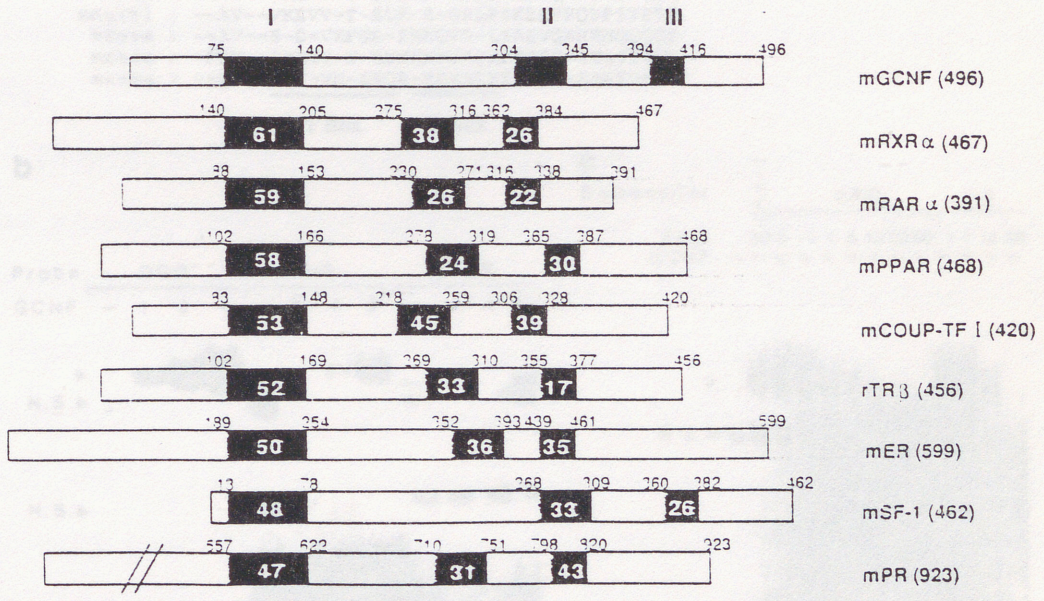
and adrenals. Mice with null mutations of the SF-1 gene did not have any testis, ovaries, or adrenals which pointed to an important developmental role for SF-1 in these tissues (Wilson et al., 1993 and Lala et al., 1992).

Chen et al. cloned a novel orphan member of the nuclear receptor superfamily called Germ Cell Nuclear Factor (GCNF) from a testis cDNA library. It is specifically expressed at high levels in developing germ cells, round spermatids and maturing oocytes.

In order to identify genes regulated by the GCNF signaling pathway, Cooney identified the response element to which it binds (Chen et al., 1994). Comparison of the amino acid sequence of GCNF in the DBD to other members of the nuclear receptor superfamily predicted that the GCNF recognition alpha helix would bind to a core hexameric nucleotide sequence of AGGTCA. Figure 3 is a detailed comparison of the amino acid sequence of mGCNF in regions I, II, and III with several representative members of the superfamily showing that the degree of identity of this orphan to the other members was relatively low, ranging from 47-61% in the most homologous regions (Chen et al., 1994). Comparison of regions II and III shows that GCNF was more closely related to COUP-TF than RXR in the ligand-binding domain. These data clearly indicate that this factor represents a new member of a distinct subclass of the nuclear receptor superfamily. Figure 4a shows alignment of the amino acid sequence of mGCNF with several representative members of the nuclear receptor superfamily in the DBD (Chen et al., 1994). Homology in the A box to SF-1 predicted that GCNF would bind to the sequence TC/GA upstream of the half site. The extended half site sequence predicted would be TC/GAGGTCA, which is included in a direct repeat of the

FIGURE 3

Comparison of mGCNF with Several Representative Members of the Nuclear Receptor Superfamily

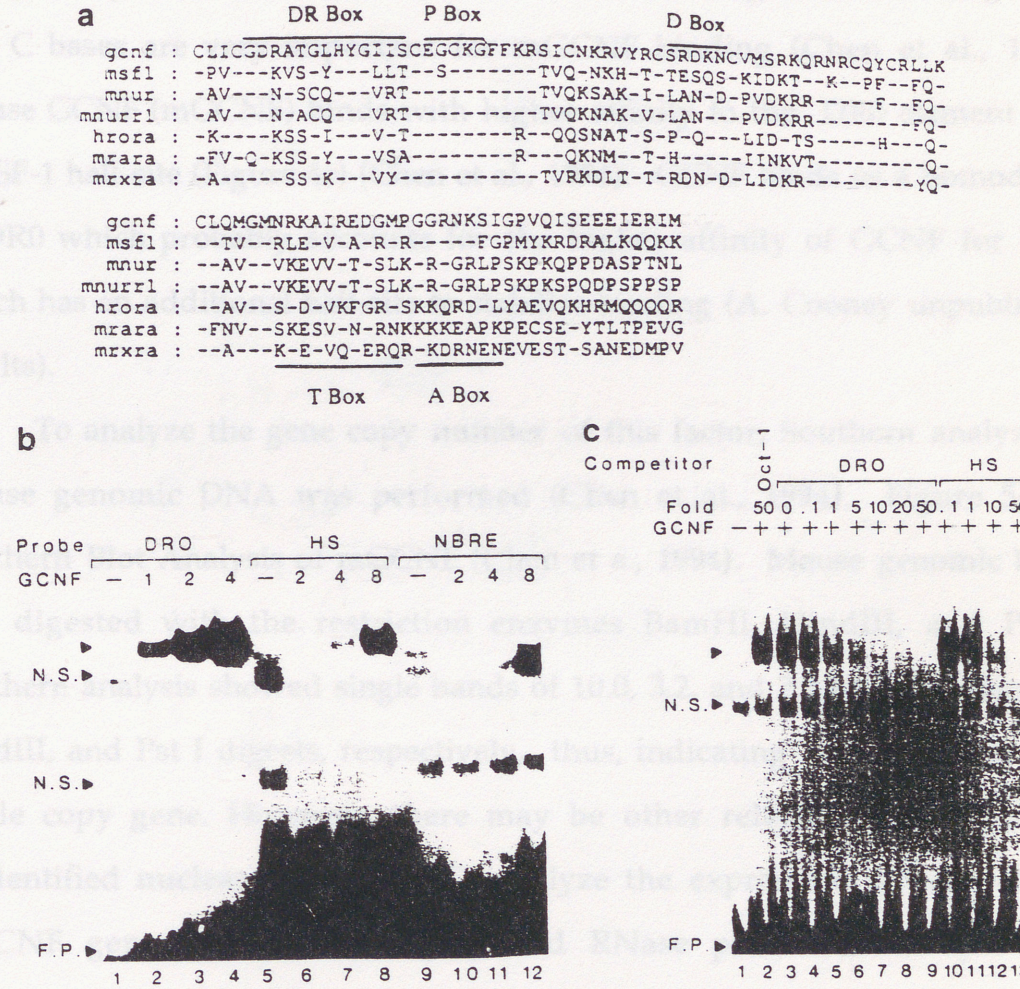


Molecular Endocrinology Vol.8: 1434-1444 (1994)

The degree of identity of this orphan to the other members is relatively low, ranging from 47-61% in the most homologous region.

FIGURE 4

Analysis of the DNA-Binding Properties of mGCNF by Gel Mobility Shift Assays



Molecular Endocrinology Vol.8: 1434-1444 (1994)

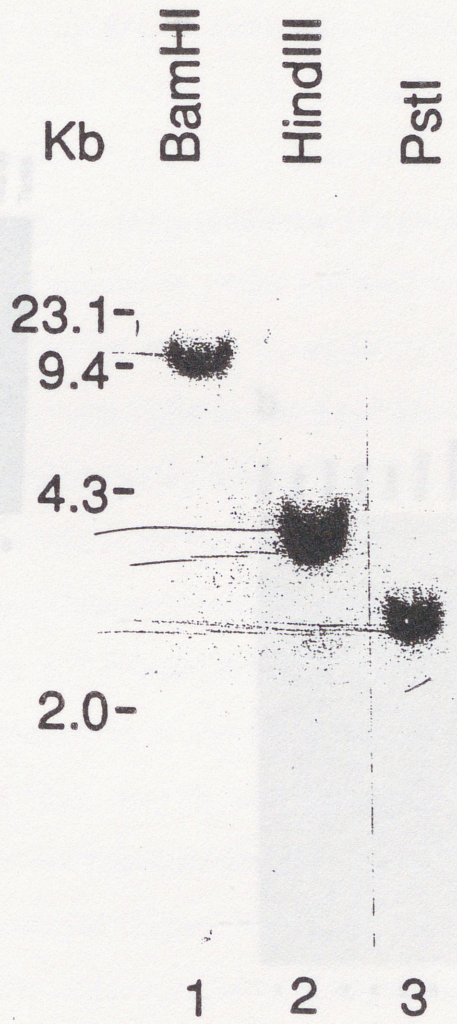
Figure 4a represents alignment of the amino acid sequence of mGCNF with several representative members of the nuclear receptor superfamily in the DBD. (b). Figure 4b represents a gel mobility shift assay of mGCNF binding to hormone response elements containing the sequence TCAAGGTCA, which is an SF-1 half site and an NBRE half site. (c). Figure 4c represents a gel mobility shift assay of GCNF binding to the DR0. Mouse GCNF (mGCNF) binds with higher

half site sequence with a 0 bp spacing, i.e. AGGTCAAGGTCA. Through the use of gel mobility shift assays (Figure 4b), Cooney found that mGCNF binds to hormone response elements containing the sequence TCAAGGTCA, which is an SF-1 half site, where as an NBRE (AAAAGGTCA) with only a 2-base-pair (bp) difference at the 5'-end shows no binding, thus indicating the T and C bases are very important for mGCNF binding (Chen et al., 1994). Mouse GCNF (mGCNF) binds with higher affinity to the DR0 element than the SF-1 half-site (Figure 4c) (Chen et al., 1994). GCNF binds as a homodimer to DR0 which probably accounts for the higher affinity of GCNF for DR0, which has an additional half-site to stabilize binding (A. Cooney unpublished results).

To analyze the gene copy number of this factor, Southern analysis of mouse genomic DNA was performed (Chen et al., 1994). Figure 5 is a Southern Blot Analysis of mGCNF (Chen et a., 1994). Mouse genomic DNA was digested with the restriction enzymes BamHI, HindIII, and Pst I. Southern analysis showed single bands of 10.0, 3.2, and 2.3 kb in the BamHI, HindIII, and Pst I digests, respectively, thus, indicating that mGCNF is a single copy gene. However, there may be other related genes encoding unidentified nuclear receptors. To analyze the expression pattern of the mGCNF gene, Northern analysis and RNase protection assays were employed. Northern blot analysis of poly $(A)^+$ RNA from eight selected adult mouse tissues revealed that mGCNF was predominantly expressed in the testis, with very little expression in the other tissues tested (Figure 6a) (Chen et al., 1994). Two different sized mRNAs, 2.4 and 7.5kb, were detected with almost the same intensity. The smaller message was exclusively

FIGURE 5

Southern Blot Analysis of mGCNF

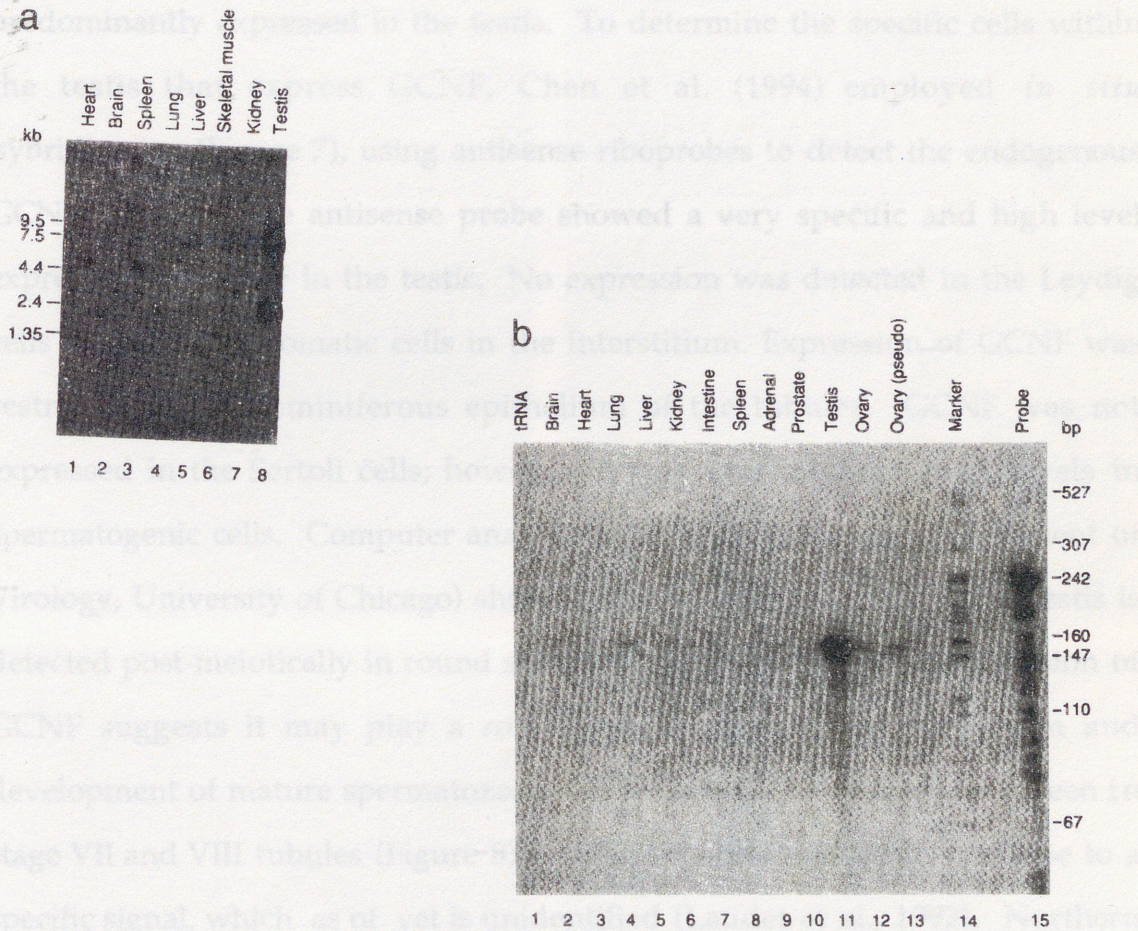


Molecular Endocrinology Vol.8: 1434-1444 (1994)

Mouse genomic DNA digested with the indicated restriction enzymes was loaded in each lane on a 0.8% agarose gel. After electrophoresis, the DNA was transferred to a nylon membrane under alkaline conditions and hybridized. The migration of the mol wt markers (kilobases) is indicated on the left.

FIGURE 6

Analysis of the Expression Pattern of the mGCNF Gene



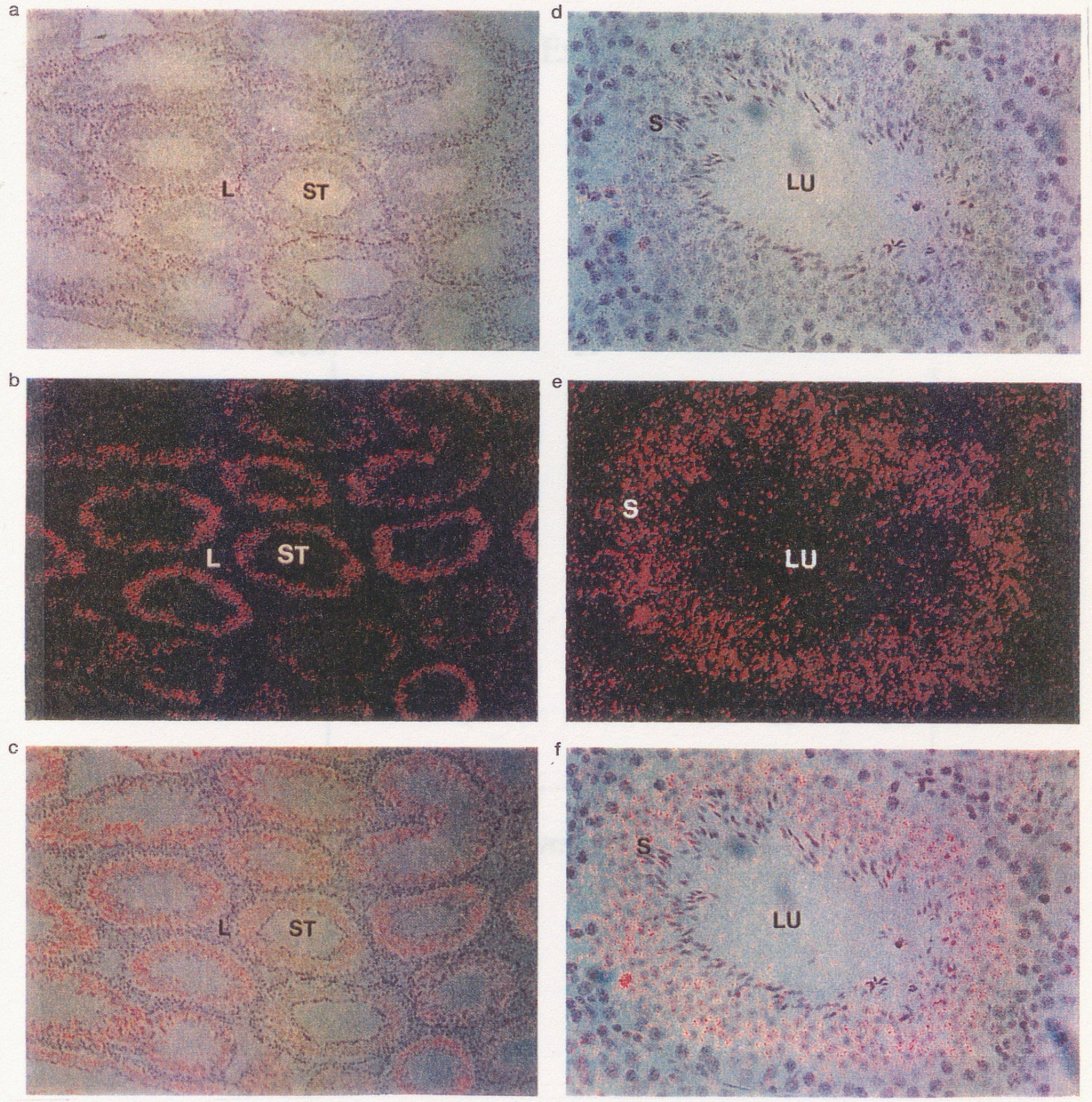
Molecular Endocrinology Vol.8: 1434-1444 (1994)

(a). is a Northern blot analysis of mGCNF RNA derived from adult mouse tissues. (b). is a RNase protection assay analysis of mGCNF RNA derived from adult mouse tissues.

expressed in the testis, whereas small amounts of the larger one could be detected in other tissues, such as the liver and kidneys. Using a very sensitive RNase protection assay, total RNA from 12 different tissues were examined (Figure 6b) (Chen et al., 1994). The results again indicated that mGCNF was predominantly expressed in the testis, with a very high relative abundance. Northern analysis and RNase protection assays revealed that GCNF was predominantly expressed in the testis. To determine the specific cells within the testis that express GCNF, Chen et al. (1994) employed *in situ* hybridization (Figure 7), using antisense riboprobes to detect the endogenous GCNF message. The antisense probe showed a very specific and high level expression of GCNF in the testis. No expression was detected in the Leydig cells or any other somatic cells in the interstitium. Expression of GCNF was restricted to the seminiferous epithelium of the tubules. GCNF was not expressed in the Sertoli cells; however, it was expressed at high levels in spermatogenic cells. Computer analysis by Dr. Niederburger (Department of Virology, University of Chicago) showed that GCNF expression in the testis is detected post-meiotically in round spermatids. The post-meiotic expression of GCNF suggests it may play a role in regulating the differentiation and development of mature spermatozoan. A peak of GCNF expression is seen in stage VII and VIII tubules (Figure 8) and is probably induced in response to a specific signal, which as of yet is unidentified (Laudet et al., 1992). Northern analysis of GCNF expression in the testis from mice between 5 to 45 days of age showed that expression of the GCNF gene was induced with the onset of puberty and spermatogenesis (Figure 9) (Laudet et al., 1992). Expression of the 7.5kb message was induced by day 20 with maximal expression by day 25.

FIGURE 7

In Situ Hybridization Analysis of Adult Mouse Testis

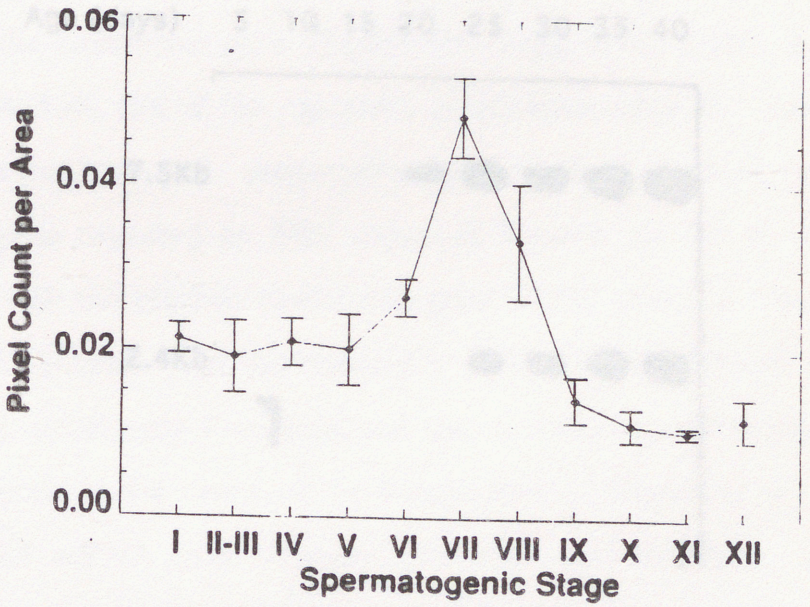


Molecular Endocrinology Vol.8: 1434-1444 (1994)

In Situ hybridization using antisense riboprobes to detect the endogenous GCNF message.

FIGURE 8

Quantitation of GCNF Expression in Spermatids

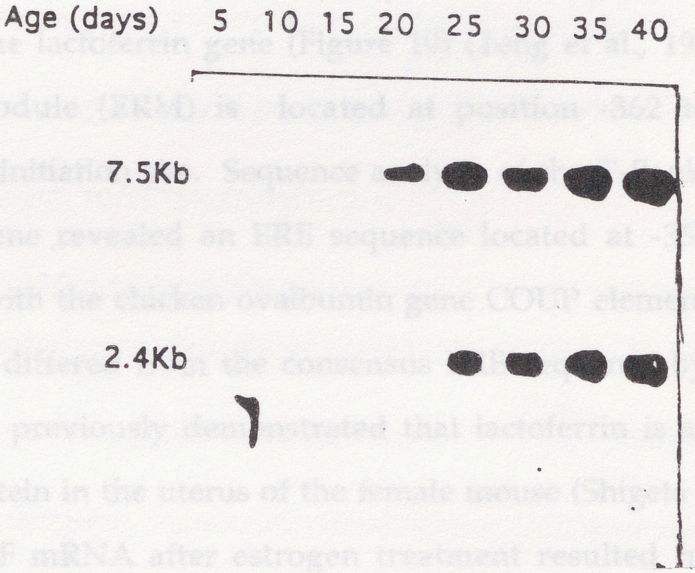


EMBO Journal Vol.11: 1003-1013 (1992)

A peak of GCNF expression is seen in stage VII and VIII tubules and is probably induced in response to a specific signal.

FIGURE 9

Northern Analysis of GCNF Expression During Puberty



EMBO Journal Vol.11: 1003-1013(1992)

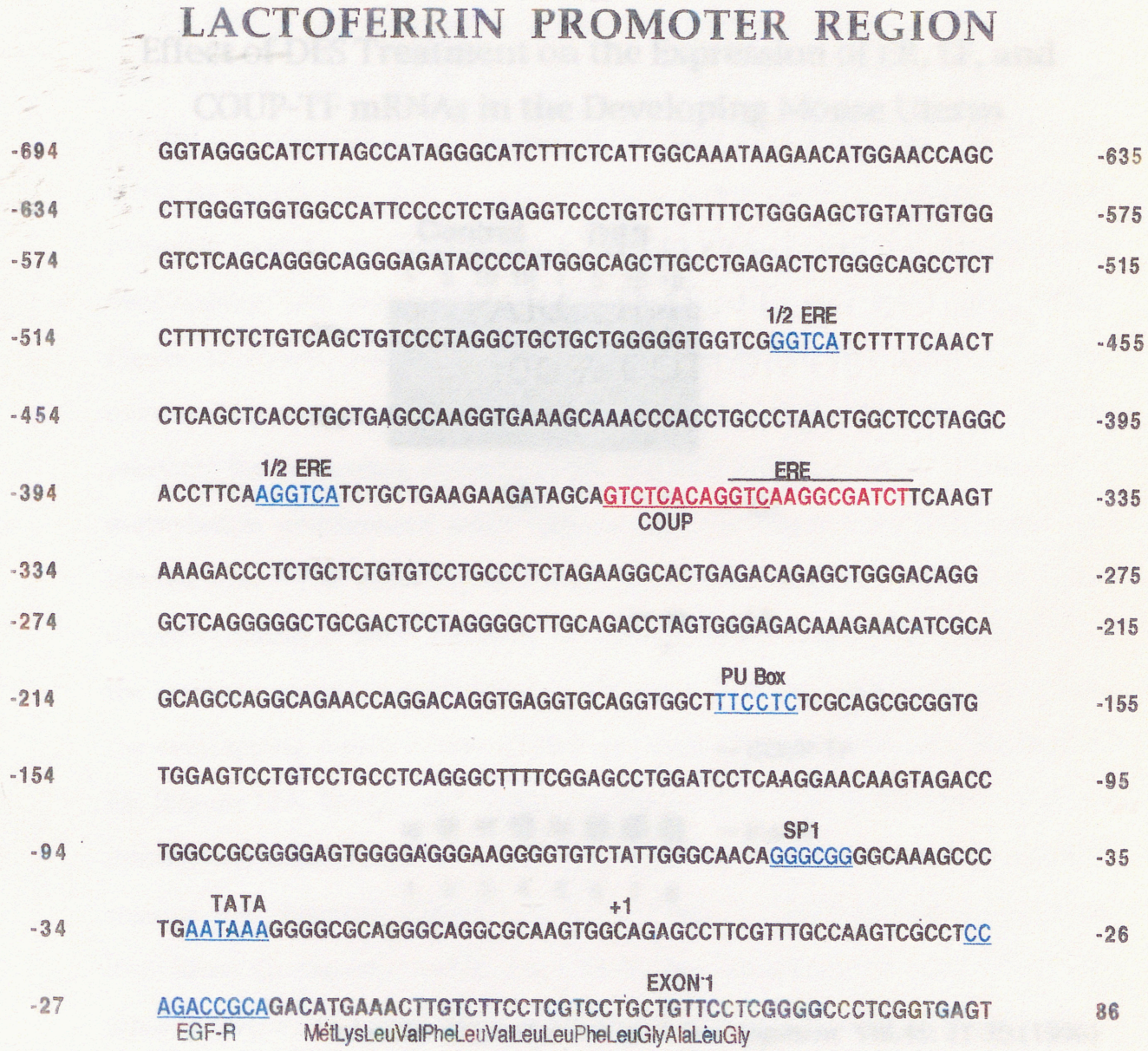
Northern Analysis of GCNF expression in the testis from mice between 5 to 45 days of age showing that expression of the GCNF gene was induced with the onset of puberty and spermatogenesis.

From this analysis of the expression of GCNF it is postulated that GCNF plays an important role during spermatogenesis and probably regulates gene expression in response to an as yet unidentified hormone signaling pathway in the testis (Cooney, 1997). GCNF was also detected in the oocytes in primary and secondary follicles (Chen et al., 1994). High level expression of GCNF is restricted to the germ cells, so this orphan receptor was named Germ Cell Nuclear Factor.

A computer search of the Genbank identified a number of potential responsive genes containing DR0 sequences in their promoter region, one of which was the lactoferrin gene (Figure 10) (Teng et al., 1989). An Estrogen Response Module (ERM) is located at position -362 to -341 from the transcription initiation site. Sequence analysis of the 5'-flanking region of the lactoferrin gene revealed an ERE sequence located at -355 to -341, which overlapped with the chicken ovalbumin gene COUP element. The lactoferrin ERE element differed from the consensus ERE sequence by one nucleotide. Shigeta et al. previously demonstrated that lactoferrin is a major estrogen-inducible protein in the uterus of the female mouse (Shigeta et al., 1996). The increase of LF mRNA after estrogen treatment resulted from a complex interplay among transcription factors acting on the estrogen response element (ERE) of the lactoferrin gene. In their 1994 study, ER and COUP-TF mRNA levels were examined and related to LF mRNA expression in various mouse tissues, including the developing uterus with/without estrogen stimulation (Figure 11). The Northern blot contained the total RNA from mouse uterus at 1, 5, 10, and 19 days of age with or without the DES treatments. The blot was probed sequentially with the cDNA to ER, LF, COUP-TF and ß-actin. The

FIGURE 10

Promoter Region of the Lactoferrin Gene

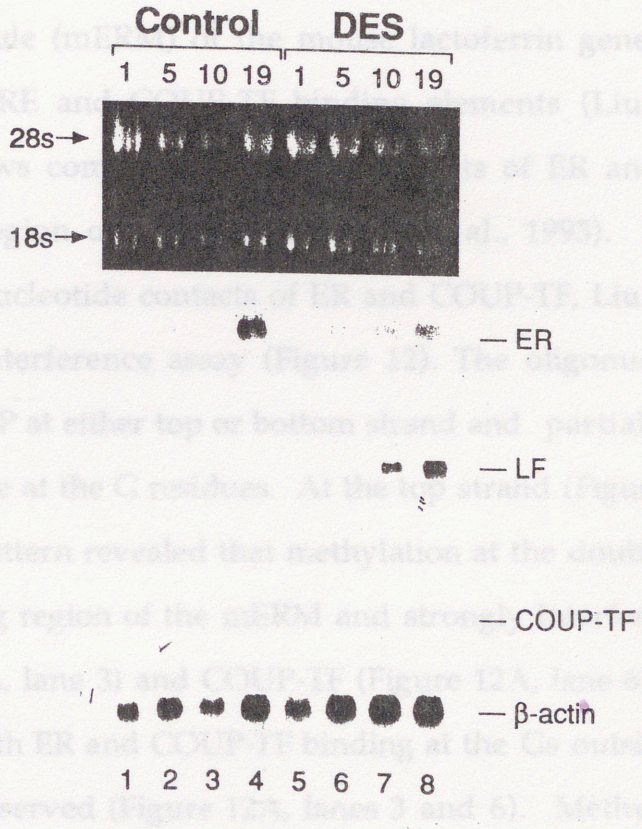


Molecular Endocrinology Vol.6: 1969-1981 (1992)

An Estrogen Response Module (ERM) is located at position -362 to -341 base pairs.

FIGURE 11

Effect of DES Treatment on the Expression of ER, LF, and COUP-TF mRNAs in the Developing Mouse Uterus



Molecular Reproduction and Development Vol.45: 21-30 (1996)

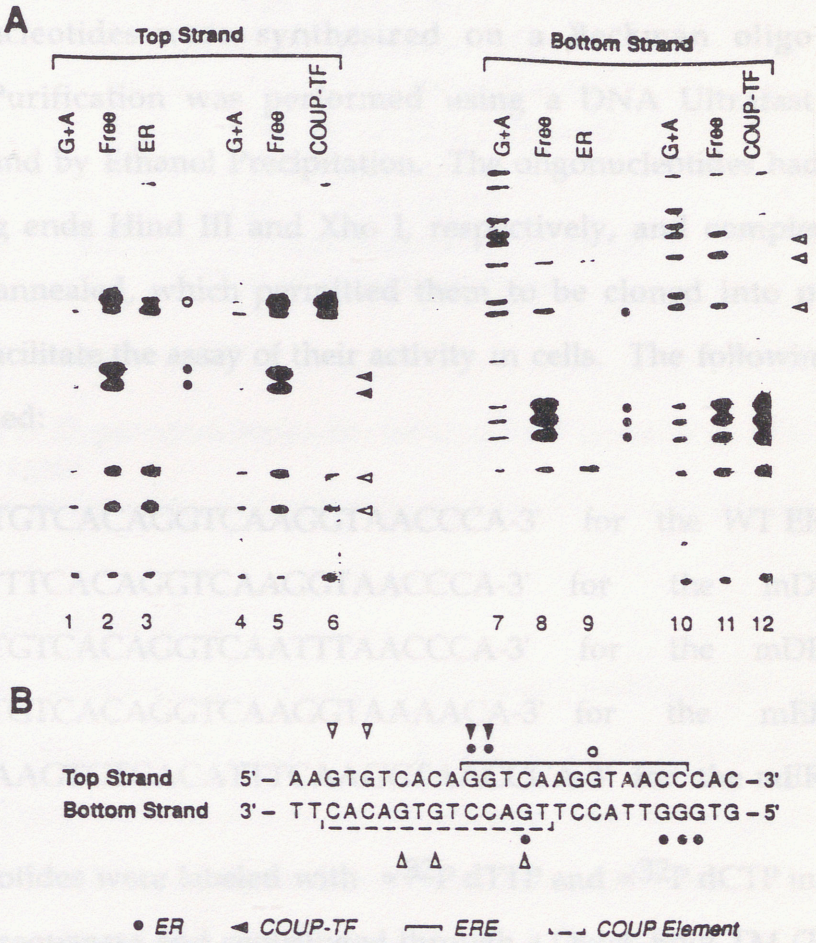
Northern blot analysis of total RNAs from either control or DES-treated mouse uterus on days 1, 5, 10, and 19 is shown. The top panel shows the ethidium bromide staining of the RNA gel before transfer to the nitrocellulose membrane. The locations of the 28s and 18s RNA are indicated. The same membrane was sequentially probed with cDNAs of ER, LF, COUP-TF, and ß-actin as indicated.

LF mRNA was not measurable in total RNA from the mouse uterus for the first 19 days of age (lanes 1-4 Figure 11). However, after DES treatment, the LF mRNA level was detected at day 5 and it greatly increased by day 19 (lanes 6-8). ER mRNA was detected from day 1 of newborn mice, and the message level steadily increased as the uterus developed. COUP-TF mRNA was present at a constant level throughout the ages examined, and DES did not affect its expression. Liu et al. previously demonstrated that the estrogen response module (mERM) of the mouse lactoferrin gene was composed of overlapping ERE and COUP-TF binding elements (Liu and Teng, 1991). Figure 12 shows common nucleotide contacts of ER and COUP-TF at the overlapping region of the mERM (Liu et al., 1993). In order to define precisely the nucleotide contacts of ER and COUP-TF, Liu et al. performed a methylation interference assay (Figure 12). The oligonucleotides were end labeled with ^{32}P at either top or bottom strand and partially methylated by dimethyl sulfate at the G residues. At the top strand (Figure 12A lanes 1 to 6) the cleavage pattern revealed that methylation at the double G resides within the overlapping region of the mERM and strongly interfered with binding of ER (Figure 12A, lane 3) and COUP-TF (Figure 12A, lane 6) binding. A weak interference with ER and COUP-TF binding at the Gs outside the overlapping regions was observed (Figure 12A, lanes 3 and 6). Methylation of the three individual Gs regions within the COUP element interfered with COUP-TF binding (Figure 12A, lane 12) (11). Methylation of the two Gs at the 3' end of the ERE, of the bottom strand, strongly interfered with ER binding (Figure 12A, lane 9). The G outside the consensus ERE that also served as the contact site for ER binding made the complexes more stable. Figure 12B shows the

nucleotide sequence of the COUP and ERE region indicating contact sites (Liu et al., 1993).

FIGURE 12

Common Nucleotide Contacts of ER and COUP-TF at the Overlapping Region of the mERM



Molecular and Cell Biology Vol.13, No.3: 1836-1846 (1993)

Figure 12A shows methylation interference at the top strand and the bottom strand of the DNA. Lanes 3 and 9, DNA recovered from the ER complexes; lanes 6 and 12, DNA recovered from the COUP-TF complex (C1); lanes Free, unbound DNA recovered from the ER (lanes 2 and 8) and COUP-TF (lanes 5 and 11) reactions; lanes G + A, chemical sequencing of the same DNA fragment as the marker. Figure 12B shows nucleotide sequence of the COUP and ERE region. The nucleotide contact sites are indicated.

CHAPTER 3

DESIGN OF THE STUDY

Synthesis and Labeling of Oligonucleotides

Oligonucleotides were synthesized on a Beckman oligo DNA synthesizer. Purification was performed using a DNA Ultrafast (AMA) Cleavage Kit and by Ethanol Precipitation. The oligonucleotides had 5' and 3' overhanging ends Hind III and Xho I, respectively, and complementary strands were annealed, which permitted them to be cloned into promoter constructs to facilitate the assay of their activity in cells. The following oligos were synthesized:

5'-AAGTGTCACAGGTCAAGGTAACCCA-3' for the WT ERM,

5'-AATTTTCACAGGTCAAGGTAACCCA-3' for the mDR1,

5'-AAGTGTCACAGGTCAATTTAACCCA-3' for the mDRO,

5'-AAGTGTCACAGGTCAAGGTAAAACA-3' for the mERE,

and 5'- AAGTGTCACATTTCAAGGTAACCCA-3' for the mERM.

The oligonucleotides were labeled with $\propto^{32}$P dTTP and $\propto^{32}$P dCTP in a fill in reaction using sequenase and centrifuged through a Quick Spin TM (TE) G-25 Sephadex column (Pharmacia) to remove unincorporated nucleotides. The labeled probes were used in gel mobility shift assays to detect DNA binding. Binding of a protein or transcription factor to the radioactively labeled DNA

causes it to migrate slower upon gel electrophoresis and results in the appearance of a retarded band using autoradiography to detect the radioactive signal.

Protocol for Labeling Nucleotides

3 µl of oligo (50 ng)
1 µl of 0.1M DTT
5 µl of 5X sequenase buffer
5 µl of $\propto^{32}$P dTTP (3000 Ci/mmol)
5 µl of $\propto^{32}$P dCTP(3000 Ci/mmol)
1 µl of 2mM dATP
1 µl of 2mM dGTP
0.25 µl of 5X sequenase (enzyme) USB
3.75 µl of deionized water

- Incubate for 30 minutes at 37°C
- Vortex probe
- Spin probe in G-25 pre-spun sephadex column for 5 minutes @ 5000 rpm
- Vortex probe again
- Count 0.5 µl of probe using a scintillation counter + 5 µl scintillation fluid

In vitro Translation of GCNF

In vitro translated protein was used in gel mobility shift assays to analyze DNA binding of GCNF. *In vitro* translated mGCNF was synthesized in a TNT T3- Coupled Reticulocyte Lysate System from Promega (Madison, WI) using the NSR 13 DNA encoding for recombinant mGCNF.

Protocol for *in vitro* Translation of GCNF

25 µl of T_3 TNT Rabbit Reticulocyte Lysate
2 µl of 10X TNT reaction buffer
1 µl of TNT T_3 RNA polymerase (40u/µl)
1 µl of Amino Acid mix minus Leucine (1mM)
1µl of Amino Acid mix minus Methionine (1mM)
3 µl of NSR 13 DNA (7.11µg)
16 µl of deionized water

- Incubate for 1 hour at 30°C
- Add 7.5μl of glycerol mix
- Freeze with liquid nitrogen (N_2)
- Store at -80°C

Gel Mobility Shift Assays

Gel mobility shift assays are used in the identification and investigation of DNA-binding proteins and transcription factors. Gel mobility shift assays provide a powerful tool for the detection of factors binding to specific DNA sequences. It relies on the ability of a protein to bind to a radiolabelled DNA fragment *in vitro*, followed by electrophoretic separation of DNA-protein complexes from the unbound DNA on non-denaturing polyacrylamide gels (Latchman, 1993). Gel mobility shift assays of GCNF DNA binding were performed by incubating *in vitro* translated mGCNF lysates with $\propto^{32}$P- labeled oligonucleotide probes. The DNA protein complexes were then separated by electrophoresis in a 5% polyacrylamide gel (46.05 ml dH_2O, 7.5ml Bio-Rad 40% polyacrylamide/ bis gel solution 19:1, 6ml 5X TBE (1X TBE = 89mM Tris base, 89mM boric acid, 2mM EDTA pH 8.0), 400μl of 10% APS (10 grams of ammonium persulfate and 10ml of deionized water), and 50 μl of Bio-Rad TEMED reagent in 0.25X TBE and electrophoresed at 200 volts on a gel electrophoresis system at room temperature. The gels were dried on a gel dryer system for 1 hour at 80°C and exposed to Kodak Biomax MS Scientific Imaging Film (8 x 10 in) overnight at -70°C for $\propto^{32}$P probes. Dose-dependent analysis of GCNF binding to the Lactoferrin DR0 was performed by adding increasing amounts of *in vitro* translated GCNF of increasing amounts of excess unlabeled oligonucleotides which were pre-incubated for 5 minutes.

Protocol for Dose Dependent Analysis of GCNF DNA Binding

Materials

probe
NSR 13 *in vitro* translated GCNF
blank lysate
deionized water (dH_2O) (2μg/μl)
20% ficoll
polydIdC•polydIdC (sigma)
buffer D
KMD

Units in microliters (μl)

probe	protein	polydIdC	buffer D	KMD	dH_2O	blank	ficoll
1μl	-	8μl	8μl	3μl	15μl	4μl	1μl
1μl	-	8μl	8μl	3μl	11μl	8μl	1μl
1μl	-	8μl	8μl	3μl	7μl	12μl	1μl
1μl	4μl	8μl	8μl	3μl	15μl	-	1μl
1μl	8μl	8μl	8μl	3μl	11μl	-	1μl
1μl	12μl	8μl	8μl	3μl	7μl	-	1μl

Total volume = 40 microliters per lane

Master Mix

probe = 8μl
polydIdC•polydIdC = 64μl
buffer D = 64μl
KMD = 24μl
ficoll = 8μl

--add dH_2O
--add 21 μl of the master mix
--add various amounts of proteins
--incubate for 15 minutes at room temperature
--load onto 5% Polyacrylamide Gel (PAG) and run for 1 hour at 200 volts
--dry on a gel dryer for 1 hour @ 80°C
--expose to Kodak Biomax MS Scientific Imaging Film and store overnight in
--70°C refrigerator

Protocol for Competition Analysis of GCNF DNA Binding

Materials

probe
10X, 50X, and 100X competitor (comp = unlabeled oligonucleotides)
NSR 13 *in vitro* translated GCNF
blank lysate
polydIdC•polydIdC (2μg/μl)
buffer D
KMD
dH_2O
20% ficoll

Units in microliters
Sample protocol using WT ERM with mERE

probe	protein	polydIdC	buffer D	KMD	comp	dH_2O	ficoll	
1μl	12μl	8μl	8μl	3μl	-	1μl	1μl	BLANK
1μl	12μl	8μl	8μl	3μl	-	1μl	1μl	GCNF
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	10X
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	50X WT
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	100X
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	10X
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	50X mERE
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	100X
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	GCNF
1μl	12μl	8μl	8μl	3μl	1μl	-	1μl	BLANK

Total volume = 34 microliters per lane

Master Mix

probe = 15μl
polydIdC = 120μl
buffer D = 120μl
KMD = 45μl
ficoll = 15μl

--add competitor or dH_2O to each tube
--add 21 microliters of the master mix to each tube
--incubate for 5 minutes at room temperature
--add protein
--incubate for 15 minutes at room temperature
--load onto 5% PAG and run at 200 volts for 1 1/2 hours

CHAPTER 4
RESULTS AND DISCUSSION

It has been shown that GCNF binds to response elements which are configured as direct repeats of the consensus half site AGGTCA core sequence with a 0 base pair (bp) spacing (DR0) and that it binds to this element as a homodimer. A computer search of Genbank identified a number of potential responsive genes containing DR0 sequences in their promoter region, one of which was the lactoferrin gene. In the promoter region of the lactoferrin gene (Figure 13), there is an estrogen response module (ERM) located at -362 to -341 base pairs (Teng et al., 1992). The ERM contains four half sites juxtaposed to generate a number of repeats; an inverted repeat, an estrogen response element (ERE), a direct repeat with one base pair spacing (DR1), and a direct repeat with 0 base pair spacing (DR0). Analyses have been undertaken in this research thesis to determine whether GCNF can regulate lactoferrin gene expression by binding to the wildtype ERM. The mutation strategy is made by substituting T bases for the G bases and A bases for the C bases seen in red in Figure 14 all performed by inserting the mutated sequences on a Beckman oligo DNA Synthesizer. Our prediction (Figure 14) of GCNF binding to the ERM wildtype and to the mutated sequences are as follows: GCNF should bind to the mDR1 and mERE sequences and should not bind to the mDR0 and mERM sequences. Figure 15 shows the response elements of the lactoferrin ERM and the mutations, in red, made within each sequence.

FIGURE 13
Sequence of the Lactoferrin Promoter Within the Lactoferrin Gene

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Within the lactoferrin gene an Estrogen Response Module (ERM) is found located at -362 to -341 base pairs. Four half sites are found within the ERM: an Estrogen Response Element (ERE), a Direct Repeat with one base pair spacing (DR1), and a Direct Repeat with 0 base pair spacing (DR0).

FIGURE 14

Response Elements and Mutations of the Lactoferrin ERM

RESPONSE ELEMENTS AND MUTATIONS OF THE LACTOFERRIN ERM

ERM_{WT}	AAGTGTCACAGGTCAAGGTAACCCA
$DR1_{M}$	AATTTTCACCAGGTCAAGGTAACCCA
$DR0_{M}$	AAGTGTCACAGGTCAATTTAACCCA
ERM_{M}	AAGTGTCACATTTCAAGGTAACCCA
ERE_{M}	AAGTGTCACAGGTCAAGGTAAAACA

LEGEND

WT - Wildtype
DR1 - Direct Repeat with one (1) base pair spacing
DR0 - Direct Repeat with zero (0) base pair spacing
ERE - Estrogen Response Element
ERM - Estrogen Response Module

The mutations made within each response element are highlighted in red.

FIGURE 15

Prediction of GCNF Binding to the ERM Wildtype and Mutant Sequences

PREDICTION OF GCNF BINDING TO THE ERM MUTANT SEQUENCES

ERM Sequence	GCNF
WT	+
DR1m	+
DR0m	-
EREm	+
ERMm	-

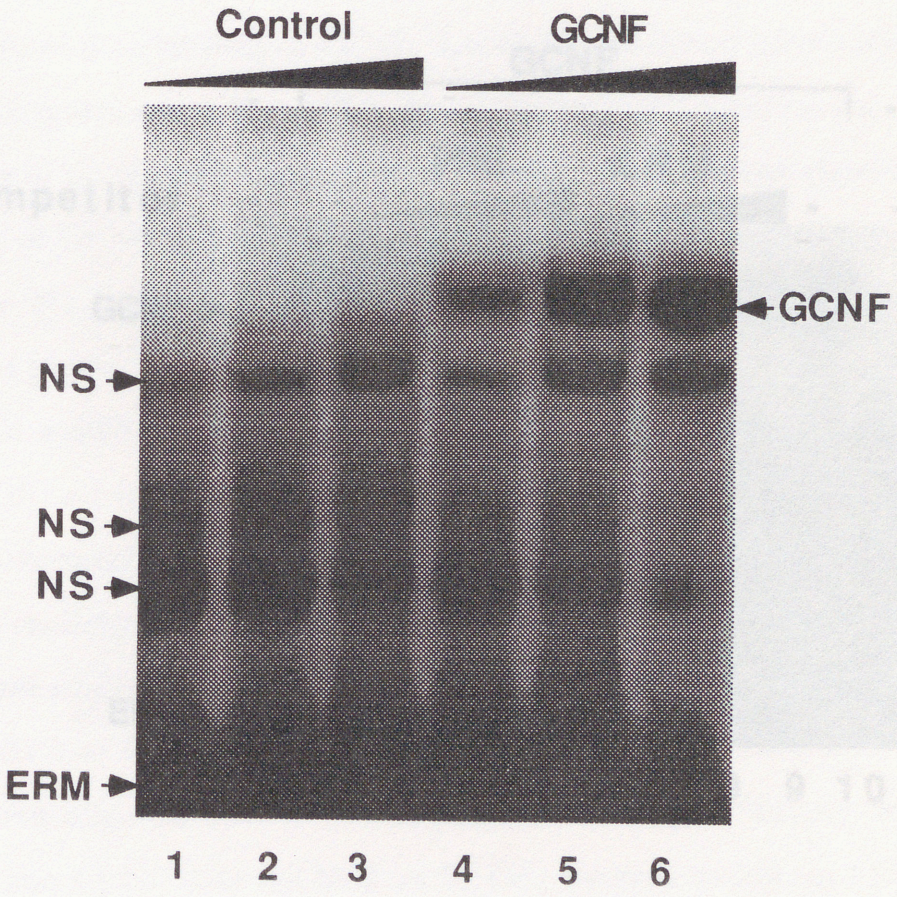
Plus signs indicate GCNF binding and minus signs indicate non GCNF binding. The prediction is that the wildtype (WT) , mDR1, and mERE sequences GCNF would bind to and the mDR0 and mERM sequences GCNF would not bind to.

ERM and the mutations, in red, made within each sequence. GCNF should bind to the mDR1 and mERE mutated sequences because the mutations have been made outside of the region of where GCNF is predicted to bind. GCNF should not bind to the mDR0 and mERM mutated sequences because the mutations have been made within the predicted region where GCNF is predicted to bind. In addition to testing for binding of GCNF to the lactoferrin promoter ERM, each of the four mutated half sites were also analyzed by gel mobility shift assays to determine which half sites and elements are important for GCNF binding. The first step was to show GCNF binding to the ERM wildtype. In a gel mobility shift dose response assay, increasing amounts of *in vitro* translated GCNF were added and compared to control lysate (Figure 16) using the WT ERM probe. No bands were seen in the region of where GCNF binds (lanes 1-3) using control lysate but increasing binding was seen (lanes 4-6) using *in vitro* translated GCNF. Non-specific bands were also observed binding to the WT ERM probe but not in the region of where GCNF binds. Thus, GCNF binds to the WT ERM probe.

To establish the specificity of binding to the lactoferrin ERM observed in Figure 16, a competition analysis using increasing amounts of unlabelled WT ERM oligonucleotide (lanes 6-8) and progesterone response element (PRE) oligonucleotide (lanes 3-5) were used to challenge GCNF binding to the WT ERM probe (Figure 17). Lanes 1 and 10 are control lysates without *in vitro* translated GCNF and lanes 2 - 9 are lysates with *in vitro* translated GCNF. The WT ERM unlabelled oligonucleotide competed for GCNF binding to the WT ERM while the PRE did not compete, thus demonstrating specificity of GCNF binding to the WT ERM.

FIGURE 16

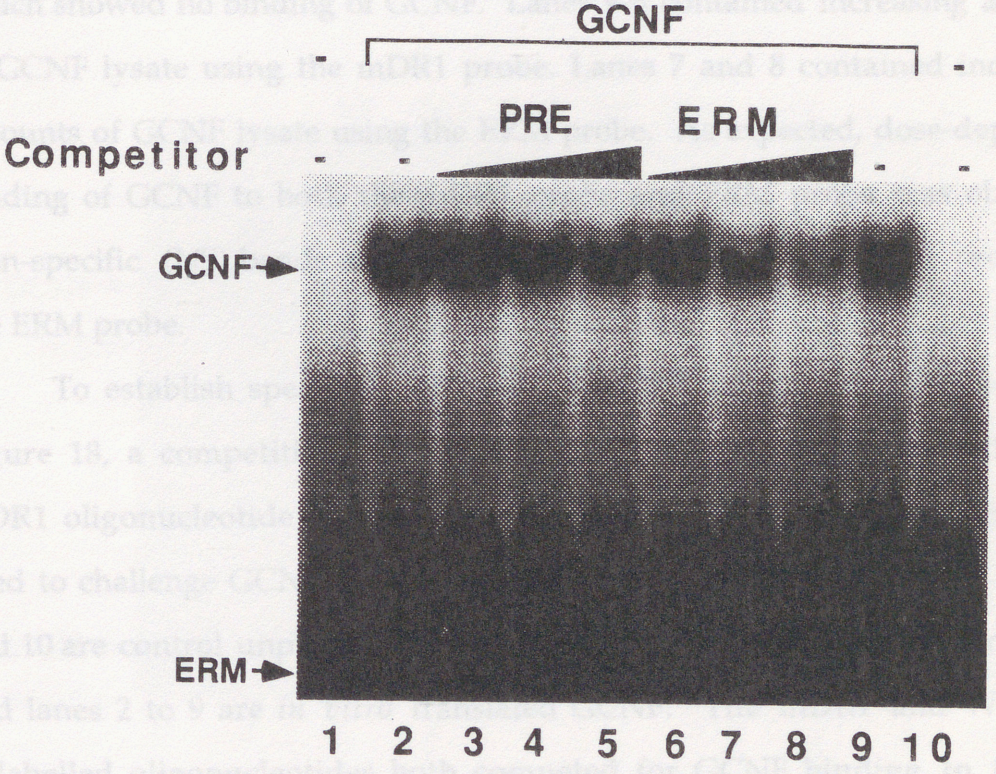
Gel Mobility Shift Dose Response Assay Showing GCNF Binding to the ERM Wildtype



Lanes 1-3 show no binding of GCNF using control lysate and lanes 4-6 show binding of GCNF using *in vitro* translated GCNF. Non-specific bands are also observed.

FIGURE 17

Gel Mobility Shift Assay Showing Competition Analysis of GCNF Binding to the WT ERM with Unlabelled PRE or ERM



Lanes 3-6 show unlabelled Progesterone Response Element (PRE) not competing for binding of GCNF and lanes 4-6 show unlabelled WT ERM competing for binding of GCNF.

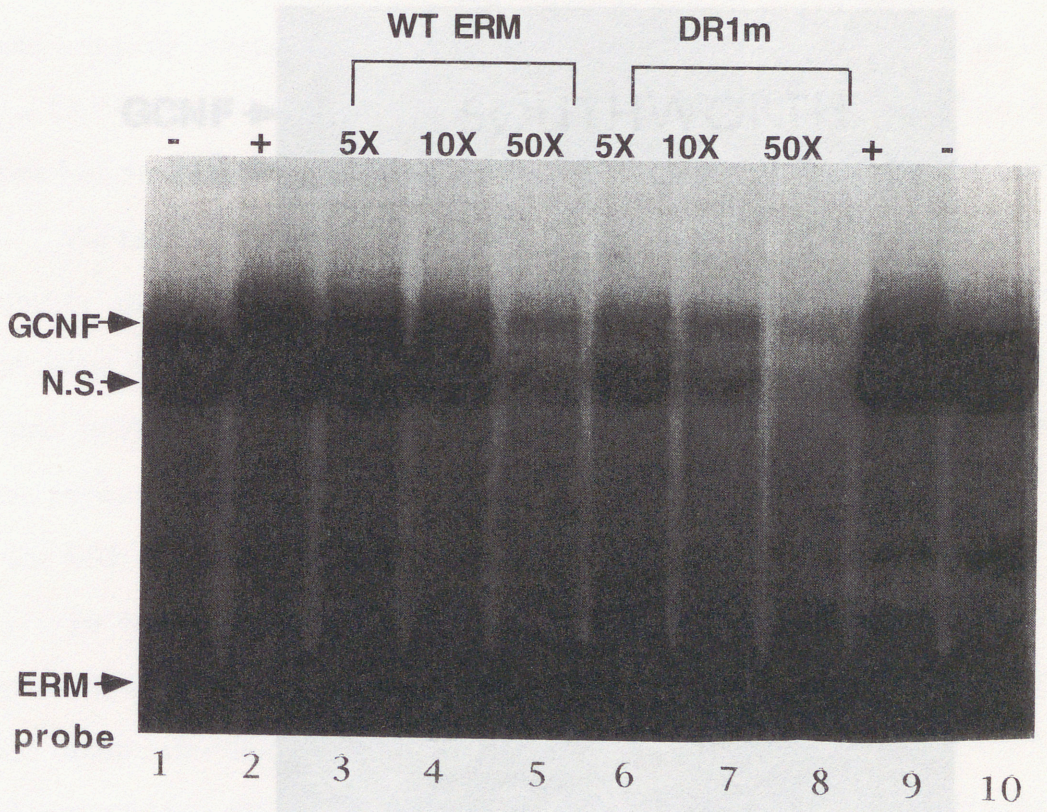
After testing for binding of GCNF to the WT ERM, the next step was to test for binding of GCNF to the mutated ERM sequences. The mDR1 probe has a mutation that destroys the DR1 element leaving the DR0 and ERE intact. In a gel mobility shift dose response assay using the mDR1 probe (Figure 18), increasing amounts of *in vitro* translated GCNF were added and compared to the control lysate. Lanes 1-6 were performed using the mDR1 probe and lanes 7 and 8 were performed using the ERM probe as a control. Lanes 1-3 contained increasing amounts of control unprogrammed lysate which showed no binding of GCNF. Lanes 4-6 contained increasing amounts of GCNF lysate using the mDR1 probe. Lanes 7 and 8 contained increasing amounts of GCNF lysate using the ERM probe. As expected, dose-dependent binding of GCNF to both the mDR1 probe and ERM probe was observed. Non-specific (NS) bands are also observed binding to the mDR1 probe and the ERM probe.

To establish specificity of binding to the mDR1 probe observed in Figure 18, a competition analysis using increasing amounts of unlabelled mDR1 oligonucleotide (lanes 6-8) and ERM oligonucleotide (lanes 3-5) was used to challenge GCNF binding to the WT ERM probe (Figure 19). Lanes 1 and 10 are control unprogrammed lysates without *in vitro* translated GCNF and lanes 2 to 9 are *in vitro* translated GCNF. The mDR1 and WT ERM unlabelled oligonucleotides both competed for GCNF binding to the WT ERM. Thus, GCNF does not bind to the DR1 element within the ERM.

Gel mobility shift assays showing dose responsive binding of mDR0 and mERM are not shown because GCNF could not bind to these probes. The mDR0 probe has a mutation that destroys the DR0 but leaves the mDR1

FIGURE 18

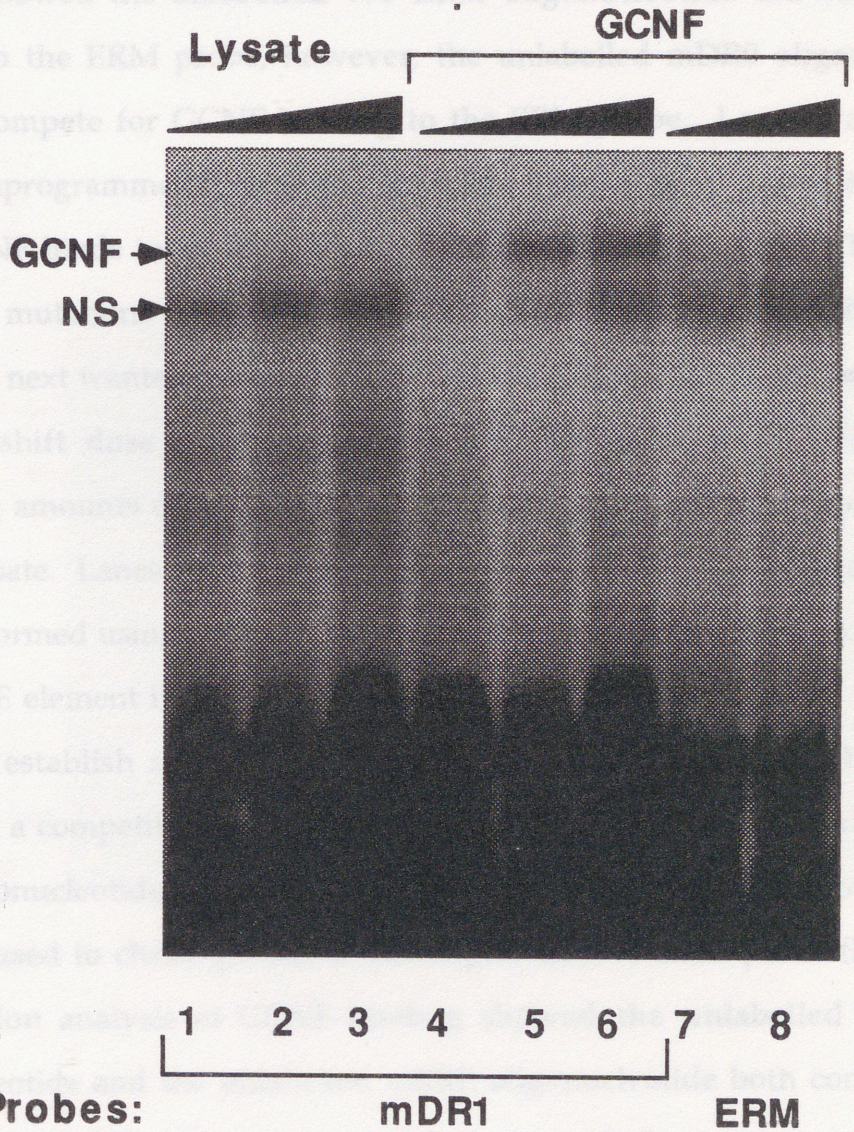
Gel Mobility Shift Dose Response Assay of mDR1 with the WT ERM



Lanes 1-3 show control lysate not binding to the mDR1 probe and lanes 4-6 show GCNF binding to the mDR1 probe. Lanes 7 and 8 show GCNF binding to the ERM.

FIGURE 19

Gel Mobility Shift Assay Showing Competition Analysis of the WT ERM with the mDR1



Lanes 3-5 contain increasing amounts of unlabeled WT ERM oligonucleotide showing binding of GCNF to the ERM and lanes 6-8 contain increasing amounts of unlabeled mDR1 showing binding of GCNF to the ERM.

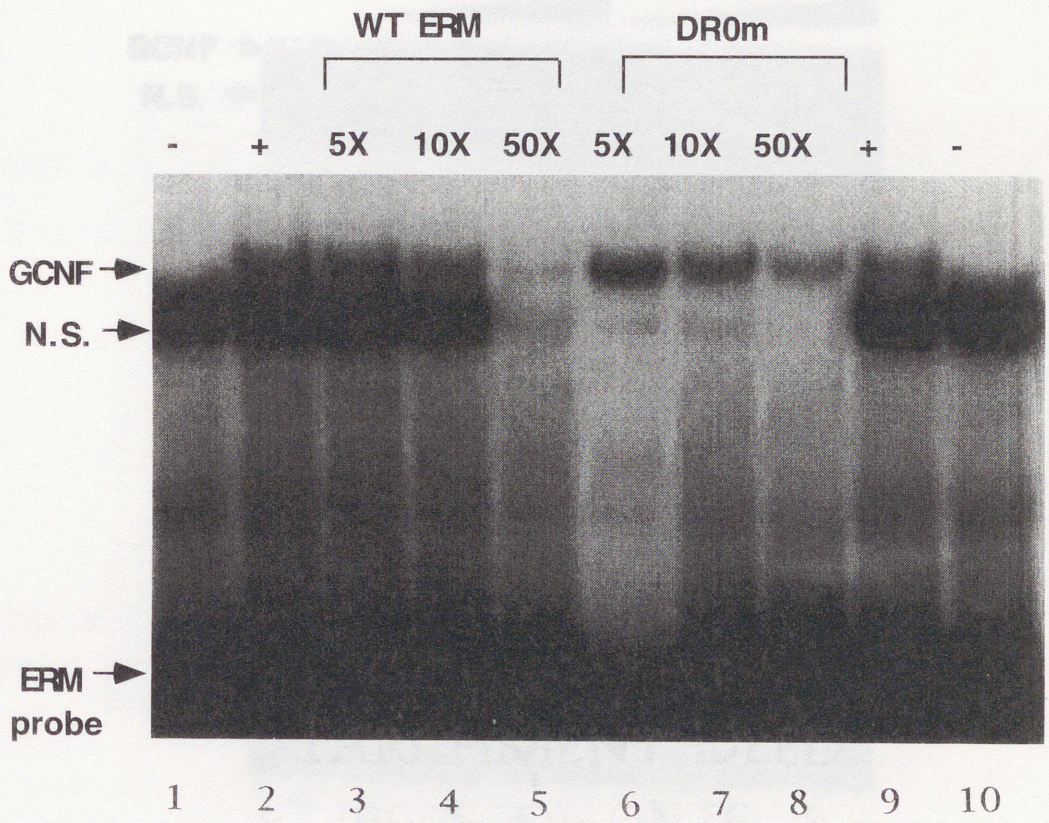
and ERE intact. Thus, to test for GCNF binding to the mDR0 probe, competition analysis was employed using *in vitro* translated GCNF. Increasing amounts of unlabelled WT ERM oligonucleotide (lanes 3-5) and unlabelled mDR0 oligonucleotide (lanes 6-8) were used to challenge GCNF binding to the WT ERM probe (Figure 20). Competition analysis of GCNF binding showed the unlabelled WT ERM oligonucleotide did compete for binding to the ERM probe, however, the unlabelled mDR0 oligonucleotide did not compete for GCNF binding to the ERM probe. Lanes 1 and 10 are control unprogrammed lysates and lanes 2 to 9 are *in vitro* translated GCNF. Thus GCNF binds to the DR0 element in the Lactoferrin ERM. The mERE probe has mutations that destroy the ERE but leave the DR1 and DR0 intact.

We next wanted to test for GCNF binding to the mERE probe. In a gel mobility shift dose response assay using the mERE probe (Figure 21), increasing amounts of *in vitro* translated GCNF were added and compared to control lysate. Lanes 1-4 were performed using the mERE probe and lanes 5-7 were performed using the ERM probe as a control. Thus, GCNF doesn't bind to the ERE element in the lactoferrin ERM.

To establish specificity of binding to the mERE probe observed in Figure 21, a competition analysis using increasing amounts of unlabelled WT ERM oligonucleotide (lanes 3-5) and unlabelled mERE oligonucleotide (lanes 6-8) was used to challenge GCNF binding to the WT ERM probe (Figure 22). Competition analysis of GCNF binding showed the unlabelled WT ERM oligonucleotide and the unlabelled mERE oligonucleotide both competed for binding to the ERM probe. Lanes 1 and 10 are control unprogrammed lysates and lanes 2 to 9 are lysates with *in vitro* translated GCNF.

FIGURE 20

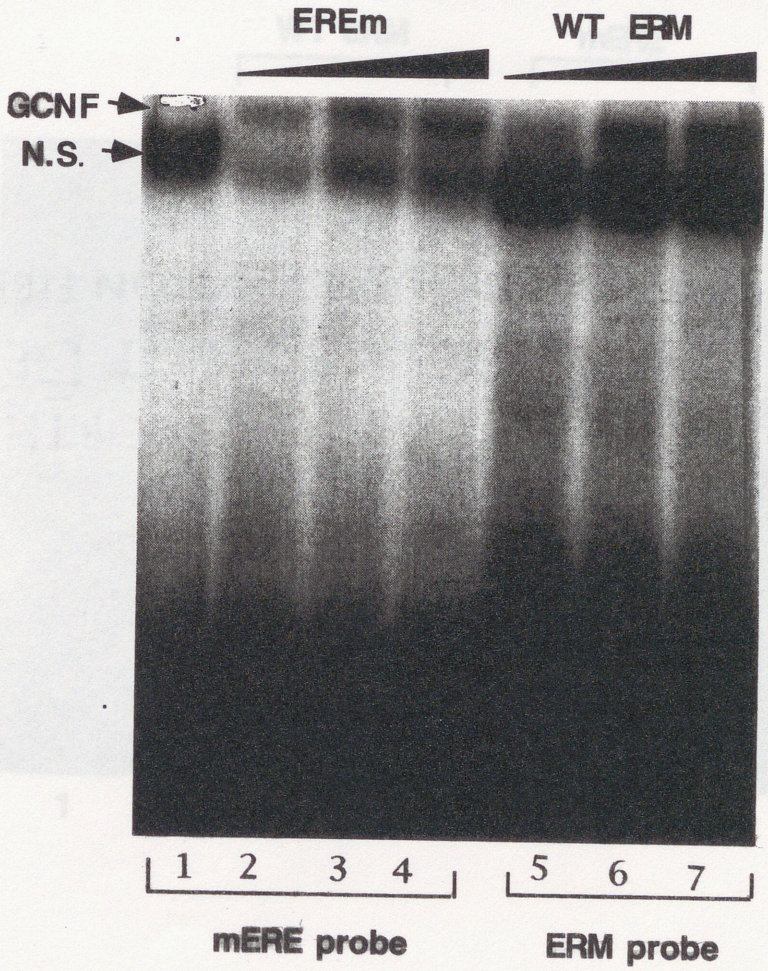
Gel Mobility Shift Assay Showing Competition Analysis of GCNF Binding to the WT ERM with the mDR0 and WT ERM



Lanes 3-5 show increasing amounts of unlabeled WT ERM oligonucleotide showing binding of GCNF to the ERM and lanes 6-8 show increasing amounts of unlabeled mDR0 oligonucleotide showing no competition of GCNF binding by mDR0.

FIGURE 21

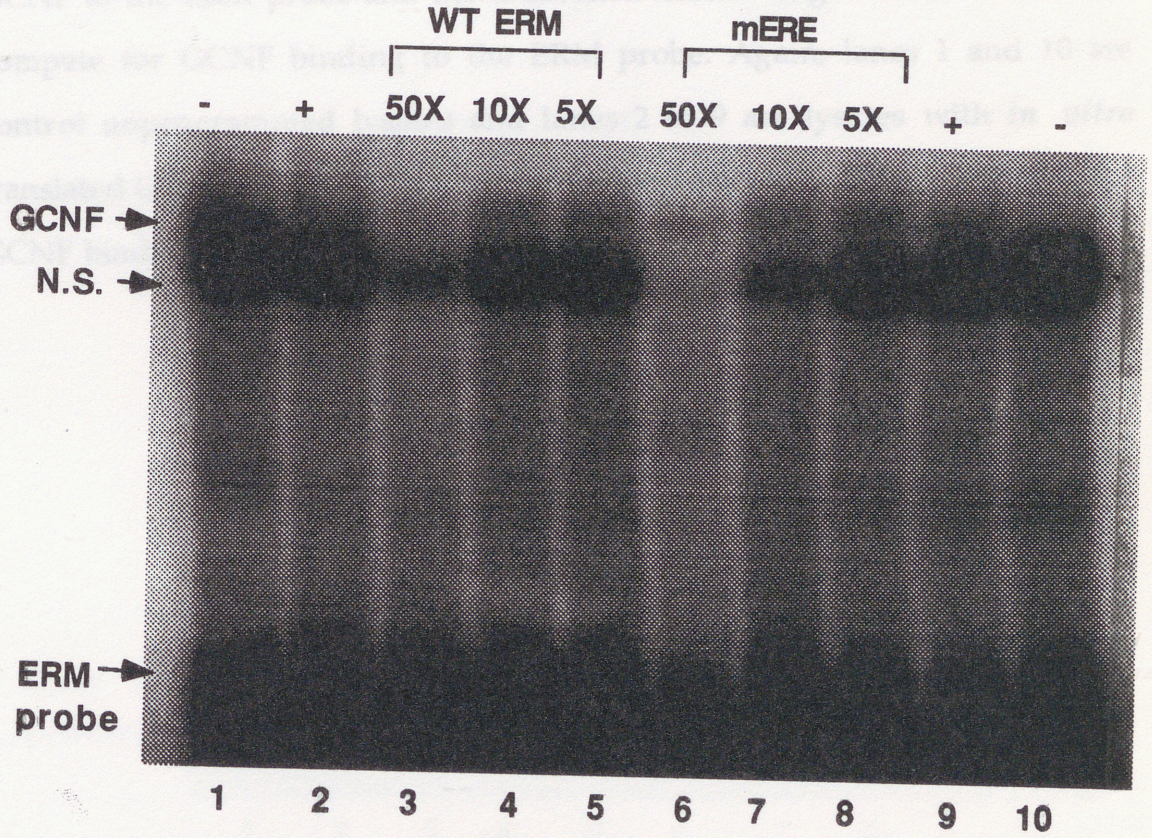
Gel Mobility Shift Assay Showing Dose Response Analysis of GCNF Binding to the mERE and the WT ERM



Lanes 2-4 show increasing amounts of *in vitro* translated GCNF binding to the mERE probe. Lanes 5-7 show increasing amounts of *in vitro* translated GCNF binding to the WT ERM probe.

FIGURE 22

Gel Mobility Shift Assay Showing Competition Analysis of GCNF Binding to the WT ERM by mERE

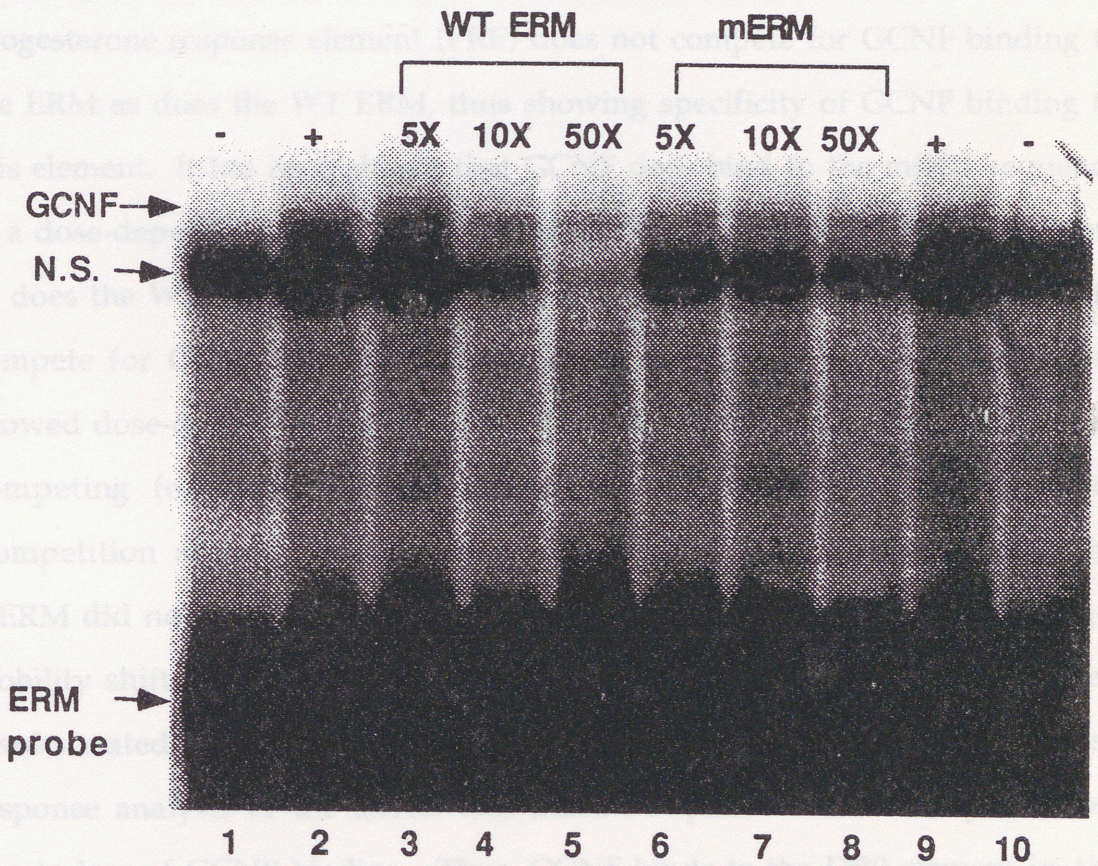


Lanes 3-5 contain decreasing amounts of unlabeled WT ERM oligonucleotide demonstrating GCNF binds to the ERM probe and lanes 6-8 contain decreasing amounts of unlabeled mERE oligonucleotide demonstrating GCNF binds to the ERM probe.

The last step was to test for GCNF binding to the mERM probe which contains a mutation that destroys all these response elements. In a gel mobility shift competition assay using *in vitro* translated GCNF, increasing amounts of unlabelled WT ERM oligonucleotide (lanes 3-5) and unlabelled mERM oligonucleotide (lanes 6-8) were used to challenge GCNF binding to the WT ERM probe (Figure 23). Competition analysis of GCNF binding showed the unlabelled WT ERM oligonucleotide did compete for binding of GCNF to the ERM probe and the unlabelled mERM oligonucleotide did not compete for GCNF binding to the ERM probe. Again, lanes 1 and 10 are control unprogrammed lysates and lanes 2 to 9 are lysates with *in vitro* translated GCNF. Thus, the central half of the Lactoferrin ERM is required for GCNF binding.

FIGURE 23

Gel Mobility Shift Assay Showing Competition Analysis of the WT ERM with the mERM



Lanes 3-5 show increasing amounts of unlabelled WT ERM oligonucleotide demonstrating GCNF binding to the ERM probe. Lanes 6-8 show increasing amounts of unlabelled mERM oligonucleotide demonstrating GCNF binding to the ERM probe.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The results in this research project using gel mobility shift assays have shown that GCNF binds in a dose-dependent manner to the estrogen response module (ERM) in the promoter region of the lactoferrin gene. The progesterone response element (PRE) does not compete for GCNF binding to the ERM as does the WT ERM, thus showing specificity of GCNF binding to this element. It has been shown that GCNF does bind to the mDR1 sequence in a dose-dependent manner and the mDR1 does compete for GCNF binding as does the WT ERM as predicted. The mDR0 sequence was shown not to compete for GCNF binding as predicted. A gel mobility shift assay also showed dose-dependent binding of GCNF to the mERE sequence as well as competing for GCNF binding in a competition analysis as predicted. Competition analysis with the mERM using the ERM probe showed the mERM did not compete for GCNF binding. Results obtained from the gel mobility shift assays of the different response elements were the predicted results stated earlier in this thesis. Gel mobility shift assays showing dose response analysis of the mDR0 and mERM sequences were not performed due to loss of GCNF binding. Thus, GCNF binds to the DR0 element in the ERM and not either DR1 or ERE elements.

Future experiments that should be performed are: Functional analyses of the estrogen receptor response from each reporter and functional analyses

of GCNF interaction with the estradiol response of each reporter. Polymerase chain reaction (PCR) of the lactoferrin promoter and introduction of mutations into each of the half-sites, and characterization of GCNF, ER, and COUP-TF binding to the lactoferrin promoter and linker mutants.

After performing all of these experiments successfully, then and only then will it be significantly proven that the lactoferrin gene is a candidate GCNF responsive gene and that it seems to be a good model system for determining the regulation of gene expression by GCNF.

APPENDIX

A. RECIPES FOR SOLUTIONS USED

DTT	3.09 grams of DTT in 20 ml of 0.01M sodium acetate (pH 5.2)
KMD	Potassium Manganese with DTT

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