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REGULATION OF METALLOTHIONEIN EXPRESSION IN THE RAT PROSTATE

BY

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"Brains first and then hard work, that's the way to build a house," said Eeyore.

A.A. Milne

To my mother who gave me love and support

throughout my education

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ABBREVIATIONS

METALS AND ELEMENTS

Pb	Lead
Ag	Silver
Bi	Bismuth
P	Phosphorous

UNITS OF MEASURE

ng	Nanogram
ug	Microgram
mg	Milligram
g	Gram
kg	Kilogram
ul	Microliter
ml	Milliliter
L	Liter
nm	Nanometer
mm	Millimeter
cm	Centimeter
M	Molar
mM	Millimolar
uM	Micromolar
Da	Dalton

UNITS OF RADIATION

Ci	Curie
uCi	Microcurie
Bq	Bequerel
cpm	Counts per minute
dpm	Disintegrations per minute

MISCELLANEOUS

A	Adenosine
T	Thymine
G	Guanine
C	Cytosine
UTP	Uracyl triphosphate
NTP	Nucleotide triphosphate
dNTP	Deoxynucleotide triphosphate
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
U	Units
^{o}C	Degrees in centigrade
Tris	Tris (hydroxymethyl) aminomethane
Pipes	Piperazine-N, N'-bis (2-ethane Sulfonic Acid)
xg	X gravitational force
kb	Kilobase
rpm	Rotations per minute
OD	Optical density
N	Normality
V	Volts

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ABSTRACT

Metallothioneins (MT) are low molecular weight proteins that bind metal ions. The protein has been discovered in many species ranging from mammals to yeast. In mammals MT is mainly found in the liver and kidney and it is believed that MT is involved in metal homeostasis (zinc, copper), and detoxification (cadmium, mercury). Metallothionein genes have been cloned and characterized and the mechanisms of gene induction by various factors have been well established.

The prostate contains the highest concentration of zinc of any organ found in mammals. The exact function of this metal for reproduction is unknown, although zinc deficiency can lead to infertility as well as other physiological abnormalities. The rat prostate is divided into three distinct lobes: lateral, dorsal, and ventral. This study investigated the regulation of MT in the rat prostate, specifically with respect to each lobe.

The rat MT-I mRNA was analyzed by using a radioactively labelled rat MT-I cDNA which was hybridized to prostatic mRNA bound to nitrocellulose (Northern Blot analysis). In addition, nuclear runoff assays were completed to determine whether zinc transcriptionally regulates the MT-I gene in the prostate.

The results indicated that the level of MT-I mRNA is higher in the lateral prostate than in the liver. The amounts found in the dorsal lobe are comparable to the liver but the ventral lobe did not contain detectable amounts of MT mRNA. Castration reduced the levels of MT mRNA in all tissues studied. In addition, the amounts of MT mRNA increased in the

prostate as the animal reached sexual maturity indicating that hormones may contribute to the regulation of the gene in this organ. However, a single injection of dihydrotestosterone did not result in any detectable change of MT mRNA in the lateral lobe.

Zinc and cadmium usually increase the accumulation of MT mRNA, via transcription initiation, in tissues. This was also the case for kidney, liver, and dorsal prostate. In the lateral prostate, however, only zinc was able to induce the accumulation of MT mRNA.

INTRODUCTION

1. METALLOTHIONEIN

Metallothioneins (MTs) are low molecular weight proteins found in many eukaryotic species. Metallothionein is able to bind many heavy metals ions such as the essential zinc (Zn) and copper (Cu) and the nonessential cadmium (Cd) and mercury (Hg). It is evident that this metal-binding ability plays an important role in its function. Although cells without MT are perfectly viable, it does appear that the protein is able to protect cells and organisms from toxic metal poisoning. In addition, MT is very likely involved in the metabolism of the nutrients Cu and Zn and may therefore contribute to the regulation of many Zn or Cu containing enzymes.

The regulation of MT synthesis has been well studied (for a review see Kagi and Nordberg, 1979). Metals and hormones act on MT gene transcription. Even other substances such as interferon, lipopolysaccharide, cyclic adenosine monophosphate (cAMP) affect MT synthesis either directly or indirectly. This regulation takes place at several levels including protein stabilization, gene amplification, gene methylation or changes in cellular physiology during development.

Apart from its metal homeostasis and protection role, MT may have additional functions. There is evidence that the protein is an important part of the cell's protection against

acute stress. It may regulate inter- and intracellular Zn distribution or may act as an efficient free radical scavenger serving to protect cells against various forms of radiation.

A. Nomenclature

Metallothioneins were first discovered and isolated as Zn and Cd binding proteins from horse kidney (Margoshes and Vallee, 1957). Since then, it has been isolated from a variety of vertebrate species including human (Pulido et al., 1966), rat (Shaik et al., 1971), and mouse (Nordberg et al., 1975). Metallothionein-like proteins have been found in invertebrates and plants (Kagi and Nordberg, 1979). The amount of MT produced varies from tissue to tissue with the highest levels being reported in liver and kidney.

The MT's exist in at least 2 isoforms which have been designated as MT-1 and MT-2 depending on the elution position from DEAE cellulose. To classify a protein as an MT it must comply with the following properties:

a) low molecular weight (6000-7000 Da),

b) high content of cysteine residues (approx. 33 mole %),

c) few aromatic and hydrophobic amino acids,

d) high content of heavy metals (4-12 atoms/mole) bound by thiolate bonds,

e) a relative good homology with the primary

mammalian MT structure.

Various proteins can therefore be classified under the general heading MT. Primate metallothionein have several functional as well as nonfunctional pseudogenes. The human MT (hMT) family consists of at least twelve genes separated into the MT-1 and MT-II classes. At least seven of these genes are functional and are clustered on chromosome 16 at 16q22 (Heguy et al., 1986; Karin et al., 1984). The remaining pseudogenes are dispersed on at least four other autosomal sites (Schmidt et al., 1984). Many of the genomic human genes and/or cDNAs have been cloned and sequenced including hMT-IA (Richards et al., 1984), hMT-IIA (Karin and Richards, 1982), hMT-IE (Schmidt et al., 1985), hMT-IF and hMT-IG (Varshney et al., 1986), and hMT-IB (Heguy et al., 1986). Many pseudogenes have been described which do not encode fully functional proteins but show considerable homology to MT. These are usually designated as ψMTs with an alphabetical letter following it e.g. ψMT-IIb (Karin and Richards, 1982). The mouse genome codes for only two functional isoforms mMT- I and mMT-II, located on chromosome 8. The MT-I lies 6 kilobases (kb) downstream of MT-II resulting in a coordinately regulated pair (Searle et al., 1984).

In rats, only two known functional proteins, again MT-I and MT-II, have been identified. The rat MT-I cDNA has been isolated and Southern analysis suggested that possibly four other MT related sequences exist (Andersen et al., 1983). Andersen and colleagues (1986) have now

characterized the rat MT-I genome. This species, like primates, has a MT-I multigene family. In addition to the MT-I structural gene, these authors have identified three pseudogenes. These have been designated ψMT-Ia, ψMT-Ic, and ψMT-Ib. The first two are apparently retrogenes which arose from reverse transcription of the MT-I mRNA. The ψMT-Ia and ψMT-Ic are therefore not functional as ψMT-Ic has lost its 3′ sequences which were homologous to the MT-I gene past the sixth nucleotide 3′ of the stop codon. The pseudogene ψMT-Ia possesses no intervening sequences and has a repeated polyadenylation signal within 4 bp of the transcription initiation site. The other pseudogene, ψMT-Ib, does possess some of the 5′ regulatory elements and the adenylation signal for a poly (A+) tail. However, no MT-Ib transcripts could be detected *in vitro* or *in vivo*. Thus the rat multigene MT-I family is quite different from the unique mouse MT-I gene (Searle *et al.*, 1984). The calculated age of appearance of the rat pseudogenes suggests that at least ψrMT-Ia appeared after the evolutionary divergence between rats and mice (Andersen *et al.*, 1986). The existence of many rat MT isoforms, some of which are pseudogenes and their occurrence on one chromosome, is typical of mammalian multigene families. It is not known whether the many isoforms appeared because of selective advantage or due to random mutation.

The MT sequences have also been cloned from other various species such as horse (Kojima *et al.*, 1979), sheep

(Peterson and Mercer, 1986), sea urchin (Nemer et al., 1985), and Drosophila melanogaster (Lastowski-Perry et al., 1985). A lower eucaryotic MT has also been identified from the yeast Saccharomyces cerevisiae which is often referred to as a copper-thionein (Butt et al., 1984).

B. Structure

The MT protein secondary structure determined for mammals are remarkably similar. All have sixty-one or sixty-two amino acids in a single polypeptide chain. The twenty cysteines are always present in Cys-X-X-Cys, Cys-X-X-X-Cys and Cys-Cys sequences. The amino- and carboxylterminal residue are N-acetylmethioneine and alanine, respectively (Kagi and Nordberg, 1979). The molecular weight of the protein ranges from 6000-7000 daltons depending on its metal content with unbound MT having the lower weight (Kagi et al., 1974).

The tertiary structure has proven to be more elusive to define although a variety of biophysical techniques such as UV spectroscopy, NMR and X-ray crystallography have been helpful. The amino acid folding results in two distinct domains composed of the carboxyl-terminal and the amino terminal half of the polypeptide chain. The carboxyl terminal end of the molecule (alpha domain, A) consists of amino acids 31-61 while the amino terminal (beta domain, B) extends from residues 1-30, with 30 and 31 acting as bridges (Winge and Miklossy, 1982). The alpha cluster, with its eleven

cysteines, binds four atoms of Zn or Cd, or five to six atoms of Cu. The beta domain contains nine cysteines which binds three atoms of Zn or Cd, or six atoms of Cu (Abrahams et al., 1986). The metals are bound to the protein by a deprotonated cysteine side chain through thiolate bonds. The sequence of Cys-X-X-Cys seems to be very important, because hexapeptides containing such a thiol sequence are able to bind Cd(II) or Zn(II) with spectral properties indistinguishable from MT (Vasak et al., 1984). Both Zn and Cd are bound in the +2 valence state resulting in a tetrahedrally coordinated complex; whereas Cu, which binds in the +1 state, forms trigonal structures. This results in an altered folding pattern and a different tertiary structure (Garvey et al., 1982; Nielson and Winge, 1985). The metals do not bind with equal strength (Hunziker and Kagi, 1985) and their affinity for the protein has been shown to be: Zn(II) < Pb(II) < Cd(II) < Cu (I), Ag(I), Hg(I), Bi(III). Therefore, Zn can be easily displaced by other metal ions. The other metals can be removed by lowering the pH to yield the metal free protein, apometallothionein (thionein). Different metals also seem to prefer one domain over another. In vitro, the alpha domain is usually filled first by Zn and Cd whereas Cu saturates the beta domain first (Kagi and Nordberg, 1979). Once one metal has been bound in a domain it promotes the complete filling of that domain. In the natural state MT rarely has a homogenous composition of metals and the binding

of one type of ion does not prevent or hinder the binding of another (Nielson and Winge, 1985). In fact, if two different MTs are artificially 'loaded' with either Zn or Cd and the proteins are mixed, the result is an intermolecular exchange of metals, producing MTs with mixed compositions (Nettesheim et al., 1985). These same studies demonstrated that the exchange reactions are rapid and occur between Zn-MT and Zn dependent enzymes. This is most likely the case in vivo.

C. Regulation by metals

It has been known for sometime that MT expression is influenced by metals. In 1964 Piscator reported that MT levels increase in rabbit liver following Cd administration. Early studies concentrated on protein analysis, reporting that radioactive Zn injections bound specifically to a Zn thionein (Squibb et al., 1977). Further studies demonstrated that the rate of MT synthesis, as measured by ^{35}S cysteine incorporation, was greatly increased following the administration of Cd or Zn (Kagi and Nordberg, 1979).

The fact that the increased MT protein synthesis was inhibited by actinomycin D (an inhibitor of RNA synthesis) and that both mRNA levels and translational activity seemed to be maintained coordinately, led (Enger et al., 1979) to postulate that metals were primary inducers of thionein mRNA. Utilizing the newly discovered MT cDNAs, much information has been compiled on the regulation of MT by metals (Suzuki and Yamamura, 1980; Richards and Cousins, 1975; Bremner and

Davies, 1975). The MT messenger RNA accumulation was found to increase following Cd administration (Ohi et al., 1981) This could be accounted for by: 1) increase of mRNA half-life, 2) increased rate of transcription and/or 3) gene amplification. To distinguish between these possibilities, nuclei were isolated from both Cd-treated and control mouse liver and kidney. These nuclei were incubated with radioactive UTP so that the nascent mRNAs could be elongated (nuclear run-off assay). The isolated labeled RNA was hybridized to a nitrocellulose immobilized plasmid containing the MT sequence. The results of these experiments indicated that the synthesis of new MT mRNA begins within one hour of metal administration with maximal synthesis occuring at four to six hours (Durnam and Palmiter, 1981). This is also true for induction by Zn, Cu or Hg although the fold change over control levels may differ. The variation in the efficiency of induction by different metals may be partially determined by the metal-tissue interaction or by the gene itself. For example, in liver, Zn and Cd induce MT production best, while Cu functions only at high doses and Hg is a weak inducer at any dose. In kidney, Hg and Cd induce well while Zn is a good inducer only at high doses and Cu inducing poorly at any dose (Durnam and Palmiter, 1981).

Stuart and colleagues (1985) have identified the specific sequences that interact with metals on the MT gene promoter. Analysis of 5′ deletion mutants revealed the bases

involved in heavy metal regulation. The first metal regulatory element (MRE) was mapped between -60 and -42 relative to transcription start site. Since specific disruption of this MRE did not entirely eliminate metal response, additional homologous elements were sought and discovered further upstream (Stuart et al., 1985). This twelve base pair sequence (C-Y-T-T-T-G-C-R-Y-Y-C-G, R=purine, Y=pyrimidine) is found five times in the mouse MT promoter and is shared with other species (rat MT-I and hMT-IA, Richards et al., 1984). Although one such sequence (when inserted upstream of the heterologous herpes tk gene) offers some metal response, there is a much greater response when two or more copies are present (Searle et al., 1985). Mouse MT-I and II genes respond equally well to Zn and Cd as does the human MT-IIA gene. A difference was found for hMT-IA gene which was strongly inducible only by Cd (Richards et al., 1984).

In addition to the sequences that respond specifically to increases in metal concentrations, there are elements that are implicated in basal level expression. Haslinger and Karin (1985) discovered several distinct basal recognition sequences which include the TATA box, a GC rich region located between nucleotides -68 to -57 and at least two distinct basal level elements (BLEs). In the human MT-IIA gene, an enhancer is located around -90 and then again further upstream between positions -162 and -169. Deletion or substitution of this GC box decreases the

transcriptional activity of the hMT-IIA promoter but this does not alter the inducibility of the gene by Cd suggesting that the GC box is not directly needed for metal response. The enhancer elements can compete with the simian virus 40 (SV 40) enhancer for 1 or more cellular factors in vivo (Scholer et al., 1986). One of these factors is a novel activator protein (AP1) which interacts with both the MT-IIA BLE and a 72 base pair repeat of the SV 40 enhancer region. The other protein binds to the GC region of hMT-IIA and has been named SP1 (Lee et al., 1987). How the enhancer elements function is still not completely known although the basal enhancer functions whether or not a MRE element is present (Haslinger and Karin, 1985). However, the MREs are not capable of responding to metals if the two basal enhancer elements are eliminated. Therefore, MREs confer metal inducibility only when they are adjacent to an already active enhancer. The presence of an enhancer element in the promoter of the hMT gene may be important for cases of acute myelomonocytic leukemia. Twenty-five percent of these patients present a rearrangement of the MT gene cluster, including promoter, split from chromosome 16. This may cause activation of a putative oncogene present on the short arm of the chromosome 16 (le Beau et al., 1985; Haslinger and Karin, 1985).

Although these proteins which regulate basal expression have been purified, the method by which metals interact with the DNA is still not well understood. The most likely method

would require a protein that binds both the DNA (MRE) and the inducing metal. This idea has been substantiated by the discovery of a protein Mer^R that mediates the induction of mercury resistent phenotypes in bacteria (O'Halloran and Walsh, 1987). It appears to be a DNA-binding metalloregulatory protein that plays a central role in a heavy metal response system.

Experiments on changes in chromatin structure, due to induction by Cd, have helped to identify the binding sites for potential regulatory proteins. Expression of both rat and mouse MT-I genes changes the nuclease hypersensitivity of its 5' regulatory region. In mouse, the strongest nuclease sensitive digestion sites are centered around position -225 and - 30. After the animals or cell cultures have been treated with Cd, there is an increase in nuclease hypersensitivity within the region -30 to -60 (Senear and Palmiter, 1982). In rat, the induction of the MT gene by Cd or Zn also resulted in the appearance of a new hypersensitive area near the start site of transcription, a region near the MREs (Taplitz et al., 1986).

At least one class of these cellular factors acts as a positive regulatory mechanism. A bacterial system in which an MT plasmid had to compete for regulatory factors with a competitor plasmid led to a sequential increase of the indicator MT expression (Seguin et al., 1984). This suggests that a limiting factor for the MT gene induction is a positive regulatory protein that binds to the same

sequences as required for maximal induction by heavy metals.

Although much has been discovered concerning the regulation of the MT gene by heavy metals, the knowledge is by no means complete. It is still unknown whether one factor that binds both metal and DNA eludes us or whether a cascade of events causes gene activation. It is evident that certain sequences termed MREs are necessary for MT gene sensitivity to metals and that the increase in MT is due to increased transcription.

D. Regulation by hormones

Since the discovery of the MT gene's sensitivity to metals, researchers noticed that MT synthesis increased in stress situations (Oh et al., 1978). Subsequent experiments determined that glucocorticoids stimulated the accumulation of Zn in many cells - a phenomenon that was inhibited by actinomycin D (Failly and Cousins, 1978). The direct effect of dexamethasone on MT was then studied. Seven hours after an injection of the hormone, maximal translatable MT mRNA was measured and the synthesis was clearly observable within four hours of exposure. This preceded the uptake of Zn (Etzel et al., 1979; Karin and Herschman, 1981). It appeared that Zn and dexamethasone induce MT by mechanisms independent of one another. It was then shown that the induction of translatable MT mRNA by glucocorticoids is independent of concomitant protein synthesis. It was a primary response similar to that seen

with metals (Karin et al., 1980). Accumulation of mRNA increases 10-fold by eight hours in cultured mouse cells (Mayo and Palmiter, 1981). Finally, in vitro nuclear runoff studies were done to show that glucocorticoids cause transcriptional activation of the mMT-I gene (Hager and Palmiter, 1981). The induction was most prominent in the liver and less effective in other tissues. The kidney MT gene was an especially weak responder which may be due to that tissue's lower number of glucocorticoid receptors. Genomic and cDNA probes were utilized to delineate exactly how glucocorticoids exert their influence. Karin et al., (1984) first performed gene transfer experiments with a Hind III fragment that contains the intact hMT-IIA gene. Results indicated that this fragment contained the sequences which allow for expression and induction of hMT-IIA by either metals or glucocorticoids. Deletion experiments found that a distinct sequence within the promoter (from -236 to -268 of the transcription start site) was responsible for the hormone response (Karin et al., 1984). Furthermore, the glucocorticoid receptor itself appears preferentially to bind to a region mapped between -265 and -245. This sequence acts both as a regulatory element necessary for glucocorticoid induction of transcription and as a binding site for the hormone-receptor complex.

The mouse genes, MT-I and MT-II, are strongly inducible by hormones in vivo (Yagle and Palmiter, 1985) but once gene manipulations are done (in vivo and in vitro) the

response to hormones is often selectively lost. For example, after fusing the MT promoter regulatory region with thymidine kinase genes and microinjecting this into fertilized eggs to obtain transgenic mice, the resultant MT-Tk expressing tissues were responsive to metals but not to glucocorticoids (Palmiter et al., 1982). Similarly, hybrids containing human growth hormone structural sequences fused to the MT promoter and presumptive control region of the mMT-I gene were not responsive to dexamethasone when used to transfect mouse cells (Pavlakis and Hamer, 1983). Transfection of MT-I genes causes some change either in the promoter sequence (perhaps another sequence necessary for glucocorticoid regulation lies further upstream than the segment used in the transfection experiment which included up to 1.7 kb of the 5'flanking DNA) or in the native chromatin structure which becomes inappropriate for DNA-receptor interaction (Mayo et al., 1982). Perhaps the latter occurs by changes in methylation of DNA but this has so far not been experimentally substantiated.

Other hormones have also recently been shown to increase both MT proteins and MT mRNA in rat liver (Cousins et al., 1986). It is believed that glucagon and epinephrine are primary regulators of MT gene expression possibly acting via a cAMP pathway. In fact Bt_2cAMP (a synthetic cAMP analog) acts to increase MT mRNA transcription as actinomycin D inhibits the response. Insulin induces MT synthesis. The response to insulin can be only partially blocked by a protein

kinase C inhibitor indicating an alternative pathway that does not involve protein kinase C. Nor is this additional route protein kinase A, since a phosphodiesterase inhibitor also has no effect on insulin induced MT transcription (Imbra Karin, 1987). In fact, evidence suggests that the pathway also includes cAMP (Cousins et al., 1986).

E. Regulation by other factors

A variety of factors that induce MT synthesis has been organized into an 'acute- phase response' group because they are released during stress. A hot environment caused relatively little increase in rat hepatic MT; whereas, injection of carbon tetrachloride has a substantial stimulating effect. All induced stresses which included cold and strenuous excercise had an observable but statistically insignificant effect on MT. However, a distinct redistribution of Zn from plasma to other Zn pools was observed (Oh et al., 1978). Even starvation can cause sufficient stress resulting in an increased MT production. This supports the theory that MT is an important Zn sequester (Bremner and Davies, 1975). Not only physical trauma to tissue but also injection of inflammatory agents such as bacterial endotoxin (LPS) will cause MT to be produced (Sobocinski and Canterbury, 1982). This effect was demonstrated by Durnam and colleagues (1984) to be independent of metals and glucocorticoids. For example, adrenalectomized rats still respond to LPS with an increased MT accumulation and LPS

stimulation of MT precedes Zn accumulation within tissues. These authors reported that a region responsible for LPS regulation lies within the region -185 to -350 base pairs (bp) 5' of the transcription start site (regions not responsible for metal regulation). A factor released from LPS stimulated macrophages also induces MT synthesis although this factor has not yet been identified (Iijima et al., 1987).

Tumor promoting agents, 12-O-tetradecanoylphorbol-13-acetate (TPA) and ultraviolet radiation (UV), induce MT synthesis (Angel et al., 1986). The induction of MT mRNA by TPA can be completely blocked by a protein kinase C inhibitor, indicating that TPA acts as a primary inducer (Imbra and Karin, 1987). The inhibitor does not alter the response of the MT gene to Cd or dexamethasone (dex). Also, TPA induces the 'UV response' which is characterized by induction of a particular set of proteins and generation of a UV resistant state after treatment of cell lines with UV, X-rays, mitomicin C (MMC) or TPA (Imbra and Karin, 1987). The mechanism by which TPA induces the MT gene is different than that responsible for Cd induction because deletion mutants that do not have metal responsive elements are still responsive to TPA. These observations indicated that MT may indeed play an important role in stress situations such as radiation exposure. Of particular interest are recent discoveries concerning the effect of immune related substances on MT expression. Interleukin-I (IL-I) is responsible for initiation and modulation of many immune and

inflammatory responses. Karin and associates (1985) have demonstrated that a significant induction of MT-IIA in response to IL-I occurs in the cell lines: Hep G2, a hepatoma line; HL-60, a promyelocetic cell line; and primary human fibroblasts isolated from foreskin. Furthermore, S1 protection assays showed that the MT-IIA mRNA is initiated at the same start site as heavy metal- and glucocorticoid-induced mRNAs.

Interferon (IFN) induces the expression of mRNAs for the histocompatibility locus antigens (HLA) genes. Treatment of a neuroblastoma cell line with IFN resulted in a three to five fold increase in transcription in both HLA class I genes and MT-IIA. Significantly, in the comparison of regions upstream of the MT-IIA, 2 HLA class I genes and 1 HLA class II gene revealed a homology of about 30 bp. These sequences may be involved in regulating transcription of IFN induced genes (Friedman and Stark, 1985). This region of homology lies -142 to -147 bp upstream of the TATA box of the class I gene and -600 or -561 bp upstream of the MT-IIA or HLA-DR TATA box, respectively. It has been shown that the MT mRNA accumulation due to IFN acts by mechanisms other than those initiated by metals (Morris and Huang, 1987). Interestingly, IFN-alpha does not cause substantial increases in MT transcription in either HeLa or RD-114 cells whereas IFN-gamma does stimulate MT-II gene activation in HeLa cells. This indicates that the response of the MT gene is specific to the different IFNs (Kusari and Sens, 1987).

Apparently, many of these MT inducing substances, which includes TPA and IL-1, appear to act through protein kinase C to induce the transcription of MT-IIA. These activators either directly or indirectly increase protein kinase C activity which in turn either directly phosphorylates a target intermediate protein or starts a series of phosphorylations resulting in the activation of trans-acting factor(s) that stimulate the MT genes.

Additional compounds and elements have been shown to regulate the MT gene. These include sodium butyrate (Birren and Herschman, 1986), gold compounds (Butt et al., 1986), D-penicillamine (Heilmaier et al., 1986) and ascorbic acid (Onosaka et al., 1987). How these inducers work has not yet been elucidated.

F. Gene amplification and methylation

Regulation of MT includes not only transcription, mRNA half-life, translation and protein half-life, but also changes in either gene copy number or its methylation state. Amplification of MT genes was discovered in Cd resistant cell lines (Rugstad and Norseth, 1975; Gick and McCarty, 1983). These cell lines can be easily established by a step-wise increase of Cd in the medium. It was found that Friend leukemia cells that were made Cd resistant in this manner had more MT-I mRNA, a higher MT mRNA transcription and a 6 fold gene amplification over non-resistant cells (Beach and Palmiter, 1981). All cell lines that have amplified

MT genes are responsive to heavy metals but not necessarily to glucocorticoids, e.g. the Cd resistant mouse sarcoma cells S180 were essentially unresponsive to steroids (Mayo and Palmiter, 1982). Apparently the primary gene structure is not sufficient for transcriptional regulation by hormones since these amplified cells possess the putative glucocorticoid binding site. Thus, the gene may somehow be altered making the receptor binding site either unaccessible or nonfunctional. Koropatnick et al., (1985) reported that the extra copies of MT-I genes in mouse liver, which result from continuous exposure to Cd, appear as early as 6 hours after Cd treatment and persist for up to 3 weeks in the absence of further heavy metal treatment. In these experiments the amplification was observed only in liver, suggesting a pharmacokinetic organ specific effect.

Cadmium is not the only metal ion that will lead to amplification of MT genes. For example, the Saccharomyces cerevisiae copper MT-gene (CUP1) can be amplified by stepwise selection of CUP1r variants which exhibit copper resistance (Fogel and Welch, 1982). Certain potent tumor promoters such as aplysiatoxin and even moderate tumor promoters such as mezerin can cause an enhanced frequency of Cd resistance in Chinese hamster lung cells. This enhancement was shown to be due to MT gene amplification (Hayashi et al., 1983). Richards and colleagues (1984) have suggested that MT-I is preferentially involved in the protective response to Cd cytotoxicity. However, it is most likely

that both proteins, MT-I and MT-II, function in a Cd binding capacity (Gick and McCarty, 1982; Nordberg and Kojima, 1979). Both proteins have high affinity for the metal and both isoforms were nonpreferentially expressed in Chinese hamster ovary (CHO) cells after selection for MT gene amplified Cd resistant variants (Crawford et al., 1985). Certain cell lines were found that do not express MT genes even when exposed to Cd or glucocorticoids (Compere and Palmiter 1981). Compere and Palmiter (1981) then compared the cleavage patterns of the MT-I gene in the W7 cell line with restriction digests from cells that do express MT. They found that the Hpa-II sites in the vicinity of the MT-I gene in the W7 cells were protected indicating methylation. Furthermore, these authors demonstrated that the W7 MT-I gene did respond to Cd after incorporation of 5-azacytidine into DNA, a cytidine analogue that cannot be methylated. Since UV radiation of Cd sensitive S49 mouse cells can cause a substantial increase in the appearance of Cd-resistant variants, Lieberman and associates (1983) investigated whether UV also affects the methylation pattern of the MT gene. The gene mapping data illustrated that a region of at least 2.5 kb spanning the MT-I gene was extensively demethylated after UV treatment.

The MT gene methylation may play an important role during development. Two fish cell lines express their MT genes differentially according to the age of the animal donor. A cell line from Rainbow trout hepatoma expresses MT

normally whereas a cell line from Salmon embryo does not. This lack of expression is due to hypermethylation (Price-Haughey et al., 1987). This suggests that hypo- or hypermethylation of the MT genes may change during development but the mechanism by which methylation causes unresponsiveness in genes is unknown. It is possible that it interferes with chromosomal or nucleosomal packing or perhaps even with the binding of RNA polymerase.

G. MT in diseases

Several diseases that involve a defect in the metabolism or handling of copper have been found in humans. Menkes disease or Kinky Hair Syndrome was first described by Menkes and associates in 1962. It is an X-linked genetic disorder characterized by cerebral degeneration, seizures, growth retardation, hypothermia and abnormally textured hair. The disease manifests itself as a defect in copper absorption and an abnormal internal copper distribution. This leads to a metal depletion in liver and brain and excessive accumulation in kidney and several other tissues. Apparently, Menkes cells, in an environment of ample copper, exhibit an increased affinity for and/or retention of copper perhaps due to a defective transport process (Goka et al., 1976). Metallothionein has been studied in this disease because of is copper-binding ability. The MT is overproduced in Menkes' cells but it is now believed that the actual genetic defect lies in a factor other than MT. This factor appears to act

at an early stage of the metal regulatory pathway (Hamer, 1986; Leone et al., 1985).

Wilson's disease is distinct from Menkes but also involves copper metabolism. This is a recessive autosomal trait expressed in early adulthood and is characterized by an overaccumulation of copper and copper-MT in the liver and brain. However, the accumulation of copper-MT shows only minor abnormalities (Chan et al., 1980) (Evans et al, 1973). Thus it appears that, in both Menkes' and Wilson's diseases, regulation of the MT gene is only indirectly affected due to an altered copper metabolism rather than a directly altered MT gene structure or expression (Hamer, 1986).

2. A) PROSTATE: Structure and function

It has been known for many years that the lobes of the prostate are unique in both structure and function. In the rat, three distinct areas or lobes can be identified: ventral, dorsal, and lateral. The coagulating gland is sometimes referred to as yet another lobe, the anterior prostate. These differences were reported as early as 1935 by Korenchevsky and Dennison. The dorsal and lateral lobes are often considered together as one organ and are referred to as the dorsolateral prostate. These two lobes are separated by a thin mid line septum with each lobe being completely enclosed in its own capsule. The ventral lobe, lies posterior to the other two and is physically quite separate, joined only by a narrow connection (Gunn and Gould,

1957).

The dorsal lobe of the rat prostate is composed of apocrine and holocrine type acini whereas the lateral tip contains only apocrine-type acini (Gunn and Gould, 1957). Both cell types are consistant with the secretory role of the gland. Brandes and Groth (1961) made detailed electronmicroscopic studies of the dorsal and ventral lobes and coagulating glands. Their results are comparable to the lateral lobe fine structure, studied intensely by Schrodt (1961). The epithelial cells of the ventral and dorsal prostate show pronounced dilatation of the cisternae of the endoplasmic reticulum (ER). A few microvillae are present at the luminal surface of the cells which also possess large ungranulated Golgi complexes. In contrast, the lateral lobe have numerous microvilli at their luminal surface, dense secretory granules in the Golgi complex and a flattened cisternae of the ER. The lateral lobe also localizes its high concentrations of Zn at the luminal border of the epithelium, in the nucleoli, and in the stroma near the base of the epithelial cells (Schrodt, 1961).

In 1921 Bertrand and Vladesco reported that the prostate contains extraordinary amounts of Zn. Extensive experiments in the 1950s and 1960s determined that the rat dorsolateral prostate contained more Zn than any other tissue (Mawson and Fischer, 1951; Gunn and Gould, 1958; Mann, 1964). Following 1945, radioactive Zn was available and made it possible to experiment with Zn uptake. Radioactive Zn is

preferentially concentrated in the rat dorsolateral prostate (Gunn and Gould, 1955) and this ability is drastically decreased following castration which led to the assumption that Zn uptake is under hormonal control (Gunn and Gould, 1958). These experiments found that suitable doses of androgens (50 ug) when given to mature castrated rats for 1 week prevented both the atrophy and decrease of prostatic Zn^{65} uptake. Immature rats in these same experiments were unable to concentrate Zn^{65} to the remarkable degree that mature prostates could. This, in immature rats, could not be altered with the administration of androgens. Surprisingly, these authors demonstrated that estrogen was able to bring the capacity of the immature dorsolateral prostate to concentrate Zn up to the level found in the intact fully mature rat. Furthermore, androgen injections in intact male rats did not influence the rate of Zn uptake, in fact it actually somewhat depressed it (Kar et al., 1956).

The function of this high concentration of Zn is largely unknown. It has been well recognized that Zn is important for reproduction (Halstead et al., 1972). Zinc is present in the prostatic secretion. These secretions contribute a large amount of Zn to the seminal plasma in which sperm, themselves rich in Zn, are further enriched (Mann, 1964). Zinc may either be important for acceleration of sperm motility or in the synthesis of citric acid, or as an anti-bacterial agent. Its overall function remains to be elucidated. A number of Zn metalloenzymes are known: alkaline

phosphatase; lactic dehydrogenase; and carbonic anhydrase (Vallee, 1959). That Zn is necessary for prostatic enzymes is well accepted but these metalloenyzmes account for only a small fraction of the total Zn in the prostate (Fischer et al., 1955). Another important enzyme, 5-alpha-reductase, is also found in the prostate. This enzyme metabolizes testosterone to dihydrotestosterone (DHT) and it is believed that this enzyme controls the concentration of DHT (Wallace and Grant, 1975). Low levels of Zn stimulate the reduction of testosterone whereas high concentrations of Zn actually inhibit 5-alpha-reductase activity. Therefore, a narrow range of Zn concentrations in the prostate seems to be needed for the function of this enzyme.

The complete function of the prostate can only be speculated upon. In humans, research has been concentrated on prostatic secretions. They are very rich in proteolytic enzymes, the strongest being fibrinolysin which plays a role in the liquidification of seminal plasma. The most typical secretions are citric acid and acid phosphatase, these provide a sensitive and reliable chemical test for the assessment of prostatic function (Foti et al., 1977; Yam, 1974).

B) Prostatic Disease

Prostatic carcinoma (CaP) is a common cancer. This disease affects one out of three males over 70 years of age rising from 10% at age 60 to 50% at age 80 (Robbins, 1974; Silverberg and Luber, 1983). Prostatic cancer accounts for

10% of the deaths due to cancer in men and considerable research has been conducted on the aetiology of CaP (Ernster et al., 1979; Isaacs, 1984). The conclusions are contradictory and much uncertainty remains as no statistical correlation has been made between CaP and various factors including genetic, ethnicity, sexual behaviour or diet (Alderson, 1981). However, there does seem to be a possible increase in CaP following long exposure to Cd e.g. battery workers (Lemen et al., 1976) but this data may not be significant (Kolonel and Winkelstein, 1977).

The human prostate is also subject to a unique condition known as benign prostatic hyperplasia (BPH) first described by Le Duc in 1939. This disease does not appear to be a 'precancerous' condition and it is totally unrelated to CaP incidence (Greenwald et al., 1974). Investigators have argued whether BPH is an epithelial or stromal disease in which the stroma induces the glandular epithelium to undergo hyperplasia. Steroids do not appear to have a direct relationship with BPH (McNeal, 1978). Many studies have been conducted to determine whether the Zn concentrations change when the human prostate becomes diseased (Okada et al., 1983; Leake et al., 1984). Although the total amount of Zn is increased in a hyperplastic prostate and is decreased in carcinoma, this seems to be more a result of the condition than a cause of it (Gyorkey et al., 1967).

In 1963, Dunning discovered a spontaneous adenocarcinoma originating in the dorsal lobe of the prostate of an

aged Copenhagen rat. This transplantable rat tumor model has allowed for detailed biochemical experiments on growth, differentiation, and androgen regulation of prostatic carcinoma (Pollack et al., 1985; Matuo et al., 1984; Hierowski et al., 1984). This rat tumor now serves as the model in the study of prostatic cancer.

This thesis describes the regulation of MT in the rat prostate. It was discovered that each prostatic lobe expressed the MT gene differently with regard to amount of mRNA and metal ion used. Transcription of the MT gene in the dorsal prostate increased in response to both Zn and Cd whereas the lateral lobe MT gene responded only to Zn. The ventral lobe was shown to possess very little MT mRNA in either control or Zn induced conditions. Only Cd caused a visible increase in ventral lobe MT mRNA. The significance of these results will be discussed.

MATERIALS AND METHODS

A. Animal Treatment

1. General.

Male Sprague Dawley rats were used for every experiment. The adult rats weighed approximately 280-350 g which corresponds to an age bracket of 8-10 weeks. At this age and weight the animals are considered to be sexually mature. For age related MT analysis, immature rats were used which were 3-6 weeks of age. A minimum of 3 animals per group were used for every experiment. The rats were maintained on a normal diet and all physical manipulations were minimized to avoid any unnecessary stress.

Adult males were castrated via the scrotal route with the rats under light ether anaesthesia. The animals were then randomized for experiments which were conducted 7 days following surgery. This seven day hiatus ensured atrophy of androgen dependent tissue and removal of existing testosterone levels. During this time the animals were maintained in plastic cages.

2. Innoculations.

The metals and hormones were injected on a mg/kg body weight (bw) concentration. Each animal was weighed before the innoculation and the dose calculated. The animals were then killed at times appropriate for determining the effects of the injected substance on MT mRNA synthesis. Control animals and those receiving metals were injected

intraperitoneally (ip) while hormone treated rats were injected subcutaneously. The doses used for Cd and Zn are standard concentrations for induction of MT (Ohi et al., 1981; Durnam and Palmiter, 1981).

The injection solutions were:

$CdSO_4$ (2.5 mg/kg), dissolved in 0.9% saline

$ZnSO_4$ (5.0 mg/kg), dissolved in 0.9% saline

Dihydrotestosterone, DHT, (1.2 mg/kg), dissolved in 10% ethanol and peanut oil

Estrogen (1.2 mg/kg), dissolved in 10% ethanol and peanut oil

controls, sterile 0.9% saline

3. Tissue extraction

The rat prostate is composed of three lobes and each is anatomically quite distinct. The identification of each lobe during dissection is important as the lobes also differ with respect to their cellular products. The surgery was done on decapitated rats and begun by cutting the abdominal wall. The prostate was easily located at the base of the bladder. The entire prostate including the seminal vesicles was first removed by cutting the urethra and by gently pulling the organ away from associated adipose tissue and blood vessels. Once the prostate was removed, the bladder was dissected off and all additional connective tissue and fat was carefully cut away. The seminal vesicles were removed. The 3 lobes of the prostate were individually removed. The

ventral lobe was the easiest and largest to distinguish and was carefully removed away from the dorsolateral prostate. The doses of DHT and estrogen have been reported to effect zinc uptake (Gunn and Gould, 1958). Previous work in our laboratory has demonstrated no effect of 10% ethanol and peanut oil on MT expression. Thus, this was eliminated as a control group. The lateral lobes could be identified as two small pink overlapping tongues of tissue that cover the apex of the dorsal lobe. These 2 lobes were carefully excised leaving only dorsal tissue which can then be cut to remove the urethra. All experimental tissues were placed on dry ice as quickly as possible to prevent endogenous nuclease activity and stored at -70°C. For some experiments, livers and kidneys were also dissected.

These methods of prostatic dissection were more difficult to perform on castrated rats because the tissue had undergone atrophy. Seven days after castration, the most drastic reduction in size occured in the ventral lobe which typically lost 5-fold of its weight; the dorsolateral lobes decreased 3 to 4 fold in size; and the seminal vesicles also were drastically reduced. The dorsolateral lobes decreased 3 to 4 fold in size. No apparent changes were noticed in liver or kidney weight.

B. Extraction of Total Ribonucleic Acids

1. General

Extreme care was used during these procedures to

prevent RNase contamination. All glassware was rinsed in 0.1 N NaOH followed by double distilled water just prior to use. Rubber surgical gloves were worn whenever RNA was handled. All buffers and reagents were filtered through a Millipore filter (45 uM pore size). All plastic ware (Eppendorf, Beckman and Falcon tubes) were assumed to be essentially RNase free as delivered by the manufacturer. All of the procedures were done at room temperature but isolated RNA was kept on ice at all times. The isolation techniques employed were described by Chirgwin et al, 1979.

a) Solutions for extraction (reagents are given in final concentrations):

I. Lysis Buffer (GITC), sterile filtered

Guanidinium isothiocyanate (Sigma)	4 M
N-lauryl sarcosine (sodium salt)	0.5%
Sodium citrate	25 mM
Beta mercaptoethanol (Sigma)	0.7%
100% Antifoam A (added after filtration)	0.4%

II. Centrifugation cushion, sterile filtered

Cesium chloride, CsCl, (Gibco)	5.7 M
Ethylenediaminetetraacetic acid, disodium salt (EDTA)	0.1 M

III. Paraffin or mineral oil (drugstore grade)

IV. Chloroform/isoamylalcohol (24:1)

V. Phenol/chloroform (1:1) This mixture was prepared fresh each time using redistilled phenol.

b) Procedure for RNA extraction:

Frozen tissue was crushed with a hammer to obtain a homogenous mix of organs. Tissue, 100-1000 mg, was placed in a small (50 ml) Falcon polypropelene tube and homogenized with a Polytron in 10-20 ml GITC/g of tissue. The homogenization was performed at high speed for approximately 1 minute to allow for complete disruption of cells and release of nucleic acids. Insoluble debris was removed by centrifuging the homogenate at 10,000 rpm in a swinging bucket rotor (Beckman JS-13) for 30 minutes at room temperature (RT). The supernatant was pipetted away from the pellet and carefully layered on top of 3 ml of 5.7 M CsCl in a 13.5 ml ultracentrifugation tube (Beckman Quickseal). Any extra space in the tube was filled with mineral oil. The tubes were carefully weighed and adjusted to within 0.01 g of each other. The tubes were sealed and placed in a Beckman Ti50 or Ti75 rotor and centrifuged at 36,000 rpm at 20 ^{o}C for least 16 hours. This CsCl centrifugation allowed pelleting of relatively pure RNA free from contaminating DNA or proteins.

Following the spin, the Quickseal tubes were cut and the oil was carefully removed with a pipette. The GITC was removed (leaving about 1 ml of CsCl), the tube was cut off within 1 cm of the bottom and the rest of the CsCl was poured off leaving the pellet on the wall. The walls of the centrifuge tube were wiped clean around the pellet and

then inverted to air dry for approximately 15 minutes allowing most of the moisture to evaporate. The dried RNA pellet was then dissolved in 100-1000 ul of sterile water and transfered to an Eppendorf 1.5 ml tube. Prostate and kidney samples were ethanol precipitated by adding 5.0 M NaCl (final concentration 0.1 M) and 2.5x volume absolute ethanol. For maximal RNA precipitation these solutions were placed at -20°C for at least 12 hours. Liver samples were usually cleaned by extraction with hydrophobic solutions. A liver RNA sample dissolved in 1 ml of water would be extracted once with an equal volume of phenol/chloroform (solution IV). This would be briefly mixed, centrifuged to separate the 2 phases and the upper aqueous phase containing the RNA was then transferred to a fresh tube. The lower organic phase was "reextracted" with an equal volume of water. The two aqueous phases were combined and then extracted with an equal volume of chloroform. The aqueous phase was again transferred to a new tube and ethanol precipitated as described above. The ethanol precipitated samples were centrifuged in an Eppendorf centrifuge for 30 minutes at 12,000 rpm, 4°C. The RNA pellet was dissolved in sterile water (400 ul) and reprecipitated as before. The recentrifuged pellet was dissolved in 100-600 ul of sterile water and stored at -70°C. To determine the concentration of the RNA solutions, a dilution was read spectrophotometrically at OD_{260}. To determine the purity of the sample the OD_{280} was measured and the ratio 260/280 was

found to be uniformly around 2.0, indicating that proteins were not contaminating the RNA (Marmur, 1961).

C. Gel electrophoresis and transfer of RNA (Northern Blot)

1. General

Several methods of determining RNA molecular weights by gel electrophoresis have been developed. Gel electrophoresis is an excellent procedure that provides a relatively simple method for characterizing RNA with high resolution. The RNA is transferred to nitrocellulose or diazobenzyloxzmethane (DBM) paper resulting in a Northern Blot. These Northerns are usually hybridized to radioactive cDNAs and exposed to X-ray film. Formaldehyde, 2.2 M, was selected as the denaturant because it is easily available; needs no further purification; is less toxic than methyl mercury; and recovery of biologically active RNA was not required. The methods employed herein were essentially as described by Lehrach et al., 1980, and Maniatis et al., 1982.

a) Reagents for electrophoresis (All reagents are analytic grade unless otherwise specified and are listed in final concentrations):

I. Formamide deionized with Biorad AG 501-X8 resin to pH 7.0, stored at -20 °C in small aliquots each of which was used only once.

II. Running Buffer: (10X stock, sterile filtered)

	morpholinopropanesulfonic acid, sodium salt (MOPS)		0.4 M
	Sodium acetate		100 mM
	EDTA		10 mM
III.	Agarose Ultra pure DNA grade (Biorad)		
IV.	Formaldehyde	(12.3 M stock)	37%
V.	Loading buffer:	glycerol	50%
		EDTA	1mM
		bromophenol blue (Sigma)	0.4%
		xylene cyanol (Sigma)	0.4%

b) Procedure for electrophoresis:

The gel was prepared by melting 2.25 g agarose (1.5%) in 108 ml distilled water. This was cooled to 60°C, 15 ml 10x Running buffer and 27 ml formaldehyde were added to give final concentrations of 1x and 2.2M, respectively. This was stirred and immediately poured onto the prepared electrophoresis plate. The RNA sample was prepared by mixing the following:

RNA (8-20 ug) in	5.5 ul water
10X running buffer	1.0 ul
formaldehyde	3.5 ul
formamide	10.0 ul

The sample was incubated at 55°C for 15 minutes after which 2 ul of loading buffer was added and the sample carefully loaded into a gel well. The gel was run at 30 V at room temperature for 12-16 hours.

c) Procedure for marker lanes:

Following the electrophoresis the marker lane was stained in 5 ug/ml ethidium bromide for 1 hour. This lane contained 8 ug of RNA from one of the samples used in the experiment. This marker lane(s) was visualized with UV light and photographed (Polaroid film type 57). The two major ribosomal RNA bands could be seen indicating that no digestion of the RNA had occurred. The 28s and 18s band also gave two points which acted as molecular weight markers of 5.1 kb and 1.9 kb, respectively.

2. Transfer of RNA to Gene Screen Plus (NEN)

a) Reagents used for transfer of RNA (reagents are given in final concentrations):

I. Salt-phosphate buffer (SSPE, 20X stock, pH 7.7)

NaCl	3.6 M
NaH_2PO_4	0.2 M
EDTA	20 mM

II. Salt-citrate buffer (SSC, 20X stock, pH 7.0)

NaCl	3.0 M
$Na_3C_6H_5O_7$	0.3 M

b) Procedure for gel transfer (Northern Blot):

The gel was washed in several changes of distilled water for about 0.5 hours and then placed on a sheet of Whatman 3M filter paper which was saturated with 20x SSPE or 10x SSC. The ends of this sheet of Whatman paper hung in a dish and

acted as a wick during the entire transfer procedure. A piece of Gene Screen Plus membrane was cut to the exact gel dimensions, briefly submerged in 6x SSPE and then laid on the gel taking care to avoid any air bubbles. Several pieces of similarly cut Whatman were placed on top of the membrane, followed by approximately 15 cm of paper towels weighted down by a 500 g weight. The transfer was allowed to proceed for at least 12 hours. During this time, the 20x SSPE passed through the gel, past the Gene Screen Plus, into the paper towels by capillary action and the RNA eluted from the gel onto the membrane where it was retained. Following transfer the membrane was submerged in 6x SSPE for 2 minutes with gentle shaking and then air dried. The RNA was covalently bound onto the membrane by baking it at 80°C for 2 hours after which the membrane is then stored at 4°C between Whatman paper until needed.

3. Transfer of RNA to diazobenzyloxymethane (DBM) paper

a) General:

This technique, as described by Alwine et al., (1977) allows for covalent linkage of RNA guanosine groups to the amino groups of diaminobenzyloxymethane which has been immobilized on cellulose membranes. It gives slightly cleaner Northerns i.e. less background, and they can be rehybridized more often.

b) Procedure for synthesis of DBM paper:

i) procedure and synthesis of nitrobenzyloxymethyl (NBM) paper:

Dissolve 1.5 g of N-(3-nitrobenzyloxymethyl) pyridinium chloride (BDH Biochemistry) in 18.5 ml of 2.5% sodium acetate trihydrate. Three sheets of Whatman 540 paper (20x20 cm) were separately dipped in this solution to saturation and allowed to drip dry. The sheets were baked at 68 °C for 15 minutes and then at 130°C for 40 minutes. After washing the paper in several changes of distilled water for up to 30 minutes they were washed in acetone for a total of 20 minutes. A final set of water washes was done before air drying the NBM paper.

ii) Reduction of NBM to aminobenzyloxymethyl paper (ABM):

The sheets were treated for 45 minutes at 60°C in 160 ml of a 20% sodium dithionite solution. The paper was then extensively washed with water followed by several changes of 30% acetic acid and finally with several more changes of water. The sheets were hung to dry and stored at 4° C until needed.

iii) Activation of ABM-cellulose to diazobenzyloxymethyl paper (DBM) and transfer of RNA:

Once the gel has been run, about 1.5 L of 200 mM sodium acetate pH 4.0 was prepared and cooled to 4° C. The gel was rinsed several times with distilled water and set up on a Whatman "wick" as previously described with the ends

hanging in the sodium acetate solution. Sodium nitrite, 32 mg, was dissolved in 100 ml water and 12.5 ml concentrated HCl was added and raised to 120 ml. The solution was cooled to 4°C. The appropriately cut ABM-cellulose was rinsed in the latter solution for 30 minutes at 4°C after which the membrane was washed with the acetate buffer. The paper began to activate just as it started to turn yellow-orange. It was best to slosh the membrane vigorously in the acetate solution for about 30-60 seconds at which point the cellulose was placed on the gel and paper towels and weights were added as described for Gene Screen Plus. The transfer was continued at 4°C for at least 12 hours by which time the DBM-cellulose had turned orange. After transfer the DBM paper was stored in 1% glycine, sealed in a plastic bag and kept at 4°C. The glycine reacted with unbound active groups significantly reducing background during hybridization.

D. Plasmid preparation

1. General

The MT plasmid used in these experiments was supplied courtesy of Dr. Harvey Herschman (University of California, Los Angeles). The plasmid, pA210 contains the cDNA made to the rat metallothionein-1 gene inserted into pBR322 at the Pst 1 site (Andersen *et al.*, 1983). In order to obtain enough plasmid for the experiments the plasmid was introduced into *E. coli* HB 101 using the calcium shock technique (Mandel and Higa, 1970). The plasmid was

amplified and isolated to obtain pure DNA.

2. Transformation

a) Reagents for Transformation Final Concentration:

I. L Broth (LB):

NaCl	10 gm/L
Bactotryptone	10 gm/L
Yeast Extract	5 gm/L
pH to 7.5 with NaOH and autoclave	
$CaCl_2$	50mM
Tris.HCl (pH 8.0)	10 mM

Tris.HCl/EDTA (TE):

Tris.HCl 10 mM with 1mM EDTA

b) Procedure for transformation:

An overnight HB 101 bacterial culture, 1 ml, was added to 100 ml of LB in a 500 ml flask. The bacterial cells were grown at 37°C with vigorous shaking yielding an OD_{550} of approximately 0.53. A 3 ml aliquot was chilled on ice for 10 minutes in a 15 ml Corex (Corning) tube and then centrifuged in a Beckman JS 13 at 4000xg for 5 minutes at 4 C. The supernatant was discarded and the cells were resuspended in 1.5 ml of ice cold transformation buffer. This suspension was kept in an ice bath for 15 minutes and recentrifuged as above. Transformation buffer, 200 ul, was added to the cells, gently swirled and stored at 4°C for 20 hours. During this period the efficiency of transformation

increases fourfold to sixfold (Dagert and Ehrlich 1979). The plasmid DNA, 30 ng, in TE (pH 8.0) was stirred into the cell solution, and stored on ice for 30 minutes. The bacteria in this solution were then heat shocked at 42 °C for 2 minutes. This technique provides an osmotic shock to the bacteria which is believed to cause the bacteria to swell creating holes in the membrane, permitting the plasmid to enter the cells. A 1 ml aliquot of LB was added and the cells were incubated at 37 °C without shaking for 30 minutes allowing the bacteria to recover and begin to manufacture the appropriated enzyme. This mixture was spread evenly (about 300 ul/plate) onto agar plates containing tetracycline (50 ug/ml, see below) and incubated at 37°C for 12-24 hours. Bacteria that contained the plasmid will be tetracycline resistant due to the tet^R gene thus permitting for simple screening of transformants. The plates with bacterial colonies were stored at 4°C for up to 3 weeks. A colony of transformed bacteria was incubated overnight in LB. An aliquot, 0.85 ml, was gently mixed with 0.15 ml of sterile glycerol and frozen at -70 °C. These bacteria can be stored for many years.

3. Amplification and isolation of plasmid.

a) Reagents for amplification and isolation of plasmid (reagents are given in final concentrations):

I. L Broth (as previously described)

II. Lysozyme (Boehringer) 20 mg/ml in 10 mM Tris.HCl (pH 8.0) freshly prepared

III. Tetracycline (Boehringer) 12.5 mg/ml stock solution in 50% (v/v) ethanol/water

IV. Chloramphenicol (Boehringer) 34.0 mg/ml stock solution in 100% ethanol

V. Bacterial wash buffer (STE):

NaCl	0.1 M
Tris.HCl (pH 7.8)	10 mM
EDTA	0.1 mM

VI. Bacterial lysis buffer (STET):

NaCl	0.1 M
Tris.HCl (pH 7.8)	10 mM
EDTA	0.1 mM
Triton X-100	0.5%

b) Procedure for amplification in rich medium:

The procedure is as described by Maniatis et al., (1982). All LB solutions contained 50 ug/ml tetracycline. A single transformed bacterial colony was transfered to 10 ml of LB medium and incubated at 37°C overnight with vigorous shaking. The L broth, 25 ml, was innoculated with 0.1 ml of the overnight culture (in a 100 ml flask) and incubated at 37°C with shaking until the OD_{600} was 0.6

(about 5 hours). The 25 ml culture was then poured into 500 ml of LB medium in a 2 L flask and shaken for exactly 2.5 hours when 2.5 ml of chloramphenicol was added (final concentration of chloramphenicol was 170 ug/ml) and further incubated for 12-16 hours at 37°C. Chloramphenicol inhibits protein synthesis and genomic DNA replication but allows for plasmid replication. Thus, plasmid numbers were amplified. The cells were then harvested by centrifugation at 6000xg for 10 minutes at 4°C.

c) Procedure for bacterial lysis and plasmid isolation:

The cells from a 500 ml culture were resuspended in 10 ml of STET in a 50 ml flask. Lysozyme, 1 ml freshly made, was kept on ice for a 2 minutes then mixed and the flask was held with constant agitation over an open flame until the liquid just began to boil. The flask was immediately immersed in boiling water for 40 seconds. The viscous contents were transferred to an ultracentrifuge tube (Beckman Ti75) and centrifuged at 25k rpm for 30 minutes at 4°C. Most of the bacterial proteins and bacterial DNA pellet out leaving RNA and plasmid DNA in the supernatant.

The supernatant was then subjected to centrifugation in a CsCl, ethidium bromide gradient. Exactly 1 g CsCl was added to every ml of plasmid solution and gently mixed until dissolved. For every 10 ml of the CsCl solution 0.8 ml of ethidium bromide (10 mg/ml in water) was added giving a final density to the CsCl of 1.55 g/ml and a final

concentration of 600 ug/ml ethidium bromide. This was loaded into a 30 ml Beckman ultracentrifuge tube and centrifuged in a Beckman Ti50 at 45k rpm for about 42 hours at 20°C.

Usually one band of DNA could be visualized under UV light. If two bands were discernible, the lower supercoiled plasmid band was drawn off using a 20 gauge needle and syringe. The DNA band was usually removed in about 2 ml of solution. The ethidium bromide was removed by repeated extraction with an equal volume of isoamyl alcohol, each time separating the phases by centrifuging at 1500xg for 3 minutes. The lower aqueous phase was pipetted into a fresh glass tube until no color remained. This extraction was repeated 4-6 times. The DNA was then dialyzed against several 5 liter changes of TE (pH 8.0) for at least 12 hours. Dialysis tubing with a molecular weight cutoff of 1000 was prepared by boiling for 10 minutes in a large volume of 2% sodium bicarbonate, 1 mM EDTA then rinsing in sterile water.

The DNA was precipitated in the presence of 0.1M NaCl and 2x volume absolute ethanol for 12 hours at -20°C. The DNA was dissolved in sterile water and an OD_{260} was spectrophometrically read to determine purity and concentration. A 0.7% agarose minigel was also prepared and run in Tris acetate buffer (0.04 M Tris acetate, 0.01 M EDTA) to ensure that no bacterial DNA was contaminating the plasmid preparation. The average yield of plasmid DNA was

about 200 ug/L bacteria.

4. Nick Translation.

a) Reagents for nick translation:

This procedure was done using a Nick Translation kit (Amersham) which contains the following solutions:

I. Nucleotide solution:

dATP	(100 mM)
dGTP	(100 mM)
TTP	(100 mM)

in a Tris.HCl (pH 7.8), $MgCl_2$

2-mercaptoethanol buffer

II. DNA polymerase I (100 U/10 ul)

III. DNase I (2 ng/10 ul)

in a Tris.Cl (pH 7.5), $MgCl_2$

glycerin, BSA buffer

IV. $-^{32}P$ dCTP (NEN, specific activity 3000 Ci/mmol)

V. Stopping buffer:

EDTA	30mM
sodium dodecyl sulfate (SDS)	3%
sonicated salmon sperm DNA	600 ug/ml

b) Procedure for nick translation:

The technique incorporates $\alpha^{32}P$ dCTP into double stranded DNA. The enzymes cause a "nicking" in the DNA by DNase I and subsequent repair by DNA polymerase I which

incorporates the "cold" nucleotides from solution I as well as the radioactive dCTP.

A 100-200 ng of plasmid DNA was mixed in 7 ul of sterile water. Then, 4 ul of solution I is added along with 70 uCi (7 ul of ^{32}P dCTP). Finally, 2 ul of solution II was added, mixed and briefly centrifuged to concentrate the liquids at the bottom of the Eppendorf tube. The mixture was maintained at 15°C for 1.5 hours and the reaction was stopped by adding 20 ul of solution V at 68°C for 15 minutes. The labeled DNA was separated from unincorporated nucleotides by G-100 Sephadex chromatography using TE (pH 7.6) as eluant. The fraction containing the labeled DNA was monitored with a Geiger counter and collected into Eppendorf tubes. Plasmid DNA elutes faster than the free nucleotides. Both are quite evident as distinct radioactive peaks in the column. A 5 ul aliquot was counted by adding it to 5 ml of scintillation fluid (Aquasol, NEN) and measuring the cpm with a beta counter. A final specific activity was typically $30x10^6$ Bq/ug DNA ($1.8x10^6$ dpm/ug DNA).

E. Detection of specific mRNA bound to either Gene Screen Plus or DBM-cellulose (Hybridization technique)

1. General

Specific mRNA species can be detected using a ^{32}P labeled plasmid probe containing the cDNA sequence of interest. The prepared membrane was first pre-hybridized in the hybridization buffer to help eliminate non-specific

binding to either Gene Screen Plus or DBM cellulose. Subsequently, the denatured labeled probe was added for hybridization. After hybridization, further washing reduced non-specifically bound probe. The specific mRNA can be visualized by autoradiography.

a) Reagents for hybrization (reagents are given in final concentrations):

I. Denhardts' 50x stock solution

ficoll (Gibco)	1%
polyvinylpyrrolidone (Sigma)	1%
Bovine serum albumin (BSA) (Gibco)	1%

II. Hybridization buffer:

SSPE (as previously described)	5x
Denhardts'	5x
SDS	0.5%
formamide (v/v)	50%

III. Salmon sperm DNA (Boehringer) 150 ug/ml

b) Procedure for hybridization:

The membrane was placed in a Seal-a-Meal bag with 10-30 ml of hybridization buffer. Salmon sperm DNA, 150 ug/ml was added before sealing the bag. This was incubated at 42°C in a shaking water bath for at least 2 hours as a pre-hybridization step. During this time the cDNA was nick translated as previously described. Once the radioactive probe was ready, the pre-hybridization buffer was

removed and replaced with fresh (including new salmon sperm DNA). The labeled plasmid was denatured by heating at 100°C and then immediately added to the bag to give a final concentration of 1-2x10^6 cpm/ml. The sealed bag was placed in a shaking water bath at 42°C for at least 12 hours.

The now radioactive membrane was washed to remove non-specifically bound radioactivity in 2-3 changes of 2xSSC; 0.1% SDS at 68°C. The filter was immediately placed in a new bag and sealed to keep it moist so that it could be rehybridized another time if desired. The bag was then placed in an X-ray film cassette along with a Dupont Cronex intensifying screen and Kodak X-AR X-ray film at -70°C for at least 12 hours.

F. Nuclear isolation and runoff

1. General

This procedure allows for direct measurement of transcription. Nuclei are isolated from tissue of appropriately treated animals in such a way that the transcription mechanisms are still viable. These nuclei are incubated in the presence of a suitable buffer containing all nucleotides and ^{32}P-UTP. The nascent mRNAs are then completely transcribed allowing for quantitative analysis of the number of mRNAs that were being synthesized at the time of death. These labeled mRNAs can then be hybridized to membrane immobilized cDNAs followed by autoradiography.

2. Reagents and procedure for nuclear runoff analysis

a) Reagents for nuclear isolation and transcription (all reagents are given in final concentrations):

I. Sodium butyrate — Stock solution is made by adding 5.44 ml of 10.0 N NaOH to 5 ml of 10.88 M butyric acid

II. Nuclei buffer A, pH 7.0 (NA):

Pipes (Sigma)	10 mM
hexylene glycol (methylpentanediol)	130 mM
sodium butyrate	30 mM
thiodiglycol (v/v)	1%
$MgCl_2$	2 mM

III. Nuclei buffer B, pH 7.5 (NB):

Pipes (Sigma)	10 mM
hexylene glycol	130 mM
sodium butyrate	30 mM
$MgCl_2$	2 mM

IV. Nuclei buffer C, pH 8.0 (NC):

glycerol (v/v)	40%
Tris.HCl (pH 8.0)	50 mM
$MgCl_2$	5 mM
EDTA	0.1 mM

V. Iodoacetamide (IAM) — a 0.5 M stock solution in buffer NB was made freshly before each use.

VI. Phenylmethyl-
sulfonylfluoride (PMSF) 1.0 M stock solution was made freshly in 50% ethanol

b) Procedure for isolation of nuclei:

Up to 1g of tissue was cut up with cold scissors and placed in 40 ml of ice cold NB buffer supplemented with stock IAM and PMSF (added at a final concentration of 1 mM). This was homogenized in a chilled blender for 60 seconds and the the homogenate was filtered through several layers of cheesecloth into a cold flask. Next, the cheese cloth was rinsed with 30 ml of NB buffer containing 1 mM IAM and 1 mM PSMF. The filtrate was divided into 2 centrifuge tubes and spun in a Beckman JS 13 or JA 20 rotor for 10 minutes at 4000 rpm, 4°C. The supernatant was discarded and the tube was inverted to allow the pellet to dry slightly. Each pellet was resuspended in 15 ml of NB buffer (plus 1 mM IAM and 1 mM PMSF). A detergent, NP-40, was added to the kidney nuclei at a final concentration of 0.2%. This detergent helps to destroy cellular membranes and therefore optimized the isolation of kidney nuclei. The tube contents were poured into a cold glass pestle homogenizer and the homogenate was slowly mixed by using 3-10 gentle strokes. The solution was poured back into the centrifuge tubes, spun, and resuspended as described 3 more times. One last homogenization wash was done for kidney nuclei in the absence of NP-40. The pellet was now resuspended

in 400 ul of NC buffer/g tissue. A small aliquot was taken to determine the concentration and purity of nuclei. A 1:200 dilution of nuclei in NC buffer was made and counted using a hemocytometer. The nuclei were stored at -70 °C until needed.

3. Nuclear transcription

a) Reagents for nuclear transcription reaction:

I. Translation buffer (TB) 10X stock solution:

Tris.HCL (pH 8.0)	100 mM
$MgCl_2$	5 mM
KCL	3 M

II. Stopping buffer:

Sodium dodecyl sulfate (SDS)	1%
EDTA	10 mM

b) Procedure for transcription reaction:

Equal number of isolated nuclei (6.6×10^6 in a total reaction volume of 200 ul) was mixed with 20 ul of 10x TB, 0.5 mM each ATP, GTP, CTP and 100 uCi (γ-^{32}P)-UTP (760Ci/mmole). The nuclei were added last and immediately incubated at 25°C for 25 minutes. The reaction mixture was stopped by adding 2 ml of Stopping Buffer and 200 ul of 2 M sodium acetate (pH 5.0) for a final salt concentration of 0.2 M. An equal volume of water saturated phenol:$CHCl_3$(2:1) was added and shaken. This mixture was incubated at 55 °C for 5 minutes then cooled on ice for 5 minutes. In order to separate the water soluble and water insoluble phases, the

solution was centrifuged at 10,000xg for 10 minutes at 4 C. The upper aqueous layer was removed and pipetted into a clean corex test tube (Corning). The nucleic acids were then precipitated a -20 °C for at least 12 hours by the addition of 2.5 volumes of ethanol. The precipitate was pelleted at 10,000xg at 4°C for 10 minutes and the pellet was dried under vacuum and dissolved in 0.5 ml of buffer (0.3 M NaCl, 0.1% SDS, 1 mM EDTA, 10 mM Tris.HCl pH 7.5).

The labeled RNA was separated from free unincorporated nucleotides by chromatography on a G-50 Sephadex column. The radioactivity was monitored with a Geiger counter and the appropriate fractions were collected. The radioactivity of the RNA was determined by scintillation counting and the nucleic acid was precipitated with the addition of sodium acetate and ethanol as before. After centrifuging the RNA, it was resuspended in sterile water to give approximately $5x10^6$ dpm/75 ul. This sample was immediately frozen and stored at -20°C until analysis could be started on the same day.

It should be noted that the radioactivity in these reactions is high and care was taken at all steps to ensure that exposure to harmful radiation was kept to a minimum.

4. Immobilization of plasmid DNA onto nitrocellulose.

The plasmid DNA, 5 ug, was dissolved in 90 ul sterile water to which was added 10 ul of 1 N NaOH as a denaturant. The DNA was boiled for 5 minutes after which 11 ul of 10 N

HCl was added and mixed well. 489 ul of 6x SSC was added and the nucleic acids were spotted onto a 6x SSC prewetted nitrocellulose membrane using a BioRad dot blotter. Each sample was rinsed with 6x SSC and the vacuum was left on for about 10 minutes. The hybridots were baked at 80°C for 2 hours to ensure that the DNA was securely fixed onto the membrane.

5. Hybridization of radiolabeled RNA to plasmid DNA.

a) Reagents for hybridization of radiolabeled RNA to plasmid DNA (reagents are given in final concentrations):

I. Hybridization Buffer

deionized formamide	50%
SSC	5x
SDS	0.1%
EDTA	1 mM
Tris.HCl (pH 7.5)	10 mM
Poly (A+) RNA	0.01 ng/ml
Denhardts'	4x
E. coli DNA	10 ng/ml

II. Wash buffer:

SSC	5x
SDS	0.1%
EDTA	1 mM
Tris.HCl (pH 7.5)	10 mM

b) Procedure for hybridization:

The plasmid DNA hybridots were incubated for 30 minutes at 45 C in 0.5 ml of the hybridization buffer. This was done in small sealed Sears seal-a-meal bags. The buffer was replaced with fresh buffer and 9.8×10^6 dpm of ^{32}P labeled RNA from each time point was added to a bag containing a dot blot. Hybridization was conducted for 72 hours with the small bags clamped in a gently shaking 42°C water bath.

The hybridots were subsequently washed to remove nonspecifically labeled RNA by 4 changes of 20 ml/filter of wash buffer, at 42 C for 30 minutes each. Next all remaining single stranded RNA was digested by incubating the filters in 0.3 M NaCl with 10 ug/ml RNase A for 10 minutes at 37°C. The previous solution was then changed to 0.3 M NaCl and the filters were further washed for 20 minutes. The hybridots were air dried, sealed in Saran wrap and placed on Kodak AR X-ray film with intensifying screens at -70°C for 48 hours.

RESULTS

A. Recovery of total ribonucleic acids

Total ribonucleic acid was isolated to measure the products of gene transcription. The methods used were essentially as described by Chirgwin et al., (1979) and as outlined in Materials and Methods. Tissue samples, 0.5-2.0 g depending on organ size, were homogenized. The yield of RNA varied considerably between tissues ranging from 2 mg/g in prostate to 1 mg/g in liver. Some of the differences may be accounted for by the many steps whereby RNA can be lost; a suboptimum volume of GITC; ethanol precipitations; phenol:chloroform extractions (in liver). Thus it was difficult to assess the variation in tissue RNA levels of the diversely treated animals.

B. MT-gene regulation in the rat kidney, liver and prostate

1. MT-gene expression during postnatal development

It first was determined whether the amount of MT mRNA in the prostate changed as a rat matured. As illustrated in Figure 1a the accumulation of MT (450 nucleotides) mRNA increased in dorsolateral prostate from age 3 weeks to age 6 weeks. The level of RNA was about 5.0 times greater at 6 weeks of age than in the first 3 weeks postpartum. The dorsal and lateral lobes were pooled (10 rats per time point) because they were too small to be dissected apart at these

ages. In fact, a fairly linear increase in gene expression was evident with the maximum amount of MT mRNA occuring in the sexually mature rat prostate (Figure 1b). Densometric scanning of Northerns as seen in Figure 1b was performed for all the Northerns. The fold changes indicated is the result of these scans. The increase in message was most likely due to newly synthesized RNA species and this will be further clarified in the discussion. No ventral prostate MT mRNA was detected (Figure 1a) even after a weeklong exposure indicating that the MT gene was differently expressed in each lobe of the prostate.

2. MT-gene expression in intact animals

The basal MT gene expression was examined in various rat tissues. The results are illustrated in Figure 2. The lateral prostate (L), ventral prostate (V), dorsal prostate (D), kidney (K), liver (L), and pancreas (P) were dissected from intact and untreated adult rats (five animals per group). The basal level of MT expression was highest in the lateral prostate. The amount of MT mRNA in dorsal prostate, liver and pancreas was about 4, 6, 8 fold lower than in the lateral prostate, respectively. The relative level of MT mRNA in kidney and ventral prostate were too low to be measured in this Northern.

3. MT-gene expression in liver, kidney and prostate after Zn treatment: 1 day post castration

Since the levels of circulating androgens falls quickly following castration, an experiment was conducted to compare MT mRNA in the liver, kidney and prostate from 1 day castrated rats. In addition, 1 day castrated animals were injected with 5 mg/kg $ZnSO_4$ and killed 5 hours later. One injection of Zn induced the MT mRNA accumulation in all tissues, except ventral prostate and dorsal (Figure 3, lanes f,h,i and j). In 1 day castrated animals, Zn increased the amount of MT mRNA about 8 fold in the lateral lobe (Figure 3, lane f), 5 fold in the kidney (Figure 3, lanes h), and about 7 fold in the liver (Figure 3, lane j). The level of MT mRNA did not change appreciably in the dorsal lobe after Zn administration.

4. MT-gene expression in liver and kidney in castrated rats after Zn treatment: 7 days post castration

In these experiments, the rats were castrated and tissue atrophy of the prostate was allowed to proceded for 7 days. As control tissues for MT expression, liver and kidney samples were examined. At this time, the rats were given 5 mg/kg $ZnSO_4$ and killed 6 or 24 hours later. The expression of MT mRNA in normal animals was seen only in liver (Figure 4, lane a). The level of liver MT mRNA was considerably reduced in castrated animals that had received NaCl (Figure 4, lane b) indicating that castration had an effect on liver MT gene expression. Six hours after a single injection of Zn, a large increase in MT gene product was

detected in both kidney and liver (Figure 4, lanes c). This accumulation of MT mRNA decreased to the levels of castrated controls by 24 hours (Figure 4, lanes d).

5. MT-gene expression in the liver and kidney tissue of rats treated 7 days after castration: 48 hours after Zn, hormone, or Cd

Seven day castrated rats were injected with various substances to test their effect on MT gene expression in the kidney (Kid) and liver (Liv) after 48 hours. A large amount of total RNA (20 ug/lane) and a long film exposure allowed the MT mRNA to be detected in normal kidney (Figure 5, Kid, lane a) and liver (Figure 5, Liv, lane a). The tissues, pooled from 5 animals per group, were analyzed 48 hours after treatment. In kidney, only Cd treatment resulted in a visible induction of MT mRNA accumulation above normal levels (Figure 5, Kid, lane d). Zinc, DHT, estrogen, or DHT + Zn (Figure 5, Kid, lanes b,c,e and f) were not able to return the amount of MT mRNA and maintain it at the normal rat kidney levels after 48 hours. Castration also affects MT gene expression in the liver. In Figure 5, LIV, lane b and c, the accumulation of the MT mRNA is lower than that found in normal animals even though the rats had received injections of Zn or DHT. Zinc does induce the level of MT mRNA but not to the same extent as Cd (Figure 5, LIV, lanes b vs. d). The dose of estrogen administered also caused an increase in MT mRNA (Figure 5, LIV, lane e).

Furthermore, while $ZnSO_4$ alone caused a slight increase in MT mRNA accumulation, and DHT resulted in little increase, the experiment illustrated in Figure 5 (LIV, lane f) demonstrates that the level of MT gene expression increases almost to normal when Zn and DHT are injected simultaneously.

6. MT-gene expression in prostate tissue after Zn treatment: 7 days post castration

Seven days after castration, the effect of Zn was examined on the prostate. Ten animal prostates were pooled per treatment. This was necessary because of the small size of the prostate after castration. A 5 times higher concentration of MT message was found in the lateral lobe relative to the dorsal for control rats (Figure 6a and 6b). No expression of MT was detected in ventral prostate (Figure 6a). The effect of castration was most evident in lateral prostate as the accumulation of MT mRNA was approximately 25% of that produced in normal animals (Figure 6b, treatment B). The dorsal lobe production was depressed to about 50% of normal (Figure 6b, treatment B). Six hours after a 5 mg/kg injection of $ZnSO_4$ the MT gene was induced more than twice over castrated levels in both dorsal and lateral lobes (Figure 6b, treatment C). However, by 24 hours post Zn injection, the two lobes differed in their MT gene expression (Figure 6b, treatment D). In the dorsal lobe the accumulation of MT mRNA continued to increase above the intact rat prostatic amount

whereas in the lateral lobe the MT mRNA level had fallen below the concentration found in tissue from castrated control rats.

7. MT-gene expression in the prostates of intact rats 6 hours after an injection of Cd or Zn

Since it is well known that MT-gene expression is regulated by Cd in most tissues (Durnam and Palmiter 1981), we investigated the Cd effect on MT expression in the prostate. In the dorsal lobe of intact rats a 5.0 mg/kg dose of $ZnSO_4$ induced the MT gene to produce approximately 6 times more than the untreated control (Figure 7, D, lane b). A treatment of 2.5 mg/kg $CdSO_4$ also increased the MT mRNA content in this prostatic lobe but to a slightly lesser degree than Zn (Figure 7, D, lane c). In contrast, the lateral lobe MT gene responded well to Zn (Figure 7, L, lane b). Cadmium did not cause an increase in MT messenger accumulation (Figure 7, L, lane c). For the first time a small amount of MT mRNA was seen in ventral prostate 6 hours after an injection of Cd (Figure 7, V, lane c) indicating that the MT gene is indeed inducible in the ventral prostate but that the mRNA levels are normally too low to detect when using total RNA samples.

8. Transcriptional regulation of the MT gene from 7 day castrated rats treated with Zn

Because Northern analysis measures the accumulation of

mRNA which is a function of gene transcription as well as half-life, nuclear runoff experiments were performed to assess MT gene induction. Figures 8a illustrates the nuclear runoff analysis of kidney tissue from castrated rats given 5 mg/kg $ZnSO_4$ and sacrificed 6 or 24 hours later. The procedure is given in detail in the Materials and Methods section.

The in vitro transcribed nuclear mRNA from kidney tissue was hybridized to 5 ug of nitrocellulose immobilized cDNAs for rMT-I, Plasmid AT-153, Placental lactogen II (rPL, courtesy of Drs. M-L. Duckworth, H.G. Friesen), M-40, and alpha actin. A negative effect of castration on MT gene transcription is noted relative to normal rat kidney expression (Figure 8a, rMT, row c). Six hours after a single injection of Zn, the MT gene had increased its transcription over the castrated level (Figure 8a, rMT, row b) and by 24 hours the amount of transcription had fallen from the maximum at 6 hours (Figure 8a, rMT, row d). The background hybridization was controlled by the pAT-153, rPL, and pM-40. These can be used to normalize the results for MT expression in each row which indicated that Zn had no effect on alpha actin expression.

The effect of Zn on the transcription of the MT gene in lateral and dorsal prostate are illustrated in Figure 8b and 8c, respectively (the same experiment as Figure 8a). The nuclei were isolated from normal, or 7 day castrated animal tissue. The castrated animals were treated with Zn for 6 or

24 hours. Castration results in a decrease in the rate of MT gene transcription in the lateral lobe (Figure 8b, rMT, row b) and in the dorsal lobe (Figure 8c, rMT, row b). This is apparent after subtracting the background of pAT for each row from the MT signal. Six hours after Zn treatment the MT transcription increased in the lateral lobe (Figure 8b, rMT, row c) which then decreased by 24 hours (row d). The MT gene induction in the dorsal lobe, over castrated levels, is also evident six hours after an injection of $ZnSO_4$ (Figure 8c, rMT, row c). By 24 hours this transcription rate was still higher than that occuring in the castrated state (Figure 8c, rMT, row d).

9. MT-gene expression in the prostate tissue of castrated rats given a single 1.2 mg/kg dose of DHT over time

This experiment indicates the effect of DHT on prostatic MT-gene expression in 7 day castrated rats killed 6 or 24 hours after an injection of DHT. In the lateral lobe (L) castration reduced the amount of MT mRNA production relative to the concentrations in the normal rat lateral prostate tissue (Figure 9, L, lane a). In the lateral lobe no change was seen 6 hours after administration of the hormone (Figure 9, L, lane c). However, by 24 hours post injection, a slight increase (approximately 2 fold) in MT mRNA accumulation could be detected (Figure 9, L, lane d). In the dorsal lobe (Figure 9, D) the level of MT mRNA in

castrated rat tissue (Figure 9, D, lane b) was comparable to the amount in normal rat dorsal prostate (Figure 9, D, lane a). The effect of DHT on the MT signal was not seen 6 hours after the DHT dose (Figure 9, D, lane c) but by 24 hours the amount of MT mRNA was slightly above saline treated castrated rat levels (Figure 9, D, lane d). In this experiment no ventral prostatic (V) MT mRNA was detected under any of the conditions.

10. Expression of M-40 mRNA

The M-40 mRNA (900 NTS) is an androgen dependent prostate specific gene. It is expressed more abundantly in rat lateral prostate than in the dorsal prostate (Matusik et al 1986). Its induction by androgens was used as a control to determine that the prostate was responding normally. Seven days after castration, the level of M-40 mRNA decreased relative to the amount found in intact rat lateral lobe (Figure 10, L, lanes b) but had little effect on M-40 expression in the dorsal prostate (Figure 10, D, lanes b). An increase in M-40 mRNA was not evident 6 hours after a single 1.2 mg/kg dose of DHT in either lobe (Figure 10, D and L, lanes c) but a higher accumulation was seen by 24 hours (Figure 10, D and L, lanes d). A very low level of ventral prostatic (Figure 10, V) pM-40 mRNA can be seen in normal rats (Figure 10, V, lane a) but castration eliminated this signal which was not recovered with the dose of DHT used either 6 or 24 hours later (Figure 10, V lanes c

and d).

11. A schematic representation of the results on MT gene regulation.

In order to summarize the data a schematic diagram illustrates the major findings. The basal level of MT mRNA increases in the order: kidney, liver and dorsal prostate, lateral prostate. Castration decreases the level of MT mRNA in all tissues examined except for ventral prostate, with the most drastic reduction in MT message being seen in the lateral prostate. It is evident that the regulation of MT gene expression is different in prostate vs. kidney or liver. In the kidney and liver both Zn and Cd induce the MT gene to produce a substantial amount of MT mRNA, well in excess of the amounts found in normal animals. In the intact rat prostate Cd induced the accumulation of MT mRNA only in the ventral and dorsal prostate. In fact, the level of MT mRNA may have decreased slightly in the lateral lobe following Cd administration. Zinc was able to increase the amount of MT mRNA in all tissues except the ventral prostate.

FIGURES

Figure 1a. MT-gene expression during postnatal development.

Total RNA (10 ug/lane), ten rats per group, from dorsolateral (DL) and ventral (V) prostate was electrophoresed in a denaturing formaldehyde agarose gel. The RNA was then transferred onto Gene Screen Plus for subsequent MT mRNA detection. The membrane was hybridized to a ^{32}P-CTP labeled rMT-I cDNA in a formamide based hybridization buffer, overnight at 4 C. To remove excess probe and nonspecific binding, the filter was then washed in 2XSSC, 0.1% SDS twice for 30 min at 68 C. The salt concentration was then brought down to 0.2XSSC with which the filter was washed for 15 min. The washed Northern was sealed in plastic and placed against X-ray film with a Dupont Cronex intensifying screen and exposed for 24 hours. Lanes (a) represent RNA from 3 week old rats, (b) 4 weeks, (c) 5 weeks and (d) 6 weeks of age.

Figure 1b. Fold induction of MT mRNA in the developing dorsolateral rat prostate.

The autoradiogram from the Northern in Figure 1a was scanned by a densitometer (Biorad) and the changes seen in the accumulation of MT mRNA were diagrammed relative to the level seen at 3 weeks of age (which was given an arbitrary unit of 1).

Figure 1a

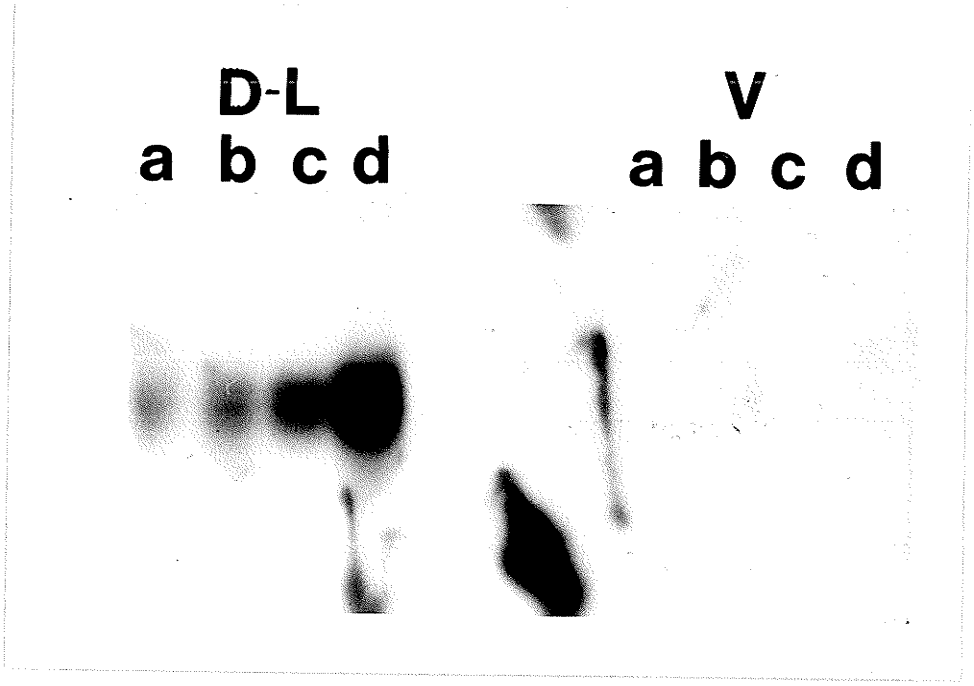


Figure 1b

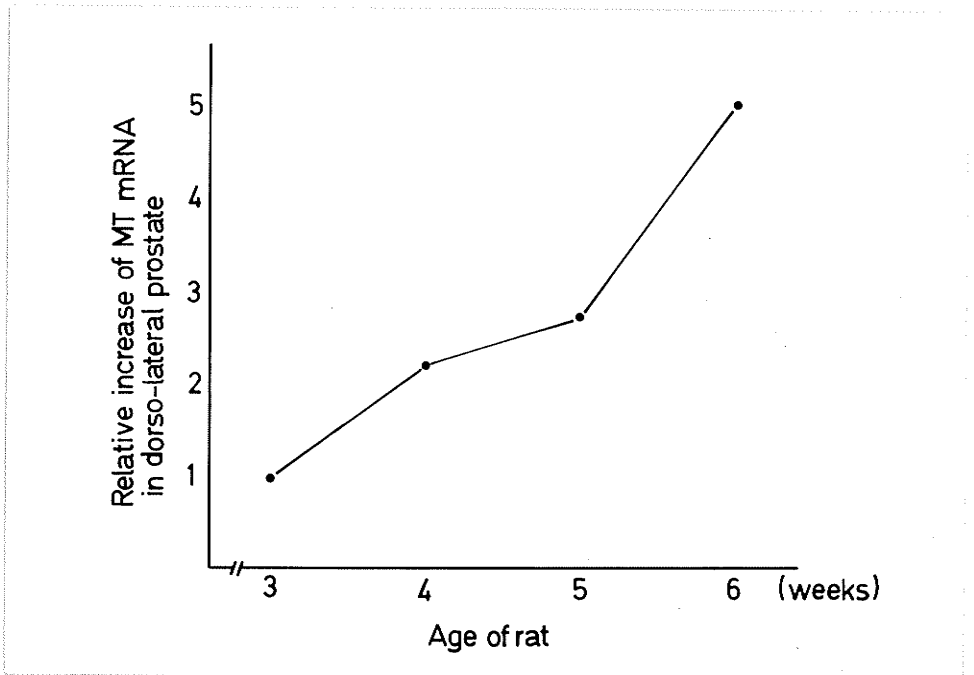


Figure 2. MT-gene expression in various organs of intact, untreated rats.

Total RNA, 20 ug/lane were electrophoresed on 1.5 % agarose gels using formaldehyde as a denaturant. These tissues were taken from adult, intact control rats. The RNA was transferred to nitrocellulose and probed with the rMT-I cDNA. The autoradiograph is a result of a 24 hour exposure to X-ray film at -70 C with an intensifying screen. The RNA in the lanes are designated in the following manner: lane a) normal lateral prostate; lane b) normal ventral prostate; lane c) normal dorsal prostate; lane d) normal kidney; lane e) normal liver; and lane f) normal pancreas.

Figure 2

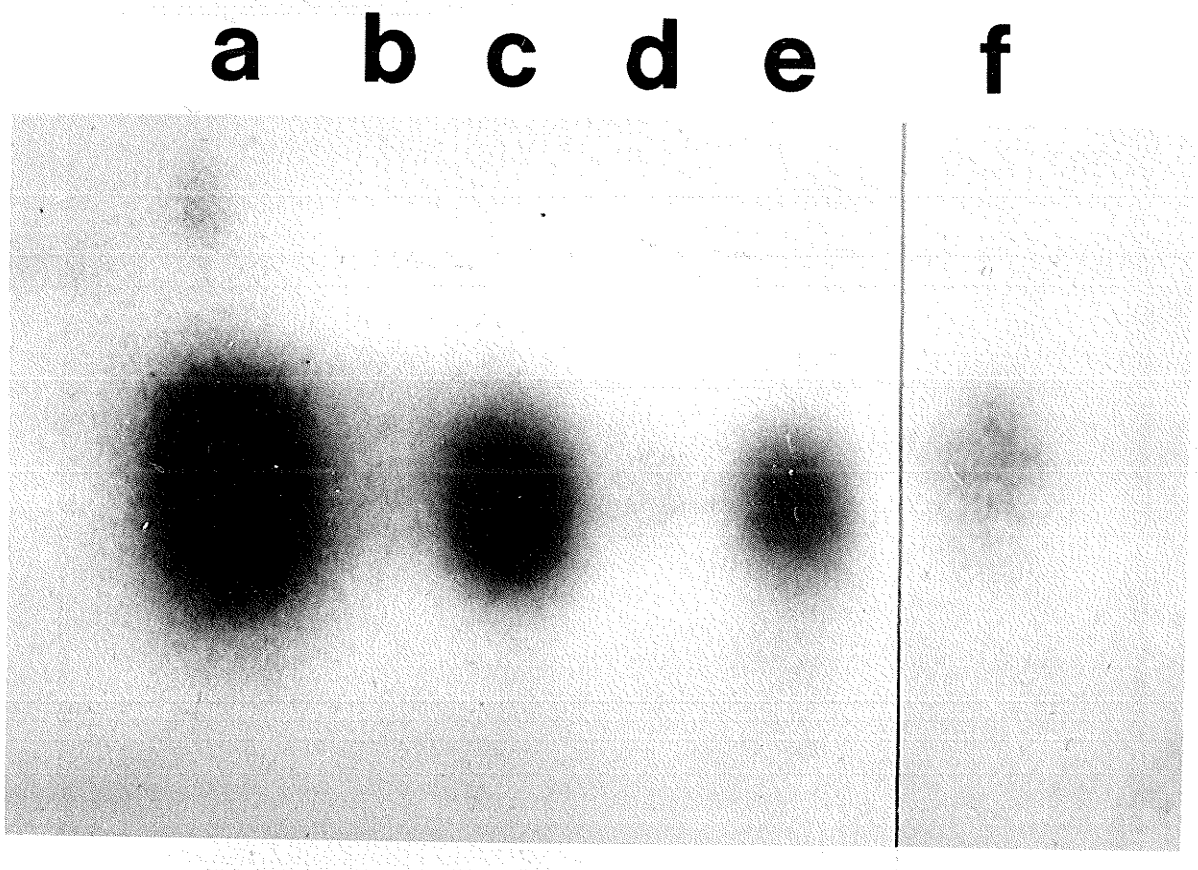


Figure 3. MT-gene expression in liver, kidney and prostate from animals castrated for 24 hours before and after treatment with Zn.

The animals had been castrated 24 hours prior to the experiment. Total RNA, 20 ug/lane, was electrophoresed on 1.5% agarose gels in formaldehyde denaturing conditions. Transfer of nucleic acids to Gene Screen Plus and hybridization was performed as described in the text. Exposure on X-ray film was for 48 hours. Lanes a-e refer to RNA taken from rats injected with saline and killed 5 hours later. Lanes f-j represent RNA from rats injected with 5 mg/kg $ZnSO_4$ and killed 5 hours later. The tissues used are as follows: (a,f) lateral prostate; (b,g) ventral prostate; (c,h) dorsal prostate; (d,i) kidney; and (e,j,) liver.

Figure 3

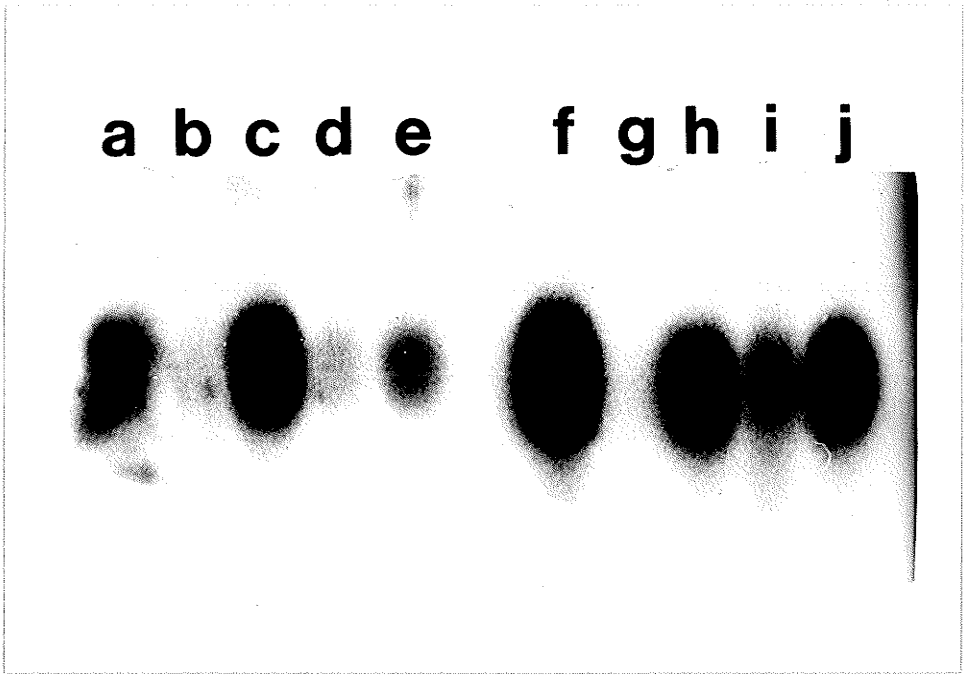


Figure 4. MT gene expression in kidney (kid) and liver (liv) from castrated rats injected with Zn.

Total RNA, 8 ug/lane, was electrophoresed and transferred to Gene Screen Plus as described previously. The rats, 5 animals per group, were injected with 5 mg/kg $ZnSO_4$ and killed either 6 or 24 hours later. The Northern was hybridized to a nick translated MT-I probe. The lanes represent the following samples: a) normal untreated; b) castrated, injected with NaCl and analysed 6 hours later; c) castrated, given Zn and analysed 6 hours later; and d) same as -c- but analysed after 24 hours. Exposure time on X-ray film was for 20 hours.

Figure 4

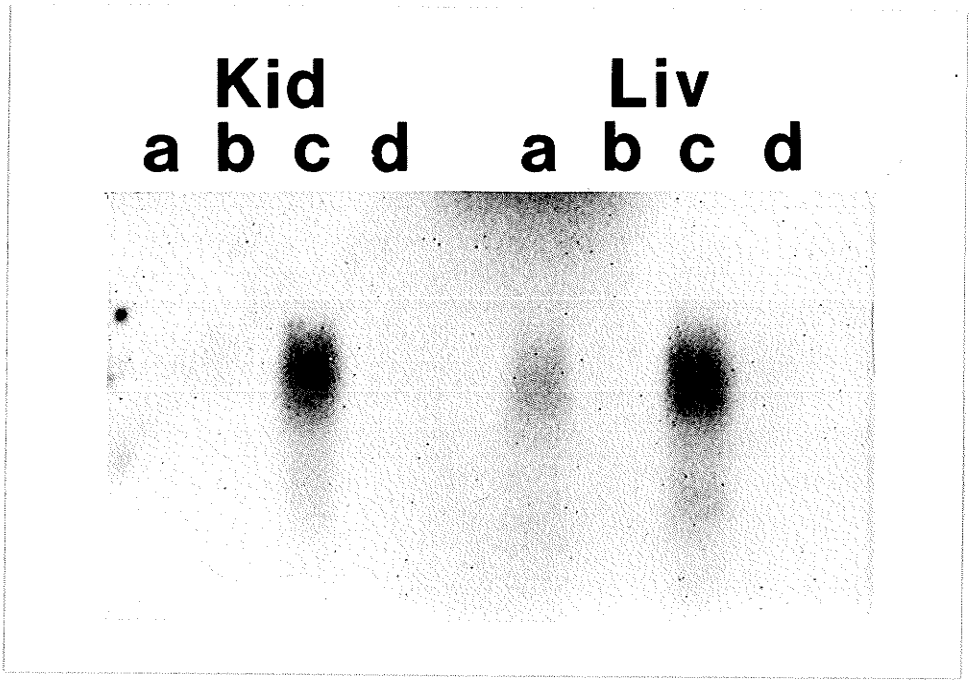


Figure 5. Accumulation of MT mRNA in the kidney (kid) and liver (liv) of castrated rats 48 hours after a single injection of a metal or hormone.

Total RNA, 20 ug/lane, was electrophoresed, transferred onto Gene Screen Plus and hybridized to a rat MT-I cDNA. All RNA was isolated from 5 animals per group, 48 hours after treatment. The lanes represent the following injections: a) normal untreated; b) castrated + 5 mg/kg $ZnSO_4$; c) castrated + 1.2 mg/kg DHT; d) castrated + 2.5 mg/kg $CdSO_4$; e) castrated + 1 mg/kg estrogen; f) castrated + 5 mg/kg $ZnSO_4$ + 1.2 mg/kg DHT. The autoradiogram was a 96 hour exposure.

Figure 5

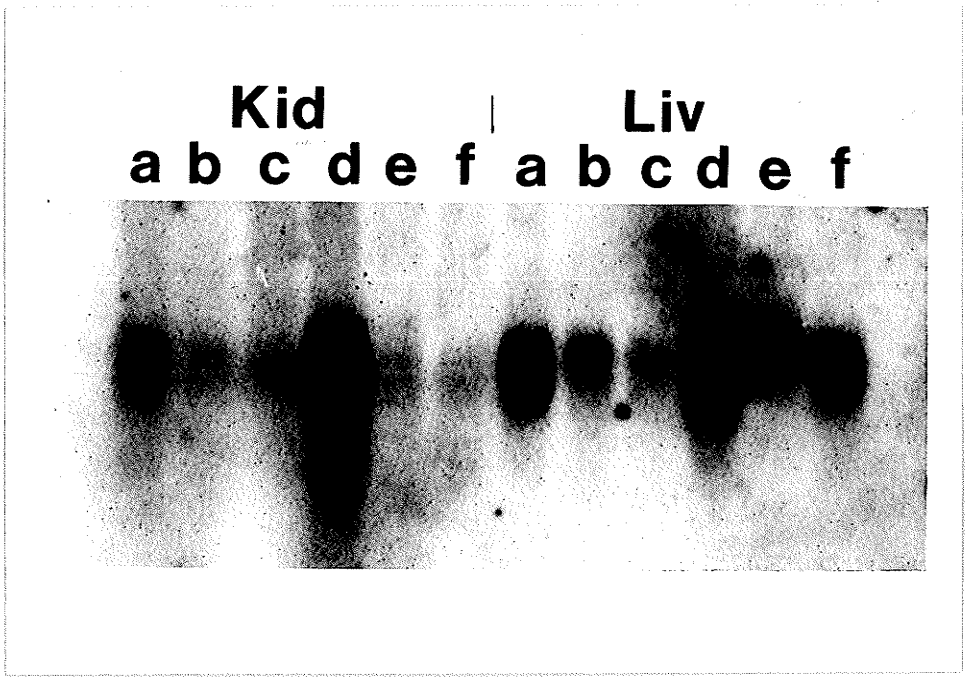


Figure 6a. Accumulation of MT mRNA in the prostate of castrated rats injected with Zn.

This autoradiogram shows MT mRNA from dorsal (D), ventral (V) and lateral (L) prostates. These animals (5 per group) were injected with 5 mg/kg $ZnSO_4$ and RNA was isolated 6 or 24 hours later. All treatments, except intact controls were performed 7 days after castration. Total RNA, 8 ug/lane was separated on formaldehyde agarose gels and transferred to Gene Screen Plus as described earlier. Hybridization to a nick translated rMT-I cDNA was also performed as described. The lanes represent the following: a) normal untreated; b) castrated, injected with NaCl and killed 6 hours later; c) castrated, given Zn and killed 6 hours later; and d) same as -c- but killed after 24 hours. The length of exposure on X-ray film was 20 hours.

Figure 6b. Relative change in MT mRNA in prostates of castrated rats after injection with Zn.

The autoradiogram in Figure 4a was scanned with a densitometer (Biorad). The changes in MT mRNA accumulation are in arbitrary units relative to normal dorsal levels which were designated as 1 (A-hatched bar). A) Normal untreated prostate; B) castrated, saline treated and analysed after 6 hours; C) castrated, Zn treated, analysed after 6 hours; and D) castrated, same as -c- but analysed 24 hours later.

Figure 6a

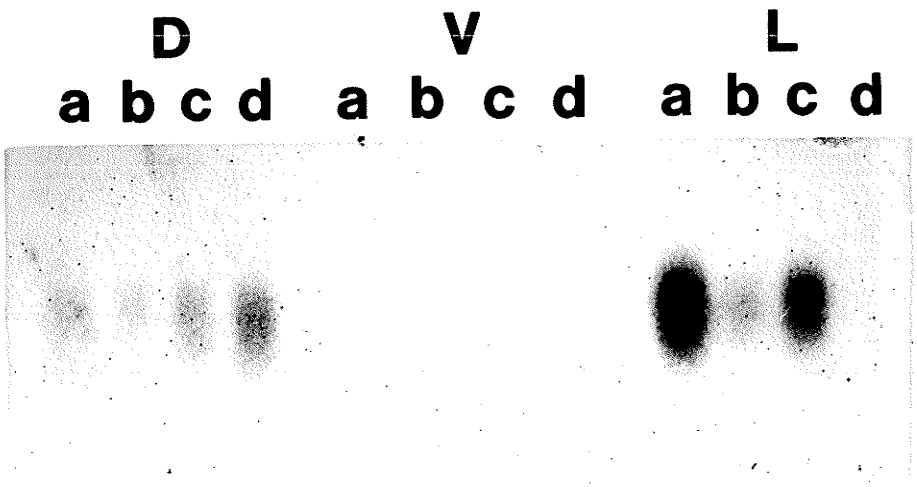


Figure 6b

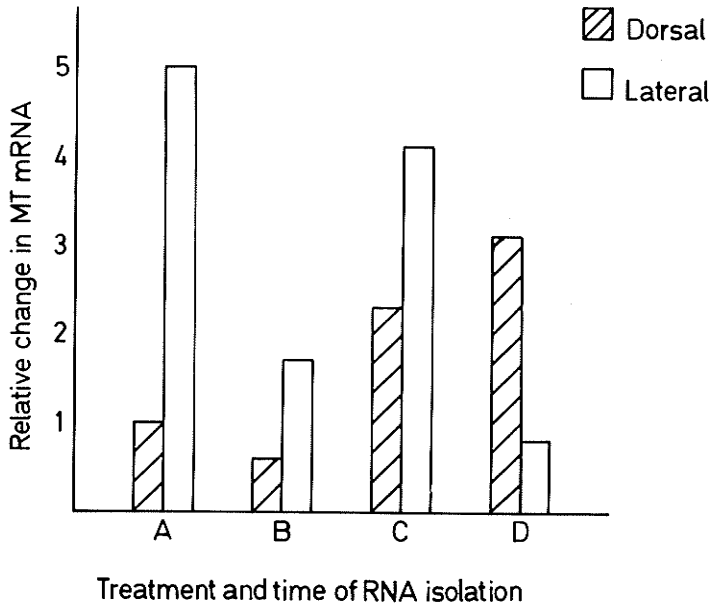


Figure 7. The MT mRNA accumulation in the prostate of normal rats 6 hours after an injection of Zn or Cd.

Northern analysis of total RNA (8 ug/lane, 5 animals per group) from dorsal (D), ventral (V), and lateral (L) prostates. These animals were all normal intact rats which were injected with 5 mg/kg $ZnSO_4$ (lanes b) or 2.5 mg/kg $CdSO_4$ (lanes c) and killed 6 hours later. Lanes (a) represents RNA from normal untreated animals. The probe was a nick translated MT-I cDNA which was hybridized to the blot. This X-ray film was exposed for 18 hours.

Figure 7

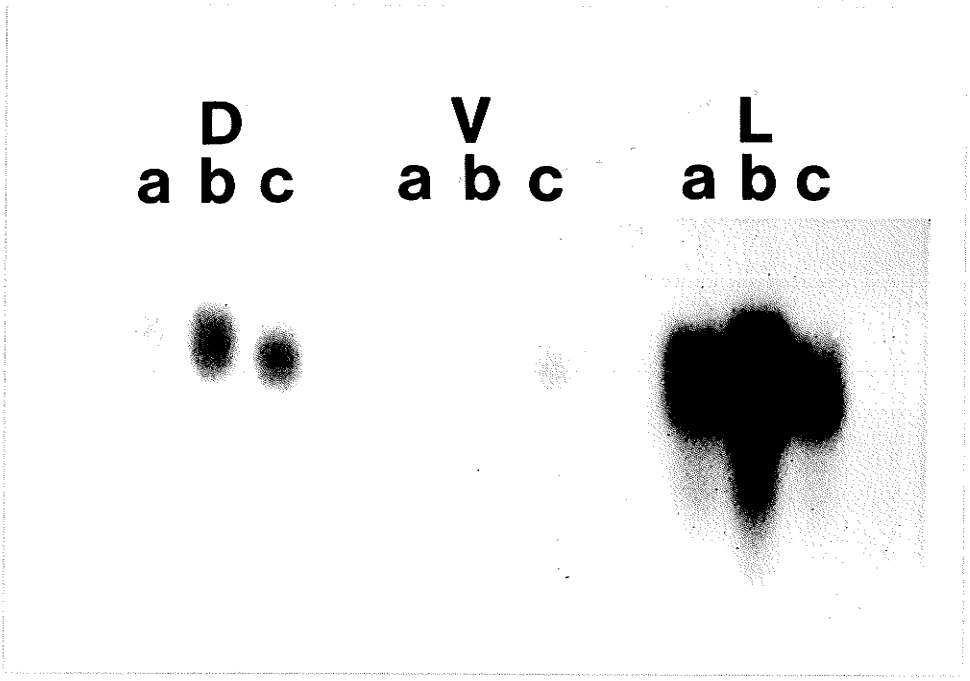


Figure 8. Nuclear Runoff of mRNA from the kidneys, lateral and dorsal prostates of castrated rats injected with $ZnSO_4$

The nuclei were isolated from various rat tissues after treatment. The RNA in these nuclei were subjected to in vitro transcription using ^{32}P-UTP as described in the Materials and Methods section. The radioactive RNA was then hybridized to nitrocellulose immobilized cDNAs (5 ug/dot) for 72 hours. The nitrocellulose strips were then washed and placed on X-ray film using intensifying screens. The autoradiographs were exposed for 48 hours at -70 C.

Figure 8a. Kidney nuclear runoff

The rows represent RNA from animals treated in the following ways: row a) normal kidney; b) kidney from 7 day castrated rats injected with $ZnSO_4$ for 6 hours; c) kidneys from 7 day castrated rats; d) kidneys from 7 day rats treated with $ZnSO_4$ for 24 hours. The columns indicate which cDNAs were fixed to the nitrocellulose: rMT, rat metallothionein; pAT, plasmid AT; rPL, rat placental lactogen; pM-40, plasmid containing the cDNA for M-40; and alpha actin used as a consitutively expressed control. The signal seen for pAT, rPL, and pM-40 represent backgound levels for each row.

Figure 8b. Lateral prostate nuclear runoff

The rows represent RNA from animals treated in the following manner: row a) normal lateral prostate; b) lateral

prostate from rats that were castrated for 7 days; c) lateral prostate from 7 day castrated rats given a $ZnSO_4$ injection for 6 hours; d) same as -c- but killed 24 hours after the $ZnSO_4$ administration. Only the rat metallothionein (rMT) and pAT are illustrated.

Figure 8c. Dorsal prostate nuclear runoff

This figure demonstrates the data from the nuclear runoff performed on nuclei from dorsal prostatic tissue. The lanes and columns represent the same type of treatment as above: row a) normal dorsal prostate; b) 7 day castrated dorsal prostate; c) 7 day castrated plus zinc for 6 hours; and row d) 7 day castrated plus $ZnSO_4$ for 24 hours.

Figure 8a

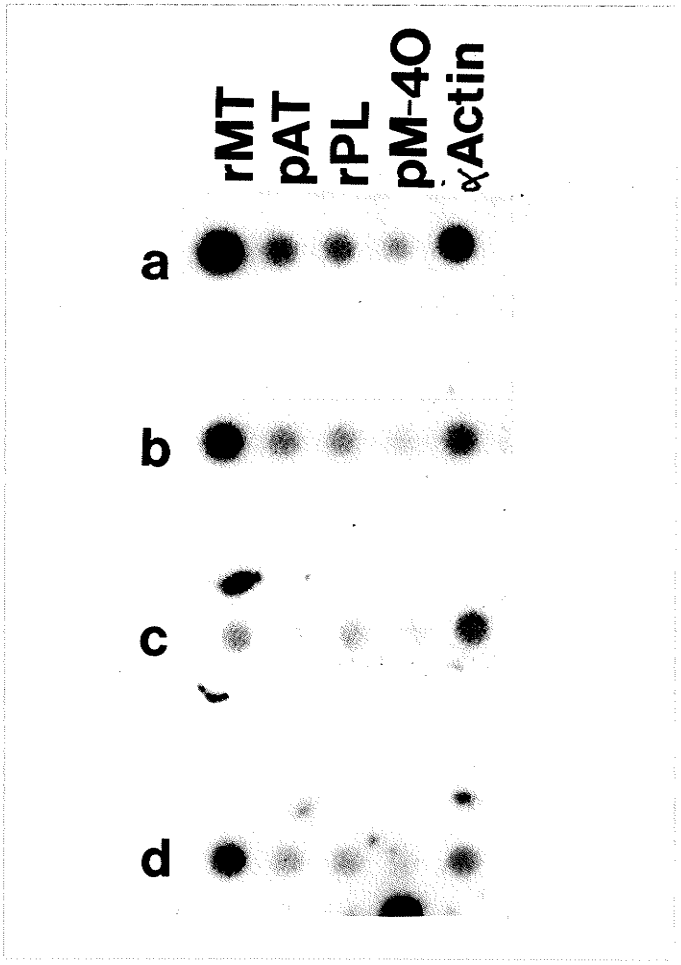


Figure 8b

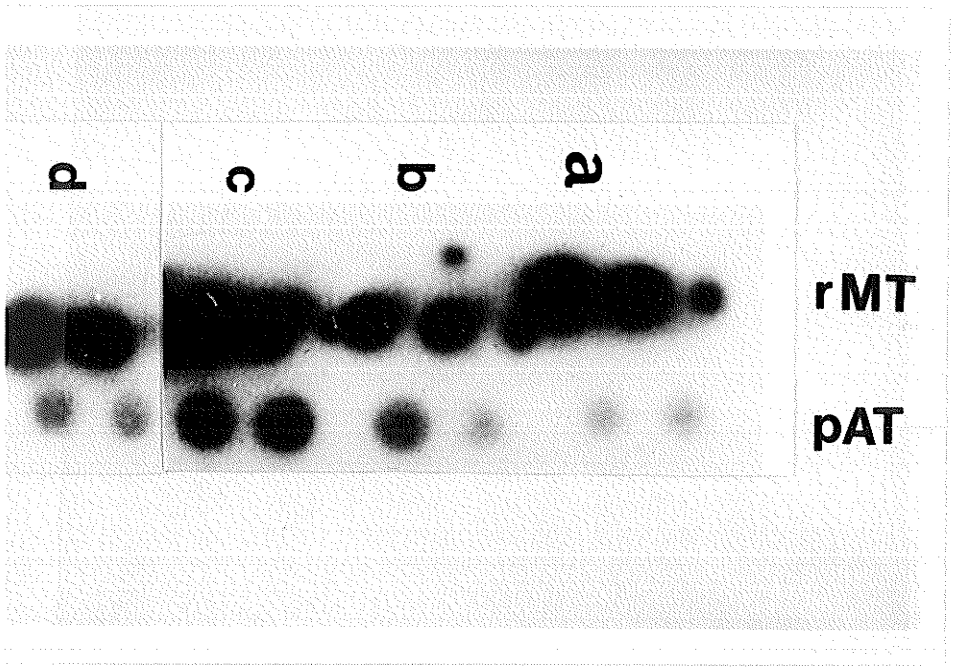


Figure 8c

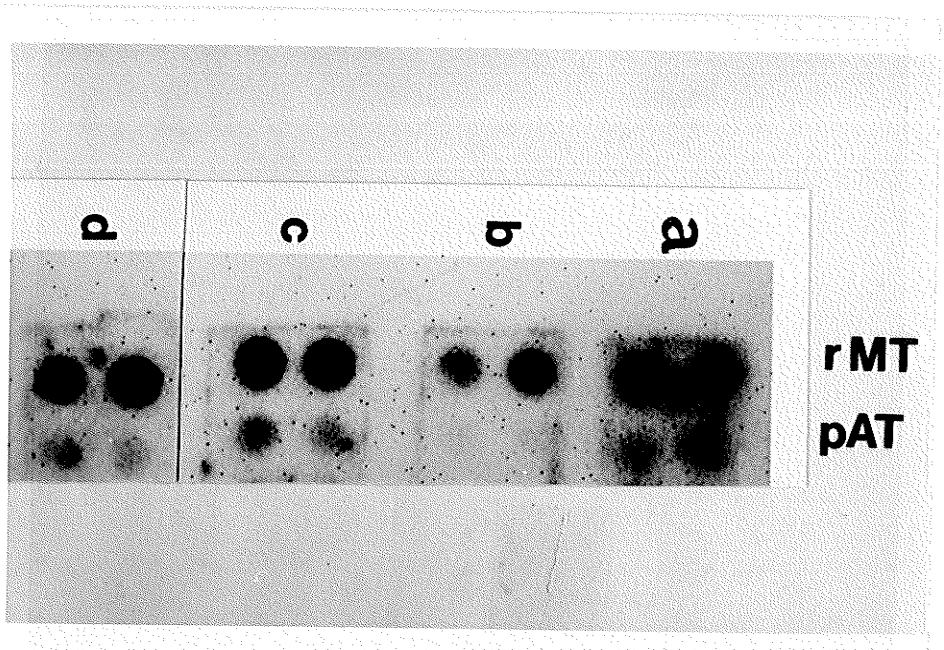


Figure 9. A time course of MT mRNA accumulation from castrated rat prostates after an injection of DHT.

The rats were injected with 1.2 mg/kg DHT and killed either 6 or 24 hours later. Five animals were used per group. The prostates were dissected into their individual lobes: Dorsal (D); Ventral (V); and Lateral (L). The RNA was isolated and 8 ug/lane of total RNA was electrophoresed and transferred to DBM paper. Hybridization to MT-I cDNA was as described. The DBM blot was exposed to X-ray film for 20 hours. The entire Northern blot is shown as a representative sample of MT mRNA size. The 18s and 28s ribosomal RNA bands were visualized with ethidium bromide staining. The lanes represent RNA from rats treated in the following manner: a) normal untreated; b) castrated + NaCl after 6 hours; c) castrated + 1.2 mg/kg DHT after 6 hours; d) same as -c- but killed after 24 hours.

Figure 9

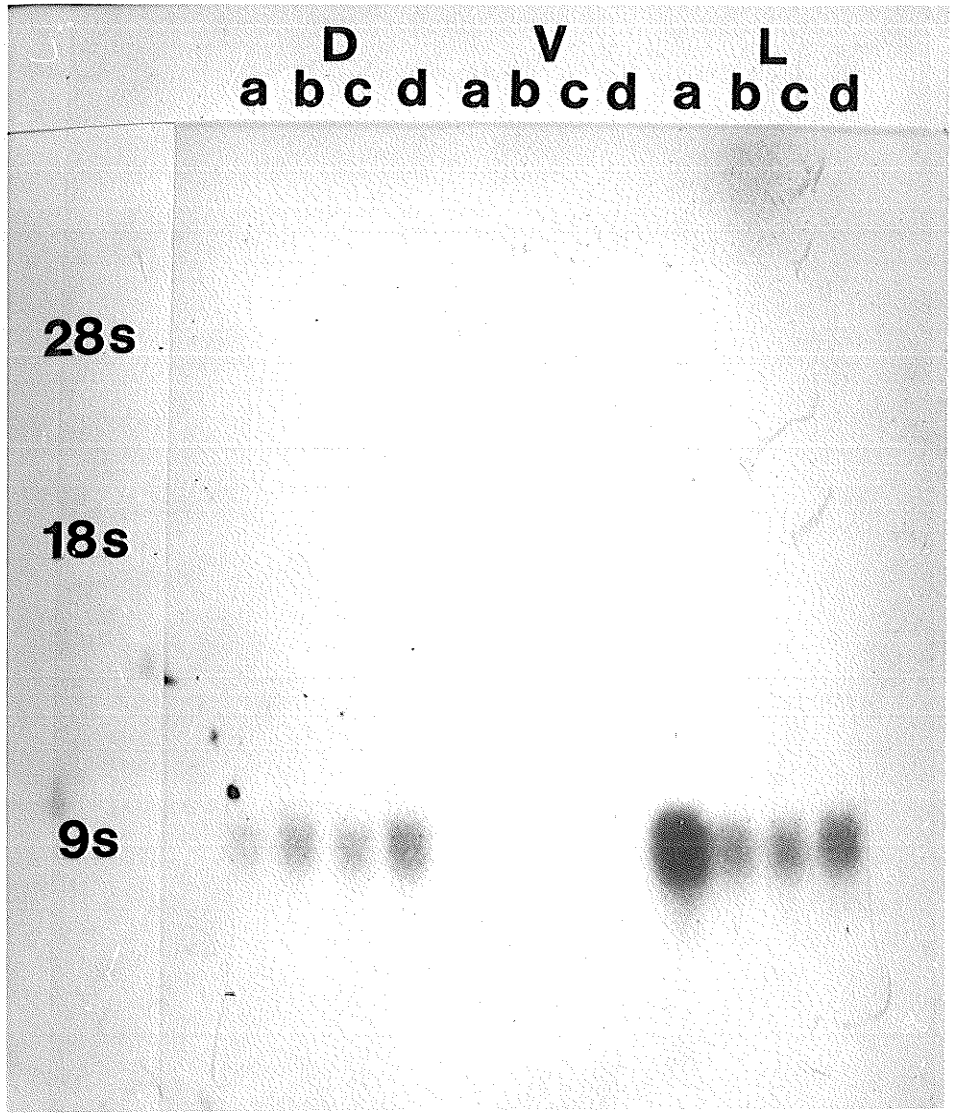


Figure 10. Androgen induction of M-40 mRNA

The same blot as in Figure 9 was used after the MT-I radioactive signal had been stripped off. The DBM was rehybridized with a nick translated pM-40 clone using the same methods as described for MT. The lanes represent the same conditions as listed in Figure 9: a) normal; b) castrated + saline after 6 hours; c) castrated + DHT after 6 hours; d) castrated + DHT after 24 hours. The film was exposed for 20 hours.

Figure 10

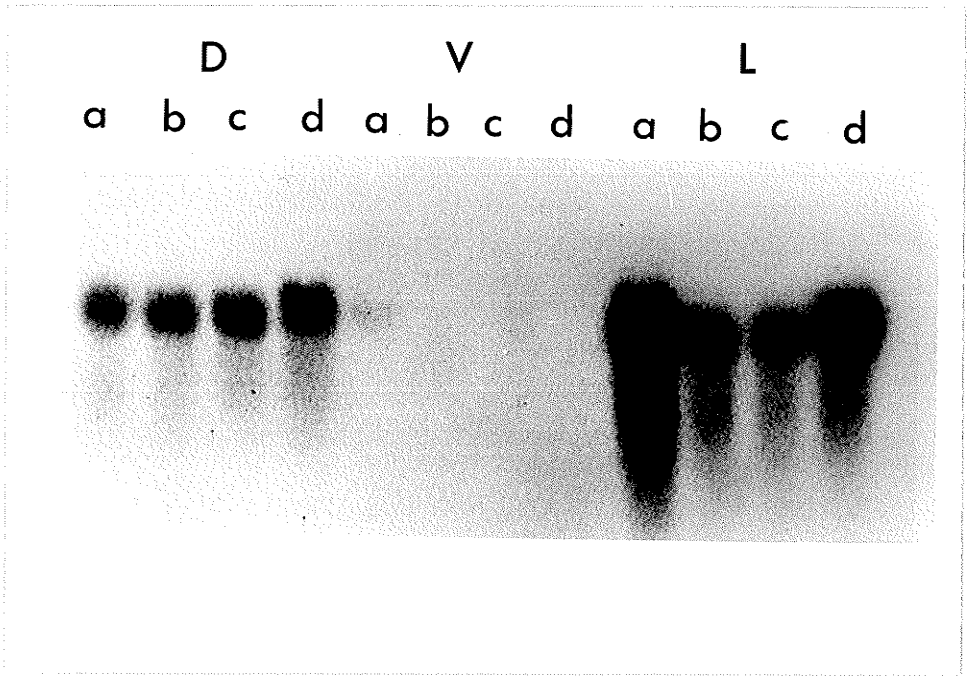
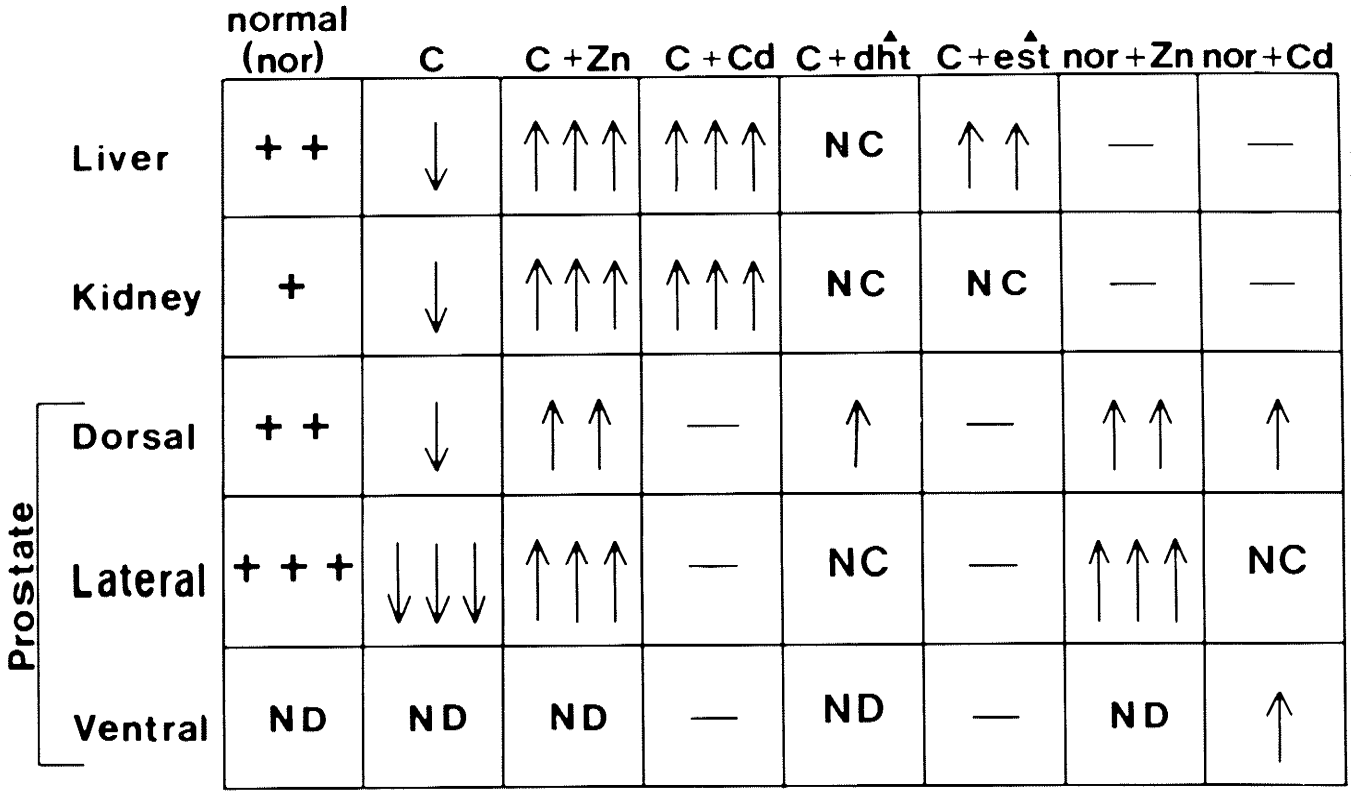


Figure 11. A schematic summarization of the results on MT gene regulation in liver, kidney and prostates of rats.

The amount of MT mRNA found in normal animal tissue was arbitrarily set relative to kidney which is represented as one (+) sign. The arrows indicate either increases (upwards pointing) or decreases (downwards pointing) in MT mRNA content. The changes in each tissue's MT mRNA content for intact rats are diagrammed with respect to their individual normal control levels. Likewise, the changes in castrated and treated rat tissues are given relative to each tissue's castrated (C) level with the exception of all normal rat experiments which are given relative to intact control. One, two and three arrows indicates a small to high change in MT mRNA content. All other parameters are drawn on the figure.

A schematic summarization of the results



▲ Analysis was 48 hours after injection
\+ Amount of MT mRNA relative to kidney (+)
C Effect of castration
ND No MT mRNA detected
NC No change relative to control
— Not tested
↑↓ Change in MT mRNA relative to respective control

Figure 11

DISCUSSION

It was quite evident that the expression of MT in the prostate was dependent on the age of the rat (Figure 1). The observed increase in MT mRNA closely followed a similar increase in Zn content that occurs in rodent prostate during development as described by Gunn and Gould in 1958. They found, in Wistar rats, that the capacity of the dorsolateral prostate to concentrate Zn^{65} increases as the age of the animal increases, with maximum efficiency of uptake occurring at about 12 weeks of age. The effect of various hormones on the Zn^{65} uptake in the immature rat prostate also has been studied. Interestingly, intact young (6 weeks) rat prostates increase their Zn uptake if the animals are injected with 10 ug estrogen/day for 1 week; whereas, 50 ug of testosterone had little effect over control values. This was also true of the prostates of castrated young rats. Whether the increase in MT was a cause or effect of higher Zn content is uncertain (Gunn and Gould, 1958). The high content of Zn during rodent adolescence appears to be typical of male reproductive organs. For example, the testes of 5-7 week old rats actually have more total Zn and MT than is present in their livers and kidneys combined (Brady and Webb, 1981).

Substantial variations in MT gene expression occurs during embryogenesis and development. Most *in vivo* studies with mammals have concerned themselves with changes in MT

levels in fetal and neonatal animals. For instance, the amount of Zn and likewise the amount of MT in rat liver is highest from approximately day 16 of gestation to parturition. Thereafter MT levels fall to the lower adult level by 24 days of age (Panemangalone et al., 1983). Investigations on sea urchin embryos also have discovered changes in MT levels during development. The highest MT gene expression occurs at the 20 hour mesenchyme blastula stage after which the levels of MT decrease. Following this stage, MT appears preferentially expressed in tissues of ectodermal origin (Nemer et al., 1984). It was postulated by Hamer in 1986 that two mechanisms may account for these developmental changes in MT expression. First, changes in methylation or chromatin modification could alter the accessibility of the MT gene to regulatory factors. Second, different cell types may change their MT gene regulatory factors as they differentiate. The change in MT level seen in maturing rat prostate also may be a result of tissue differentiation although the role of changing testosterone levels during puberty is very likely an influential factor. The adult high level of MT may act as one of the Zn storage systems in the prostate.

The high level of MT expression in the dorsolateral prostate was compared to other tissues. It has been reported that the liver contains and expresses the highest amounts of MT (Waalkes and Klaassen, 1985). The only other reproductive organ studied in this manner has been the testes

which has basal MT transcription levels comparable to liver (Durnam and Palmiter 1981). The ability of metals to induce the MT gene in various organs also has been investigated. Liver and kidney MT transcription appeared to increase after metal treatment. However, the MT mRNA content and regulation in the prostate had not yet been studied. The results presented in this thesis indicate that the mature prostate of normal rats contains levels of MT mRNA comparable (dorsal lobe) and higher (lateral lobe) than those found in the liver (Figure 2). However the fold increase in MT mRNA after Zn administration was the same for the prostate and liver.

Although much has been written on the effect of castration on reproductive organs, little information exists on the changes that occur in other tissues. It has been shown that castration does affect the weight of the liver when compared to normal rats (Korenshevsky and Dennison, 1935). The lack of circulating androgens distinctly decreases the MT mRNA content in the liver (Figure 4). Androgens have a profound effect on general anabolic processes and the removal of this steroid undoubtedly decreases the transcription of many proteins. Liver MT appears to be one such gene that is affected by circulating testosterone.

It is known that hormones such as glucocorticoids substantially alter hepatic MT mRNA and that the steroids act at the transcriptional level with some effect on mRNA stability (McKnight and Palmiter, 1979). A single dose of

glucagon also elevates hepatic MT bound Zn levels which influences overall Zn kinetics and involves changes in rates of MT synthesis (Cousins et al., 1986). It is possible that in vivo androgens directly influence hepatic MT although, in vitro, testosterone is a poor inducer of MT in hepatic cell culture (Bracken and Klaassen, 1987). One should also not rule out the possibility that secondary factor(s) may be responsible or that the half life of the mRNA may be severely decreased due to castration.

Castration did affect MT gene transcription in the kidney as demonstrated in the nuclear runoff experiment (Figure 8a). Whether androgens directly induce kidney MT gene transcription is unknown.

Castration also drastically reduced the amount of MT mRNA in the rat lateral prostate. Seven days after castration the transcription of MT message appeared to decrease by 75% (Figure 8b). This decrease seemed to begin as early as 24 hours following the surgery because the level of lateral prostate MT mRNA seen in Figure 3 (lane a) appeared to be lower than the dorsal amount (lane c). The normal intact rat lateral prostate contained more MT mRNA than the dorsal lobe (Figure 2). The dorsal prostate of castrated rats responded to a lesser degree in that the suppression of MT mRNA production was not as great as in the lateral lobe (Figure 6). It is well known that the lateral prostate has a striking ability to concentrate administered Zn (Gunn and Gould, 1955). This ability is drastically

reduced after castration by about 70% (Gunn and Gould, 1958). Therefore, the reduction in MT mRNA in lateral prostate could be due to the decrease in Zn concentrations.

No ventral prostate MT mRNA was detected in total RNA samples. Thus, it was not possible to measure whether castration had any effect on MT gene expression in this lobe. Ethidium bromide staining of these total RNA samples indicated that the amounts loaded onto the gels were the same for all prostatic lobes.

Castration produces many changes in the rat prostate. The first reported detailed analysis of ultrastructure changes that occur post castration was conducted by Harkin in 1957. This author noted a marked and sustained collapse of the rough endoplasmic reticulum (RER) by the seventh day after castration. In addition, Brandes and Groth (1963) showed that the protein synthetic capacity of the rat ventral prostate was less than 40% within 2 days following orchiectomy. In all lobes, the tubules decrease in number, size, convolutions and contain fewer secretory vesicles in the narrowed lumina. Even the layers of smooth muscle surrounding the tubules becomes thinner and the muscle cells themselves atrophy (Korenchevsky and Dennison 1935). Changes also occur in the castrated prostate on a molecular level. Several proteins are regulated by androgens and may decline to about 1% of that found in normal animals: Testosterone reversed these changes in castrated animals (Parker et al., 1978; Parker et al., 1980; Dodd et al., 1983; Matusik et al., 1985;

Dodd et al., 1986). Proteins may be androgen stimulated or androgen repressed. Androgen repressed proteins are elevated immediately after castration before tissue necrosis begins (Saltzman et al., 1987; Leger et al., 1987). Thus the results we have noted for MT expression in the prostate indicate that androgens also influence its regulation.

As illustrated in the summary, Figure 11, Zn had a large effect on MT expression in the examined tissues of intact or castrated rats (with the exception of ventral prostate). The response of the MT gene to a single injection of Zn reflects the results already published for kidney and liver of normal animals (Durnam and Palmiter 1981). The maximal increase in MT message was seen 6 hours after a single ip injection of $ZnSO_4$ Levels of MT mRNA returned to castrated control levels by 24 hours. Zinc also was able to induce the MT gene in the lateral prostate above castrated saline-treated levels, although the increase in MT mRNA at 6 hours after injection did not equal the amount found in the lateral prostate from normal rats. The slightly higher amount of MT mRNA seen from castrated rat lateral tissue when compared to the amount seen 24 hours after a Zn injection could be due to induction by stress because the tissue from castrated rat tissue was removed 6 hours after a NaCl injection (Figure 6a).

The dorsal prostate of castrated rat responded differently to Zn. An increase in MT mRNA was not discernible until 24 hours after injection of the metal. Zinc uptake in

the dorsal prostate is much less efficient than in the lateral. In fact, the dorsal lobe becomes saturated with Zn^{65} 20-40 minutes after injection whereas the lateral lobe continues to accumulate Zn^{65} until 24 hours post injection (Gunn and Gould, 1956). From these Zn^{65} uptake studies, we would expect a prolonged rise in MT in the lateral lobe and a quick short rise in the dorsal; our results are the opposite. Apparently the ability of the MT gene in the dorsal prostate of castrated rats to increase its production of MT mRNA after a single dose of Zn was slower than in the lateral lobe. These differences between the two prostatic lobes with respect to MT gene expression indicates how important it is to distinguish between these lobes during experiments. It must be emphasized that the rat prostate is neither functionally nor histologically, an homogenous organ.

The MT gene in the prostate reacted differently to Cd than it did in the kidney and liver (Figure 11). Both renal and hepatic tissue showed an MT mRNA induction even 48 hours after castrated rats were given single injection of $CdSO_4$. An induction of MT mRNA also was observed in the dorsal prostate of intact rats 6 hours after an ip dose of Cd was administered (Figure 8). However, no MT gene response was recorded in the lateral lobe (Figure 8). This is one of the few observations of a tissue in which the MT gene reacted at the mRNA level to Zn but not Cd. The MT gene in the testes also does not respond to Cd (Durnam and Palmiter, 1981). However, it is not known whether Zn induces the MT gene in this organ.

The effects of Cd on the reproductive organs have been well studied although the effect of Cd on the testes has been the most thoroughly recorded. The metal has a devastating effect in mammalian testes at various dosage levels (Parizek and Zahor, 1956). The same authors discovered that the damage to the testes of rats produced by Cd could be prevented by simultaneous administration of Zn; However, this protection was not permanent. In the late 1950s and early 1960s several experiments were conducted to determine how the rat testes and dorsolateral prostate handle Cd. It was first determined that injections of Cd in both intact and castrated rats interferes with Zn uptake into the dorsolateral prostate. This effect is unique to this organ as Cd does not decrease Zn uptake to this extent in other rat tissues (Gunn et al., 1961).

Since the advent of molecular biology techniques it is now known that Cd is often associated with MT and that Cd does not induce MT in the mouse testes (Durnam and Palmiter 1981). Since MT is often considered to be a Cd detoxifying agents, then its lack of induction by Cd in the testes argues against this protective function. Why Cd does not induce the testicular MT gene has not been elucidated. The reasons may be similar in the lateral prostate. It is possible that the MT gene unresponsiveness to Cd in the lateral prostate is a pharmacokinetic one. It has been shown that Cd reduced testicular blood flow by 2-9% of control values within 12 hours of administration. These experiments also

noted a much decreased blood flow in the ventral prostate but very little change in the dorsolateral lobe (Waites and Setchell, 1966). In addition injections of radioactive Cd into rat prostates showed a difference in the ventral and dorsolateral retention of the metal. The ventral lobe was able to retain significant amounts of Cd above control values with the dorsolateral prostate retaining only minimal amounts (Aughey et al., 1975). Since prostatic MT may play an important role in the homeostasis of Zn, this role may be so important that the commonly seen Cd detoxifying mechanism of MT is not functional in the rat lateral prostate. Cadmium may be unable to compete with the high levels of Zn found in the lateral prostate although the response to added Zn argues against this theory.

Ultrastructural analysis of prostates from rats fed low doses of $CdCl_2$ for long periods (40 weeks) revealed little change in the tissues compared to control animals. Only low levels of Cd were retained in the ventral prostate in this instance (Brandes and Groth, 1963). Therefore, the mechanism of Cd toxicity to the prostate is unknown even though the histological change in prostatic tissue exposed to Cd is well documented (Levy et al., 1973; Levy and Clack, 1975). Metallothioneins are not the only Cd binding proteins. Partially characterized proteins have been isolated from rat testes that are low in molecular weight but possess amino acid compositions quite different from MT (Waalkes et al., 1984; Deagen and Whanger, 1985). Such proteins may also

exist in the prostate and their regulation of Cd cannot be ruled out.

The protective role of MT against Cd poisoning is well known. Cells that produce higher or lower than normal levels are resistant or sensitive to Cd toxicity, respectively (Crawford et al., 1985; Compere and Palmiter, 1981). It also is known that MT may protect against toxic levels of other metals (Durnam and Palmiter, 1984). The testes are one of the organs most sensitive to Cd poisoning. Injections of low doses of Cd results in a selective testicular necrosis for rodents (Parizek and Zahor, 1956). It has been hypothesized that this hypersensitivity to Cd could be due to unresponsive MT genes since Cd does not induce MT mRNA synthesis in mouse testes (Durnam and Palmiter, 1981). Occupational Cd exposure has been associated with prostatic cancer (Ernster et al., 1979). Primary cultures of prostatic cells from species ranging from rat to man are susceptible to $CdCl_2$ resulting in cellular transformation (Aughey et al., 1975). Cadmium carcinogenicity has been well documented (Kazantzis, 1963) but direct in vivo mutagenesis of the prostate by Cd has not been unequivocally established. Other reproductive organs have not been analyzed with respect to their MT content or MT gene expression.

Recently, by using indirect immunoperoxidase procedures and indirect immunogold labeling techniques with anti-MT, two independant groups have reported results similar to those presented in this thesis (Umeyama et al.,

1987; Bataineh et al., 1986). Both groups found that MT protein can be found in dorsal and lateral prostate; specifically in the nucleus, nucleolus, rough endoplasmic reticulum, secretory vesicles and secretory products. The Japanese group also found that an injection of Cd did not change the staining pattern or intensity of MT in the lateral lobe. Our results demonstrate that Cd did not induce the MT gene in the lateral prostate confirming, at a molecular level, these previous reports.

It has been shown that androgen therapy to castrated rats can restore both prostatic weight and Zn^{65} uptake to normal levels (Gunn and Gould, 1958). This was accomplished by daily injections of at least 50 ug/rat testosterone for one week. However, 50 ug of testosterone/day for 1 week administered to castrated rats increases Zn^{65} uptake in the dorsolateral prostate only half as efficiently as 25 ug testosterone/day for 1 week (Gunn and Gould, 1958). These researchers also found that giving intact mature rats testosterone actually depressed Zn^{65} uptake. In the same paper, they reported that increasing doses of estradiol also increased the ability of the dorsolateral prostate to take up Zn^{65} while the prostatic weight actually decreased. The single injection of 50 ug/rat of DHT was used in the experiment illustrated by Figure 9 was insufficient for restoring prostatic MT gene expression.

A slight increase of MT mRNA was seen in both lateral and dorsal lobes 24 hours post DHT injection leading to

an accumulation that was between 1 to 2 fold above castrated saline-treated levels. This could be explained by an increase in MT mRNA half life as was reported for increased MT mRNA due to induction by another steroid, dexamethasone (Mayo and Palmiter, 1981). Whether DHT could induce MT mRNA accumulation in intact rats, or whether higher doses given for longer periods to castrated rats would have an effect on MT mRNA levels remains to be studied. However, the dose of DHT used was sufficient to induce the androgen regulated M-40 gene almost back to normal (Figure 10).

Many prostatic proteins have been identified that depend on the hormonal status of the tissue for their expression. The ventral prostate has been the most studied lobe to date. Here, testosterone, has been shown to stimulate not only mRNA synthesis but also DNA synthesis both in vitro and in vivo (Mistry, et al., 1982; Coffey, 1974). Dorsal and lateral prostate proteins are less well characterized, although a few abundant mRNAs from these tissues have been cloned. Two of these have been named RWB (Dodd et al., 1986) and M-40 (Dodd et al., 1983). The expression of M-40 is highest in the lateral lobe and lowest in the ventral lobe of normal rats. Both RWB and M-40 mRNA decrease after castration and both genes are inducible by androgen therapy. The M-40 gene is unique in that it is also inducible by Zn in the absence of androgens (Matusik et al., 1985). The induction by Zn and the high level of expression of both MT and M-40 suggest they play an important role for Zn homeostasis in this tissue.

Most MT gene inducers appear primarily to act at the transcriptional level (Durnam and Palmiter 1981; Friedman and Stark, 1985; Hager and Palmiter, 1981). It is assumed that other agents (e.g. sodium butyrate, gold thiomalate), for which nuclear runoff has not yet been done also regulate MT gene transcription (Birren and Herschman 1986). In liver and kidney it is well established that metals regulate the MT gene by interacting with the MT promoter (Durnam and Palmiter 1981; Stuart et al., 1984). We examined this response in the prostate (using the kidney as a control). Zinc induced transcriptionally MT expression in the kidney (Figure 8a). Likewise, a similar transcriptional change was seen in the prostate (Figure 8b and 8c). It is very likely that all increases in MT mRNA, seen in the rat prostate after $ZnSO_4$ treatment, were the result of MT promoter activation. We did not investigate the effect of Cd on transcription in the prostate since the lateral lobe did not show a mass accumulation of MT mRNA to Cd treatment (Figure 7).

In all of the studies, equal quantities of RNA were compared. The ethidium bromide stained gels reflected equal loading of intact RNA. The changes in MT mRNA levels were measured on the autoradiograms by densitometric scanning exposure time and specific activity of the probes would vary among experiments. Thus, one cannot directly compare different Northerns. The large range in MT mRNA levels among different tissues resulted in some lanes on the autoradiograms to become saturated, reaching the limitation levels with

saturated levels of MT mRNA would give an underestimate of the true change. Thus, the relative difference are inaccurate since they do not reflect the true magnitude of the response.

In summary, the levels of MT mRNA are highest in the rat lateral prostate compared to any other tissue. The amount of MT mRNA in the dorsal prostate is comparable to that found in the liver. Castration reduces the accumulation of MT mRNA in the lateral and dorsal prostate, kidney and liver. The lateral prostate's MT gene is very responsive to Zn in both castrated and normal animals but the gene is not induced by Cd in this organ.

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