BioMetals **14:** 385–395, 2001.

Review

Zinc physiology and biochemistry in oocytes and embryos

Kenneth H. Falchuk* & Marcelo Montorzi

*Center for Biochemical and Biophysical Sciences and Medicine, and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, 02115, USA; *Author for correspondence (Tel: (617) 432 - 1367; Fax: (617) 566-3137; E-mail: kenneth_falchuk@hms.harvard.edu)*

Received 2 February 2001; accepted 28 May 2001

Key words: eggs, embryos, oocytes, transcription factors, transport proteins, yolk platelets, storage, zinc

Abstract

The essential role of zinc in embryogenesis was identified through studies of its presence in eggs and embryos, the effects of its deficiency and its role in metallo proteins required for organ development and formation. The *Xenopus laevis* oocyte zinc content varies during oogenesis. It increases from 3 to 70 ng zinc/oocyte as it progresses from stage I to VI. The oocyte zinc is derived from the maternal liver as part of a metallo-complex with vitellogenin. The latter transports the metal in plasma and into the oocyte. Once internalized, most of the zinc is stored within yolk platelets bound to lipovitellin, one of the processed products of vitellogenin. About 90% of the total zinc is associated with the yolk platelet lipovitellin while the remaining 10% is in a compartment associated with hitherto unknown molecule(s) or organelle(s) of the cytoplasm. The bi-compartmental distribution remains constant throughout embryogenesis since the embryo behaves as a closed system for zinc after fertilization. The yolk platelet zinc is used after the tadpole is hatched while we proposed that the 10% of the zinc in the non-yolk platelet pool is the one used for embryogenesis. It provides zinc to newly synthesized molecules responsible for the development of zinc-dependent organ genesis. Interference with the availability of this zinc by the chelating agent 1,10-phenanthroline results in the development of embryos that lack dorsal organs, including brain, eyes and spinal cord. The extensive teratology is proposed to be due to altered or absent zinc distribution between the cytosolic pool and zinc-transcription factors. The data identify the components of a zinc transport, storage and distribution system in a vertebrate organism.

Introduction

Zinc is a constituent of many molecules involved in protein, lipid and carbohydrate metabolism. It also participates in the synthesis of viral, prokaryotic and eukaryotic nucleic acids. It is present in all living cells. Its function at the cellular level has depended on a large body of phenomenological information available on the effects of its deficiency (Vallee & Falchuk 1993). Thus, zinc deficiency induces proliferative arrest in many cell types, suppresses growth of plants and animals and causes congenital malformations in offspring of zinc-deprived animals. The teratology in vertebrate embryos is striking. In the mouse, the plasma zinc pool is responsible for delivering zinc to the growing fetus (Dreosti *et al.* 1968). Within 24 h of maternal zinc deprivation in the diet, the amount of available zinc is depleted and the embryo is deprived of the metal. The consequences include high mortality or malformations that involve nearly all organ systems. The most pervasive malformations are those of dorsal organs such as the head structures, neural tissues such as brain and spinal cord, as well as musculo-skeletal abnormalities (Keen & Hurley 1989). The most sensitive period for teratology appears to be in the early developmental stages, in humans corresponding to the first trimester.

There is limited knowledge regarding the basis for its role in cellular proliferation, differentiation and growth, in general, and for the teratology arising from

its deficiency, in particular. Similarly, there is sparse information on zinc uptake and distribution within cells. Finally, the foundation for understanding the metabolism of zinc in the oocyte and embryo is at its inception despite the acceptance that the embryo is a sensitive target of zinc deficiency (Falchuk 1998). To provide the basis for that understanding, we will review the information that is available on the content and distribution of zinc during oogenesis and embryogenesis as well as the underlying molecular events that are dependent on the metal and are crucial for embryogenesis.

Egg metal content

Eggs from oviparous animals behave as a closed systems while those from mammals behave as open systems. Thus, once eggs from e.g. sea urchin or frog are fertilized, they are deposited into a sea, river, lake or placed on the undersurface of leaves or other sites to develop into embryos. In those environments, these fertilized eggs must have all of the necessary nutrients or constituents to form a complete embryo without depending on an external and variable supply (Davidson 1990; Nomizu *et al.* 1993). Therefore, during maturation in the maternal ovary, the egg must acquire all essential chemical substances, including metals, for use after fertilization. In this sense, it is a closed system. On the other hand, mammalian eggs take up zinc, and likely other metals and nutrients, from the fallopian fluid once they enter the tube and begin dividing as fertilized eggs (Hurley & Shrader 1975; Gallaher & Hurley 1980). Moreover, once implantation has taken place, and the maternal blood supply is linked to that of the placenta, exchange of nutrients occurs between mother and fetus and, therefore, the latter is open to and dependent on a maternal source of metals, vitamins, etc.

The composition and biochemistry of individual eggs from many oviparous animals have been studied for years. Over the past decades, it also has become possible to obtain single eggs from mammals. Therefore, sea urchin, frog and mouse eggs can be individually collected and prepared for metal analysis by obviating spurious metal contamination using buffers, water, glass and plastic ware rendered metal-free. The collection of eggs, their preparation and the operational conditions for analyses have been reported (Nomizu *et al.* 1993; Vallee & Falchuk 1993). Briefly, the frogs are stimulated to ovulate by injection with chorionic gonadotropin. Hundreds of eggs are readily collected as they are spawned. Sea urchins are injected with KCl and they immediately release eggs, also in the hundreds. Mice are induced to ovulate with chorionic gonadotropin and ten or more eggs and embryos are collected within the fallopian tubes.

Table 1. Zinc content of a white blood cell and an individual mature egg.

Species	Zinc content, ng/egg or cell	Relative amounts
Human leukocyte	0.01	1
Mouse	1	100
Sea urchin	20	2000
Xenopus laevis frog	70	7000

The zinc contents of single eggs from mice, sea urchin and frog are shown in Table 1. The zinc content calculated for a single leukocyte (a polymorphonuclear white blood cell) is shown for comparison as an example of a fully differentiated adult cell. All three eggs have higher zinc content than the white blood cell. This suggests a higher requirement for zinc and/or the existence of a storage site, quite distinct from that of adult cells. Moreover, the two closed systems, sea urchin and frog eggs, have a much higher zinc content than the open system, the murine egg.

The information shown in Table 1 has been used to select a suitable system to study zinc metabolism during oogenesis and embryogenesis. The mature *Xenopus laevis* egg offers the advantage over the other two egg types that its zinc content is higher. Another advantage is that large numbers of oocytes at each stage of oogenesis and embryos at different points of development can be readily obtained and used for study of metal metabolism.

As part of that study, the contents of other metals in *Xenopus laevis* eggs also has been determined (Table 2).

The amounts of each metal in the egg differ. There are two metals, magnesium and calcium that are higher than zinc while four are less. Two are not detected. The metal contents can be arranged in the following order: Mg>Ca>Zn>Fe>Mn>Cu>Ni.

Zinc during oogenesis

The frog ovaries are located within the abdomen forming the most immediate visible structure on entering

Table 2. Metal content of oocytes.

Metal	Content, ng/oocyte*
Mg	329 ± 32
Ca	161 ± 15
Zn	70 ± 3
Fe	33 ± 4
Mn	10 ± 1
Cu	2 ± 0.1
Ni	0.2 ± 0.03
Co	ND
Cd	ND

*Average value $\pm$ standard deviation; $n = 10$; ND = not detected.

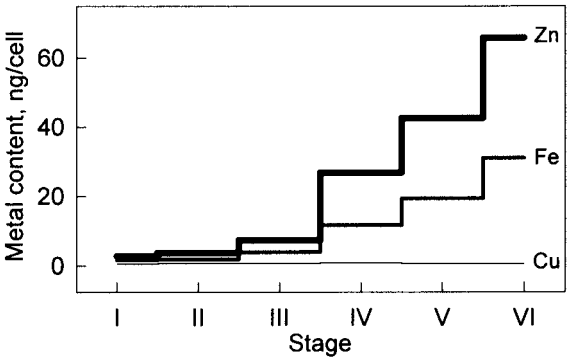

Fig. 1. Changes in metal content during oogenesis. As oocytes mature, they take up metals from maternal plasma. Zinc and iron increase during the entire oogenic period while copper attains its maximal value by stage I.

the cavity. It is composed of six lobes each with hundreds of oocytes at all stages of development. Their removal does not injure the oocytes and they are easily exposed by dissection of the fibrous membrane encasing the oocytes.

Oocytes can be separated on the basis of the six stages of their maturation since their size and morphological appearance differ from 50 to 1300 μm in stages I and VI, respectively (Hansen & Riebessell 1991). Their color changes from clear and transparent in the earliest stage to green/black pigment in the last stage. The zinc content of oocytes varies as a function of the stage of maturation. During the initial stages of maturation (stages I to III), the zinc content increases from 2 to 7 ng/oocyte (Figure 1). However, from stage III to VI it increases to approximately 70 ng/oocyte, a 35-fold increment from its original value. The contents of iron and copper are shown for comparison. Similar increase in iron is observed during oogenesis though the total amount is always less than zinc at all stages. The maximum copper content is attained during stage I and remains constant throughout oogenesis.

The zinc content of stage VI oocytes does not vary in eggs of any given frog though it varies in those of different frogs. The range of values in eggs of different frogs is about 65 to 133 ng/egg, a two-fold variation (Nomizu *et al.* 1993). Assuming an average egg volume of 1 μl (Hansen & Riebessell 1991) and a zinc content of 70 ng the concentration of zinc in the oocyte is approximately 1 mM.

The final zinc content of stage VI oocytes is achieved over a period of about 3 years, the amount of time that is required to terminate oogenesis. During the first three stages of oogenesis, the increases are quantitatively less than during the last three stages (Figure 1). This behavior corresponds exactly to the pre-vitellogenic and vitellogenic phases of oocyte development. These phases are descriptive of the rate of uptake of the protein vitellogenin by the oocyte during oogenesis. The concurrent uptake of zinc and vitellogenin by the oocyte is due to the relationship between the two processes.

Zinc is transported in plasma by, and is taken into the oocyte bound to, vitellogenin. Estrogen induces the liver to synthesize the phospho-glyco-lipo-metallo-protein vitellogenin. Within two weeks of the hormonal stimulation, the frog liver up-regulates its synthesis of the protein and secretes it into the plasma in large quantities. The protein is purified from the serum by chromatography on a Mono Q column and identified on the basis of its molecular weight and amino acid composition, specifically its high serine content with about 30% phospho-serine (Montorzi *et al.* 1995). Vitellogenin is a metallo protein that contains one g/at of zinc per 220 kDa monomer but no other group IIB or transition metal and 1.5 mol of calcium per monomer (Table 3). Since vitellogenin is a dimer, its total metal content, therefore, is 5 mol of metal, 2 mol of zinc and 3 mol of calcium per molecule. These data demonstrate that zinc is transported in plasma bound to vitellogenin.

The zinc protein is taken up from the plasma by oocytes through receptor-mediated endocytosis (Wallace 1978; Wallace *et al.* 1983; Wallace & Jared 1968; Banaszak *et al.* 1991; Hansen & Riebessell 1991). The receptor is a 115-kDa membrane protein (Stifani *et al.* 1990). The receptor – vitellogenin complex is internalized in vesicles by endocytosis. These fuse with other vesicles and lysosomes to form multivesicular bodies and process the protein into lipovitellin and

Table 3. Metal content of vitellogenin, lipovitellin and phosvitin.

Metal	Vitellogenin (mol/220kDa)	Lipovitellin (mol/141kDa)	Phosvitin (mol/30kDa)
Zn	1.02	1.06	0.20
Ca	1.50	ND*	2.10
Mg	0.15	0.10	3.0
Cd	ND	ND	ND
Mn	0.06	ND	0.05
Fe	0.15	0.10	0.5
Co	ND	ND	ND
Ni	ND	ND	ND
Cu	0.09	0.03	ND

* ND = Not detected

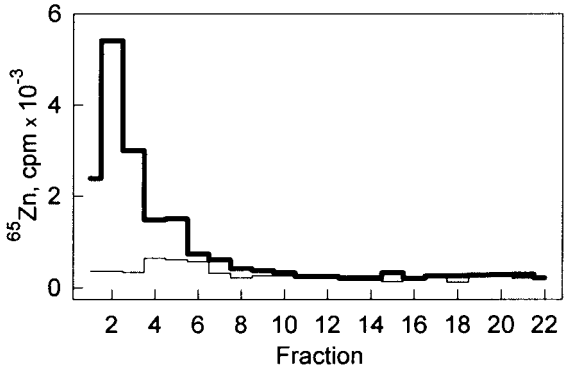

Fig. 2. Stage II oocyte ^{65}Zn vitellogenin uptake. Oocytes were incubated for 30 min with either ^{65}Zn vitellogenin (bold line) or free ^{65}Zn (thin line). Oocytes were homogenized and the constituents separated in a sucrose gradient. Fractions 0–5 contain cytosol, ribonucleoprotein particles and other small organelles. Free zinc does not enter the oocyte. In contrast, when zinc is bound to vitellogenin, it enters and is distributed only in the low density fractions.

phosvitin. The latter fuse further into yolk platelets of progressively larger size and weight. The vesicles, multivesicular bodies and yolk platelets can be separated in a sucrose gradient on the basis of their differing densities. Yolk platelets localize in sucrose densities between 1.20 and 1.24 g/ml. They are distributed bi-modally with the lighter ones concentrating at 1.21 g/ml and the heavier ones at 1.23 g/ml. As the oocytes mature, more of the yolk platelets sediment to the higher density. One of the resultant effects of this organelle formation is the entry of zinc into the oocyte and its appearance in yolk platelets.

This conclusion has been confirmed experimentally. The chelating agent 1,10-phenanthroline (OP) removes the zinc from vitellogenin in a concentration dependent manner. When dialyzed against 10^{-6} M OP, the metal protein complex remains intact while at 10^{-3} M all of the zinc is removed (pK for removal is 4.8). The metal removal by OP is very rapid and the apoprotein is formed within minutes of incubation with the chelating agent. A ^{65}Zn species has been generated from the apo vitellogenin and used to study the protein's role in zinc uptake by oocytes *in vitro* (Falchuk *et al.* 1995). Neither oocytes in the previtellogenic (stage II) or in the vitellogenic phase (stage IV) take up free ^{65}Zn during an incubation period of 24 h (Figure 2). However, both stage II and IV oocytes take up a zinc-vitellogenin complex but their distribution of zinc differs. Within 30 min of incubation, ^{65}Zn-bound vitellogenin enters both the stage II and IV oocytes. In stage II oocytes, all of the ^{65}Zn is localized entirely to the cytosolic fluid and remains in that space throughout the entire period of observation (Figure 2). In contrast, the distribution of ^{65}Zn within the more developed stage IV oocytes mirrors the time course and the formation of the vesicles, multivesicular bodies and yolk platelets. It changes in the first 300 min of incubation (Figure 3). After 30 min, the major peak of zinc is found in the 1.10–1.12 g/ml gradient fraction, the region of multivesicular bodies. After 90 min, a second zinc peak appears in the 1.20–1.24 g/ml region, where light and heavy yolk platelets are found. In the course of the next 300 min, over 90% of the zinc localizes to the yolk platelet region. This distribution remains unchanged for the next 20 h of the experimental period. The zinc content of heavy yolk platelets, 13.8 fg/platelet, is about 3 times greater than that of light platelets, 3.2 fg/platelet (Figure 4). Thus, within hours of uptake, zinc is transferred from the multivesicular bodies into a yolk platelet pool during the vitellogenic phase of oogenesis. At the mature

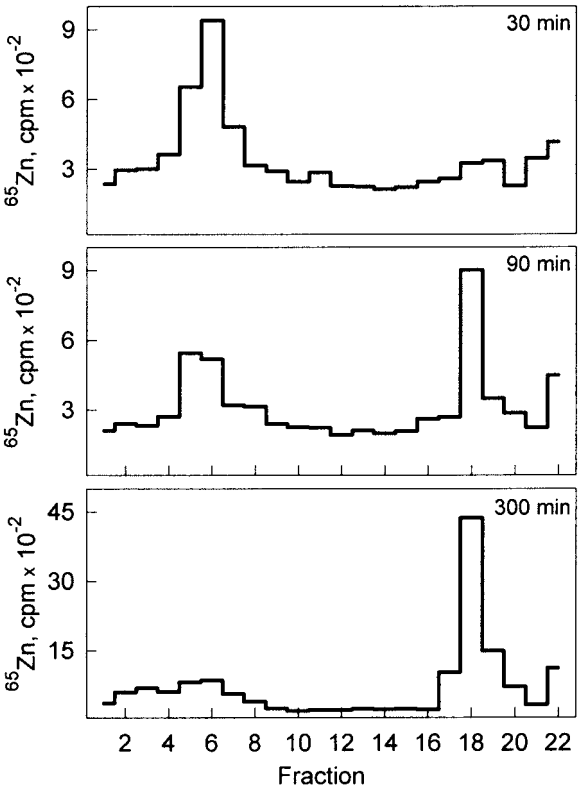

Fig. 3. Stage IV oocyte ^{65}Zn vitellogenin uptake. Stage IV oocytes were incubated and the components separated as described in Figure 2. Free zinc does not enter the oocyte. Within 30 min, ^{65}Zn vitellogenin has entered and is found within the fractions containing multivesicular bodies. During the next 300 min nearly all of the ^{65}Zn vitellogenin is distributed to the region of yolk platelets (fractions 17–20).

stage, about 10% of the zinc is present in a low density fraction (pool I) while 90% is found within yolk platelets (pool II).

Processing of zinc-vitellogenin in the oocyte

The generation of both zinc pools described above from the same process of vitellogenin uptake implies that in stage II oocytes, vitellogenin does not undergo the same processing as in stage IV, and, therefore, is not transferred into yolk platelets in the early compared with latter stages. It is not known if stage II oocytes take up vitellogenin through receptor-mediated endocytosis, as in stage IV, but rather an alternate, non specific pathway, as shown with albumin and other proteins that enter the oocyte (Opresko 1991).

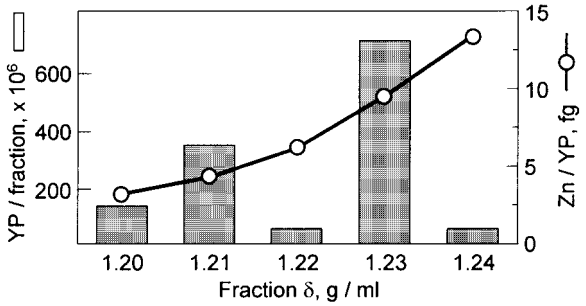

Fig. 4. Yolk platelet zinc content. As yolk platelets mature their density increases. This results in two populations. The light platelets distribute to a density of about 1.21 g/ml sucrose while the heavy organelles are concentrated around 1.23 g/ml. The zinc content of platelets increases progressively as the platelets mature.

Alternatively, zinc could be released from vitellogenin in the cytosol following entry and be transferred to other molecules, analogous to the behavior of iron as it is exchanged between transferrin and ferritin. A number of molecules in the stage II oocytes are potential candidates as zinc acceptors in the cytosolic pool. The 7S and 42S ribonucleoprotein particles are distributed in the pertinent sucrose gradient regions (Denis & Le Maire 1972) where zinc is located in the stage II oocytes. Both particles contain zinc and one of the proteins, TFI IIA, of the 7S one, is a zinc protein (Hanas *et al.* 1983; Miller *et al.* 1985). Moreover, TFI-IIA is synthesized during stages I and II and zinc must be available in the cytosol at that time to fully load the apoprotein. Metallothionein is another zinc acceptor that can distribute zinc to other proteins and it has to be given consideration in the embryo.

The different behavior of vitellogenin taken up by stage IV oocytes leads to its storage in oocyte special organelles. At this stage, vitellogenin does enter the oocyte through the receptor-mediated endocytosis as already described. In the multivesicular body, vitellogenin dissociates from its receptor and it is cleaved into two proteins, lipovitellin and phosvitin. This cleavage is a necessary step for further fusion of the multivesicular bodies to form first light and then heavy yolk platelets that contain condensed and crystalized lipovitellin and phosvitin complexes (Wallace & Jared 1968; Wallace & Opresko 1983; Opresko & Karf 1987). Lipovitellin and phosvitin are readily differentiated on the basis of their solubility properties, electrophoretic mobilities, amino acid composition and sequences, high phosphoserine and lipid contents (Banaszak *et al.* 1991; Montorzi *et al.* 1995; Falchuk *et al.* 1995). Both yolk platelet proteins are solubilized

by 1 M NaCl and can be separated from each other by differential precipitation with 66% ammonium sulfate. Lipovitellin precipitates by this treatment while phosvitin remains in the supernatant. Lipovitellin can then be resolubilized and obtained in pure form following chromatography on Sepharose 6B (Montorzi *et al.* 1995). The yolk platelet zinc is associated entirely with lipovitellin. In fact, the only metal that is bound to lipovitellin in stochiometric amounts is zinc, 1 mol/141 kDa (Table 3). The other major metal of vitellogenin is calcium and it is in the domain that is cleaved into phosvitin. At some point during the uptake and processing of vitellogenin by the oocyte and its assembly in the yolk platelets, phosvitin acquires magnesium (Table 3). The zinc is tightly bound to lipovitellin since it survives the purification procedure that includes extensive dialysis. Furthermore, to remove the zinc requires use of 10^{-3} M OP (other chelating agents will also work) or exposure to acid at pH below 5. The removal is completed within minutes. X-ray absorption fine structure analysis (XFAS) identified the zinc coordination sites in both vitellogenin and lipovitellin (Auld *et al.* 1999). The amino acid ligands for vitellogenin and lipovitellin have been shown to be two histidines and two other N/O ligands. Recently, lipovitellin from the chicken has also been shown to be a zinc protein (Groche *et al.* 2000).

Distribution of zinc and other metals in the embryo

While it requires three years for the oocyte to mature and complete its uptake and storage of zinc into cytosolic and yolk platelet pools, its utilization of some of that zinc takes place over a period of days. Once the egg is fertilized, the two pools remain constant during the entire cleavage and gastrulation periods as well as until the embryo has hatched, a period of less than 48 h depending on the temperature. Beyond that stage, the zinc is progressively transferred out from the yolk platelets. We have proposed that the pool that is used to deliver zinc to any newly formed apoprotein is the cytosolic pool. Any transfer of zinc from the yolk platelet pool would appear in the cytosolic fraction and would have been readily detected. Based on our finding that the oocyte zinc content is about 60–70 ng, the low density pool I contains 6–7 ng while the yolk platelet pool II has the remainder. The zinc content of a typical fully differentiated cell contains 0.01 ng (Table 1). Therefore, if the differentiated phenotype is associated with this quantity of zinc, there is sufficient zinc in the cytosolic pool alone for about 7 million cells.

The distribution of zinc in two pools, i.e., the cytosol and the oocyte/egg yolk platelet, differs from the distribution of other metals (Figure 5). Iron is mostly distributed in the region of the mitochondria and vesicular bodies. Copper is mostly in the low density with a second smaller fraction in the region of the heavy yolk platelets/nuclei. Calcium, magnesium and manganese are in both a low density fraction and the yolk platelet one. Manganese is also found in the region of mitochondria.

Role of zinc in nucleus and transcription underlying development

The zinc in the embryo must be distributed to newly formed apoproteins that function in the generation of the phenotype. When there are insufficient quantities of zinc in the embryo, the phenotype is totally disturbed and in some organism over 80% of all organs are either malformed or are not made at all. The molecular basis for these phenomena is only now emerging and will be reviewed here with the intent of setting the stage to understanding how the zinc and zinc proteins of the oocyte described above are ultimately used to direct the formation of organs by the embryo. A major role of zinc is to regulate chromatin structure and function (Vallee & Falchuk 1981, 1993). At the level of the nucleus, zinc content determines the types and amounts of chromatin binding proteins and their effect on its organization and capability to be transcribed. When zinc is present in normal amounts, a full complement of histones is associated with nuclear DNA. When zinc is reduced in the cell, new histones are not formed and instead basic polypeptides are synthesized (Stankiewicz *et al.* 1983; Mazus *et al.* 1984; Czupryn *et al.* 1987). The consequence is chromatin condensation and an overall reduction in total mRNA formation. Moreover, different proteins are transcribed leading to a different phenotype. These findings led to the formulation of a hypothesis that zinc regulates the expression of a defined set of genes (Vallee & Falchuk 1981). One of the types of proteins requiring zinc for function was proposed to be gene regulatory molecules acting at the level of transcription. Nuclear zinc interacts with these regulatory proteins to activate (or in some cases repress) transcription of particular genes, which in turn, determine the types of proteins formed. The

Table 4. Zinc transcription regulatory proteins.

Protein	Zinc, g at mole	Reference
X. laevis TFIIIA	2	Hanas *et al.* 1983
X. laevis TFIIIA	7–11	Miller *et al.* 1985
Glucocorticoid Receptor (407–556) Fragment	2	Freedman *et al.* 1988
Estrogen Receptor (185–250) Fragment	2	Schwabe *et al.* 1990
Yeast GAL4 (1–147) Fragment	2	Johnston 1987; Pan *et al.* 1990
HIV tat protein	2	Frankel *et al.* 1988
A. nidulans ALCR (7–58) Fragment	2	Sequeval *et al.* 1994
Yeast CYP1(HAP1) (49–126) Fragment	2	Timmerman *et al.* 1994
Yeast PPRI (1–118) Fragment	2	Ball *et al.* 1995
LIM domain (lin-11, RBTN, ISI-1)	2	Li *et al.* 1991; Archer *et al.* 1994
Hela cell SP1 (614–778) Fragment	3	Kuwahara *et al.* 1990
K. lactis LAC9 (1–128) Fragment	2	Halvorsen *et al.* 1990

model described has been amply confirmed. At least 10 transcription factors have now been identified by direct analyses of zinc to require the metal for function (Table 4). The first of these, TFIIIA, has a primary structure with highly conserved sequences comprised of two cysteine and two histidine residues separated by variable numbers of amino acids in 9 repeat units of about 30 amino acids. The Cys and His residues in each of these conserved repeat units could serve as zinc ligands forming tetrahedral coordination complexes with one zinc atom (Miller *et al.* 1985). In the case of TFIIIA, the presence of zinc generates an intervening compact loop structure containing the DNA binding domain of the protein in the sequence intervening between the pair of Cys and His residues. This is the 'zinc finger' DNA binding motif. The number of zinc atoms needed for DNA binding is 2–3 since a peptide containing 'fingers 1–3' binds almost as tightly to specific DNA fragments as the nine 'finger' molecule (Liao *et al.* 1992). While the actual number needed in the intact molecule has yet to be resolved experimentally, it is noteworthy that 2 or 3 zinc atoms is the most frequent number required for function by all of the other transcription factors examined to date (Table 4).

Following the insight on zinc binding motifs, hundreds of transcription factors have been identified with homologous sequences comprised of different combinations of Cys and His residues. These are presumed to be zinc binding sites and the proteins to require zinc for function. None have been isolated to homogeneity and submitted for metal analysis since they are present in exceedingly small quantities in the cell. They must be considered, therefore, putative zinc proteins. Many of the ones listed in Table 4 have been cloned as fragments containing the DNA binding domain of the entire molecule. These fragments bind specific DNA sequences only when zinc is present and associated with the fragment.

In the absence of zinc, a number of regulatory proteins either might not be formed at all or, if formed, might remain as apomolecules that would lack function. On the other hand, other genes might be activated and their transcription products result in inhibitory polypeptides. Together, such effects on synthesis of functional proteins and gene repression and/or activation could produce the phenotype of zinc deficient cells and organisms. Thus, failure to express or to generate active factors by providing zinc to apoproteins

Table 5. Putative zinc transcription factors involved in development.

Gene	Tissues expressing gene	Effect of absence or mutation	Reference
Scratch	Neural precursor cells	Decreased eye photoreceptors and neural loss	Roark *et al.* 1995
Castor	Delaminated CNS neuroblasts, ventral midline glial precursors	Reduction in CNS axonal network	Mellerick *et al.* 1992
Spelt	Terminal pattern elements	Absence of terminal elements	Kuhnlein *et al.* 1994
Krox20	Hindbrain	Loss of rhombomers 3 and 5, fusion of trigeminal, facial and vestibular ganglia	Swiatek *et al.* 1993
Kitz-1	Olfactory epithelial cells	? Malformation	Bernard *et al.* 1994
SKr2	Schwan cells, cephalic and neural crest derived tissues	? Malformation	Schutz *et al.* 1994
Zic	Early embryonic stage neural tube and granule cells of developing cerebellum	Neural tube defects	Nagai *et al.* 1997
Zfh-4	Midbrain	? Malformation	Kostich *et al.* 1995
Ovo	Female gametes	? Altered gametogenesis	Mevel-Ninio *et al.* 1991
Kr	Abdominal segments	Absence of thoracic and abdominal segments	Redeman *et al.* 1988
MZF1	Hematopoietic cells	Altered hematopoiesis	Perrotti *et al.* 1995
Egr-1	Hematopoietic cells	Altered hematopoiesis	Krishnaraju *et al.* 1995
Snail *Escargot,* *Worniu*	Central Nervous System	Defective neuroblast asymmetric divisions	Cai *et al.* 2001
Forkheak genes, *Homeodomain* *box A5,* *Gli,* *Pod1*		Defective lung morphogenesis	Costa *et al.* 2001

at critical junctures in the process of organogenesis could lead to failure to produce tissues or organs. The identification of the specific zinc transcription factors that could be altered and are responsible for the phenotype characteristic of zinc deficiency has yet to be carried out. A number of transcription factors categorized as containing one form of zinc binding domains or another have been recognized (Table 4). While they belong to the large group that has not been certified to contain zinc by direct analysis, their functions in development and the effects of their absence or mutation are of particular interest. Specifically, the effects are those known to be targets of zinc deficiency, namely formation of tissues of the nervous, reproductive, musculoskeletal and hematopoietic systems. These genes link a functional alteration in a putative zinc transcription factor and a specific developmental abnormality. Hence, they provide the experimental tools to study

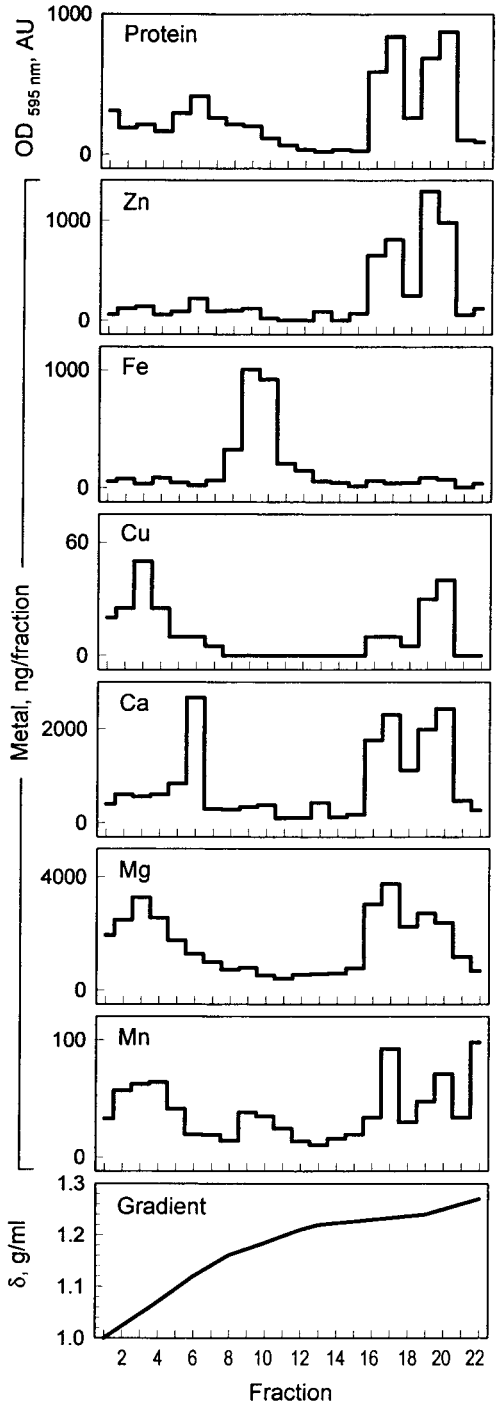

Fig. 5. Distribution of metals in oocyte compartments. Homogenates were centrifuged in a sucrose gradient from 1.0 to 1.25 g/ml (lower panel). Protein was measured by the Bio Rad total protein method. Metals were analyzed by atomic absorption spectrometry (Nomizu *et al.* 1993). The majority (80%) of the protein is in the yolk platelets localized to fractions 17–22. Nearly 90% of the zinc is located in those organelles. Other metals also found in the platelets include copper, calcium, magnesium and manganese. All of these metals are also distributed in less dense fractions containing cytosol, mitochondria and multivesicular bodies.

the distribution of zinc in the embryo and the molecular events that result when zinc is not incorporated into crucial proteins.

The first steps toward this objective have been taken with *Xenopus laevis*. Curtailment of zinc available from the putative cytosolic stores has been accomplished with incubation of the embryos in solutions containing the chelating agent OP. These studies provide support for the proposal that the cytosolic pool, in fact, is the one that distributes zinc to apoproteins during embryogenesis. An OP concentration of 10^{-3} M is required to remove zinc from lipovitellin, as described above. Incubating embryos with OP at concentrations of 100-fold lower does not remove zinc from lipovitellin, yet results in embryos that lack formation of head structures, including the brain and eyes among other classic teratology of zinc deficiency (Jornvall *et al.* 1993; Montorzi *et al.* 2000). About 74% of the embryos hatch. The embryos are smaller, manifest craniofacial malformations, including microcephaly. They do not form head structures; the brain and eyes are absent and there is extrusion of ocular bud regions. The spine and tail regions evidence blebs. Somites and heart are absent. The malformed embryos survive for about 24–48 h after hatching. The stages of embryogenesis that are most sensitive to the teratogenic effects of the chelating agent are 7–15, the period when migration of germ cell lines and their organization into future organs is achieved.

Additional information is emerging relating transcription factors directly involved in development that is considered to be zinc dependent. These would be molecules that could be affected in zinc deficiency. Thus, as described above, zinc deficiency results in pattern of activation and/or repression of a set of genes that is distinct from that of zinc sufficient cells. This manifests in the formation of particular gene products together with the synthesis of others. These products function as necessary components of proliferation and are presumed to be necessary for development as well. Failure to express them at a critical juncture in the process of organogenesis or to supply and incorporate zinc into these macromolecules could result in abnormal phenotypes.

The identification of the specific zinc transcription factors that could be affected and which could be responsible for the pathology of zinc deficiency has yet to be carried out. A number of transcription factors, expressed in cells and tissues of developing embryos, are examples of the many candidates that continue to emerge in the literature (Table 5). They have been cat-

egorized as belonging to the class of zinc dependent transcription factors because of the existence in their primary amino acid sequences of 'zinc binding motifs' that are homologous to those of the known zinc transcription factors (Table 4). While these have not been isolated or characterized as actual zinc proteins, their functions in developmental processes are of particular interest. The ones shown (Table 5) are selected on the basis of their apparent involvement in the formation of tissues, such as the nervous, reproductive, musculoskeletal and hematological systems, all known to be targets of zinc deficiency (Keen & Hurley 1989). In some instances, gene mutation or knock out experiments have been carried out and resulted in lack of expression of normal gene products accompanied by either abnormal or even absent anatomical structures and/or organs.

Concluding remarks

The gene products listed in Table 5 serve to generate an initial list of specific examples with which to study the biochemical functions and characteristics of their transcription products in both zinc sufficient and deficient organisms. Towards this end, the information on the content, uptake and distribution of zinc in *Xenopus laevis* together with the effects of chelating agents on its development provides a suitable biological system to study the relationship between zinc, transcription factors, differentiation and organogenesis.

Acknowledgements

This work was supported, in part, by the Endowment for Research in Human Biology, Inc. and the Bert and Natalie Vallee Foundation, Boston, Massachusetts.

References

Archer VE, Breton J, Sanchez-Garcia I, Osada H, Forester A, Thompson AJ, Rabbitts TH. 1994 Cysteine-rich LIM domains of LIM–homeodomain and LIM- only proteins contain zinc but not iron. *Proc Natl Acad Sci USA* **91**, 316–320.

Auld DS, Falchuk KH, Zhang K, Montorzi M, Vallee BL. 1996 X-ray absorption fine structure as a monitor of zinc coordination sites during oogenesis of *Xenopus laevis. Proc Natl Acad Sci USA* **93**, 3227–3231.

Ball LJ, Diakun GP, Gadhavi PL, Young NA, Armstrong EM, Garner CD, Laue ED. 1995 Zinc coordination in the DNA binding domain of the yeast transcription activator PPR1. *FEBS Lett* **358**, 278–282.

Banaszak L, Sharrock W, Timmins P. 1991 Structure and function of a lipoprotein: lipovitellin. *Annu Rev Biophys Chem* **20**, 221–246.

Bernard O, Ganiatsas S, Kannourakis G, Dringen R. 1994 Kizl, a protein with LIM zinc finger and kinase domains, is expressed mainly in neurons *Cell Growth Diff.* **5**, 159–1171.

Cai Y, Chia W, Yang X. 2001 A family of snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate *Drosophila* neuroblast asymmetric divisions. *EMBO J* **20**, 1704–1714.

Costa RH, Kalinichenko VV, Lim L. 2001 Transcription factors in mouse lung development and function. *Am J Physiol* **280**, L823–L838.

Czupryn M, Falchuk KH, Vallee BL. 1987 Zinc deficiency and metabolism of histones and nonhistone proteins in *Euglena gracilis*. *Biochemistry* **26**, 8263–8269.

Davidson EH. 1990 How embryos work: A comparative view of diverse modes of cell specification. *Development* **108**, 365–389.

Denis H, Le Maire M. 1983 Thesaurisomes, a novel kind of nucleoprotein particle. *Subcell Biochem* **9**, 263–297.

Dreosti IE, Tao S, Hurley LS. 1968 Plasma zinc and leukocyte changes in weanling and pregnant rats during zinc deficiency. *Proc Soc Exp Biol Med* **127**, 169–174.

Falchuk KH. 1998 The molecular basis for the role of zinc in developmental biology. *Mol Cell Biochem* **188**, 41–48.

Falchuk KH, Montorzi M, Vallee BL. 1995 Zinc uptake and distribution in *Xenopus Laevis* oocytes and embryos. *Biochemistry* **34**, 16524–16531.

Frankel AD, Chen L, Cotter RJ, Pabo CO. 1988 tat protein from human immunodeficiency virus forms a metal linked dimer. *Science* **240**, 70–73.

Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB, Yamamoto KR. 1988 The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature (London)* **334**, 543–546.

Gallaher D, Hurley LS. 1980 Low zinc concentration in rat uterine fluid after 4 days of dietary deficiency. *J Nutr* **110**, 591–593.

Groche D, Rashkovetsky LG, Falchuk KH, Auld DS. 2000 Subunit composition of the zinc proteins alpha- and beta-lipovitellin from chicken. *J Protein Chem* **19**, 379–387.

Hanas JS, Hazuda D, Bogenhagen DF, Wu FY-H, Wu C-W. 1983 *Xenopus* transcription factor A requires zinc for binding to 5S gene. *J Biol Chem* **258**, 14120–14125.

Hansen P, Riebessell M. 1991 *The early development of Xenopus Laevis*. Berlin: Springer-Verlag: 1–18.

Halvorsen YC, Nandabaln K, Dickson RC. 1990 LAC 9 DNA-binding domain coordinates two zinc atoms per monomer and contacts DNA as a dimer. *J Biol Chem* **265**, 13283–13289.

Hurley LS, Shrader RE. 1975 Abnormal development of preimplantation rat eggs after three days of maternal dietary zinc deficiency. *Nature (London)* **254**, 427–429.

Jornvall H, Falchuk KH, Geraci G, Vallee BL. 1993 1,10-phenanthroline and *Xenopus laevis* teratology. *Biochem Biophys Res Comm* **200**, 1398–1406.

Johnston M. 1987 Genetic evidence that zinc is an essential cofactor in the DNA binding domain of GAL4 protein. *Nature (London)* **328**, 353–355.

Keen CL, Hurley LS. 1989 Zinc and reproduction: Effects of deficiency on foetal and postnatal development. In: Mills CF. ed. *Zinc in Human Biology*. London: Springer-Verlag: 183–220.

Kostich WA, Sanes JR. 1995 Expression of zfh-4, a new member of the zinc finger-homeodomain family, in developing brain and muscles. *Dev Dyn* **202**, 145–152.

Krishnaraju K, Nguyen HQ, Liebermann DA, Hoffman B. 1995 The zinc finger transcription factor Egr-I potentiates macrophage differentiation of hematopoietic cells. *Mol Cell Biol* **15**, 5499–5507.

Kuwahara J, Coleman JE. 1990 Role of zinc (II) ions in the structure of the three finger DNA binding domain and the SP1 transcription factor. *Biochemistry* **29**, 8628–8631.

Kuhnlein RP, Frommer G, Friedrich M, Gonzalez-Gaitain M, Weber A, Wagner-Bernholz JF, Gehring WJ, Jackle H, Schuh R. 1994 spalt encodes an evolutionary conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J* **13**, 168–179.

Li PM, Reichter J, Freyd G, Horvitz HR, Walsh CT. 1991 The LIM region of a presumptive *Caenorhabditis elegans* transcription factor is an iron-sulfur and zinc containing metallodomain. *Proc Natl Acad Sci USA* **88**, 9210–9213.

Liao X, Clemens KR, Tennant PE, Wright JM, Gottesfeld M. 1992 Specific interaction of the first three zinc fingers of TFIIIA with the internal control region of the *Xenopus* 5S RNA gene. *J Mol Biol* **223**, 857–871.

Mazus B, Falchuk KH, Vallee BL. 1984 Histone formation, gene expression and zinc deficiency in *Euglena gracilis. Biochemistry* **23**, 42–44.

Mellerick DM, Kassis JA, Zhang SD, Odenwald WF. 1992 castor encodes a novel zinc finger protein required for the development of a subset of CNS neurons in *Drosophila*. *Neuron* **9**, 789–803.

Mevel-Ninio M, Terracol R, Kafatos FC. 1991 The ovo gene of *Drosphila* encodes a zinc finger protein required for female germ line development. *EMBO J* **10**, 2259–2266.

Miller J, McLachlan AD, Klug A. 1985 Repetitive zinc binding domains in the protein transcription factor IIIA from *Xenopus oocytes. EMBO J* **4**, 1609–1614.

Montorzi M, Falchuk KH, Vallee BL. 1995 Vitellogenin and Lipovitellin: zinc proteins of *Xenopus Laevis* oocytes. *Biochemistry* **34**, 10851–10858.

Montorzi M, Burgos MH, Falchuk KH. 2000 *Xenopus laevis* embryo development: arrest of epidermal cell differentiation by the chelating agent 1,10 phenanthroline. *Mol Reprod Dev* **55**, 75–82.

Nagai T, Aruga J, Takada S, Gunther T, Sporle R, Schugart K, Mikoshiba, K. 1997 The expressions of the mouse Zicl, Zic2 and Zic3 gene suggest an essential role for zic genes in body pattern formation. *Dev Biol* **182**, 299–313.

Nomizu T, Falchuk KH, Vallee BL. 1993 Zinc, iron and copper contents of *Xenopus laevis* oocytes and embryos. *Mol Reprod Dev* **36**, 419.

Opresko LK. 1991 *Xenopus Laevis*: Practical Uses in Cell and Molecular Biology. *Methods Cell Biol* **36**, 117–132.

Opresko L, Karpf RA. 1987 Specific proteolysis regulates fusion between endocytic compartments in *Xenopus* oocytes. *Cell* **51**, 557–568.

Pan T, Coleman JE. 1990 GAL4 transcription factor is not a 'zinc finger' but forms a Zn(II)2Cys6 binuclear cluster. *Proc Natl Acad Sci USA* **87**, 2077–2081.

Perrotti D, Melotti P, Skowedki T, Casella I, Peschle C, Calebretta B. 1995 Over-expression of the zinc protein MZI inhibits hematopoietic development from embryonic stem cells. Correlation with negative regulation of CD34 and cmyc promoter activity. *Mol Cell Biol* **15**, 6075–6087.

Redemann N, Gaul U, Jackle H. 1988 Disruption of a putative cys-zinc interaction eliminates the biological activity of the Kruppel finger protein. *Nature (London)* **332**, 90–92.

Roark M, Sturtevant MA, Emery J, Vaessin H, Grell E, Brier E. 1995 scratch, a pan-neural gene encoding a zinc finger protein related to snail, promotes neuronal development. *Genes Dev* **9**, 2384–2398.

Schutz B, Niessing J. 1994 Cloning and structure of a chicken zinc finger cDNA: Restricted expression in developing neural crest cells. *Gene* **148**, 227–236.

Schwabe JWR, Neuhaus D, Rhodes D. 1990 Solution structure of DNA binding domain of the oestrogen receptor. *Nature (London)* **348**, 458–460.

Sequeval D, Felenbok B. 1994 Relationship between zinc content and DNA binding activity of the DNA- binding motif of the transcription factor ALCR in *Aspergillus nidulans. Mol Gen Genet* **242**, 33–39.

Stankiewicz A, Falchuk KH, Vallee BL. 1983 Composition and structure of zinc deficient *Euglena gracilis* chromatin. *Biochemistry* **22**, 5150–5156.

Stifani S, Nimpf J, Schneider WJ. 1990 Vitellogenesis in *Xenopus laevis* and chicken: cognate ligands and oocyte receptors. The binding site for vitellogenin is located on lipovitellin I. *J Biol Chem* **265**, 882–888.

Swiatek PJ, Gridely T. 1983 Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene krox20. *Genes Dev* **7**, 2071–2084.

Timmerman JE, Guiard B, Schechter E, Delsuc MA, Lallemand JY, Gervais M. 1994 The DNA-binding domain of the yeast Saccharomyces cerevisiae CYP1 (HAP1) transcription factor possesses two zinc ions which are complexed in a zinc cluster. *Eur Biochem* **225**, 593–599.

Vallee BL, Falchuk KH. 1981 Zinc and gene expression. *Philos Trans R Soc Lond B Biol Sci* **294**, 185–197.

Vallee BL, Falchuk KH. 1993 The biochemical basis of zinc physiology. *Physiol Rev* **73**, 79–111.

Wallace RA. 1978 *The Vertebrate Ovary*. In: Jones RE. ed. New York: Plenum: 469–502.

Wallace RA, Jared DW. 1968 Estrogen induces lipophosphoprotein in serum of male *Xenopus laevis. Science* **160**, 91–92.

Wallace RA, Nickol JM, Ho T, Jared OW. 1972 Studies on amphibian yolk. X. The relative roles of autosynthetic and heterosynthetic processes during yolk protein assembly by isolated oocytes. *Dev Biol* **29**, 225–272.

Wallace RA, Opresko L, Wiley HS, Selman K. 1983 The oocyte as an endocytic cell. *Ciba Found Symp* **98**, 228–248.