EFFECT OF CELLULAR ZINC CONCENTRATION ON GLUCOCORTICOID INDUCED GENE EXPRESSION

by

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(ABSTRACT)

Previous studies in our laboratory have demonstrated that consumption by mammals and birds of a low zinc diet is associated with a diminished output of gene products in response to either endogenous or exogenous estrogen challenge. A recent discovery of the highly conserved zinc finger (or twist) motif in the steroid receptor superfamily suggests that zinc may play a broad role in assuring swift and timely adaptive, regulatory, and developmental responses to this entire class of lipophilic substances. To determine the effect of zinc status at the molecular level, a zinc deficit was created in cell cultures and glucocorticoid regulated and constitutive gene expression monitored.

Using a cell impermeable zinc specific chelator, diethylenetriaminepentaacetic acid (DTPA), a zinc deficient state in HeLa cells was created. This deficit was quantified by a fluorescence assay using N-(6-methoxy-8-quinolyl)-p-tolunesulfonamide (TSQ) to measure exchangeable zinc and induced coupled plasma analysis to measure total zinc. The 83% reduction in total cellular zinc, corresponding to a 65% decrease in TSQ fluorescence was rapid, nearly maximal within 4 hours,

and without significant loss of viability over 72 hours as tested by dye permeability. This was prevented by addition of zinc ions. This cellular system was used to study the effects of zinc deficits separately upon the individual steps in steroid-directed gene expression and constitutive protein synthesis. Differentiation between the effect of zinc supply upon hormone mediated and constitutive gene expression in HeLa cells was accomplished through transient transfections. By examining the expression of a glucocorticoid-responsive chloramphenicol acetyltransferase (CAT) reporter gene as compared to CAT activity derived from glucocorticoid-insensitive reporter plasmid, it was determined that glucocorticoid directed expression was reduced to a greater extent than constitutive (88% vs. 15%) when zinc concentations were reduced 80%. Furthermore, neither the binding affinities of receptors for dexamethasone nor nuclear translocation was altered by zinc concentrations. However, glucocorticoid receptor concentrations were reduced by 50%. This reduction in receptor number is sufficient to produce decreases in gene induction of the magnitude observed. Based on these results in conjunction with previous studies in our laboratory we propose that the most sensitive need for zinc may be in permitting rapid induction of newly synthesized enzymes as seen during hormone responses, rather than serving as a cofactor of metalloenzymes involved in constitutive synthesis.

Acknowledgments

I would like to thank my grandmother, Margaret Stewart. She has been an inspiration to all who have known her. Her strength and perseverance during adverse circumstances is commendable. It is with love and admiration that I dedicate this work to her.

A number of people deserve special acknowledgment for their roles in this project. I would like to express my appreciation to Drs. Ed Bunce, Pat Bender, Brian Storrie, Joe Falkinham, and John Hess for their guidance, expertise, and support throughout my time at Virginia Tech. I would particularly like to acknowledge Drs. Bunce, Bender and Storrie, whose invaluable contributions have had significant impact on many of the ideas and techniques presented here.

Finally, I would like to thank my husband, Tom, and my family and friends for their support and encouragement. In closing I would like to leave you with these words:

"Discovery is seeing what everybody else has seen,
and thinking what nobody else has thought."

-Albert Szent-Gyonrgi

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Abbreviations

BCIP: 5-bromo-4-chloro-3 indoyl phosphate

cAMP: cyclic adenosine monophosphate

CAM: chloramphenicol

DBD: DNA binding domain

DEX: dexamethasone

DNA: deoxyribonucleic acid

DMEM: Dulbecco's modified Eagle's medium

DTPA: diethylenetriaminepentaacetic acid

EDTA: ethylenediaminetetraacetic acid

ER: estrogen receptor

GH: growth hormone

GR: glucocorticoid receptor

GRE: glucocorticoid response element

HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

HSP 90: heat shock protein 90

ICP: induced coupled plasma reaction

JMEM: Joklik's minimal essential medium

MMTV: mouse mammary tumor virus

mRNA: messenger RNA

NMR: nuclear magnetic resonance

PBS: phosphate buffered saline

1,10 PHEN: 1,10 orthophenanthroline

PKC: protein kinase C

RDA: recommended dietary allowance

RNA: ribonucleic acid

SBS: supplemented bovine serum

T_3: triiodothyronine

T_4: thyroxin

TBS: tris buffered saline

TFIIIA: transcription factor III A

TPEN: *NNN-N*-tetrakis-(2-pyridylmethyl) ethylenediamine

TSQ: *N*-(6-methoxy-8-quinolyl)-*p*-tolune sulfonamide

TSH: thyroid stimulating hormone

SM-C: somatomedin C

Zn: zinc

Role of Zinc in Biological Systems

Nutritional Zinc Requirements:

In 1869 Raulin showed that zinc is indispensable for the normal growth and development of *Aspergillus niger* (Bettger and O'Dell, 1981). Birkner discovered the first indication of function for zinc in higher animals in 1919 (Mills, 1988). Almost a decade later, Somner introduced the idea that zinc is essential in higher plants (Mills, 1988). Scientists working at the University of Wisconsin demonstrated specific effects resulting from zinc deprivation in rats in 1934; Todd, Elvehjem, and Hart described the failure of growth and noted loss of hair and hair pigment in animals placed on a purified diet that contained only 0.16 mg zinc/100 g (Prasad et al, 1971; Roth and Kirchgessner, 1980, and Scheinmam et al, 1995). Nearly two decades later Tucker and Salmon showed that zinc cures and prevents parakeratosis, a thickening of the epithelial skin cells, in pigs (Mills, 1988). O'Dell and colleagues reinforced earlier studies regarding zinc and growth. They reported that zinc was essential for growth, feathering, and skeletal development in poultry (Mills, 1988). In the 1960's, Prasad and coworkers provided the first evidence for the occurrence of nutritional zinc deficiency in humans (Prasad et al, 1971; Roth and Kirchgessner, 1980, and

Scheinmam et al, 1995). The importance of zinc was further clarified during the 1970's. Highlights include the identification of acrodermatitis enteropathica as a genetic disease of zinc metabolism by Moynahan, which was followed by the identification of severe acquired zinc deficiency states and appreciation of the multifaceted clinical consequences of severe human zinc deficiency (Mills, 1988). The basic defect in acrodermatitis enteropathica has not been identified but is probably related to impaired intestinal uptake and transfer of zinc (Piotrowski, 1974). Recognition that mild human zinc deficiency syndromes occur in North America throughout the free-living population stimulated considerable interest in the clinical and metabolic consequences of human zinc deficiency (Hough et al, 1989).

Impaired growth velocity is the clinical feature of mild zinc deficiency that has received most recognition in infants, children, and adolescents. Ganji and colleges recently reviewed data collected in a nationwide food consumption survey taken in 1987-1988. The survey evaluated the nutrient intakes of 1-10 year old children in the United States. The 3-day food intake data was used to evaluate the nutrient intakes of 1-3, 4-6, and 7-10 year old American children. Nutrient intakes were compared

with 1989 Recommended Dietary Allowances (RDA) and percent RDA's were computed. When compared with RDAs, mean intakes of most vitamins and minerals were close to or above the RDA levels, except for vitamin E, calcium, iron, and zinc. Mean intakes of zinc for all age groups tested were below the RDA. Children 1-3 years exhibited only 58% of the RDA for zinc. The 4-6 and 7-10 year age groups exhibited 77% and 92% of the RDA levels, respectively (Franker et al, 1995; Henkin et al, 1984). Two studies in southern France also provide evidence suggestive of human zinc deficiency. Low serum zinc was discovered in 10% of children with growth retardation (Franker et al, 1995). It is apparent zinc is important for normal growth and development. However, much remains to be learned about changes in specific zinc dependent functions resulting from a zinc deficient state, and how these relate to clinical manifestation of zinc deficiency.

Spectrum of Zinc Deficiency in Humans

During the past two decades, a spectrum of clinical zinc deficiency in human subjects has been recognized. At one end, the manifestations of zinc deficiency may be severe, and at the other end, deficiency may be mild or marginal. The combination of clinical features, especially the skin lesions,

and severe hypozincemia make the detection of severe human deficiency relatively simple. In mild and moderate cases of zinc deficiency detection is more difficult since plasma zinc levels may be within normal ranges (Mertz, 1989).

Zinc Requirement During Pregnancy:

During times of rapid growth and development, physiological requirements for zinc increase. This increased demand is also seen during pregnancy. Pregnancy is associated with extraordinary metabolic demands on both mother and developing fetus. It has been well demonstrated that a 10-20 % decline in plasma zinc concentrations occur during the course of normal pregnancy (Mills, 1989). The reduction in plasma zinc during pregnancy is thought to be due to plasma volume expansion, the effect of rising estrogen and progesterone levels on liver zinc accumulation, and the uptake of zinc by the fetus (Mills, 1989). While part of this decline may be considered "normal", the exent of decline in some women may be sufficient to represent a risk to the fetus.

Zinc and Fetal Developement:

The development of embryos of fish (Ozoh and Jacobson, 1979), birds (Blamberg et al, 1960; Iniguez et al, 1969; O'Dell, 1968) and mammals (Apgar, 1972; Apgar and Fitzgerald, 1985; Dreosti, 1983; Dreosti et al, 1986; Hurley, 1981; Keen and Hurley, 1989; Rogers et al, 1985; Sato et al, 1985) including humans (Hambidge et al, 1975; Sever and Emanuel, 1973), is altered, and their survival is placed at risk when zinc intake is reduced. The effects on the rat embroy have been examined in the greatest detail.

During normal pregnancy, the rat fetus acquires 3% of the total maternal dietary zinc from the maternal plasma (Masters et al, 1983). One or two days of a low zinc diet can reduce the plasma zinc content of a pregnant dam to 30% of the control value (Dreosti et al, 1986). Within four days, the decrease in plasma zinc is followed by a 50% reduction of its amount in the rat uterine fluid (Gallaher and Hurley, 1980). As early as the four-cell and persisting through the blastocyst stage, the embryos of zinc-deficient females differ from controls in size and abnormalities of the blastocoel cavity (Hurley and Shrader, 1975). The resorbtion rate of embroys that survive to be implanted is 30%, while 90% of those that do

survive become malformed and are underweight (Hurley and Swenerton, 1966). The congenital malformations involve nearly all organs, including lack of formation of the head region, bone, and brain structures. The types of malformations vary depending on the time and extent of zinc deprivation. Early deprivation, (ie. in the first days of pregnancy), usually causes more marked defects of the head region, including eyes, facial structures, and central nervous system. Later exposure, in the first to second weeks, to zinc deficiency results in a more frequent incidence of skeletal malformations (Hurley and Swenerton, 1966; Keen and Hurley, 1989; Rogers et al, 1985; Warkany and Petering, 1972).

Zinc Requirment During Parturition:

Another area that has recieved much attention is zinc's role in parturition. Apgar first reported the observation of prolonged and difficult labor along with excessive bleeding in zinc deficient female rats (Apgar,1968). Failure to consume afterbirths or to care for and nurse their young were also noted (Apgar, 1968, Bunce, 1994). O'Dell and coworkers noted, in addition, that the zinc-deficient dams had significant decreases in body temperature, hypotension at or immediately following parturition, and

delayed onset of parturition, as well as increased perinatal mortality in both dams and fetuses (O'Dell et al, 1977). Some of these symptoms may be partially explained by a reduction in steroid responses during times of zinc deficiency.

The transition from gestation to labor requires the relief of the progesterone block and establishment of estrogen dominance over the uterus (Bunce et al, 1994). During gestation, progesterone suppresses the contractile activity of the uterus. Upon onset of labor, progesterone is removed by the ovarian enzyme 20α-hydroxysteroid dehydrogenase (Bunce et al, 1994). This enzyme converts progesterone into the biologically inactive C-20 hydrogenated metabolite (Mertz, 1988). The induction of this enzyme is stimulated by estrogen and mediated by prostaglandin F2α (Bunce et al, 1994). Bunce and coworkers showed that the preparturient decline in plasma progesterone in zinc deficient rats began at the same time as in control groups, but progesterone did not decline to the same exent as in control groups (Bunce et al, 1983). They also noted that plasma estrogen increased and peaked regardless of zinc status. However, induction of the estrogen regulated enzyme, 20α-hydroxysteroid dehydrogenase, was

delayed and its tardiness correlated with incomplete removal and delayed onset of parturition in zinc-deficient rats (Bunce et al, 1994). Furthermore, analysis of two other proteins sensitive to estrogen directed expression, gap junction proteins (Dylewski et al, 1986) and vitellogenin (Bunce et al, 1994), showed decreases of approximately 35% for both proteins. From these studies, it was concluded that establishment of zinc deficiency insufficient to limit growth or produce any outward sign of deficiency can still significantly curtail estrogen-directed gene expression (Bunce et al, 1994).

Distribution of Zinc within Tissues:

Athough it has been known for more than a century that metals participate in biological processes, details of the manner in which they function remained a mystery until recently. Advances in metal chemistry and biochemistry over the past three decades have allowed for a better understanding of the role of trace metals. Although these elements occur in biological matter in very low concentrations, their presence is critical for normal growth and development. Of all the metals, zinc is the most widely utilized in biological systems.

Zinc is present in all organs, tissues, fluids and secretions of the body. The average concentrations found in normal tissues of humans are shown in table 1-1, together with the proportion of total body zinc. Because of the large bulk of skeletal muscle, this tissue contains the greatest portion of the body's zinc, although the actual zinc concentration is not high. Together, bone and muscle account for more than 80% of the total body zinc (Prasad, 1993). Well over 95% of the total body zinc is intracellular. It can therefore be assumed that at least some of the important sites of zinc function are intracellular and in studies of the possible roles of zinc in human pathology, an assessment of intracellular zinc content is likely to be extremely important.

The nature of the binding of zinc within cells is much less clearly understood. The major portion of the zinc within cells is protein-bound; however, dialysis experiments and experiments with isolated proteins indicate that different proteins exhibit different affinities for zinc. It appears that the ion may have a regulatory role in certain enzymes, such as fructose-1,6-diphosphatase (Pefrosa et al, 1977), rather than as a firmly bound structural moiety. If zinc can play a regulatory role within cells, this

Table 1-1 Distribution of Zinc within the Body

Tissue	Approximate zinc concentration (μg/g wet weight)	Proportion of total body zinc (%)
Skeletal muscle	51	57
Bone	100	29
Skin	32	6
Liver	58	5
Brain	11	1.5
Kidneys	55	0.7
Heart	23	0.4
Hair	150	0.1
Blood plasma	1	0.1

The approximate zinc content of major organs and tissues in the normal adult man (70 kg) is shown (Prasad, 1993). Note that skeletal muscle accounts for the greatest proportion of zinc in the body. This is due to the large amount of this tissue and not a high concentration of zinc.

implies that there is a "pool" from which the zinc is readily available for the activation of certain enzymes. This pool may not be free, studies on methallothionien show that it may act as a zinc reservior, constantly exchanging zinc with enzymes (Karin et al, 1979, 1981; Kim and Wyckoff, 1989; Kimball et al, 1995, King et al, 1995, Luisi et al, 1991; Webb, 1972)

In every animal species so far studied, growth failure is one of the earliest signs of experimental zinc deficiency. Present evidence suggests that zinc is required in a highly available form for changes in the genetic expression of cells. Prime examples are the induction of the enzymes for DNA synthesis prior to entry into the S phase of the cell cycle (Prasad, 1993) and the induction of new proteins during tissue differentiation (Bunce et al, 1994). However, the mechanism of its involvement in these processes is still unknown and remains a fascinating field for further research.

Role of Zinc in Enzymes and Proteins:

The physical and chemical properties of zinc, including its generally stable association with macromolecules and its coordination flexibility, make it highly adaptable to meeting the needs of proteins and enzymes that carry out diverse biological functions (Vallee, 1993). In 1971 Prasad

suggested that the level of zinc in cells controls physiological processes through the formation and/or regulation of activity of zinc-dependent enzymes (Prasad, 1971). Over 300 zinc-requiring enzymes, representing more than 50 different types have been reported in microorganisms, plants, and animal (Vallee et al, 1993). Among the zinc enzymes are representatives from all six enzyme classes; oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Table 1-2 lists some of these enzymes along with the source where they were detected. Zinc is the only metal found in all classes of enzymes (Vallee et al, 1983, 1993). The level of zinc in cells can control many metabolic processes by the formation or regulation of activity of carbohydrate, fat, and protein metabolisms, as well as nucleic acid synthesis or degradation (Bunce, 1994; Schmid et al, 1995; Vallee et al, 1983, 1993).

Zinc has three functions in enzymes: catalytic, coactive (or cocatalytic) and structural (Figure 1-1) (Vallee et al, 1991, Vallee and Falchuk, 1983, 1993). A catalytic role specifies that the metal participates directly in enzyme catalysis. If the metal is removed by chelation or dialysis, the enzyme becomes inactive. This abolishment of activity is

Table 1-2 Zinc Enzymes

Name	Source
Class I: Oxidoreductases	
Alcohol dehydrogenase	yeast, vertebrates, plants
Sorbitol dehydrogenase	vertebrates
D-Lactate dehydrogenase	barnacle, bacteria
D-Lactate cytochrome reductase	yeast
Superoxide dismutase	vertebrates, plants, fungi, bacteria
Class II: Transferases	
Transcarboxylase	*P. shermanii*
Aspartate transcarbamylase	*E. Coli*
Phosphoglucomutase	yeast
RNA polymerase	wheat germ, bacteria, viruses
Reverse transcriptase	oncogenic viruses
Nuclear poly(A) polymerase	rat liver, viruses
Terminal deoxyribonucleotidyl transferase	calf thymus
Class III: Hydrolases	
Leukotriene A_4 hydrolase	human
Alkaline phosphatase	mammals, bacteria
5'-nucleotidase	bacteria, lymphoblast, plasma
Fructose-1.6-bisphosphatase	mammals
Phosphodiesterase	snake venom
Phospholipase C	*B. cereus*
Cyclic nucleatide phosphodiesterase	yeast
Nuclease	microbes
α-Amylase	*B. Subtilis*
α-D-Mannosidase	mammals, plants

Name	Source
Class III: Hydrolases (*continued*)	
Aminopeptidase	mammals, fungi, bacteria
Carboxypeptidase A	vertebrates, crustacea
Carboxypeptidase	mammals, crustacea
Elastase	*P. aeruginosa*
Collagenase	mammals, bacteria
Protein kinase C	mammals
Aminoacylase	pig kidney, microbes
Creatinase	*P. putida*
AMP deaminase	rabbit muscle
Nucleotide pyrophosphatase	yeast
Adenosine deaminase	*E.coli*, mammals
Class IV: Lyases	
Fructose-bisphosphate aldolase	yeast, bacteria
Carbonic anhydrase	animals, plants
δ-Aminolevulinic acid dehydratase	mammalian liver, erythrocytes
Glyosalase I	mammals, yeast
Class V: Isomerases	
Phosphomannose isomerase	yeast
DNA topoisomerase I	*E. coli*
Class VI: Ligases	
tRNA synthetase	*E. Coli, B. stearothermophilus*
Pyruvate carboxylase	yeast, bacteria

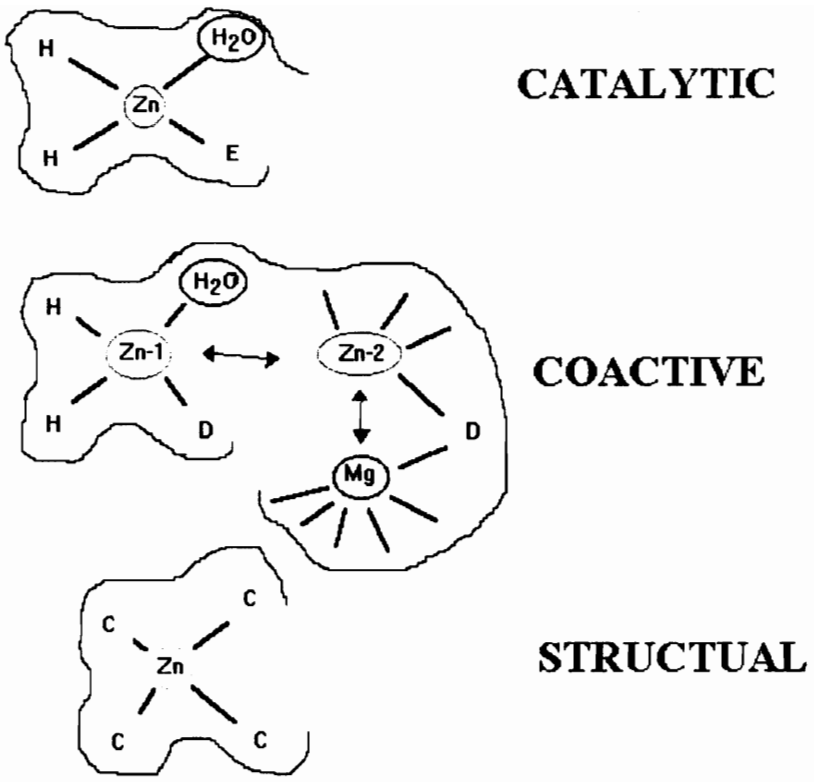

Figure 1-1 Zinc Binding Sites in Enzymes

Three amino acids (1 letter codes) and one water molecule coodinate the zinc ligand in the catalytic site. Enzymes with a coactive site contain both a catalytic site (Zn-1), and a separate coactive site (Zn-2). In such multizinc enzymes, both sites act as a single unit for catalytic activity. Zn-2 acts as a modulator and is not essential for catalysis. These Zn-2 atoms can bind to an amino acid (usually aspartic acid) sharing it as a bridge. Furthermore, one of the ligand of the Zn-2 site can also form a bridge to a third metal, in this case magnesium. In the structural zinc site shown, the metal is coordinated tetrahedrally to 4 cysteine residues.

attributed primarily to the fact that zinc itself participates directly in the catalytic process; this does not exclude the possibility that there may also be a concomitant structural change (e.g. in local conformation and/or that of the ligands). One catalytic atom per subunit is the rule. Typically, it is bound to four ligands, three of which are amino acids, a water molecule is the fourth ligand. Mechanistically, the water molecule can be ionized, polarized, or displaced. Ionization or polarization provides hydroxide ions at neutral pH, while the displacement of the water leads to Lewis acid catalysis. A coactive (or cocatalytic) zinc ion enhances or diminishes catalytic function in conjunction with another active site zinc ion in the same enzyme, but is not required for either enzyme activity or stability. In this case, an amino acid forms a ligand bridge between two zinc atoms or one zinc atom plus an atom of another metal. There are two such bridged metal atoms, zinc and magnesium, in alkaline phosphatase (King et al, 1995), and two zinc atoms in phospholipase C.(Hough et al, 1989) Structural zinc atoms are required solely for stability of the protein and can help stabilize the quaternary structure of oligomeric holoenzymes. Structural zinc atoms have been found in alcohol dehydrogenase, aspartate

transcarbamylase and protein kinase C (Hubbard et al, 1991; Webb, 1972). In addition to enzymes, two other classes of zinc proteins, the metallothioneins and the gene regulatory proteins, contain structural zinc ions.

Metallothionein is composed of 62 amino acids, including 20 cysteines, with a molecular weight of 6,700 (Vallee and Falchuk, 1993). It contains 7 gram.atoms of zinc per mole of protein. Metallothionein-like proteins and peptides have been found in numerous unicellular and multicellular organisms. They are grouped together as a family composed of three classes. Class I metallothioneins typically occur in mammalian organisms and their primary structure is highly conserved. Class II metallothioneins are found in unicellular eukaryotes, such as yeast, and their primary structures exhibit little resemblance to those of class I. Class III is present only in plants. Metallothionein is, in the absence of zinc, a random coil, and only with binding of metal ions does it form a series of turns so as to hold the ions in two types of clusters (Piotrowski, 1974). The zinc atoms in Zn_7-metallothionein are organized into two distinct metal clusters, Zn_4Cys_{11} and Zn_3Cys_9, with five and three cysteine residues acting as

bridging ligands between two metal ions in each cluster (Figure 1-2) (Vallee and Falchuk, 1993). Metallothionein is thought to play a role in Zn metabolism. A large number of physiological and pathological agents induce thionein (the metal-free protein) or metallothionein synthesis in vivo (Vallee and Falchuk, 1993). These include metal atoms (zinc, cadmium, and copper), hormones (dexamethasone, glucogon, epinephrine, and norepinephrine), cytokines (interleukin-1 and -6), interferon, as well as physical and chemical stresses (Anderson and Weser, 1978; Evans-Storms and Cidlowski, 1995; Prasad et al, 1961, Vallee and Falchuk, 1983, 1993). This induction is mediated through an increase in transcription of metallothionein mRNA (Failla and Cousins, 1978; Vallee and Falchuk, 1993). Metallothionein has been shown to be a source of metals for newly synthesized apoenzymes and postulated to serve as a regulator molecule in gene expression (Vallee and Falchuk, 1993). The capability of metallothioneins to exchange metals not only among themselves, but with other proteins shows that it is a store of zinc ions for proteins. It may also regulate the activity of certain proteins by removing the metal ions.

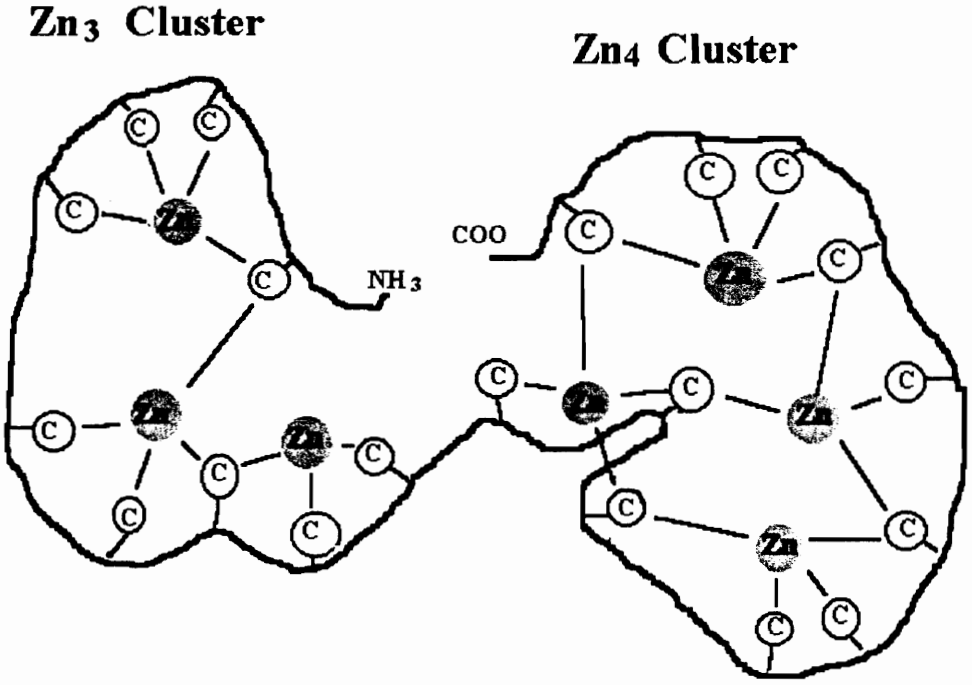

Figure 1-2 Metallothionein Zinc Clusters

Zinc clusters of metallothionein are shown. This molecule is composed of two clusters, containing 3 (Zn3) and 4 (Zn4) zinc atoms. Each metal is tetrahedrally coordinated to 4 thiolate bonds from cysteine residues. Some cysteines are shared between two zinc ions.

Zinc in Gene Regulation:

Literally hundreds of gene regulatory proteins directly involved with replication and transcription of DNA are now known or suspected to contain functionally important zinc atoms. The recognition that zinc is likely involved in regulation of their activity has attracted much attention and generated new research aimed at understanding how specific genes are expressed and what role zinc may play in this process

A functionally heterogeneous group of DNA-binding proteins that includes transcription factors, large tumor antigen, bacteriophage proteins, hormone receptors, and others have been inferred to be zinc proteins based solely on their primary structures (Vallee and Falchuk, 1993). Combinations of conserved cysteine and histidine residues separated by variable numbers of amino acids have been formulated into the popular model known as a "zinc finger". Zinc fingers, along with helix-turn-helix and basic regions, are now considered the three fundamental motifs utilized by eukaryotic regulatory proteins to bind DNA sequences. Zinc finger has become a widely used description encompassing any relatively short sequence that contains four or more cysteine and/or histidine residues and is

believed to function as a nucleic acid-binding domain. Vallee suggested that based on recent three dimensional structural analysis, DNA binding proteins can be categorized into different structurally distinct groups constituted by zinc fingers, zinc twists and zinc clusters (Figure 1-3) (Vallee et al, 1991, 1993). Each group likely consists of a family of related proteins. The intermolecular distances between the zinc ions differ, and may be characteristic of each of the three groups: 27Å for zinc fingers, 12-13Å for zinc twists, and 3Å for zinc clusters (Vallee and Falchuk, 1993). There are also important differences in the mode of binding of zinc to these proteins. The finger type requires only one ion for each site, whereas the other two require at least two. In twists and clusters, the DNA binding site is located between the two zinc ions, whereas in the finger, the binding site is located between two successive pairs of ligands on one zinc atom (fig 1-3) (Vallee and Falchuk, 1993). The functional significance of this is unknown at the present time.

TFIIIA was the first transcription factor to be identified as a zinc protein. It interacts with the 50-bp internal control region of the 5S RNA gene, thereby activating transcription by RNA polymerase III (Vallee and

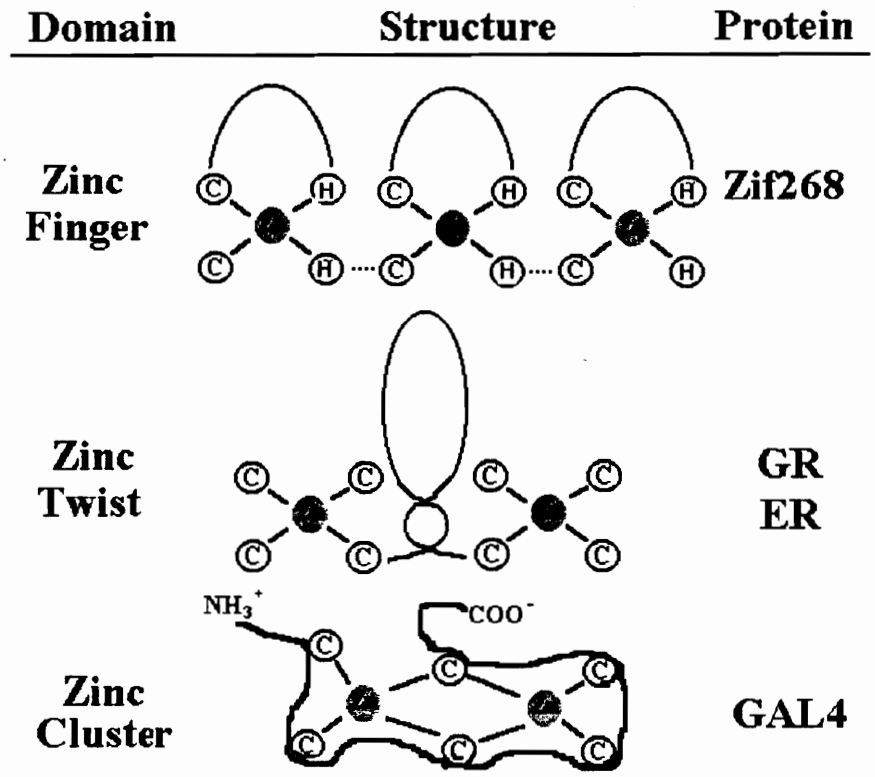

Figure 1-3 Zinc Binding Sites in Regulatory Proteins

Three distinct zinc sites are presently known. In the zinc finger type, metal is coordinated by 2 cysteine and 2 histidine residues. This structure has been determined crystallographically for Zif268 (Pavletich 1991). In both the zinc twist, found in the glucocorticoid receptor (GR) and estrogen receptor (ER), and the zinc cluster type found in GAL4 protein, the ion is coordinated to four cysteine residues.

Falchuk, 1993). In this protein the zinc is tetrahedrally coordinated by two cysteine and two histidine residues. The intervening sequence between the pairs of Cys and His residues generates a loop structure containing the DNA binding domain, resulting in the "zinc finger" DNA binding motif.

A zinc twist motif has been identified in the glucocorticoid and estrogen receptors. The primary structure of the DNA binding domain of the glucocorticoid receptor contains one His and 9 Cys residues. These cysteine-rich regions of the receptor (amino acids 440-525) serve as ligands for two zinc ions. Each metal is coordinated to four sulfur ligands on the cysteine residues. The intervening DNA binding sequence is located between and anchored by the two zinc atom complexes. Thus, the resulting DNA binding motif is helical and does not exhibit the typical finger structure seen in TFIIIA.

The structure of the DBD composed of a cluster is clearly distinct from those of the TFIIIA and Glucocorticoid receptor proteins. Gal-4 is an example of a protein containing a zinc cluster. Based on x-ray crystallographic and NMR three-dimensional structure analyses, the two

metal ions are coordinated to a sequence that contains six cysteines, two of which form bridging ligands between two of the ions.

Zinc and Gene Regulatory Proteins:

Zinc not only participates in the actions of enzymes that carry out transcription, but also is a component of the proteins that regulate this process. The recognition of this function is based on three types of observations 1) zinc induces the formation of a number of proteins. 2) zinc-deficient and -sufficient cells produce different types of transcripts, and 3) the actions of several molecules directly involved in gene activation are zinc dependent (Vallee and Falchuk, 1993).

Which, if any, of the regulatory proteins involved in development and what alteration in their function will prove to involve the biological role of zinc and its deficiency is not known at present. The zinc requirement for normal development and proliferation and the congenital malformations or other manifestations of zinc deficiency cannot be related to a decline of any one enzyme or protein product of genes activated by zinc proteins. Surely there are transcription factors involved in cell division an developmental

processes that must be sought out. Their identification along with information pertaining to the role of zinc in their activities, the genes they regulate, and an understanding of the effects of zinc deficiency on their function will further the understanding of the mechanisms underlying this most important aspect of developmental biology.

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Effect of a Zinc Specific Membrane-Impermeable Chelator on Cellular Zinc Concentrations

ABSTRACT

By using a cell impermeable zinc specific chelator, diethylene-triaminepentaacetic acid (DTPA), a reversible, non-toxic, zinc deficient state in HeLa cells was achieved. Zinc levels where measured by a zinc specific fluorophore or induced coupled plasma analysis. Cellular fluorescence decreases 65% compared with controls, as assayed with the zinc specific fluorescent molecular probe, N-(6-methoxy-8-quinolyl)-p-tolunesulfonamide (TSQ). This coorelates with a 83% decrease in total cellular zinc measured by induced coupled plasma analysis (ICP). Removal was rapid, nearly maximal within 4 hours, without significant loss in viability over 72 hours as tested by dye permeability, and readily reversible by addition of Zn^{2+}.

INTRODUCTION

Zinc deficiency is a significant pathological state involving abnormalities in the metal's metabolism. This can be due to inadequate dietary intake, increased requirements or excretion, or genetic causes (Vallee and Falchuk, 1993). The effects of zinc deficiency on the physiology and biochemistry of specific organ systems varies. In general,

cells from intestinal, dermatological, and gonadal tissues that normally undergo rapid turnover and frequent proliferation appear to be most sensitive to zinc deprivation. Therefore, pathological consequences of zinc deficiency predominate in these tissues.

In most tissues the zinc content is in the range of 10-200 μg/g wet weight (Mills, 1988; Vallee and Falchuk, 1993). About 30-40% of the total cellular zinc is in the nucleus, ~50% is in the cytoplasm and its organelles, and the remainder is in the cell membrane or wall in the case of plant cells (Vallee and Falchuk, 1993). Virtually all zinc in these compartments is bound to macromolecules in the form of zinc proteins/enzymes or nucleotides (Abbasi et al, 1980; Archer et al, 1990). In biological systems, very little, if any, zinc is free in solution. The relative concentration of free or exchangeable zinc in biological systems is thought to be 1 nM in most cells and this constitutes about 3-8% of the total zinc for the average cell (Mills, 1988). Ohno states "free" zinc in erythrocytes to be 7-8% of the total erythrocyte zinc (Clair et al, 1995). In contrast, the intracellular exchangeable erythrocyte zinc pool as examined by ^{65}Zn uptake and exchange studies, amounts to only 2.6% of the total zinc found in these cells

(Van Wouve, 1990). In most zinc deficient states, more than the free pool is depleted. For instance, the plasma zinc levels in Zn deprived pigs and rats were reduced 75% and 63%, respectively (Clair et al, 1995). In severe cases of zinc-deficiency, the redistribution of the endogenous cellular zinc in response to a reduction in exogenous zinc supply can only delay the effects of zinc deficiency. In rats, a reduction in food intake seen in times of Zn-deficiency, caused weight loss which in turn, through tissue catabolism, increased their cellular zinc supply (Mills, 1988).

In the present study, we have examined the extent to which the total and free zinc pools in cultured human cells are exchangable. To selectively remove zinc, three divalent chelators specific for the metal were tested. Free zinc pools in living HeLa S3 cells were monitored using the zinc specific fluorophore, TSQ, while total zinc pools were quantitated by induced coupled plasma (ICP) reaction. A cell impermeable zinc specific chelator, DTPA, induced a zinc deficient state in HeLa S3 cells where reductions of 65% and 83% were observed in TSQ detectable and total cellular zinc pools, respectively. This experimental system should be useful in studying the role of zinc in cellular metabolism.

MATERIALS AND METHODS

Reagents:

Zinc Specific Fluorescence Assay:

N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ, Molecular Probes Eugene, OR) was prepared according to Fliss et al. (Fliss et al, 1990). Briefly, TSQ was dissolved in ethanol by heating in a 80°C water bath and stored at a stock concentration of 45.7 mM (1.5% w/v) at 4°C. Immediately before use, TSQ working solution (100 μM) was prepared by transferring 33 μL of dissolved stock solution to 15 mL of rapidly stirring physiological saline buffered with 40 mM hepes to pH 7.3 (Fliss et al, 1990). Working solutions were protected from light and used within 30 minutes of preparation (Fliss et al, 1990; Fredrickson et al, 1987, Savage et al, 1989).

Chelators:

Diethylenetriaminepentaacetic acid, DTPA (Molecular Probes) was dissolved in distilled H_2O (10mM) (Chesters et al, 1989; McCabe et al, 1993). NNN,N,-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN)

(McCabe et al, 1993), and 1,10-orthophenanthroline (Clair et al, 1995) were dissolved in 95% ethanol at concentrations of 10 mM.

Other reagents were obtained from Fisher Scientific Company, Baxter, or Sigma Chemical Company.

Cell Culture:

HeLa cells were grown in Dubecco's modified essential memdium (DMEM) high glucose supplemented with 10% supplemented bovine serum (SBS) (Hyclone). Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown, until 70-90 % confluent, either in 100 mm tissue culture dishes or for fluorometric studies, on dry heat sterilized coverslips. When confluent, cells were passed after 10 minute exposure to trypsin-EDTA at 37°C.

Spectrofluorometric analysis of TSQ-Zn^{2+} fluorescence:

TSQ was added to cuvettes containing known amounts of Zn^{2+} or other metal cations. Fluorescence was measured at room temperature in a Perkin-Elmer 650-10S fluorescence spectrophotometer with an excitation and emission wavelengths of 380 nm and 495 nm, respectively (Chesters et

al, 1989; Fliss et al, 1990; Hennig et al, 1992; Savage et al, 1989; Zalewski et al, 1993).

Analysis of TSQ-Zn^{2+} fluorescence in cells:

Twenty-four hour old cell cultures were rinsed with HEPES-buffered saline solution. The medium was then replaced with one of the various incubation media, consisting of either DMEM and 10% SBS (control) or DMEM, 10% SBS, and chelator (0.1-600 μM) (Chesters et al, 1989; Hennig et al, 1992). The cells were incubated from 1 to 72 hours in the appropriate medium. Cells were washed twice in 10 mL of HEPES-buffered saline (pH 7.3) solution to reduce background fluorescence. Coverslips were placed cell side down in 200 μL of 100 μM TSQ solution for 90 seconds to allow the fluorescent dye to pentetrate the cells. Cells were then rinsed in saline briefly to remove any residual TSQ. Coverslips were mounted and cellular fluorescence was visualized with a Zeiss IM 35 inverted microscope equipped with a G-365 excitation filter, 395 dichtoic mirror, and a LP-420 emission filter. Images obtained with a Dage-MT1 SIT 66 video camera were digitized with a LG-3 scion framegrabber. Analysis was with the NIH Image computer program for Macintosh. Control cells (no TSQ) were also

examined. Any fluorescence due to the light scattering or natural luminescence of these cells was subtracted from all samples. All measurements are expressed as relative fluorescence with TSQ fluorescence for cells grown in DMEM plus 10 % SBS set to 100.

Determination of total zinc:

For induced coupled plasma reaction (ICP) analysis, HeLa cells were ashed by heating to dryness at 200°C in a sand bath with alternating doses (3 times each) of concentrated nitric acid and 100% hydrogen peroxide. Residue was dissolved in ion-free water and ICP analysis was performed at the Soil Testing Laboratory, Virginia Polytechnic Institute and State University.

RESULTS

Spectrofluormetric analysis

The solubility of hydrophobic TSQ in aqueous solution is low at physiological pH but increases in alkaline solutions (Fliss et al, 1990). Consequently, cellular studies with this compound are routinely done at pH 10.2 (Fredrickson et al, 1987; Savage et al, 1989). To determine the

reactivity of the probe at physiological pH, the fluorescent properties of TSQ were examined at pH 7.3.

To compare the fluorescent properties of TSQ at pH 7.3 and 10.2, the fluorescence response of TSQ (100 μM) suspension at pH 7.3 to increasing concentrations of zinc compared with the dissolved probe at pH 10.2 were examined (fig 2-1). At pH 7.3, fluorescence increased in a curvilinear fashion and saturated at a zinc concentrations of 10 μM. In contrast, dissolved TSQ fluorescence at pH 10.2 increased linearly through 25 μM zinc. These results are consistent with previous studies (Fliss et al, 1990; Fredrickson et al, 1987; Savage et al, 1989). The rate of fluorescence increase for both suspended and dissolved TSQ was fairly similar within the range of 0-10 μM Zn^{2+}. Thus showing that TSQ at physiological pH can respond in a quantitative manner in response to increases in Zn^{2+} concentration. The specificity of TSQ for Zn^{2+} over other divalent cations was examined. TSQ did not fluoresce in the presence of 5 mM Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, or Mn^{2+}, concentrations vastly higher than physiologic (table 2-1). However, these cations did show apparent weak affinity for TSQ by quenching the fluorescence of TSQ-Zn^{2+} (Table 2-1).

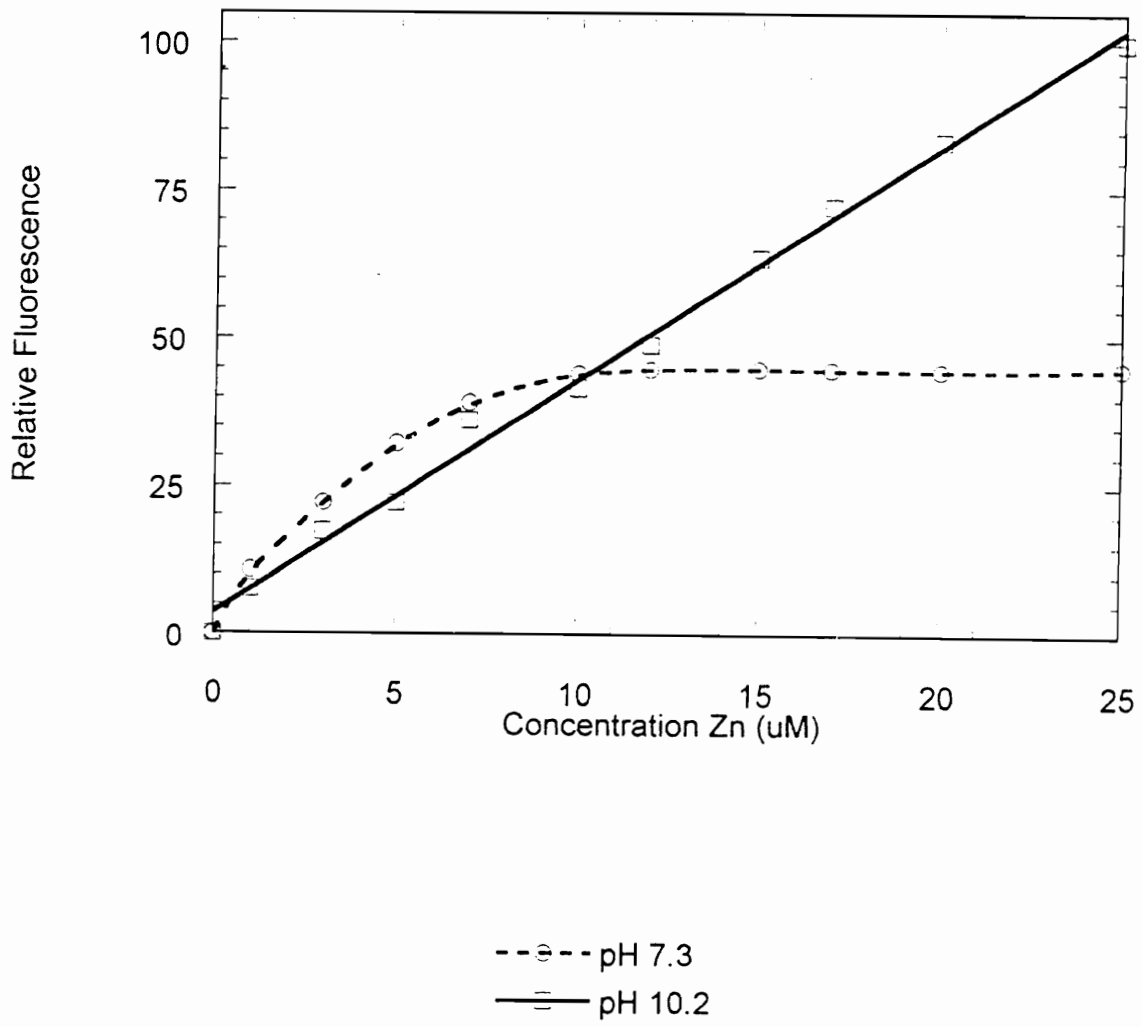


Figure 2-1 TSQ-Zn^{2+} Standard Curve

TSQ (100 μM) was exposed to increasing concentrations of zinc at pH 7.3 and 10.2. The fluorescence intensities are expressed as relative fluorescence where TSQ + 25 μM zinc (pH 10.2) is 100.

Table 2-1 TSQ Fluorescence with Divailent Cations

Cation	*Relative Fluorescence*
Zn^{2+} (10 μM)	100 ± 0.5
Zn^{2+} (10 μM) + Cu^{2+} (20 μM)	75.2 ± 0.35
Zn^{2+} (10 μM) + Fe^{2+} (50 μM)	82.7 ± 0.42
Zn^{2+} (10 μM) + Mn^{2+} (250 μM)	77 ± 0.64
Ca^{2+} (5 mM)	< 1
Cu^{2} (5 mM)	< 1
Fe^{2+} (5 mM)	< 1
Mn^{2} (5 mM)	< 1
Mg^{2+} (5 mM)	< 1

The specificity of TSQ for zinc was examined by incubation of the probe with various amount of cations. Only in the presence of zinc did any significant fluorescence occur. However, the ability of certain cations to quench TSQ-zinc fluorescence shows that they do exhibit some affinity for TSQ. TSQ concentration was 100 uM. The data represents the average and the standard deviations of 5 independent experiments.

At concentrations over 1,000-fold higher than that of total cellular levels of copper, iron, and manganese in human tissues (Kutsky, 1981; Mertz, 1986, Williams, 1984), an ~20% decrease in TSQ-Zn^{2+} fluorescence was seen. Thus, the fluorescence of TSQ is specific for zinc and other divalent cations do not appear to affect these results. At physiological concentrations no fluorescence quenching was observed. This result is consistent with those of Fliss (Fliss et al, 1990). The TSQ probe at the free divalent cation concentrations in the cell (10^{-9}- 10^{-10} M), should give a zinc specific linear fluorescence response.

The effect of various zinc specific chelators on TSQ-Zn^{2+} fluorescence was examined. Three chelators, two cell permeable (NNN,N,-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN, (McCabe et al, 1993), and 1,10-orthophenanthroline (Clair et al, 1995)) and one impermeable (DTPA, (Chesters et al, 1989; Clair et al, 1995, Fliss et al, 1990; Fredrickson et al, 1987; Hennig et al, 1992; Savage et al, 1989, Zalewski et al, 1993) were examined at a concentration of 10 μM. All three chelators equally reduced TSQ-Zn^{2+} fluorescence (figure 2-2). After 10 minutes exposure time, all chelators had diminished fluorescence by 50%. This

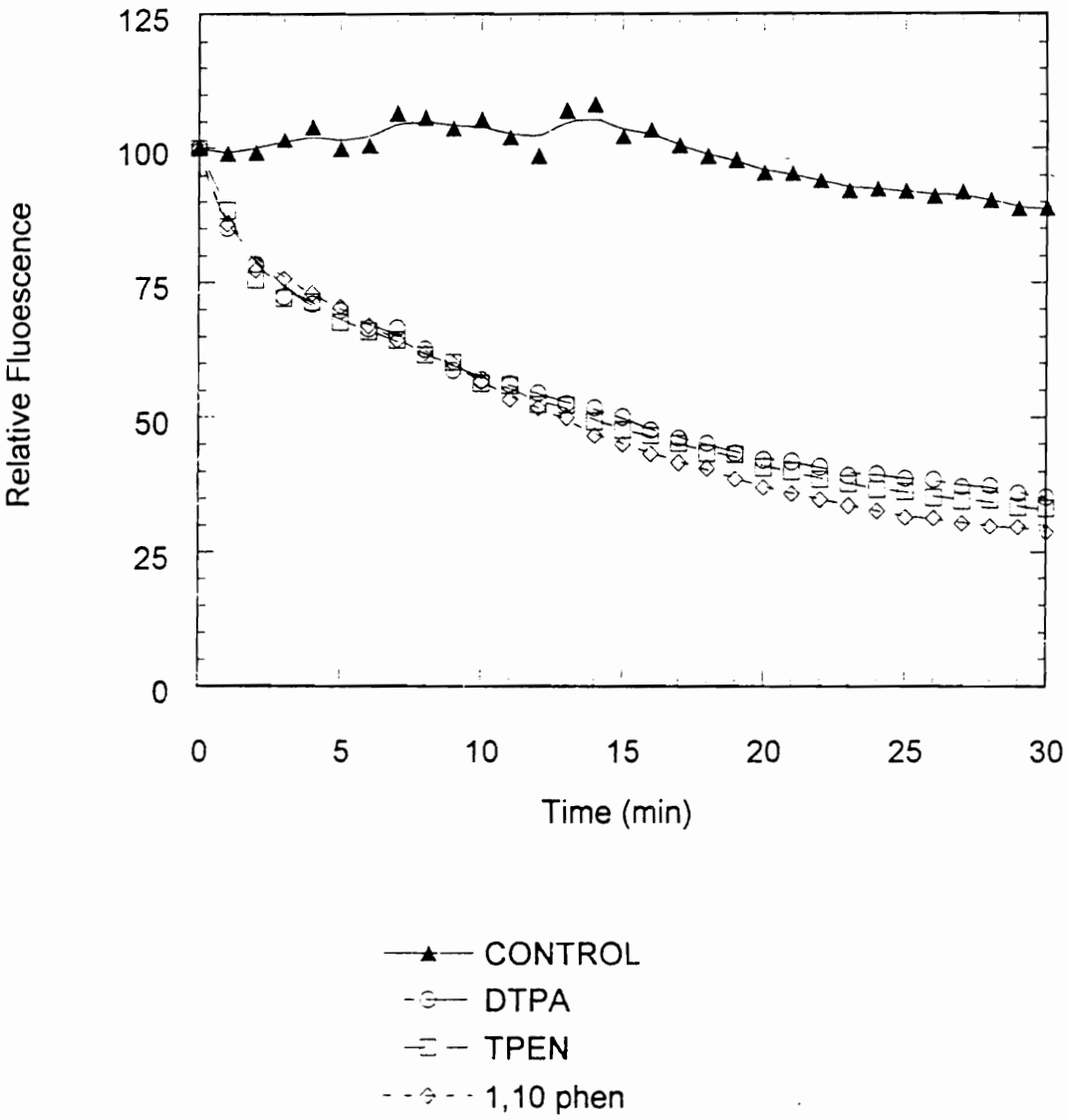


Figure 2-2 Effect of Chelators on TSQ-Zn^{2+} Fluorescence

TSQ (100μM) added to 10 μM zinc. After 1 minute 10 μM of chelator was added and fluorescence was monitored for 30 minutes. All three chelators tested were able to remove zinc from TSQ and dimish fluorescence accordingly. The control represents the fluroescence of TSQ-Zn^{2+} in the absence of chelator.

reduction was progressive and after a 30 minute incubation fluorescence was 35% of the TSQ-Zn^{2+} alone.

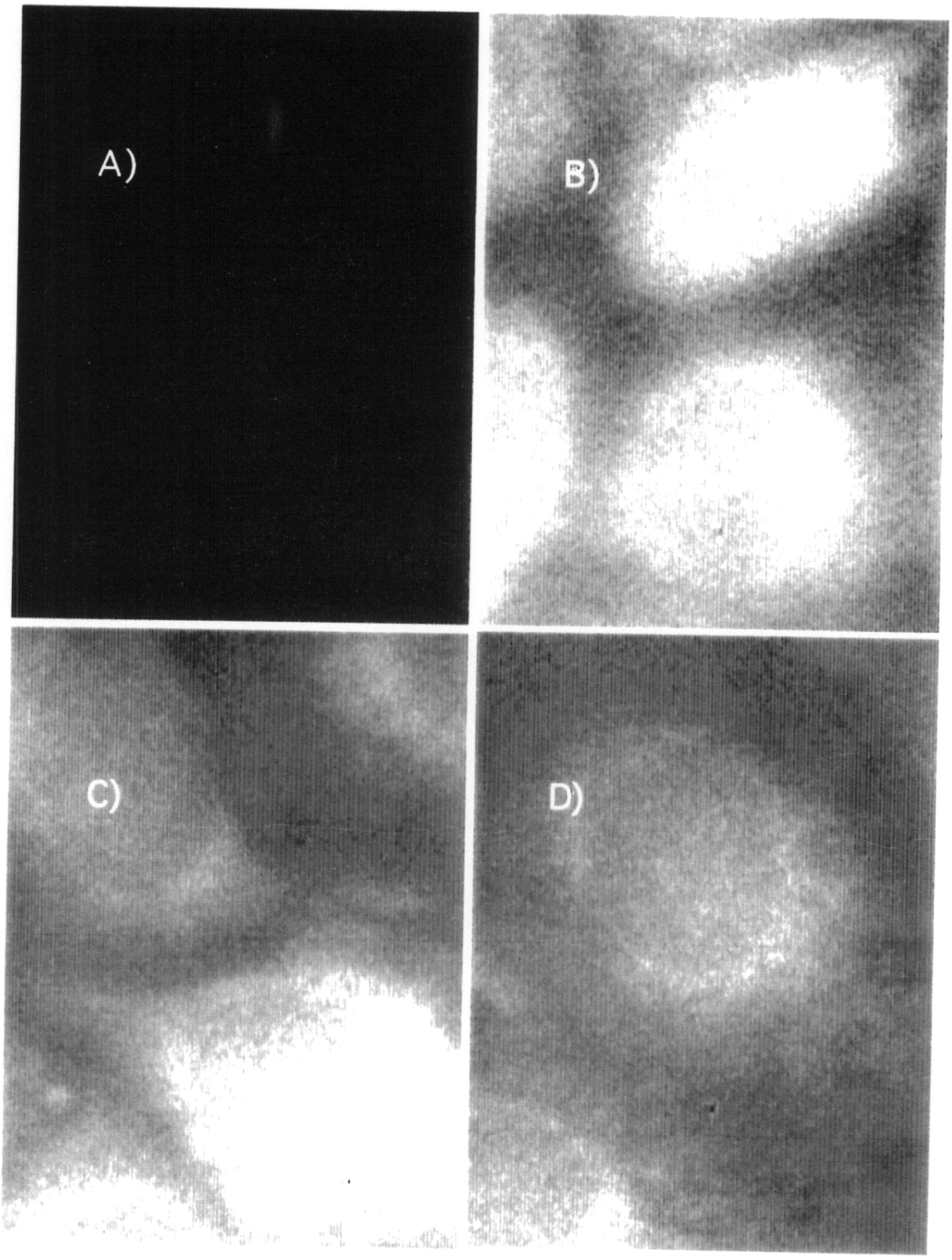
Effect of Chelators on Exchangeable and Total Zn^{2+} pools in HeLa cells

The effect of canadite Zn^{2+} chelators and TSQ on HeLa cell viability was determined. Three zinc chelators and TSQ were added to HeLa cells at concentrations of 10-600 μM and viability (dye exclusion) was monitored over 72 hours. TSQ itself at 100 μM was found not to effect viability (percent viable: 98%). DTPA at higher concentrations (600 μM) did have a slight effect, reducing viability by 25% after 12 hours exposure. At 10 μM concentration, no effect on cell viability was noted. The permeable chelators drastically effected viability at 10 μM concentration (49% viable) after only 4 hours incubation. By 12 hours all cells were dead. DTPA was used as the zinc chelator in all subsequent experiments.

To test if exchangeable zinc could be readily removed from HeLa cells, cultures were exposed to DTPA. After 2 hours incubation with 10 μM DTPA, a portion of the cells exhibited decreased TSQ-Zn^{2+} fluorescence. After 24 hour incubation cells were uniformly low in TSQ-Zn^{2+} fluorescence (Figure 2-3, 2-4). Quantitatively, TSQ fluorescence dropped

Figure 2-3 TSQ-Zn^{2+} Fluorescence in HeLa Cells

HeLa cells were exposed to TSQ (100 μM) after incubation in the absence (A, B) or presence (C, D) of DTPA (10μM). The backgound fluorescence of the cells (no TSQ) is shown (A). TSQ fluorescence is the absence of chelator is depicted (B). After 4 hours (C) incubation with DTPA, cells begin to exhibit decreased flourescence. After 24 hours (D), the cells are uniformly low in fluorecence.


A)
B)
C)
D)

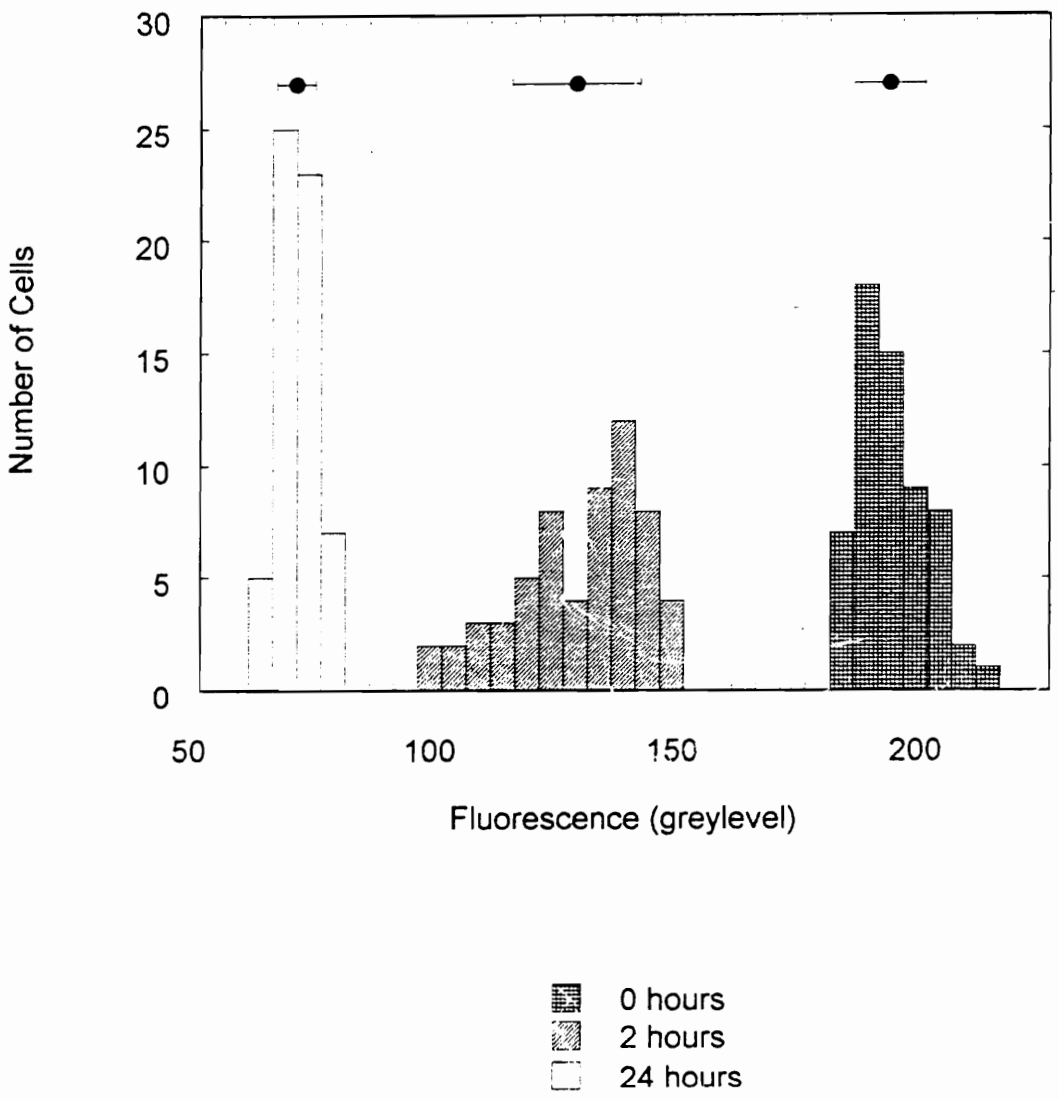


Figure 2-4 Effect of DTPA on TSQ-Zn^{2+} Fluorescence at Various Incubation Times

HeLa cells were treated with 10 μM DTPA for various times. Histograph depicts the TSQ (100μM) fluorescence intensities at 0, 2, and 24 hours after addition of DTPA. Fluorescence is expressed as greylevel (range 0-255). The means and standard deviations shown, represents the averages for each group of 60 cells illustrated in each section of the histograph.

by approximatly 40% during the first 2 hours incubation with DTPA and fell further over time until after 24 hours, fluorescence had dropped by 60% (Figure 2-5). In order to quantify the change in zinc, total zinc was determined through ICP analysis for cultures grown for 4 and 24 hours in the presence of various concentrations of DTPA (Figure 2-6). The normal zinc levels for HeLa cells was 32.02 pmol ±13.3. At 10 μM DTPA, total zinc levels were 14.26 pmol ± 2.375 (4hrs) and 4.265 pmol ± 0.9 (24 hrs). Based upon the DPTA dose-response curves little further reduction in zinc levels is expected with higher DTPA levels. Changes in exchangeable and total zibc in HeLa cells showed a similar dose-response to DTPA (figure 2-8). The reduction in both total and exchangable (TSQ-Zn^{2+} fluorescence) zinc levels is ~60% even after a few hours incubation with DTPA.

CONCLUSIONS

Through this research, it has been demonstrated that the use of the zinc specific molecular probe, TSQ, can accurately predict the amount of exchangeable zinc in a cell. By introducing a zinc specific chelator, it is possible to reduce the absolute amount of zinc (ICP) in HeLa cells by 83%, corresponding to a TSQ-Zn^{2+} fluorescence reduction of 65%.

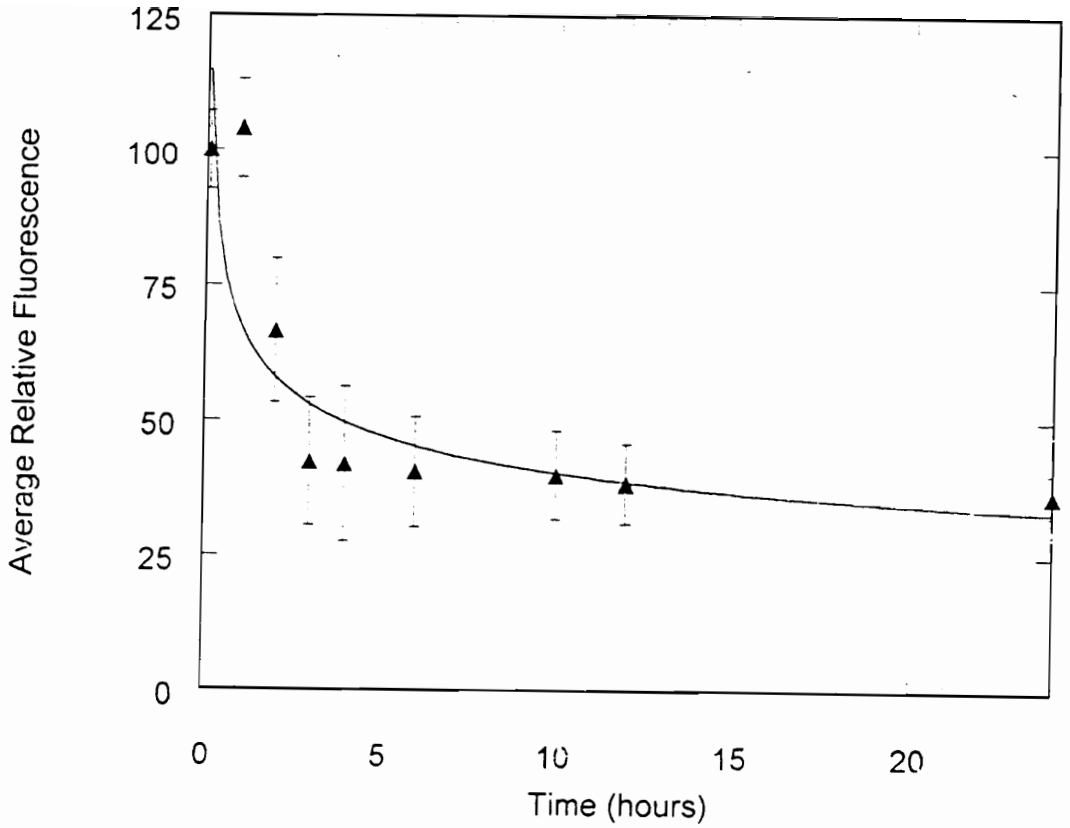


Figure 2-5 Average TSQ-Zn^{2+} Fluorescence in HeLa Cells After Exposure to DTPA

HeLa cells were exposed to DTPA (10 μM) up to 24 hours. After desired incubation, cells were exposed to TSQ as described under methods. The average cellular fluorescence is shown along with the standard deviations. Each point represents the average of 60 individual cells.

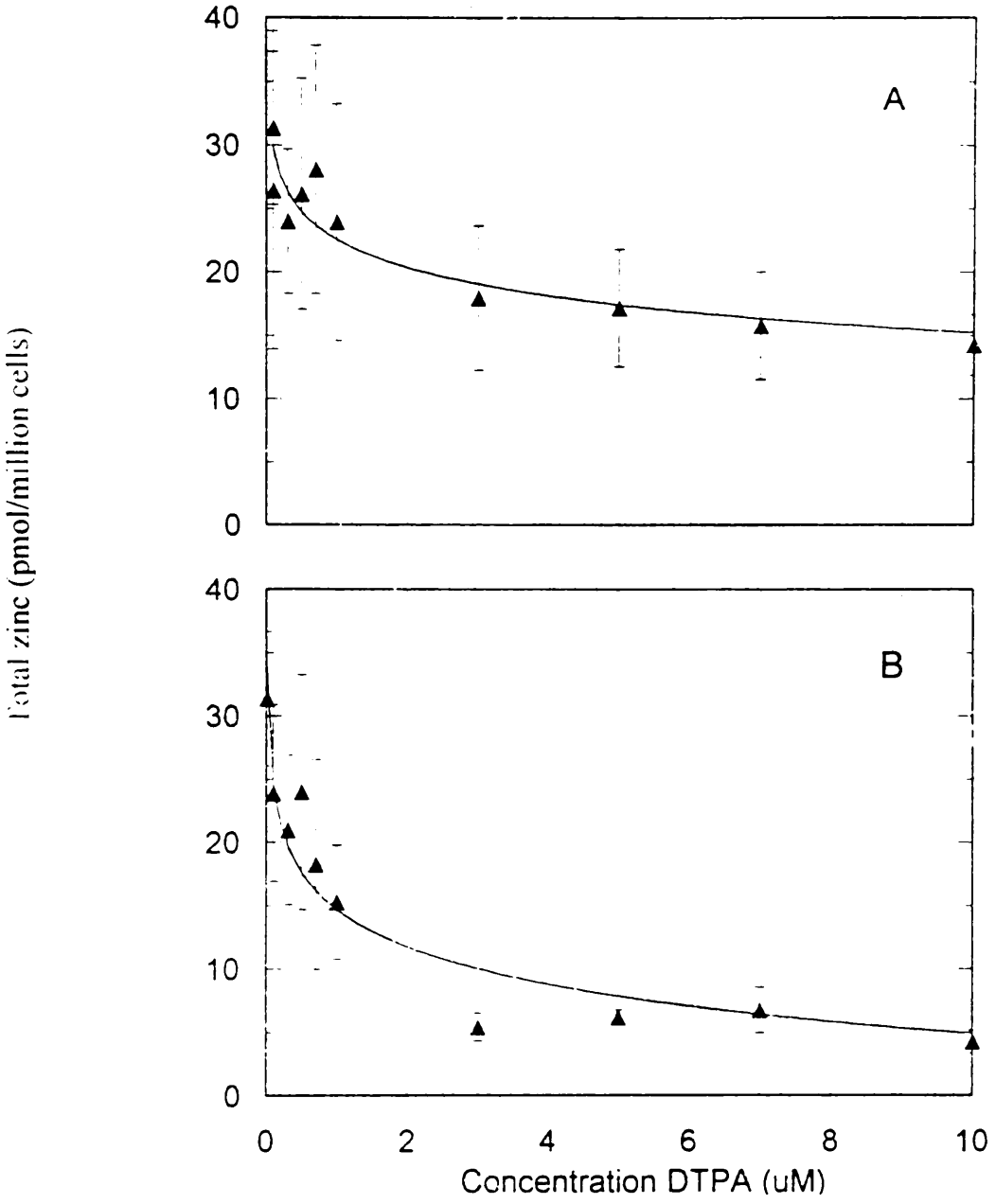


Figure 2-6 Total Zinc After DTPA Incubation

Total zinc was determined by ICP. Cells were treated with DTPA for 4 (A) or 24 (B) hours. The total zinc (pmol) per million cells is depicted along with the standard deviations. Each point represents the mean of 5 independent experiments.

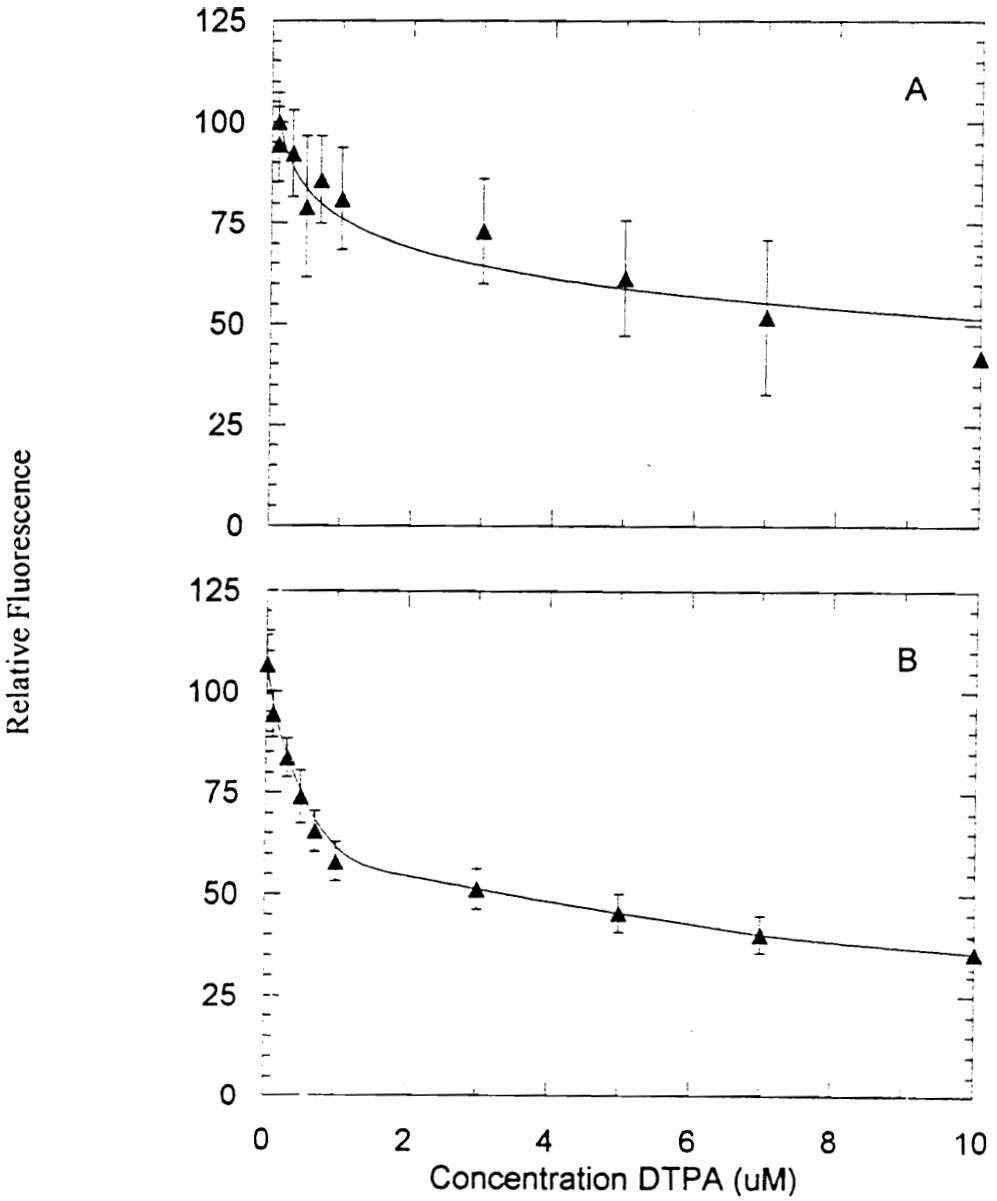


Figure 2-7 Cellular TSQ-Zn^{2+} Fluorescence

TSQ fluorescence depicts the amount of zinc that is available to react with the probe. Cells were treated with various concentrations of DTPA for 4 (A) or 24 (B) hours prior to TSQ exposure. Each point represents the mean of 60 individual cells along with the standard deviations.

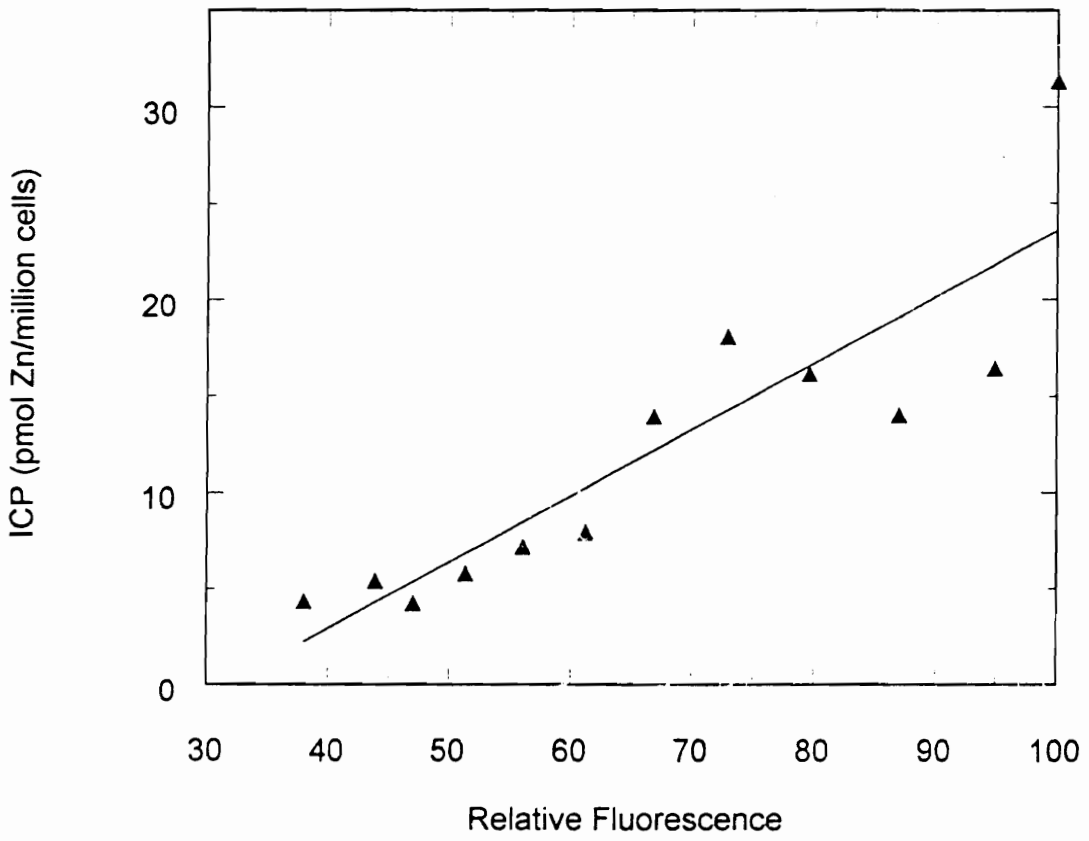


Figure 2-8 TSQ-Zn^{2+} Fluorescence vs ICP

A favorable coorolation between TSQ fluorescence and ICP shows that both are capable of predicting cellular zinc concentrations.

Reduction of cellular zinc is thought to be attributed to the turnover to zinc containing proteins and enzymes, therefore alleviating the necessity to overcome the binding constants of various proteins for zinc ions. It is suggested that through normal degradation of zinc containing enzymes and proteins, zinc ions are freed and thus can be removed by a thermodynamic flux created by the chelator.

The implications of being able to selectively remove zinc from cell cultures allows for an avenue to explore zinc dependent processes. Zinc deficiency causes numerous metabolic changes in plants and animals. In spite of the large majority of zinc metalloenzymes which have a very small dissociation constant (K_d<10^{-10} M), there is no clear evidence that the activity of any one declines to such an extent as to limit physiological function during nutritional deprivation of zinc (Karin et al, 1979, 1981; Kim and Wyckoff, 1989, Kimball et al, 1995). Two hypotheses for the physiologically limiting role of zinc have been postulated. O'Dell has suggested that zinc exerts its first limiting function at the level of the cell plasma membrane (O'Dell et al 1987). Zinc is an essential component of biomembranes and is necessary for maintenance of membrane structure and

function (Bettger and Taylor, 1986). Data indicating that high extracellular zinc levels stabilize membranes lead investigators to presume that a depression of the zinc concentration in extracellular fluid may cause adverse changes in the integrity of the cell plasma membrane (Bettger and O'Dell, 1981). This hypothesis is drawn from the observation of increased osmotic fragility and decreased zinc concentration in erythrocytes in zinc-deficient rats, (Bettger and O'Dell, 1981; Bettger and Taylor, 1986; O'Dell et al, 1987).

Another hypothesis is that zinc's critical role is involved in gene expression. The discovery of the zinc finger by Klug in 1985 as a DNA binding motif in a transcription factor protein (TFIIIA) provided a molecular role for zinc in gene expression (Miller et al, 1985). Since 1985, more than 500 proteins functioning in replication and/or transcription control in viruses, bacteria, yeast, and mammals, have been shown or are suspected to contain metal binding sites in DNA binding domains based on primary structures (Vallee and Falchuk, 1993). The presence of the zinc finger as a highly conserved motif within the DNA binding domain of many transcription factors, including the steroid receptors and numerous others

involved in growth and development, provides new insight into the function of this metal in cellular systems. Constitutive zinc metalloproteins involved in transcription and replication are relatively stable. A small reduction of these enzymes cannot account for the severely compromised metabolic functions seen in zinc deprived animals. Chesters postulated that the effects of zinc deficiency could not be explained solely by the decline of a few enzymes, but rather closely resembled a failure of individual cells to synthesize fresh groups of enzymes (Chesters et al, 1989).

In conclusion, the method proposed allows selective removal of zinc from HeLa cell cultures. This Zn-deficient state may be useful in studying the role of zinc in cellular metabolism. By reducing the amount of cellular zinc available to bind with components in the cell, it may be possible to determine the effects of specific proteins and or enzymes that rely on zinc for structure or function (Abbasi et al, 1980; Archer et al, 1990; Freedman, 1992).

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Effect of Zinc in Glucocorticoid Receptor Mediated Induction

Abstract:

The effect of cellular zinc concentrations on glucocorticoid induced gene expression was examined. Implementation of the zinc specific impermeable chelator, diethylenetriaminepentaacetic acid (DTPA), removed 83% of cellular zinc. By examining the expression of a chloramphenicol acetyltransferase (CAT) reporter gene under the control of a glucocorticoid response element (GRE), it was determined that expression was reduced 88% during times of low zinc. Constitutive expression was only reduced by 15% under similar zinc depletion. Furthermore, neither the binding affinities of receptors for dexamethasone nor translocation of receptors were altered by reduced zinc concentrations. However, the total receptor pool was decreased by 50% and reduction in receptor aggregation within the nucleus was noted during reductions of cellular zinc. This reduction in receptor number may be sufficient to produce the decreased gene induction observed.

Introduction:

Many of the clinical manifestations of zinc deficiency may be explained by alteration in hormone responses. Steroid hormones regulate a number of developmental and physiological processes that are affected during zinc deprivation by specifically controlling the transcriptional activity of genes in target tissues (Beato, 1989). Zinc has also been shown to be indispensable for the efficient transduction of these hormone responses (Vallee, 1993). Investigation of how zinc deficiency effects steroid mediated responses can lead to a greater understanding in the diagnosis of zinc deficiencies and potential complications in their treatment.

The steroid superfamily consists of ligand-activated transcription factors (Danielson et al, 1989; Krust et al, 1986). Included in this group are glucocorticoids, progesterone, estrogen, aldosterone, androgen, hormonal forms of vitamins A and D, thyroid hormones, peroxisomal activators and others yet to be identified (Freedman, 1992). They can exhibit either positive or negative control over their target genes.

The ability of target cells to respond to glucocorticoids requires the presence of specific receptors which mediate hormone action within cells

(Kothekar, 1992; Ray et al, 1991). In the inactive state, these receptors exist within the cell in association with other proteins, e.g. Hsp 90 (Alexis et al, 1992; Bodine and Litwack, 1990, Diehel and Schmidt, 1993; Renoir et al, 1990). Upon hormone binding, the hormone-receptor complex disassociates from hsp 90 (Hard et al, 1990; Baumann et al, 1993). The activated receptor (bound to ligand) translocates into the nucleus and binds as a dimer to specific DNA sequences, termed glucocorticoid response elements (GRE) (Kothekar, 1992; Ray et al, 1991).

During cellular zinc deficiency, steroid hormone responses are decreased (Bunce et al, 1994). Previous studies in our laboratory have shown that responses directly regulated by estrogen receptors are decreased 30-35% during times of low zinc (Bunce et al, 1994; Dylewski et al, 1986). The discovery of the zinc finger in its various forms as a highly conserved DNA binding motif in the steroid receptor family, and within numerous transcription factors active in embryogenesis, growth and differentiation provides a possible molecular role for zinc in steroid induced gene expression. We have proposed that even modest deficits of cellular zinc

might seriously affect growth and development through decreased hormone responses (Bunce, 1994).

These studies attempt to uncover explanations for zinc dependence in hormone-mediated responses by measuring the glucocorticoid response, receptor number, translocation, and hormone binding under conditions of zinc deficiency. A zinc deficient state was created in HeLa S3 cells through the use of a cell impermeable zinc specific chelator, diethylenetriaminepentaacetic acid (DTPA) where approximately 80% of cellular zinc was removed. Expression of a transfected glucocorticoid-sensitive CAT reporter plasmid (pGMCS) was reduced 88% in zinc deficient cells. However, expression from a transfected glucocorticoid-insensitive plasmid (pBLCAT2) was only reduced 15%. Analysis of receptor pools, showed that receptor concentration was reduced 50%, but translocation and steroid binding affinities were unaltered by changes in cellular zinc concentration. These results should be useful in gaining a better understanding for zinc's role in steroid induced gene expression.

Materials and Methods:

Cell Culture:

HeLa S3 cells were grown as monolayer cultures in a 5% CO_2 humidified atmosphere in Jokliks minimal essential medium (JMEM) supplemented with 2 mM glutamine and 10% supplemented bovine serum (SBS). Harvesting of cells was accomplished by removing the medium and incubating with versene (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 0.5 mM EDTA, 8 mM Na_2HPO_4) for 10 minutes. The cells were then replated at a 1:10 dilution. Where indicated, cells were propagated in JMEM containing diethylenetriaminepentaacetic acid (DTPA) to create a zinc deficient state, and dexamethasone (DEX) (Simon). The concentrations were 10 μM and 1 μM, respectively, unless otherwise noted.

Recombinant Plasmids:

Plasmid pGMCS, which contains the chloramphenicol acetyltransferase (CAT) reporter gene fused downstream of the mouse mammary tumor virus promoter and glucocorticoid regulatory element (GRE) and upstream of the murine sarcoma virus GRE, was the generous

gift of Dr. John Cidlowski (NIEHS, Research Triangle Park, NC) (Allgood et al, 1991, 1993). Plasmid pBLCAT2, obtained from Dr. John Cidlowski, contains a thymidine kinase promoter from herpes simplex virus fused to a CAT reporter gene (Allgood et al, 1991, 1993).

Cell Transfections:

Twenty-four hours prior to transfection, cells were replated at 1 x 10^6 cells/100 mm tissue culture dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% supplemented bovine serum (SBS). Transfections were accomplished using Transfectam™ reagent (Promega, Corp.). For transfections, cells were washed three times with DMEM without SBS and covered with 2 mL of media only. Transfectam reagent (10μL) and plasmid DNA (5 μg) were each added to 1 mL serum free DMEM and this mixture added directly to the cells. After 4 hours the medium was removed and replaced with DMEM containing 10% SBS and incubated 24 hours. At this point, the medium was removed and replaced with JMEM medium either containing or lacking steroid and/or chelator as indicated in the appropriate figure legends.

Analysis of CAT activity:

HeLa S3 cells are washed in sterile PBS and respended in 1 mL of PBS. The cells are pelleted and lysed by repetitive freezing and thawing. The extracts were then quantified for protein amounts, and 100 μL of extract was placed with 3μL ^{14}C-chloramphenicol (0.15 μCi) and 5 μL n-butyrl CoA, and 17 μL of 0.25M Tris-HCl, pH 8.0. The reaction mixture was incubated at 37°C for 6 hours. The reaction was terminated by addition of mixed xylenes. Back extractions were performed to increase sensitivity. The amount of butyrylated ^{14}C-chloramphenicol (CAM) was determined through the use of a liquid scintillation counter.

Quantitation of Receptor Pool:

The amount of receptor was determined through western blot analysis. HeLa S3 cells were harvested in 1 mM EDTA, 10 mM Tris, 0°C. The cells were disrupted with a Polytron tissue grinder. The lysates were centrifuged at 11,000 rpm for 15 minutes to obtain a nuclear fraction. The pellet was then resuspended and both fractions are centrifuged at 100,000 x g for 1 hour at 0°C. The samples were microconcentrated 3-5 fold in a Microcon 30 microconcentrator. Equal amounts of protein, as determined

by Lowry method, were added to 2x loading buffer (20% glycerol, 4.6% SDS, 0.125 M Tris, pH 6.8), heated at 100 °C for 2.5 minutes, and kept at -70 °C until electrophoresis.. The samples are electrophoresed on a 1.5 mm SDS polyacrylamide gel (8%) with a 3.5% stacking gel. The proteins were electrophoretically transferred to Immobilon™ (Millipore) membrane using a semi-dry apparatus. The resulting blots were blocked in 5% casein for 2 hours, washed in TBS containing 0.1 % Tween and 5% dry milk, and incubated overnight at 4°C with a 1:100 dilution of rabbit anti human glucocorticoid receptor antibody (Affinity Bio Reagents) in nonfat dry milk buffer. The membranes were subsequently washed in TBS-Tween containing 5 % non-fat dry milk 3 times for 10 minutes each. After washing blots were incubated for 2 hours at room temperature with a 1:5000 dilution of phosphatase-conjugated goat anti rabbit antibody in 5% milk buffer. Three subsequent washes in the TBS-Tween, dry milk buffer for 15 minutes were employed (Cidlowski et al, 1990). The phosphatase color reaction was developed by incubation of the blot in nitro blue tetrazoluim and 5 bromo-4 chloro-3 indoyl phosphate (BCIP). The colorimetric reaction was stopped by immersion of the blot in H_2O.

Steroid Binding Affinities:

HeLa S3 cells were harvested as described under cell culture. The cells were then pelleted and resuspended in 1 mL 10 mM Tris, 1 mM EDTA, 12 mM α-thioglycerol, 20 mM sodium molybdate at 4°C. The cells were disrupted with a polytron tissue grinder and centrifuged at 100,000 x g for 1 hour at 0°C. Aliquots (50-100 μL) containing equal protein, as determined be Lowry, were removed to precooled tubes containing [^{3}H]-dexamethasone at concentrations ranging from 1-400 nM. The samples were incubated 2 hours at 4°C. An aliquot was removed for quantitation of total steroid concentration. The remainder was treated with an equal volume of dextran-coated charcoal for 5 minutes at 4°C. The charcoal was pelleted by centrifugation at 11,000 rpm at 4°C. The amount of radioactive steroid remaining in the supernatant was quantified and the results are analyzed by a Scatchard plot to determine binding affinity (Scatchard, 1949).

Nuclear localization of receptors:

Coverslip cultures of HeLa S3 cells were rinsed three times, 5 min each in PBS. The cells were fixed in methanol at -20° C for 4 minutes. The coverslips were then washed three times for 5 minute in PBS. Steroid receptors were localized by incubation with 1:50 dilution of rabbit anti-glucocorticoid receptor primary antibodies for 20 minutes, followed by washes and 20 minute incubation with a 1:100 dilution of a fluorescein conjugated goat anti rabbit secondary antibody. Coverslips were mounted and fluorescence was visualized with a Zeiss IM 35 inverted microscope equipped with a 450 excitation filter, 510 dichroic, and a 560 emission filter. Images were integrated with a Cohu 4910 CCD camera for 4-10 frames and transferred to a Macintosh computer with a Scion LG-3 framegrabber. Analysis was with IPLab Spectrum 3.0 for Macintosh computers.

Results:

Steroid hormone responses are curtailed during times of low zinc (Bunce, 1994). The mechanism for zinc dependence is still unknown. To address this issue, we have introduced a glucocorticoid-responsive CAT reporter plasmid, pGMCS, into HeLa S3 cells. This is a stable cell culture line that has high affinity saturable receptors for the glucocorticoid hormones, but lacks receptors for other steroid hormones (Allgood, 1990, Melnkovych 1969, 1979). Earlier studies have shown that this system is not induced by estrogen, progesterone, or androgen (Allgood 1990). Although progestins and androgens have been reported to regulate the expression of reporter genes that are under control of DNA sequences derived from the mouse mammary tumor virus genome (Cato 1988, Ham 1988), the ineffectiveness of these steroid hormones in HeLa cells containing pGMCS, most likely reflects the absence of their respective receptors (Allgood 1990). These cells, when transiently transfected with the plasmid pGMCS, respond specifically to administration of the synthetic glucocorticoid dexamethasone with an increase in CAT activity. The effect

of zinc on the induction of glucocorticoid induced gene expression was examined.

The treatment of transfected HeLa cells with dexamethasone (1 μM) resulted in the characteristic appearance of CAT activity (Table 3-1). To determine if the glucocorticoid receptor is required for induction of CAT activity in transfected HeLa cells, the effect of the GR antagonist RU 486 was examined. Previous studies have shown this competitive antagonist inhibits the glucocorticoid-mediated induction of tyrosine aminotransferase and tryptophan oxygenase (Allgood, 1990; Liu et al, 1995) as well as the glucocorticoid-stimulated transcription of mouse mammary tumor virus (MMTV) RNA (Allgood, 1990). The addition of a glucocorticoid antagonist (RU 486, 1 μM) to dexamethasone treated cells inhibited glucocorticoid mediated transcription of MMTV RNA (Table 3-1), thus, showing that the response is mediated by the glucocorticoid receptor.

Zinc is known to be necessary for the timely and effective induction of steroid induced genes. The ion is thought to play a role in maintaining the structural integrity of the DNA binding domain in the receptors in order to effectively bind the GRE. By decreasing the available zinc in HeLa

Table 3-1 Plasmid Responses to Zinc Concentration and Dexamethasone

Treatment	Percent Conversion of CAM to Acetylated Form (%) Plasmid	
	pGMCS	pBLCAT2
Control (no addition)	0.97 ± 0.012	41.13 ± 3.03
+ 1 μM dexamethasone	14.11 ± 2.16	43.0 ± 3.14
+ 10 μM DTPA, 1 μM dexamethasone	1.7 ± 0.012	35.56 ± 2.65
+ 10 μM DTPA, 1 μM dexamethasone, 50 μM Zn^{2+}	14.02 ± 1.06	42.89 ± 3.12
+ 10 μM DTPA	0.68 ± 0.045	35.3 ± 2.84
+ 1 μM RU 486, 1μM dexamethasone	1.86 ± 0.097	42.01 ± 2.95

The results are expressed as percentage conversion of chloramphenicol to its butylated forms by chloramphenicol acetyltransferase. RU 486 is a glucocorticoid antagonist. All data represents the average of 5 independent experiments along with the standard deviations.

cells, we examined the effect on glucocorticoid receptor induced gene expression (Table 3-1). Lowering cellular zinc concentrations by 80% causes a decrease CAT activity of 88% when compared with control cells. When 50 μM zinc was added along with the DTPA, the CAT activity levels were equivalent to control values. Thus, the loss of induction was zinc specific. The effects of dexamethasone and zinc on constitutive gene expression were also examined (Table 3-1). Expression from pBLCAT showed no change in response to dexamethasone, however a 88% reduction in cellular zinc caused a 15% decrease in CAT activity. This decrease is not unexpected, since reductions in zinc have been shown to decrease the activities of zinc containing enzymes responsible for transcription. The small loss of constitutive expression does not explain the substantial drop in glucocorticoid induced expression. Rather the results demonstrate that the glucocorticoid response is more sensitive to zinc levels that the constitutive response.

In order to determine the least amount of zinc deprivation necessary to alter glucocorticoid-directed responses, HeLa cells transfected with pGMCS were exposed to increasing concentrations of DTPA, then

challenged with dexamethasone (Figure 3-1). Removal of 20% of the total zinc had little effect of CAT reporter gene activity. With further reduction of cellular zinc (>25%), CAT activity decreased rapidly. Depletion of 50% of cellular zinc caused a decline in CAT activity of 75-80%. Higher concentrations of DTPA did not effect the remaining 20% of CAT activity. Thus it appears that reductions of less than 25% of zinc concentrations do not drastically alter glucocorticoid steroid responses, however further removal of zinc causes a rapid decline in glucocorticoid responses.

A decline in the glucocorticoid response could result from a reduction in receptor pool, hormone binding, translocation, or GRE binding. We first investigated effects on the receptor pool. In order for the timely induction of GR-mediated genes, there must be a functionally active pool of receptors. After treatment with 10 μM DTPA for 24 hours, western blot analysis showed an obvious reduction in receptor concentration when compared with untreated cells (Figure 3-2). Further analysis with [^{3}H] dexamethasone binding quantified this reduction. A decrease of approximately 50% of the receptor concentration was observed. In control experiments the addition of 50 μM zinc to cell cultures reversed the effects of the chelator on receptor

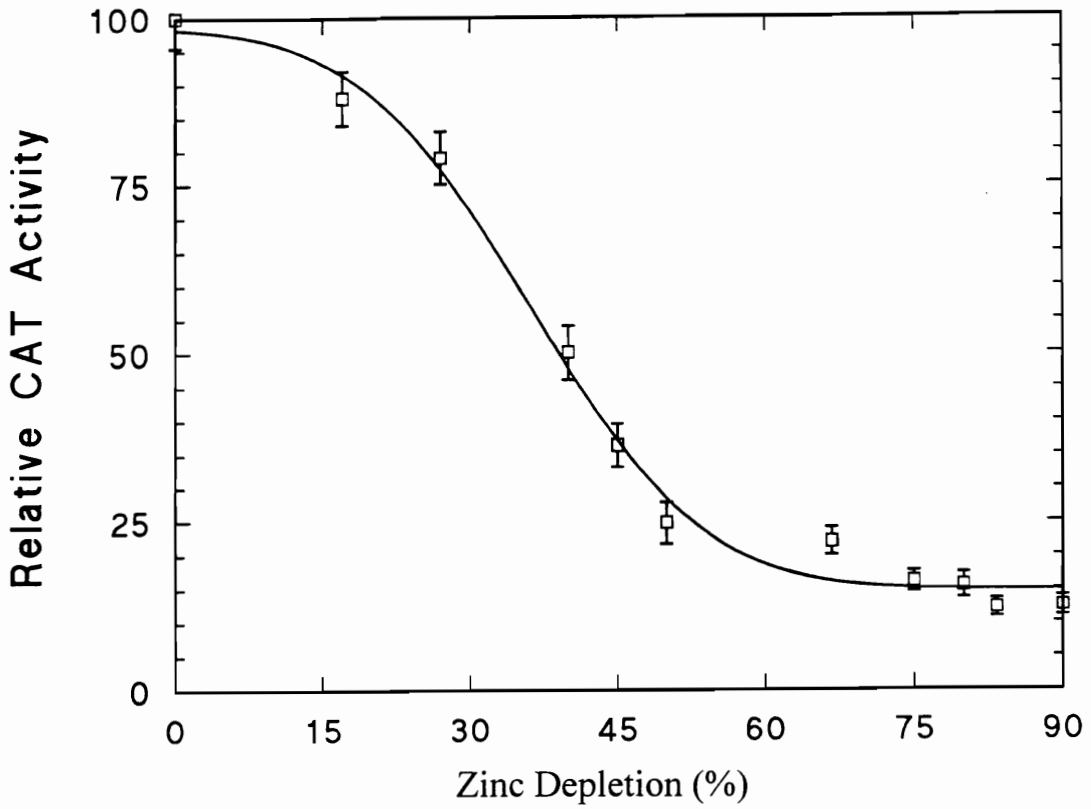


Figure 3-1 Reduction of CAT Activity by DTPA

HeLa cells transfected with pGMCS were exposed to increasing concentrations of DTPA, then challenged with dexamethasone. pGMCS responds with increased CAT activity upon administration of dexamethasone (1 μM). The reduction of activity as a result of decreased zinc, suggests that zinc is critical for effective glucoricoid mediated induction. The substanial decrease in activity with small decreases of cellular zinc suggests that GR-sensitive responses are curtails even during mild zinc deficiency.

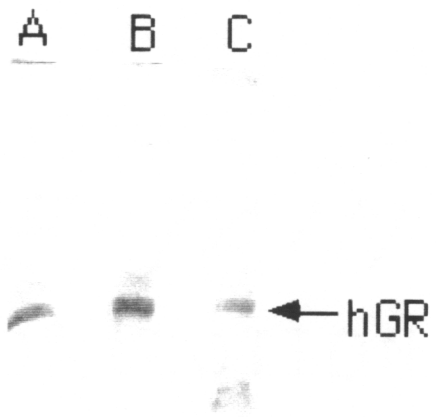


Figure 3-2 Effect of zinc status on glucocorticoid receptor concentration

Western blotting was used to determine the relative amounts of glucocorticoid receptors in HeLa cells. Lane A shows the migration of human glucocorticoid receptors. While the amount of receptors in control cells and those treated with 10 μM DTPA for 24 hours are shown in lanes B and C respectively. The amount of receptors contained in zinc deficient cells is noticeably less then that of control cells.

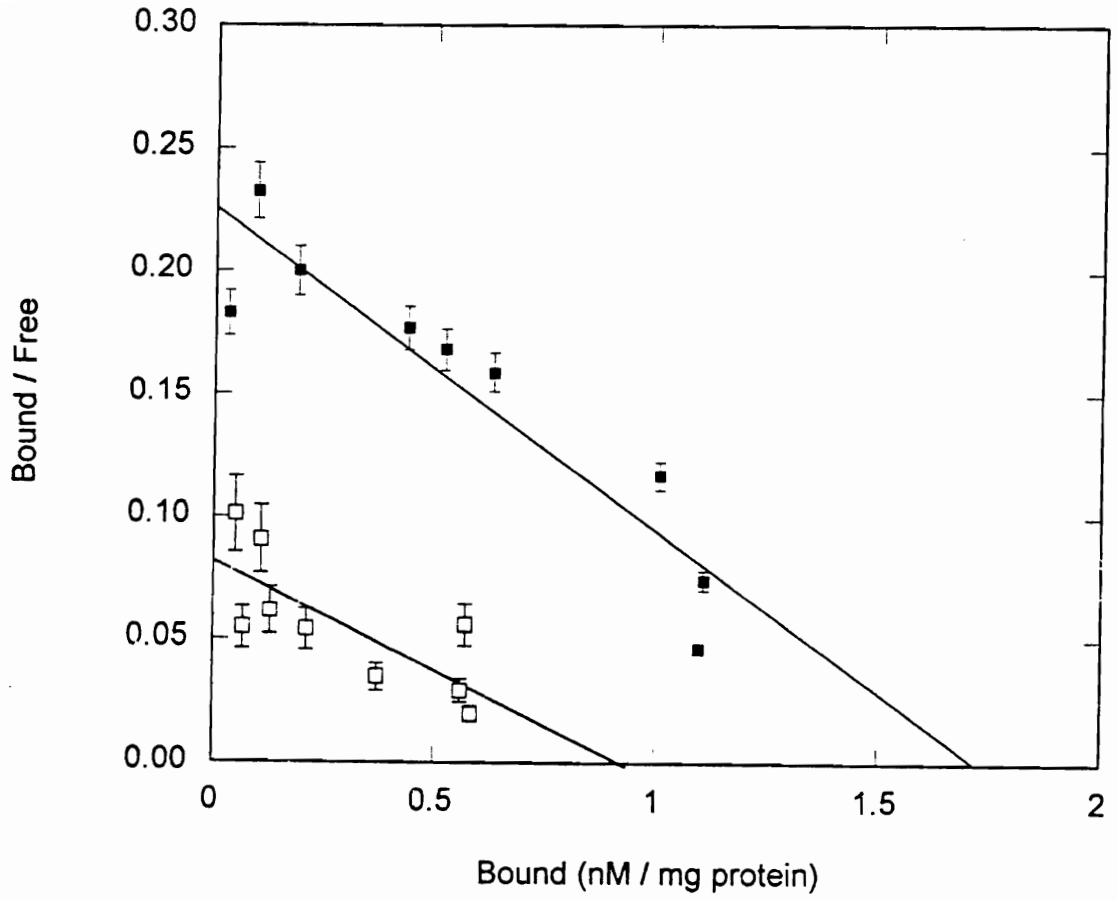


Figure 3-3 Effect of Zinc Concentrations on Binding Affinities

Cytosols were prepared from HeLa cells after 24 hour culture in control medium (■) or medium supplemented with 10 μM DTPA (□). Aliquots of extracts were incubated with [^{3}H] dexamethasone at concentrations ranging from 0-400 nM. Unliganded steroid was absorbed with dextran-coated charcoal, and bound ligand quantitated by scintillation counting. Binding data were analyzed by the method of Scatchard (Scatchard, 1949). The experiments shown are averages, along with standard deviations, of four independent experiments.

Table 3-2 Steroid Receptor Pool

Treatment	Receptors (fmol/mg protein)
control (no addition)	492 ± 9.04
+ 10 μM DTPA	206 ± 6.27
+ 10 μM DTPA, 50 μM Zn^{2+}	426 ± 12.62
+ 10 μM DTPA, 50 μM Zn^{2+} (post extreaction)	261 ± 8.60

These results were computed by Scatchard amalysis. The amount of receptor is proportional to the y-intercept of the plot. Each point represents the average of 4 independent experiments.

number, while addition of zinc to samples post extraction had no effect (Figure 3-3, Table 3-2). The results of post extraction addition of zinc demonstrate that the apparent loss of receptors is not attributable to alterations in conformation that effects steroid binding upon reduced zinc. If apoproteins lacking proper secondary conformations effecting steroid binding were present, addition of zinc to cell extracts may have corrected these defects. The reduction is thought to be attributed to either reduction in the GR gene transcription, its mRNA stability, or changes in receptor turnover. Further studies are ongoing to determine the exact cause.

To examine the steroid-binding function of these receptors, equilibrium hormone binding studies were conducted. The binding capacity of glucocorticoid receptors derived from cells of unaltered intracellular zinc concentrations exhibited high affinity dexamthasone binding, with a dissociation constant of 7.6 nM (Figure 3-3, Table 3-2). This value is consistent with previously reported dissociation constants for dexamethasone binding in HeLa cells (Allgood, 1990; Cidlowski and Cidlowski, 1981). When cells were treated with 10 μM DTPA, the binding affinity for dexamethasone, 9.7 nM, was not significantly different from

Table 3-3 Steroid Receptor Binding Affinities

Treatment	Binding Affinity (K_d, nM)
control	6.9 ± 0.37
+ 10 μM DTPA	7.8 ± 0.33
+ 10 μM DTPA, 50 μM Zn^{2+}	5.9 ± 0.45
+ 10 μM DTPA, 50 μM Zn^{2+} (post extreaction)	8.5 ± 0.50

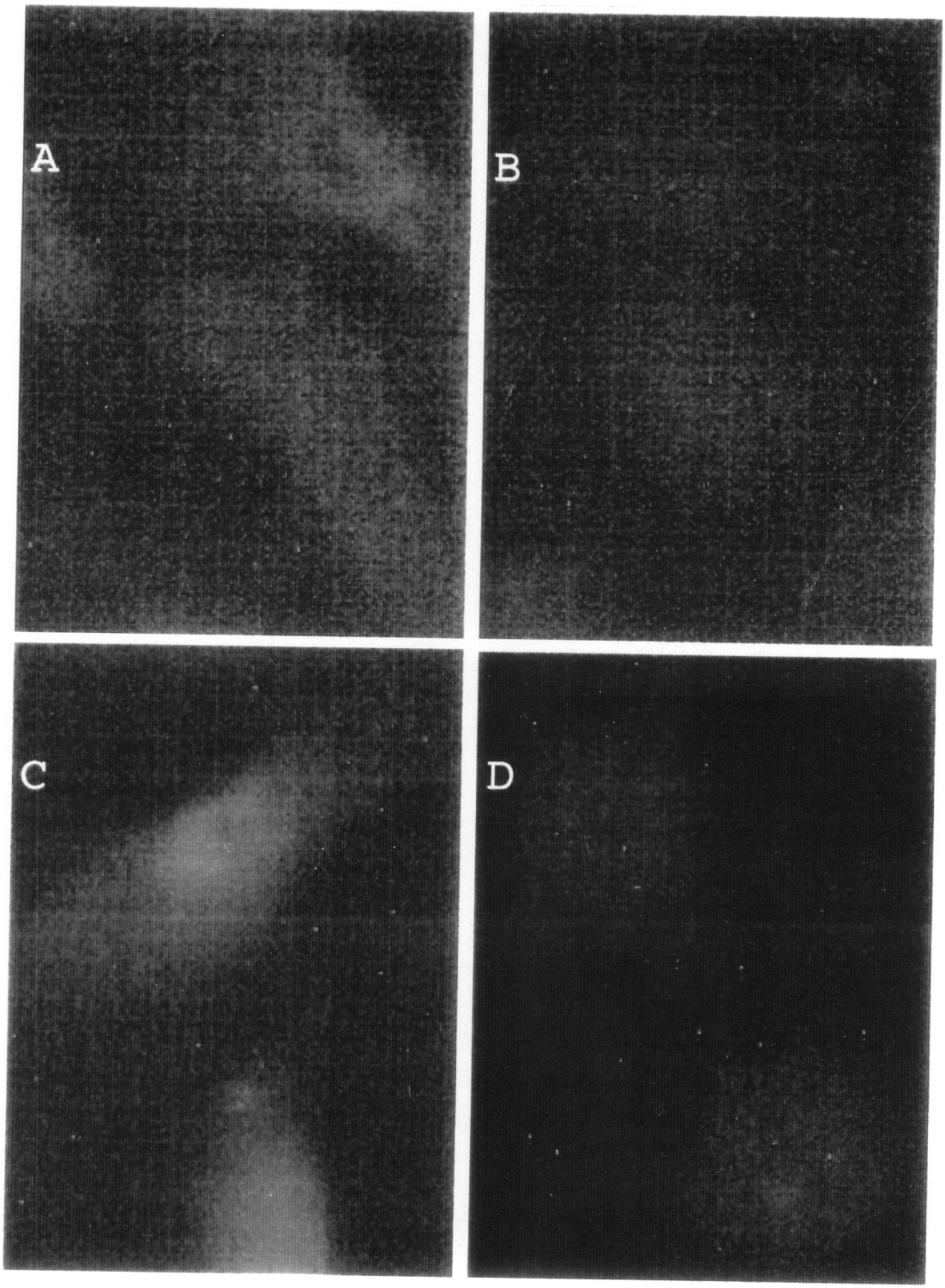
These results were computed by Scatchard analysis. The binding affinities are calculated from the slopes of each scatachard plot. Each point represents the average of 4 independent experiments.

control values. Thus, the effect on glucocorticoid-induced CAT activity is not accounted for by alteration in the ligand binding affinities.

In order for efficient induction the glucocorticoid receptor, after binding ligand, must translocate to the nucleus. Effects of zinc on translocation were examined. Upon exposure to dexamethasone, functionalreceptors will bind ligand and translocate into the nucleus. By using immunolocalization, this migration can be quantified through examination of nuclear and cytosolic staining before and after hormone exposure. Although the receptor pool was decreased during times of low zinc, nuclear localization appears to be zinc independent (Figures 3-4, 3-5). After 30 minutes exposure to dexamethasone, increases in nuclear staining were seen. In control cells a 1.6 fold increase in nuclear/cytosol fluorescence ratios was observed. Cultures exposed to 10 μM DTPA for 24 hours, exhibited a lower fluorescence due to the lowered receptor number, but the ratios of nuclear to cytosolic fluorescence shows definite migration into the nucleus (Figure 3-5). Again, these results were zinc specific, since addition of 50 μM zinc completely reversed the effects of DTPA.

Figure 3-4 Nuclear Localization of Receptors

HeLa cell were stained for receptors after 30 minute exposure to dexamethasone (1μM) using immunodection procedures described under methods. Cells were incubated in the absence (A, C) or presence (B, D) of DTPA (10 μM). The migrationof receptors is represented by the increased fluorescence in the nucleua (C). Although the fluorescence intensity is decreased in the presence of DTPA, translocation still occurs (D). The relative drop in fluorescence is indicative of a reduction in receptor number.


A
B
C
D

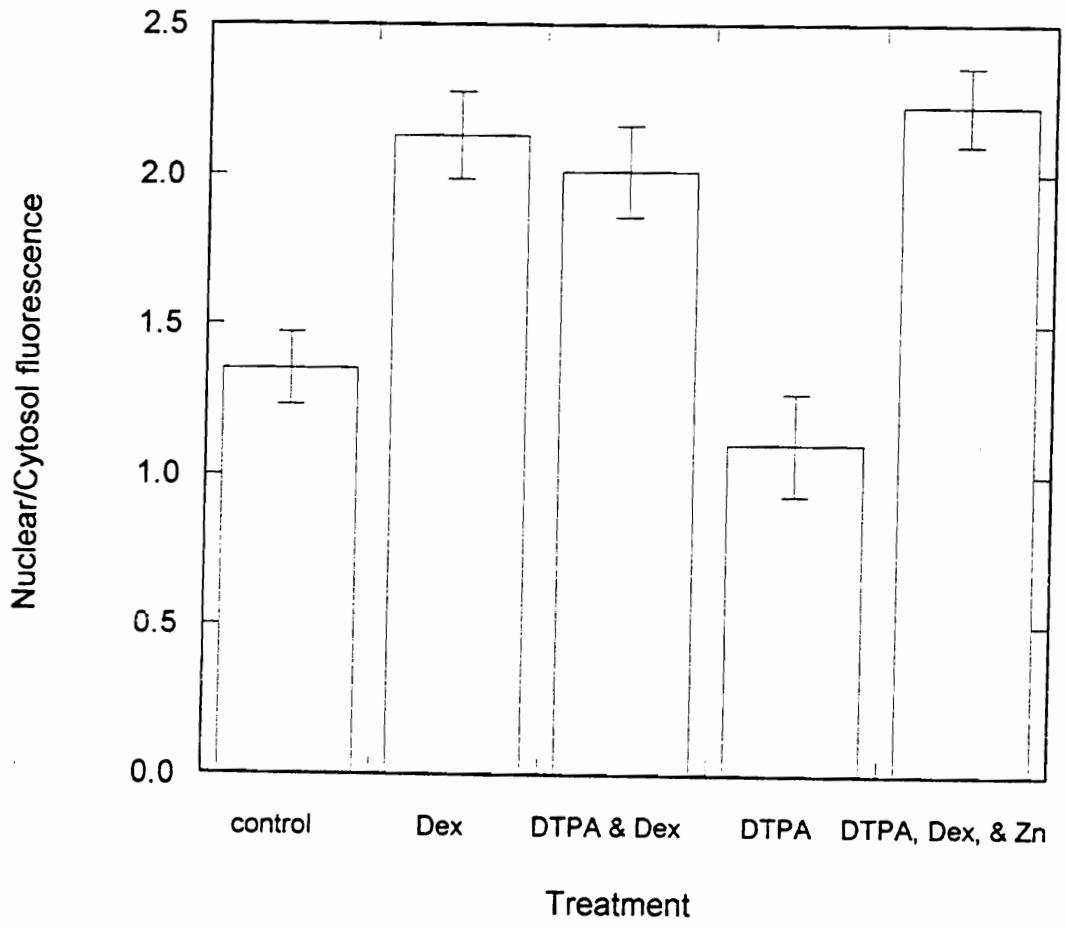
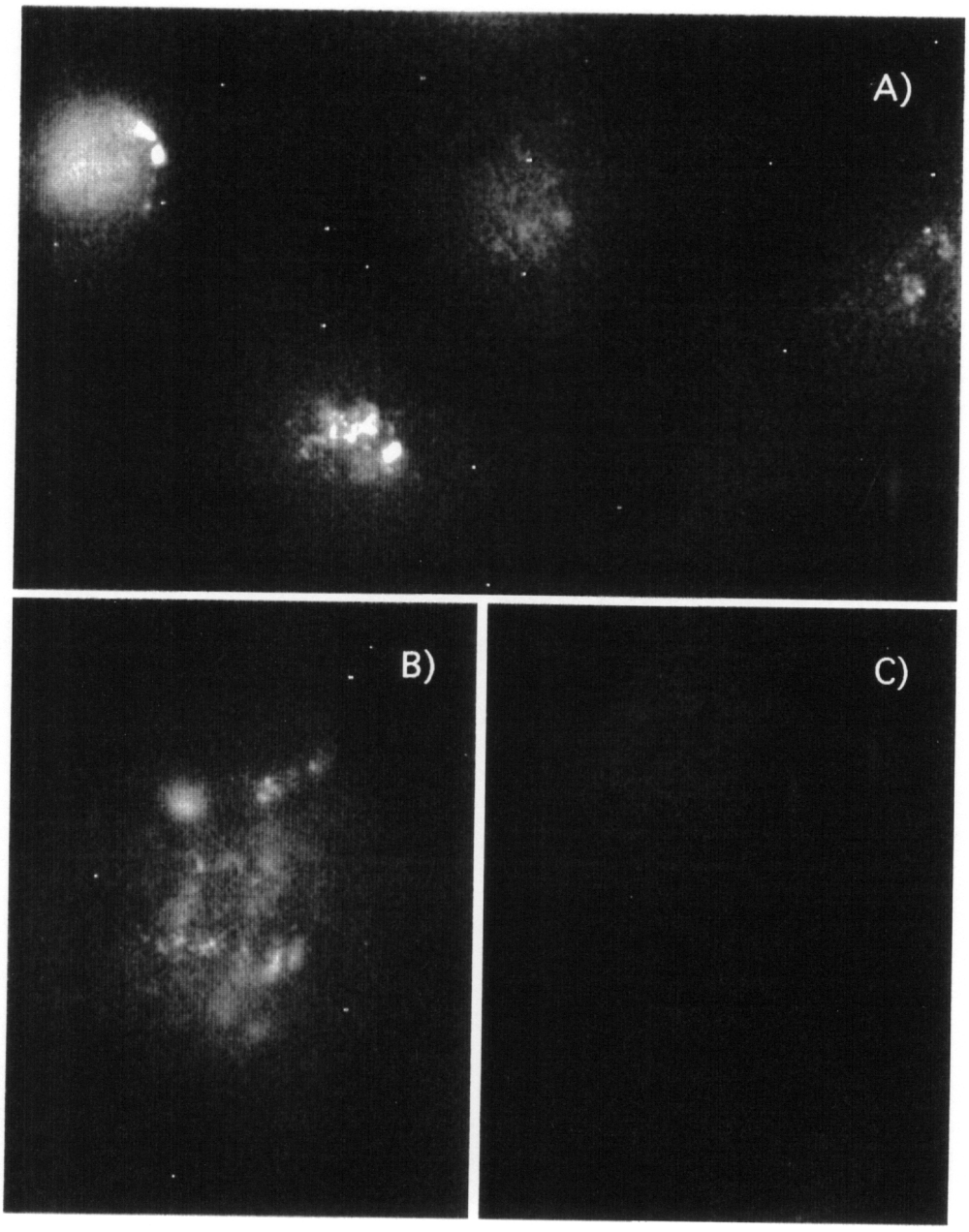


Figure 3-5 Effect of Zinc on Translocation

HeLa cell under various treatment conditions were exposed to dexamethasone for 30 minutes. Control represents the natural ratio of receptor populations before dexamethasone treatment The ratios of nuclear to cytosol fluorescence after staining with immunofluoresent techniques described under methods, are shown. A higher ratio of nuclear/cytosol is indicative of translocation. Data represents the average and standard deviations of no less than 40 individual cells from 4 separate experiments

Figure 3-6 Effect of Zinc on Receptor Aggregation

HeLa cell under various treatment conditions were exposed to dexamethasone for 4 hours. The occurance of punctate staining in the nucleus upon dexamethasone administration is shown in A. The lack of punctate staining (C) occurs when cultures are exposed to DTPA (10 μM) for 24 hours prior to steroid administration. Addition of 50 μM zinc reversed the effects of DTPA (B). The dependance on zinc of this staining suggests that zinc may play a role in the observed receptor aggregation.


A)
B)
C)

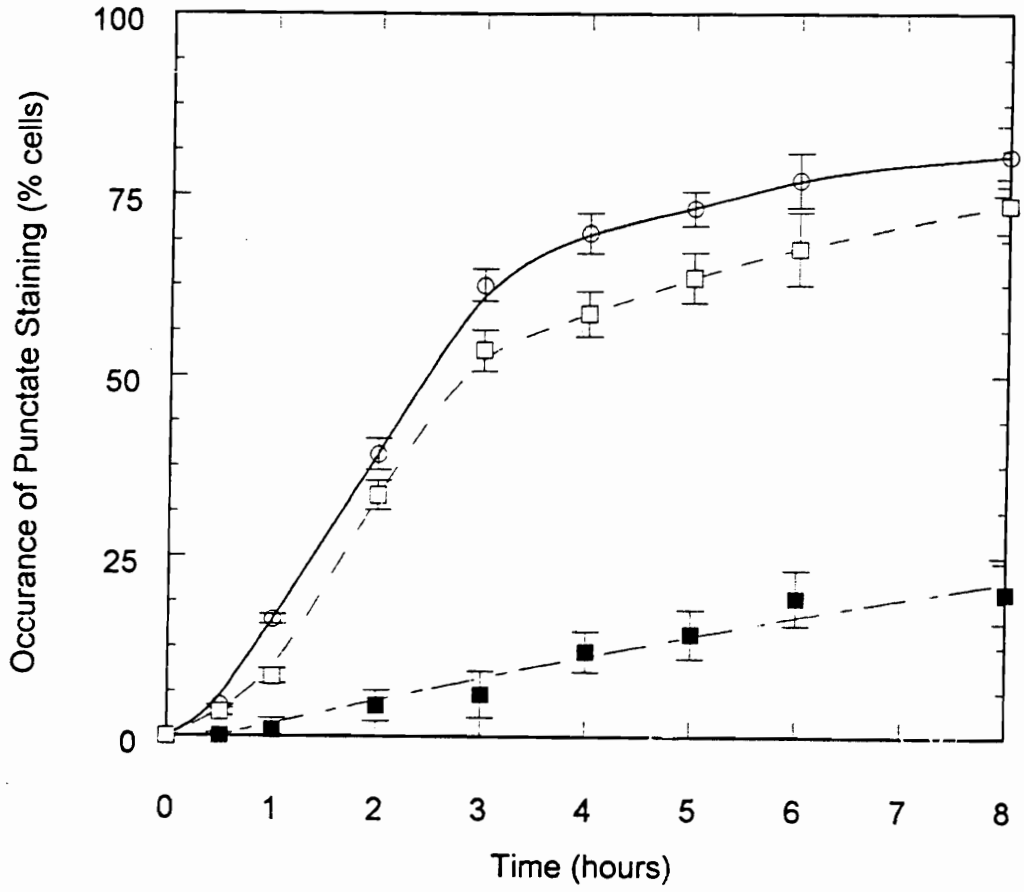


Figure 3-7 Effect of DTPA on Receptor Aggregation

HeLa cells under various treatment conditions were exposed to dexamethasone for up to 8 hours. The percentage of cells exhibiting punctate fluorescence over time is illustrated. DTPA (10 μM) drastically reduced the occurance of punctate staining (■). The addition of 50 μM zinc to cultures reverses the effect of the chelator (□). Control cell (no DTPA) are also shown (○). Data represents the average and standard deviations of no less than 40 individual cells from 4 separate experiements

After 2 hours incubation with dexamethasone, nuclear punctate staining is observed in cells with adequate zinc. The occurrence of this staining increased over the next 2 hours (Figures 3-6, 3-7). After 4 hours exposure to dexamethasone, further incubation with steroid showed little effect of this staining. It is suspected that this is a phenomenon of receptor aggregation which may be related to DNA binding. Recent studies performed with rat glucocorticoid receptors tagged on the N-terminus with green fluorescent protein showed nuclear localization after treatment with dexamethasone (Htun et al, 1995; Ogawa et al, 1995). Htun reported that dexamethasone induced a speckled intranuclear receptor localization pattern, with very bright spots at discrete locations. They concluded that the nuclear localization pattern may be related to the assembly of GR-directed transcription complexes (Htun et al, 1995).

Further experiments to determine the effect of DTPA on nuclear localization and receptor aggregation were conducted. Dose response curves with DTPA suggest that punctate staining is reduced with increasing concentrations of zinc chelator (Figure 3-8). Again, a reduction of more than 30% of total zinc causes sharp reduction in the occurrence of punctate

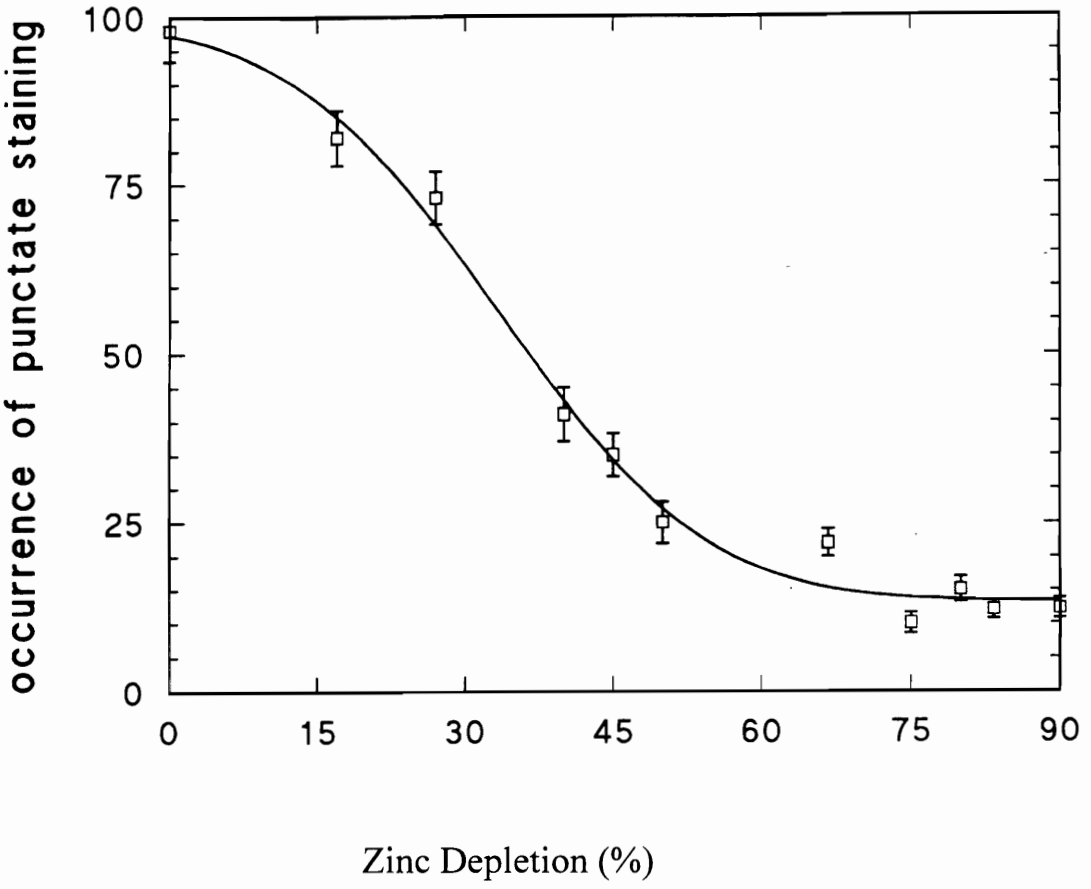


Figure 3-8 Reduction of Punctate Staining by DTPA

HeLa cells were exposed to increasing concentrations of DTPA. The occurance of punctate staining is drastically reduced. Data respresents the average and standard deviations of no less than 40 invidual cells.

staining. Further reductions of zinc beyond 50% exhibited little effect on punctate staining. These results suggest that zinc is required for receptor aggregation. The specific role of this accumulation of receptors to discretelocations within the nucleus is unknown. If it can be linked to specific DNA regions containing glucocorticoid response elements, this may explain the reduced gene expression observed during zinc deficiency. It is known that zinc ions are critical for maintaining the structural properties of DNA binding domains in steroid receptors, but in cellular systems, the regulatory role for zinc appears to be much more complicated. These studies indicate that, in moderate zinc deficits, receptor populations are drastically diminished. Furthermore, the effect of zinc reductions beyond 25% on steroid induced gene expression is drastic. If even zinc concentrations are reduced 25% or more during zinc deficient conditions, this is enough to erode hormone responses.

Conclusion:

Although the exact role of zinc in glucocorticoid induced gene expression cannot be resolved, the dependence upon zinc is undisputed. Evidence supportive of a connection between receptor number and steroid

sensitivity is abundant. A direct correlation between receptor number and cellular responsiveness to hormones has been demonstrated in a variety of cell lines (Bourgeois and Newby, 1974; Gehring et al, 1984; Vanderbilt et al, 1987) and in intact animals (Smith and Shuster, 1984; Yi-Li et al, 1989) as well as in humans (Pui et al, 1984; Iida et al, 1985). A 40% decrease in receptor number corresponded to 60% reduction in glucocorticoid-sensitive tyrosine aminotransferase activity in rats (Yi-Li et al, 1989). Similarly, 35-45% reduction in glucocorticoid receptors in human subjects have been shown to abolish corticosteroid-sensitivity (Iida et al, 1985). Thus it is possible that the 88% reduction in expression may be accounted for by a small loss of constitutive expression combined with a 50% reduction of receptors. The existence of such a relationship between hormone sensitivity and receptor concentration in target cells and tissues necessitates a thorough understanding of the mechanisms responsible for maintaining receptor levels. If zinc concentrations can alter receptor populations, this alone may account for the reduced glucocorticoid response.

In the studies performed here, a zinc specific dependence on glucocorticoid induced expression was observed. Evidence suggests that

zinc reductions may modulate receptor populations and this alteration may account for the decreased responsiveness to hormones. During times of zinc deficiency both DNA-dependent RNA polymerase and ribonuclease activities are altered. In the former, activity decreases, but in the later, activity increases (Burnstein and Cidlowski, 1993). Thus, both transcriptional activity and messenger RNA stability are decreased. However, this non-selective decrease in expression can only account for a portion of the reception decrease, zinc must play a specific role in the regulation of receptor populations.

Alterations in receptor number may be due to transcriptional, post-tanscriptional (mRNA stability), or post-translational (protein turnover) changes. We suspect the effect on DNA binding is a symptom of the decreased receptor pool. Examination of zinc's role in expression of the glucocorticoid receptor may uncover the connection between reductions in receptor populations and zinc deficiency.

The possibility of decreased expression of glucocorticoid receptor genes specifically can not be ruled out. Reductions of constitutive synthesis cannot account for the total decrease seen, however, zinc may play a role in

the timely induction of receptor genes. Alterations of transcription may be linked to zinc containing transcription factors or other methods of regulation that may be zinc sensitive. A factor that down-regulates the human GR gene has been cloned and, based on sequence analysis, thought to contain three zinc fingers (LeClerc et al, 1991). Therefore, the possibility exists that zinc could effect another factor involved in GR gene expression.

Alterations in mRNA stability will have a dramatic effect on cellular GR concentrations. Protein kinase C, a zinc containing enzyme, is though to be involved in the phosphorylation of many of the steroid receptors and the closely related vitamin D receptor. Agents which effect protein kinase C have been implicated in the down-regulation of estrogen receptor mRNA stability (Saceda et al, 1991), vitamin D receptor gene expression (Hsieh et al, 1991), and possibly GR gene expression (Burnstein and Cidlowski 1993). Furthermore, zinc has been suggested to modulate the catalytic activity of PKC (Forbes et al, 1989). If decreases in cellular zinc can decrease PKC activity and subsequently GR mRNA stability, then this may be a critical link in the understanding of the reduction of receptors concentrations observed during zinc deprivation.

A decline in cellular zinc may also cause increased degradation of nonfunctional receptors. If zinc ions are not readily available to bind to newly formed receptors, then the turnover rate of these non-functional apoproteins may be increased. This may partially explain the diminished receptor pool. Although cellular receptors lacking zinc have yet to be identified, addition of zinc to cell extracts show no increase in glucocorticoid-sensitive induction, or in receptor populations. This suggests that apoproteins lacking zinc are not present in the cell.

As shown here, in cases of nutritional zinc deficiency, steroid induced gene expression is compromised to a greater extent then constitutive expression. Our results demonstrate that the reduction in glucocorticoid response upon zinc depletion may be related to receptor number. The decrease in receptor number could result from a decrease in the transcription of its GR gene, a decrease in its mRNA stability, or an increase in receptor turnover. Translocation and hormone binding were not found to be significantly affected. The drastic effect of zinc concentrations on receptor aggregation may prove to be a critical link to uncovering the relationship between zinc and hormone responses. Receptors from zinc-deficient HeLa

cells exhibit altered aggregation properties that are zinc-dependent. The possibility of a Zn-dependent transcription regulation of the GR gene is interesting in light of recent evidence showing a down regulation of the GR gene. Experiments investigating these possibilities are in progress.

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Relationship Between Manifestations of Zinc Deficiency and Hormone Responses

Zinc Deficiency

During zinc deficiency growth retardation is one of the first symptoms observed in young developing animals. This decline in growth is both severe and rapid. For embryos, cessation of growth can be life threatening. Comprehensive studies by Apgar have shown that zinc deprivation during gestation results in low birthweights and poor survival of offsprings (Apgar, 1985).

The clinical manifestations of zinc deficiency reflect a wide range of metabolic roles for this metal. Its evolutionary selection to function in catalytic, structural, or regulatory roles in hundreds of enzymes and proteins makes detection of limiting biochemical functions difficult. The general chaos to many different systems in the body during zinc deprivation has been postulated to result from inadequate supplies of zinc-dependent enzymes. Attempts to match losses of specific enzymes with various features of zinc deficiency, however, have proved unsuccessful. Chesters suggested that the effect of inadequate zinc on RNA and DNA synthesis could not be explained solely by reduced activity of zinc-requiring

polymerases but more closely resembles a failure of individual cells to synthesize new enzymes (Chesters, 1972, 1989).

Constitutive zinc metalloenzymes are part of the basic cellular structure. Losses of 10-20% of a non-rate limiting enzyme may not severely compromise metabolic function and cellular survival. The discovery of a variety of zinc fingers as a highly conserved DNA binding motif in numerous transcription factors including the receptors for steroid and thyroid hormones, and vitamins A and D, active in embryogenesis, growth, and differentiation provided new insight into the metabolic function of this metal in cellular systems. These studies, in conjunction with past research in our laboratory, suggests that many of the clinical features of zinc deficiency may arise from an interference in the timely and swift induction of genes under the control of hormone receptors.

Previous studies in our laboratory have attempted to determine the molecular basis for abnormal parturition in zinc deficient rats. The phenomenon of delayed and difficult parturition in zinc deficient female rats was first described by Apgar in 1968 (Apgar, 1968). However, the molecular basis for the phenomenon is still under scrutiny. Research

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conducted in our laboratory showed a delayed appearance of proteins whose expression was regulated by estrogen receptors. This tardiness correlated with a delayed onset of parturition and prolongation of labor as well as aberrations in the uterine pressure cycle (Bunce, 1994). Furthermore, studies conducted on Japanese male quail showed mild zinc deficiency, insufficient to limit growth, did curtail vitellogenin synthesis, a protein whose gene is exclusively under the control of estrogen (Bunce, 1994; Kim, 1990). These results clearly show that in quail and in pregnant rats, establishment of a moderate zinc deficit insufficient to limit growth or produce any outward signs of deficiency can create circumstances in which estrogen-regulated gene expression is significantly altered (Bunce, 1994).

In order to identify the mechanism of reduced hormone responses during times of zinc depletion, we created a zinc-deficient cellular system. Using a zinc specific impermeable chelator, DTPA, HeLa cells were depleted of 80% of cellular zinc, without loss of viability. Transfections with CAT reporter plasmid either containing or lacking glucocorticoid response elements allowed discrimination between the effects of low zinc on constitutive and glucocorticoid-induced gene expression. Results clearly

demonstrated that following zinc-depletion glucocorticoid-directed expression was compromised to a greater degree than constitutive synthesis (88% vs. 20%), and that glucocorticoid receptor populations were reduced by 50%. The change in receptor concentration alone was sufficient to explain the observed decline in CAT protein activity. Disappearance of punctate staining in zinc deficient cells, however, suggest that DNA binding may also be effected. It is still unclear if the reduced puntate staining is just a phenomenon of the reduced receptor pool or if these receptors, although decreased in number, exhibit altered functions induced by the zinc deficient state.

The abundance of data suggesting that zinc deficiency causes a reduction in hormone responses warrants further examination. Reductions in signal transduction directly effect expression of genes under the control of hormones. The loss of induction of new proteins and enzymes due to ineffective hormonal signal transduction could explain many of the clinical manifestations of zinc deficiency. These findings combined with previous studies suggest that clinical symptoms of zinc deficiency may be a result of reduction in hormonally regulated gene expression.

Role of Zinc in Hormone Signaling:

Specialized endocrine cells secrete hormones, which travel throughout the bloodstream to influence target cells that are widely distributed throughout the body. Target cells respond to a particular extracellular signal by means of receptors, that bind the signaling molecule and initiate the response. Hormones can be divided into two categories: 1) lipid-diffusible molecules that pass through the plasma membrane and interact with cytosolic or nuclear receptors; and 2) molecules that bind to receptors embedded in the cell membrane. The principal representatives of lipid-diffusible molecules are steroids, thyroxin, vitamin D, and retinoic acid. Examples of hormones that bind cell-surface receptors are insulin, prostaglandins, or amino acid derivatives (epinephrine). The mechanism of signal transduction differs between these two types of hormones. For lipid soluble molecules, the receptors, after capturing ligand, bind to specific sites on DNA and modulate target genes. In the second instance, binding of ligand to surface receptors triggers an increase or decrease in cytosolic

concentrations of secondary messengers (cAMP, Ca^{2+}, or 1,2-diacylglycerol). These intracellular signals usually activate one or more transcription factors (Lucas and Granner, 1992). In both cases transactivation or repression of target genes can result from the hormone signal.

Clinical Manifestations of Zinc Deficiency Relating to Hormone Responses:

Most of the clinical manifestations of zinc deficiency, including appetite reduction, reproductive abnormalities, decreased immunocompetence, and growth retardation, can be linked to hormone-regulated responses. Higher zinc concentrations in tissues that contain hormone receptors suggests that a pool of reserved zinc ions may be necessary for normal responses. High concentrations of zinc are found in various regions of the of male and female reproductive systems and the brain. The distribution of zinc between extracellular fluid pools and tissues appears to be sensitive to changes in hormonal concentrations. Glucocorticoids have been shown to stimulate zinc uptake by HeLa cells (Davis et al, 1973), hepatic cells in culture (Fanger et al, 1986; Flynn et al, 1973), and probably the intact human liver (Forbes et al, 1989; Hough et al,

1987). Thus mechanisms may have evolved to ensure a supply of critical zinc ions during times of hormonal stimulation.

Androgen

Delayed sexual maturation and severe growth retardation observed in zinc deficient rats has been linked to diminished target cell responses induced by testosterone (McClain, 1984). Although basal levels of testosterone were equivalent in both zinc deficient and zinc adequate rats, the prostate gland, epididymis and seminal vesicle were significantly lower in weight. Similar symptoms have been documented to occur in zinc deprived adolescent boys (Sandstead et al, 1967). Supplementation with zinc was required to stimulate linear growth, maturation of the skeleton and development of the genitalia and secondary sex organs (Mertz, 1986).

Vitamins D:

Studies conducted in both rats and tissue culture have shown that vitamin D stimulates bone formation (Yamaguchi and Yamaguchi, 1986; Yamaguchi et al, 1987). Further examination uncovered that vitamin D_3 alone significantly increased alkaline phosphatase activity and DNA content

in cells. Roth and Kirchgessner (1974) found that rat serum alkaline phosphatase activity was decreased by 25% as soon as two days, and further decreased to 50% by four days of dietary zinc depletion. They concluded that the apoprotein rather than the ion was missing since preincubation of serum with zinc did not normalize activity. Thus the stimulatory effect of vitamin D on alkaline phosphatase activity is more likely to be related to induction of newly synthesized enzymes rather than activation of preexisting enzymes.

Growth Hormone:

Pituitary levels of growth hormone (GH) are reduced in zinc deficient rats (Root et al, 1979) and humans (Collip et al, 1982). This decrease in GH is thought to be a symptom of reduced induction by other hormones. Growth hormone secretion by rat pituitary tumor cells is modulated by thyroid hormone, triiodothyronine (T_3) in conjunction with glucocorticoids and somatomedin-C.

In animals, thyroid stimulating hormone (TSH) stimulates the formation of thyroxin (T_4) which can then be converted to other biologically active forms such as triiodothronine (T_3). Thyroxin and its

various conversion forms promote differentiation in embryos during development, and regulate growth in all mammals (Kutsky, 1973). In zinc deficient rats, serum T_3 level are significantly lower as compared to controls despite normal circulating levels of thyroxin and TSH (Morely et al, 1980). This suggests that conversion of thyroxin to T_3 may be impaired by zinc deficiency. If during zinc deprivation, thyroxin cannot be converted to T3, a resulting failure of growth hormone induction may result.

Somatomedin C (SM-C) is a polypeptide hormone (ie. insulin like growth factor I, IGF-I) synthesized in the liver and stimulated by growth hormone. It generates negative feedback on the hypothalamus and pituitary and acts as an intermediary in the effect of growth hormone on cartilage proliferation and consequential linear growth of skeletons (Mills, 1988). Somatomedin C actions are mediated by zinc. Due to the ion's role as a cofactor for SM-C, a high correlation between SM-C activity, weight gain and growth and normal zinc concentrations are observed.

Steroid Response: First Limiting Biochemical Function in Zinc Deficiency?

Zinc has numerous and widespread functions in man and animals, but the first limiting biochemical function, the one that occurs first during

dietary deprivation, is unknown. The studies described here show that reductions of more than 25% of cellular zinc compromise glucocorticoid-induced gene expression. Although the exact mechanism of zinc dependence cannot be determined from these experiments alone, glucocorticoid-sensitive gene expression appears to require a supply of zinc ions in order for efficient induction to occur. Occurrence of decreased steroid sensitivity when other cellular responses are normal suggests that it may be one of the first functions effected by declining zinc concentrations. If in fact, steroid responses are affective indicators of marginal zinc concentrations, identification and measurement of these functions would provide an excellent index of zinc status.

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APPENDIX A

DETAILED METHODS

TSQ PREPARATIONS

STOCK SOLUTION: 45.7 mM (1.5% w/v) TSQ

1) DISSOLVE IN ETHANOL

2) HEAT IN 80°C WATER BATH TO DISSOLVE

3) STORE AT 4°C PROTECTED FROM LIGHT

WORKING STOCK: 100 nmol/mL

1) WRAP FLASK IN ALUMINUM FOIL TO PROTECT FROM LIGHT

2) 15 mL RAPIDLY STIRRING PHYSIOLOGICAL SALINE WITH 40 mM HEPES (pH 7.3, ROOM TEMPERATURE)

3) ADD 33 µL 45.7 mM TSQ (HOT)

4) USE WITH IN 30 MINUTES

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FLUORESCENCE OF TSQ IN HeLa CELLS

USE G-365 EXCITER FILTER AND LP-420 BARRIER FILTER ON FLUORESCENCE MICROSCOPE

1) GROW HeLa CELLS ON DRY HEAT STERILIZED COVERSLIPS FOR 24 HOURS BEFORE TREATMENT.

2) PLACE COVERSLIPS IN VARIOUS TREATMENT MEDIA FOR DESIRED TIMES.

3) REMOVE COVERSLIPS FROM MEDIA

3) WASH CELLS TWICE WITH 10 mL HEPES-BUFFERED SALINE (pH 7.3)

4) EXAMINE MICROSCOPICALLY TO ENSURE ABSENCE OF BACKGROUND FLUORESCENCE

5) PLACE COVERSLIPS IN 200 µL TSQ (100 nmol/mL)

6) ALLOW UPTAKE FOR 90 SECONDS

7) RINSE OFF RESIDUAL DYE WITH TWO 10 mL HEPES-BUFFERED SALINE WASHES

8) EXAMINE FLUORESCENCE

HeLa CELL CULTURES

GENERATION TIME: 24 HOURS
MEDIA: DMEM + 10% FETAL CALF SERUM FOR ATTACHED

CULTURING TECHNIQUES:

1) TAKE OUT OLD MEDIA

2) RINSE WITH 2 mL TRYPSIN/EDTA TWICE (CHELATES Mg AND Ca)

3) ADD 1 mL TRYPSIN/EDTA

4) INCUBATE 10 MINUTES: CELLS WILL DETACH AND ROUND UP

5) ADD 10 mL MEDIA TO NEW PLATES

6) RINSE OLD PLATE WITH 9 mL MEDIA AND EXTRACT CELLS

7) ADD DESIRED AMOUNT TO NEW PLATE
   0.5 mL= 4 DAYS
   1.0 mL= 3 DAYS
   2.0 mL= 2 DAYS

MEDIA: JOKLICS MINIMAL ESSENTIAL MEDIUM (JMEM) + 2 MM GLUTAMINE + 10% SUPPLEMENTED BOVINE SERUM (SBS).

CULTURING TECHNIQUES:

1) TAKE OUT OLD MEDIA

2) RINSE WITH 2 mL VERSENE (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 0.5 mM EDTA, 8 mM Na_2HPO_4), TWICE

3) ADD 1 mL VERSENE

4) INCUBATE 10 MINUTES: CELLS WILL DETACH AND ROUND UP

5) ADD 10 mL MEDIA TO NEW PLATES

6) RINSE OLD PLATE WITH 9 mL MEDIA AND EXTRACT CELLS

7) ADD DESIRED AMOUNT TO NEW PLATES

VIABILITY TEST

MATERIALS:

1mg\mL HOECHST REAGANT

METHODS:

ADD AMOUNT TO CELL CULTURE DISH TO GIVE 2.5 µg\mL

SWIRL DISH AND INCUBATE FOR 5 MINUTES

READ FLUORESCENCE UNDER UV LIGHT WITH WATER IMMERSION OBJECTIVE

INCREASE IN STAINING, DECREASE VIABILITY.
VIABILITY TEST

SPECTROFLUOROMETRIC ANALYSIS OF TSQ-Zn^{2+} FLUORESCENCE

MATERIALS:

1mM STOCK $ZnSO_4$
100 nmol/mL TSQ WORKING STOCK
METAL CHELATORS:
1.) DTPA 30 mM STOCK IN SALINE
2.) TPEN DISSOLVED IN 95% ETHANOL
3.) 1,10-ORTHOPHENANTROLINE DISSOLVED IN 95% ETHANOL
100 μM STOCKS OF Cu, Ca, Mn, Fe
PERKIN-ELMER 650-10S FLUORESCENCE SPECTROPHOTOMETER WITH AN EXCITATION AND EMISSION WAVELENGTHS OF 380 nM AND 495 nM

METHODS: STANDARD CURVE

1) TO CUVETTE ADD:

-2 mL 100 nmol/mL TSQ WORKING STOCK
-ADD 0-25 μM Zn^{2+}

2) READ FLUORESCENCE AFTER 1 MINUTE

METAL CHELATORS/CATIONS:

1) ADD 2 mL 100 nmol/mL TSQ TO CUVETTE

2) ADD 5 OR 10 μM Zn^{2+}

3) WAIT 1 MINUTE

4) ADD APPROPRIATE ALIQUOTS FROM STOCK SOLUTIONS OF Zn^{2+} CHELATORS OR CATIONS

5) MONITOR FLUORESCENCE

HELA CELL TRANSFECTION USING TRANSFECTAM

A) PLATE CELLS
   1) PLATE CELLS DAY BEFORE TRANSFECTION
   2) GROW TO 30 -70 % CONFLUENCY THE DAY OF TRANSFECTION
   3) GROW IN DMEM 10% SBS

B) TRANSFECTAM REAGENT STOCK
   1) 1 mg REAGENT DISSOLVED IN 100% ETHANOL, ROOM TEMPERATURE FOR 5 MINUTES

      200 µL ETOH FOR 0.5 mg
      400 µL ETOH FOR 1.0 mg

   2) VORTEX SOLUTION AND STORE AT 4°C

C) TRANSFECTION
   1) RINSE CELLS 3 TIMES WITH SERUM FREE DMEM

   2) ADD 1.0 mL OF DMEM TO PLATE

   3) MAKE TRANSFECTAM SOLUTION AS FOLLOWS:

      ADD 5 µG DNA TO 1 ML DMEM (SOL'N A)
      ADD 10 µL TRANSFECTAM TO 1 ML DMEM. (SOL'N B)
      VORTEX
      MIX SOL'N A AND B. ADD DIRECTLY TO CELLS.
      (3 ML /PLATE TOTAL).

   4) LEAVE ON CELLS FOR 4 HOURS
   5) AFTER 4 HOURS, REMOVE DMEM AND ADD 5 ML/ DMEM + 10% SBS
   6) INCUBATE 48 HOURS BEFORE REMOVING CELLS

NOTES: OPTIMUM RATIO OF DNA:μL TRANSFECTAM (HELA) IS 1:2. 10 μG TO 20μL GAVE 80 %. I USE 5μG TO 10μL AND GET 55-60 %. TRANSFECTAM IS INHIBITED BY COMPONENTS IN SERUM AND TRYPSIN . DNA MUST BE AT 1μG/μL OR GREATER. SEEMS TO WORK BETTER AT HIGHER CONCENTRATION. SO TRY TO USE LEAST AMOUNT OF DNA DISSOLVED IN TE AS POSSIBLE. CELLS WERE 50 % VIABLE AT 6 HOURS WITHOUT SERUM. 4 HOUR INCUBATION INCREASES CELL VIABILITY WHILE STILL ACHIEVING GOOD EFFICIENCY.

*RATIO OBTAINED FROM PROMEGA TECH. LINE (MARK 1-800-356-9526 EXT. 1364) PROMEGA USES 2.5μG/5μL/ PLATE

ANALYSIS OF CAT ACTIVITY

1) TRANSFECT CELLS WITH pGMCS OR pBLCAT2 USING TRANSFECTAM

2) WAIT 24 HOURS

3) CHANGE MEDIA TO VARIOUS INCUBATION MEDIA

4) REMOVE MEDIA AND WASH CELLS WITH 1 mL STERILE PBS. TWICE.

5) ADD 1 mL PBS TO PLATE

6) REMOVE CELLS BY SCRAPING PLATE WITH RUBBER POLICEMAN.

7) DISRUPT CELLS BY PLACING IN DRY ICE THEN INTO 50°C WATER. (FREEZE AND THAW METHOD)

8) REMOVE 2 X 2 µL FOR QUANTIFYING PROTEIN AMOUNTS

9) MIX REAGENTS AS FOLLOWS:

   100µL CELL EXTRACT
   3 µL 14C-CHLORAMPHENICOL (0.15 µCi)
   5 µL n-Butyrl CoA
   17 µL of 0.25M Tris-HCl, pH 8.0

10) INCUBATE REACTION FOR 6 HOURS AT 37°C

11) BRIEFLY SPIN THE TUBES IN A MICROCENTRIFUGE

12) ADD 300 µL MIXED XYLENES TO TERMINATE THE REACTION

13) VORTEX SAMPLES FOR 30 SECONDS, SPIN AT TOP SPEED IN

MICROCENTRIFUGE FOR 3 MINUTES

14) TRANSFER UPPER XYLENE PHASE TO FRESH TUBE

15) ADD 100 µL 0.25 M TRIS-HCl (pH 8.0) AND BACK EXTRACT BY REPEATING STEP 14

16) BACK EXTRACT A SECOND TIME BY TRANSFERRING XYLENES AND ADDING 100 µL TRIS-HCl

17) CAREFULLY REMOVE 200 µL OF UPPER XYLENE PHASE AND TRANSFER TO A SCINTILLATION VIAL

18) ADD APPROPRIATE SCINTILLATION FLUID AND COUNT ON LIQUID SCINTILLATION COUNTER

19) CPM MEASURED REPRESENTS THE BUTYRYLATED CHLORAMPHENICOL PRODUCTS

EQUILIBRIUM STEROID HORMONE BINDING ANALYSIS

[^{3}H]DEXAMETHASONE MESYLATE (48.9 Ci/mmol) FROM DUPONT

DEXTRAN-COATED CHARCOAL: 1% ACTIVATED CHARCOAL, 0.1% DEXTRAN (MW 60,000-90,000) IN 1.5 mM MgCl2

1) REMOVE CELLS FROM PLATE IN VERSENE AND PELLET

2) HELA S3 CELLS WERE SUSPENDED IN 1 mL 10mM TRIS, 1mM EDTA, 12mM ∝-THIOGLYCEROL, 20 mM SODIUM MOLYBDATE pH 7.5, at 4° C

2) HOMOGENIZE WITH POLYTRON TISSUE TEARER

3) CENTRIFUGE AT 100,000 X G FOR 1 HOUR AT 0° C

4) DETERMINE PROTEIN CONCENTRATIONS BY LOWRY

5) ALIQUOTS CONTAINING EQUAL AMOUNTS OF PROTEIN (50-100 μL) REMOVED TO PRECOOLED TUBES CONTAINING [^{3}H]DEX AT CONCENTRATIONS FROM 1-400nM (3, 6, 9, 12.5, 25, 50, 100, 200, 400)

6) INCUBATE 2 HOURS AT 4° C

7) REMOVE ALIQUOT FOR QUANTITATION OF TOTAL STEROID CONCENTRATION

8) TREAT WITH EQUAL VOLUME OF DEXTRAN-COATED CHARCOAL FOR 5 MIN AT 4°C

9) PELLET CHARCOAL AT 4° C BY CENTRIFUGATION AT 11,000 RPM

10) QUANTITATE STEROID REMAINING IN SUPERNATANT

11) ANALYZE BY SCATCHARD

PLASMID AMPLIFICATION

1) GROW A 30 mL CULTURE OF BACTERIA CONTAINING PLASMID TO LATE LOG PHASE (OD_{600}=0.6)

2) INOCULATE 500 mL OF LB + GLUCOSE + AMPECILLIAN (37°C) WITH LOG PHASE CULTURE.

3) INCUBATE CULTURE FOR 2.5 HOURS AT 37°C AT 300 CYCLES/MIN SHAKING

4) ADD 2.5 mL CHORAMPHENICOL (34 mg/mL IN ETHANOL) FINAL CONCENTRATION 170 µg/mL.

5) INCUBATE CULTURE 12-16 HOURS AT 37°C, VIGOROUS SHAKING.

6) CENTRIFUGE AT 4000 RPM FOR 15 MINUTES 4°C

7) RESUSPEND PELLET IN 100 mL ICE COLD STE

8) CENTRIFUGE 4000 RPM FOR 15 MINUTES 4°C

9) RESUSPEND WASHED BACTERIAL PELLET IN 10 mL SOLUTION I

    SOL'N I:
    50 mM GLUCOSE
    25 mM TRIS-HCl (pH 8.0)
    10 mM EDTA (pH 8.0)

   PREPARE IN BATCHES OF 100 mL, AUTOCLAVE 15 MINUTES, STORE 4°C.

10) (OPT) ADD 1 mL FRESHLY PREPARED LYSOZYME (10 mg/mL IN 10 mM TRIS-HCl (pH 8.0)

11) ADD 20 mL SOLUTION II, CLOSE TOP AND MIX GENTLY. STORE ROOM TEMPERATURE 5-10 MINUTES.

SOL'N II
0.2 N NaOH
1% SDS

12) ADD 15 mL ICE COLD SOLUTION III, MIX, STORE ON ICE 10 MINUTES.

SOL'N III
5 M K. ACETATE (60 mL)
GLACIAL ACETIC ACID (11.5 mL)
H2O (28.5 mL)

13) CENTRIFUGE 8500 RPM FOR 20 MINUTES 4°C

14) DECANT OFF SUPERNATANT, ADD 0.6 VOLUMES ISOPROPANOL. LET STAND 10 MINUTES AT ROOM TEMPERATURE.

15) RECOVER PLASMIDS BY CENTRIFUGE 9000 RPM 15 MINUTES, ROOM TEMPERATURE.

16) DECANT SUPERNATANT AND WASH PELLET WITH 70% ETHANOL (ROOM TEMP), DRAIN OFF ETOH, VACUUM DRY PELLETS.

17) DISSOLVE PELLET IN 4.65 mL TE (pH 8.0)

18) ADD 4.6 g CsCl (1.55 g/mL)

19) ADD 0.2 mL 0.5 M EDTA (pH 8.0), ADD 0.2 mL ETHIDIUM BROMIDE.

20) CENTRIFUGE 8000 RPM FOR 5 MINUTES

21) BALANCE WITH 4.6 g CsCl/5.05 mL H_2O

22) SPIN 56,000 RPM FOR 16 HOURS AT 4°C IN VERTICAL ROTOR.

23) COLLECT PLASMID BANDS UNDER UV LIGHT USING HYPODERMIC NEEDLE

24) REMOVE ETHIDIUM WITH dH_2O SATURATED BUTANOL (EQUAL PARTS)

25) REMOVE AND DISCARD TOP PINK LAYER

26) CONTINUE UNTIL BOTH LAYERS ARE CLEAR AND COLORLESS

27) ADD 2 VOLUMES dH_2O AND 2 VOLUMES ABSOLUTE ETOH

28) STORE AT -20°C 6-8 HOURS

29) SPIN AT 11,000 RPM FOR 15 MINUTES

30) DISSOLVE IN TE (pH 8.0), READ OD AT 260 AND 280 ($1OD_{260}$ = 50 µg/mL, 260/280=1.8 PURE)

WESTERN BLOT ANALYSIS

1) GROW CELL IN VARIOUS INCUBATION MEDIA

2) COLLECT CELLS IN 1mM EDTA, 10 mM TRIS, 0°C.

3) DISRUPT CELLS WITH A POLYTRON TISSUE GRINDER

4) THE LYSATES ARE CENTRIFUGED AT 11,000 RPM FOR 15 MINUTES TO OBTAIN A NUCLEAR FRACTION

5) THE NUCLEAR PELLET IS THEN RESUSPENDED IN 1 mL EDTA-TRIS

6) BOTH FRACTIONS ARE CENTRIFUGED AT 100,000 X g FOR 1 HOUR AT 0°C

7) THE SAMPLES ARE MICROCONCENTRATED 3-5 FOLD IN A MICROCON 30 MICROCONCENTRATOR ACCORDING TO DIRECTIONS

8) REMOVE 2 X 2 µL FOR PROTEIN DETERMINATION BY LOWRY

9) 100 µG OF PROTEIN FROM EACH SAMPLE IS ADDED TO EQUAL VOLUME OF 2X LOADING BUFFER (20% GLYCEROL, 4.6% SDS, 0.125 M TRIS, pH 6.8), HEATED AT 100°C FOR 2.5 MINUTES, AND KEPT AT -70°C UNTIL ELECTROPHORESIS

10) THE SAMPLES ARE ELECTROPHORESED ON A 1.5 mm SDS POLYACRYLAMIDE GEL (8%) WITH A 3.5% STACKING GEL

11) THE PROTEINS WERE THEN TRANSFERRED TO IMMOBILON™ MEMBRANE USING A SEMI-DRY TECHNIQUE.

12) THE BLOTS ARE BLOCKED IN POLYVINYL ALCOHOL (1:1000 DILUTION) FOR 2 MINUTES

13) BLOTS ARE WASHED IN 1X TBS CONTAINING 0.1 % TWEEN AND 5 % DRY MILK FOR 5 MINUTES

14) INCUBATED OVERNIGHT AT 4°C WITH A 1:100 DILUTION OF RABBIT ANTI HUMAN GLUCOCORTICOID RECEPTOR ANTIBODY IN 5% CASIN BUFFER

15) REMOVE ANTIBODY SOLUTION AND WASH MEMBRANE IN TBS-TWEEN CONTAINING 5 % NON-FAT DRY MILK 3 TIMES FOR 10 MINUTES EACH.

16) BLOTS ARE THEN INCUBATED FOR 2 HOURS AT ROOM TEMPERATURE WITH A 1:5000 DILUTION OF PHOSPHATASE-CONJUGATED SECONDARY ANTIBODY (GOAT ANTI RABBIT) IN 5 % MILK BUFFER

17) THREE SUBSEQUENT WASHES IN THE TBS-TWEEN, 5% DRY MILK BUFFER FOR 15 MINUTES

18) DEVELOP COLOR REACTION BY INCUBATION OF THE BLOT IN NITRO BLUE TETRAZOLUIM AND 5 BROMO-4 CHLORO-3 INDOYL PHOSPHATE (BCIP)

19) STOP REACTION BY IMMERSION OF THE BLOT IN H_2O

NUCLEAR LOCALIZATION OF RECEPTORS

1) HeLa CELLS WERE GROWN ON DRY HEAT STERILIZED COVERSLIPS FOR 3 DAYS

2) REMOVE COVERSLIPS FROM MEDIA AND WASH 3 TIMES FOR 5 MINUTES IN PBS

3) FIX CELLS BY PLACING COVERSLIPS IN -20°C METHANOL FOR 4 MINUTES

4) WASH COVERSLIPS 3 TIMES FOR 5 MINUTES IN PBS

5) INCUBATE COVERSLIPS, CELL SIDE DOWN, IN 50 µL OF 1:50 DILTION OF RABBIT ANTI-GR PRIMARY ANTIBODY FOR 20 MINUTES

6) WASH COVERSLIPS 3 TIMES FOR 5 MINUTES IN PBS

7) INCUBATE COVERSLIPS, CELL SIDE DOWN, IN 50 µL OF 1:100 DILUTION OF FLUORESCEIN CONJUGATED GOAT ANTI RABBIT SECONDARY ANTIBODY FOR 20 MINUTES

8) MOUNT COVERSLIPS AND VISUALIZE STAINING USING A ZEISS IM 35 INVERTED MICROSCOPE, 490 EXCITATION FILTER AND 520 EMMISION FILTER

VITA

On May 26, 1970, Edward and Leslie Freeman were blessed with the birth of their second daughter, Kathryn Denise. Kathryn grew up in Chantilly, Virginia with her sister, Valerie. In June of 1988 she graduated from Chantilly High School and went on to attend college. In January of 1992 she earned a Bachelor of Science degree in Biology with a minor in Chemistry from George Mason University, Fairfax, Virginia. In June of 1992, she started persuing a doctorate of philosophy in Biochemistry at Virginia Polytechnic Institute and State University. On May 22,1993 she married Mr. Thomas C. Simon in Millwood Virginia. Her doctorate was completed in May of 1996.