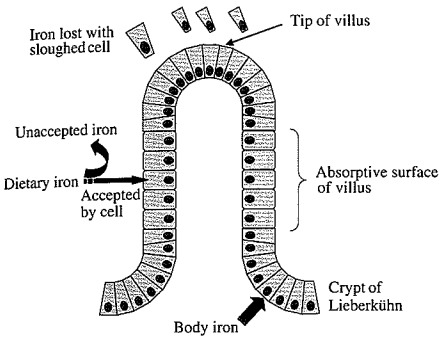

CHAPTER 5

UPTAKE OF ELEMENTS FROM A BIOLOGICAL POINT OF VIEW

ULF LINDH
Uppsala University

CONTENTS

The utilization of an element in biology is intimately dependent on its uptake into the living organism. A lot is known of the qualitative aspects of uptake; for example, common sense tells us that what originates in the geological background has to be transported through the soils and presented to plants in a convenient form for uptake. These processes are affected by physicochemical factors due to Nature itself and the increasing pressures from human activities.

From a biologist's perspective, the uptake process is extremely fascinating. Current knowledge tells us that organisms need about 20 of the naturally occurring elements found in the periodic table. Over the course of evolution, mechanisms have developed for the uptake and utilization of elements in organisms that are more or less specific for each. Additionally, those elements that are not essential or even detrimental to the organism are excluded, usually in an efficient manner. This chapter deals with the general aspects of element uptake as well as principles of exclusion. In addition, examples will be given pertaining to some major, minor, and trace elements collectively described as essential. This chapter begins with a discussion of the periodic table and what is meant by *essentiality*.

I. ESSENTIALITY OF ELEMENTS

A usually ignored fact in biology is that explanations for the behavior of elements can be found in the periodic table itself. Among the elements known to be involved in biology, 11 appear to be approximately constant and predominant in all biological systems. The human body is comprised of about 99.9% of the 11 elements, but surprisingly only 4 of them—hydrogen, carbon, nitrogen, and oxygen—account for 99% of the total. These 4 elements, the major elements, comprise the bulk of living organisms. In addition to these elements, there are the minor elements—sodium, magnesium, phosphorus, sulfur, chlorine, potassium, and calcium (also called *electrolytes*). The minor elements appear in much lower concentration than major elements. One group of elements has still to be defined: the trace elements.

From an analytical chemistry standpoint, trace elements would be described as elements appearing in low concentrations in living systems (*i.e.*, <100 mg/kg). In biology, however, trace elements would be defined by exclusion; for example, a biological approach begins by excluding the major elements as well as the minor elements. Furthermore, group 18 elements (the noble gases) are excluded due to their disinclination for chemical reactions, a property that makes these elements less likely to be a factor in biological functions. Depending on how many elements are considered naturally occurring, the trace elements thus constitute the remainder of the periodic table (*i.e.*, 73 or 75 elements). Most of the elements of the periodic table, then, are trace elements in the eyes of a biologist. Surprisingly, this exclusion definition coincides closely with the one of analytical chemistry. Most of the trace elements appear in biology at concentrations below or well below 100 mg/kg.

Essentiality is usually defined in an operational way, based on early protein chemistry. More stringent criteria have evolved as our knowledge has improved. A trace element can be considered essential if it meets the following criteria: (1) it is present in all healthy tissues of all living things, (2) its concentration from one animal to the next is fairly constant, (3) its withdrawal from the systems induces reproducibly the same physiological and structural abnormalities regardless of the species studied, (4) its addition either reverses or prevents these abnormalities, (5) the abnormalities induced by deficiencies are always accompanied by specific biochemical changes, and (6) these biological changes can be prevented or cured when the deficiency is prevented or cured. It is obvious that the number of elements recognized as essential depends on the sophistication of experimental procedures and that proof of essentiality is technically easier for those elements that occur in reasonably high concentrations than for those with very low requirements and concentrations. Thus, it can be expected that, with further improvement of our experimental techniques, additional elements may be deemed essential.

These six criteria might appear to be too stringent, and in some cases they must be modified. Most of the trace elements essential to both plants and animals are found in the first row of the transition (redox) metals. Zinc is not included in the transition metals, and it does not take part in redox reactions, which is an important property in biology; however, zinc is a good Lewis acid. All of the bulk elements are non-metals. The minor elements include metals as well as non-metals, with only one oxidation state available. Metals are the dominant components of the essential trace elements, but some very important trace elements are non-metals, such as selenium and iodine. Boron and silicon are non-redox non-metals, and both of them are acknowledged as being essential. Boron, in fact, has been shown to be essential to plants, although it is found in appreciable concentrations in animals as well. The functions of these elements will be discussed in greater detail in Chapter 6.

II. GENERAL ASPECTS OF ELEMENT UPTAKE

The uptake of elements is a process that may vary considerably depending on the complexity of the living system being considered. Unicellular organisms account for the simplest processes, but in complex organisms several aspects of the uptake process must be considered. In humans, for example, the primary uptake process takes place in the gastrointestinal tract, predominantly in the duodenum and first part of the jejunum. Elements taken up have to be transported across the mucosal cells of the intestines to reach the bloodstream, from which they are transported to the liver, where the elements are isolated and delivered into the main bloodstream. After being transported to the organs that will utilize them, these elements must then enter the cells of these organs. If the final target is not found inside the cell, then further transport across additional membranes may be required. Let us review the general principles of transport across cell membranes. A cell or an organelle cannot be entirely open or entirely closed to its surroundings. Although the cell interior must be protected from certain toxic compounds, metabolites must be taken in and waste products removed. Because the cell must contend with thousands of substances, it is not surprising that much of the complex structure of membranes is devoted to the regulation of transport.

III. THE THERMODYNAMICS OF TRANSPORT

Before considering specific mechanisms of transport, it is useful to review some general ideas. The free energy change, ΔG, for transporting one mole of a substance

from one place with concentration C_1 to another place with concentration C_2 is

$$\Delta G = RT \ln \frac{C_1}{C_2} \quad (1)$$

where R is the gas constant and T the temperature. According to Eq. (1), if C_2 is less than C_1, then ΔG is negative and the process is thermodynamically favorable. When more substance is transferred (between two finite compartments), C_1 decreases and C_2 increases, until $C_2 = C_1$. Now, the system is at equilibrium, and $\Delta G = 0$. This equilibrium is the ultimate state approached by transport across any membrane. The concentration of any substance traversing the membrane will end up the same on both sides. In kinetic terms, if the molecules enter the membrane randomly, the number entering from each side will be proportional to the concentration on that side. Once the concentrations are equal, the rates of transport in the two directions will be the same; consequently, no net transport occurs. This equalization can be sidestepped under three conditions, each of which is important in the behavior of membranes.

A substance may be bound by macromolecules restricted to one side of the membrane. It could also be chemically modified once it has crossed the membrane. Compound M could be more concentrated inside a cell than outside, but much of M could be bound to cellular macromolecules or could have been modified. This part of M is not included in Eq. (1), which only deals with the free M, implying, then, that the concentration would be equal on both sides at equilibrium. We can use oxygen in red blood cells to illustrate this principle. Measurements would indicate that the total concentration of molecular oxygen is lower in the blood plasma than in the red cells. Included in the total concentration of the red cells, however, is the portion bound to hemoglobin. The concentration of free dioxygen is still the same in the red cells and plasma at equilibrium.

A membrane is often characterized by an electrical potential governing ion distribution. The well-known principle saying that equal charges repel each other and unequal charges attract each other may now be used to show a simple example. The negatively charged inside of a cell tends to attract cations and to drive out anions. Mathematically, the free energy change for transport over a membrane is

$$\Delta G = RT \ln \frac{C_1}{C_2} + ZF\Delta\Psi \quad (2)$$

where Z is the charge of the ion, F is the Faraday constant (96,485 J mol^{-1} V^{-1}) and $\Delta\Psi$ is the transmembrane electric potential (in V). If Z is positive and $\Delta\Psi$ negative (with the inside negative relative to the outside), transport of cations into the cell is favored. The concentration difference of ions across the membrane in most cells is kept at more than ten times, implying that active transport is a major energy-requiring process in biology.

Usually Eq. 1 does not reflect the real situation well enough and must be modified accordingly:

$$\Delta G = RT \ln \frac{C_1}{C_2} + \Delta G' \quad (3)$$

where $\Delta G'$ may correspond to a thermodynamically favored reaction. Adenosine triphosphate (ATP) hydrolysis coupled to the transport might be such a situation. Equation (3) is clearly a generalization of Eq. (2) that now allows a variety of processes to participate in the transport.

Equations (2) and (3) convey the message that transport across membranes (in and out of cells) can take place against unfavorable concentration gradients. The sodium–potassium pump provides continuous import of potassium and export of sodium, thereby maintaining the concentration difference between inside and outside. Following is a review of the mechanisms by which substances are passed through membranes.

A. Passive Transport: Diffusion

Passive transport or passive diffusion occurs due to the random walk of molecules through membranes. The process is the same as the Brownian motion of molecules in any fluid and is called *molecular diffusion*. During passive transport, the diffusing substance ultimately reaches the same free concentration inside and outside the membrane. The net rate of transport, J (mol cm^{-2} s^{-1}), is proportional to the concentration difference ($C_2 - C_1$) over the membrane:

$$J = -\frac{KD_1(C_2 - C_1)}{l} \quad (4)$$

where l is the thickness of the membrane, D_1 is the diffusion coefficient of the diffusing substance in the membrane, and K is the partition coefficient for the diffusing material between lipid and water (the ratio of solubilities of the material in lipid and water). For ions and other hydrophilic substances, K is a very small number. Diffusion of such substances through lipid membranes is thus extremely slow. In agreement with Eq. (1), Eq.

TABLE I. Permeability Coefficients from Some Ions and Molecules Through Membranes

P ($cm\,s^{-1}$)	Membrane Phosphatidylserine	Human Erythrocyte
K^+	$<9 \times 10^{-13}$	2.4×10^{-10}
Na^+	$<1.6 \times 10^{-13}$	10^{-10}
Cl^-	1.5×10^{-11}	1.4×10^{-4}[a]
Glucose	4×10^{-10}	2×10^{-5}[a]
Water	5×10^{-3}	5×10^{-3}

[a] Facilitated transport. Note that whenever facilitated transport is encountered, the permeability coefficient rises dramatically.

(4) says that net transport stops when $C_2 = C_1$. If C_1 and C_2 are expressed in $mol\,cm^{-3}$ and l in centimeters, then D_1 has the dimension $cm^2\,s^{-1}$. Note that D_1 is not the same as the diffusion coefficient (D) that the same molecule would have in aqueous solution. D_1 depends not only on the size and shape of the molecule but also on the viscosity of the membrane lipid.

K, D_1, or the exact thickness of the membranes involved are not usually known, so the rate of passive transport is often described in terms of the permeability coefficient, P, which can be measured by direct experiment:

$$\mathcal{J} = -P(C_2 - C_1) \qquad (5)$$

By comparing Eqs. (5) and (4), we see that P is expressed by:

$$P = \frac{KD_1}{l} \qquad (6)$$

with the dimensions $cm\,s^{-1}$.

Table I shows the permeability coefficients for a number of small molecules and ions in membranes. The low P values of the ions are expected, because ions, as already mentioned, have low values of K; however, the relatively large permeability value for water is conspicuous. Biological membranes are not, in fact, very good barriers against water, the reasons of which are not entirely clear, but they have the obvious advantage of allowing the ready exchange of water with their surroundings.

B. Facilitated Transport: Accelerated Diffusion

The functional and metabolic needs of cells often require transport that is more efficient than passive diffusion. The adequate handling of catabolically produced CO_2 is coupled to the red blood cell exchange of HCO_3^- and Cl^-. In respiring tissues, CO_2 enters the red cell and is converted to HCO_3^- by carbonic anhydrase, an enzyme that is zinc dependent. HCO_3^- leaves the red cell, and Cl^- enters. The HCO_3^- is transported to the lungs in the plasma where it enters the red cell, and Cl^- is driven out. Inside the red cell, HCO_3^- is again converted to CO_2 by carbonic anhydrase, leaves the cell, and is exhaled. The permeability coefficients for Cl^- and HCO_3^- in red cell membranes are about $10^{-4}\,cm\,s^{-1}$, or about 10 million times greater than the permeability coefficients for such ions in pure lipid bilayers such as the artificial phosphatidylserine membrane described in Table I. Some special mechanism, then, is required to account for this difference. Two general types of facilitated transport, or facilitated diffusion, are known. One type, which is responsible for the rapid transport of Cl^- and HCO_3^- through red cell membranes, involves pores formed by transmembrane proteins (Figure 1A). The other type is mediated by transmembrane carrier molecules (Figure 1B).

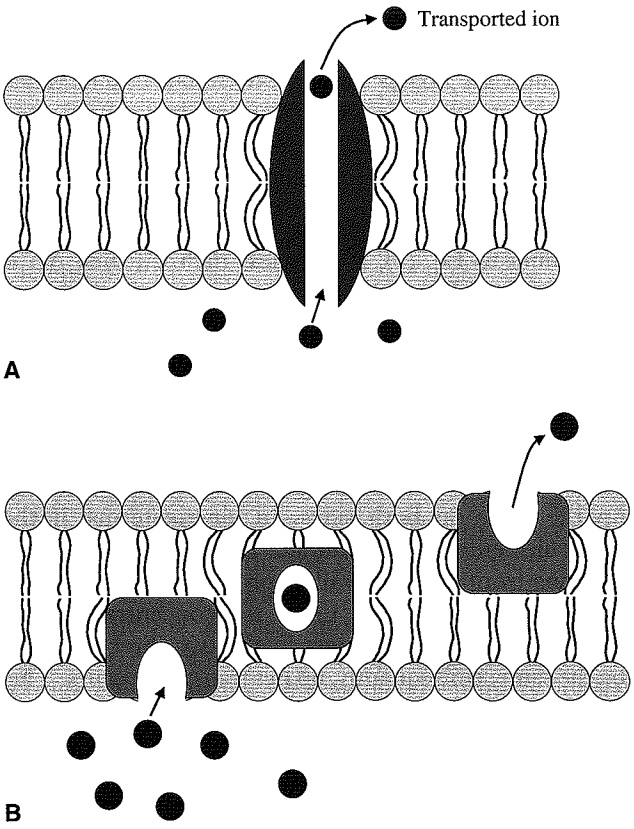

FIGURE 1 (A) Facilitated transport by pores. (B) Facilitated transport by carrier molecules.

Pore-facilitated transport is an important process. An example is the band 3 integral protein that exists as a dimer in the red cell membrane and serves as an anion channel that exchanges Cl^- and HCO_3^-. This protein probably spans the membrane 12 times. Exit of the HCO_3^- is balanced by an influx of Cl^-, which means that, in the absence of Cl^- ions, the transport of HCO_3^- stops. The band 3 protein does not simply form a hole in the membrane for the passage of ions; rather, the pore is very selective and exchanges HCO_3^- and Cl^- on a 1:1 basis. Such facilitated transport, however, is not necessary for O_2 or CO_2. These small, nonpolar molecules are allowed to move rapidly through the membrane by passive diffusion.

A common example of carrier-facilitated transport is the antibiotic valinomycin (from *Streptomyces*), which is a polymer with an approximately spherical shape. Its outer layer has numerous methyl groups; thus it is hydrophobic. Inside the sphere are collections of nitrogen and oxygen that can bind (chelate) a potassium ion. This structure, however, cannot chelate other cations, so it is specific for potassium. Due to its hydrophobic exterior, valinomycin easily passes through a membrane, in contrast to the ion itself. In mathematical terms, the valinomycin increases the factor K in Eq. (6).

The measurable difference between passive and facilitated diffusion is, of course, the transport rate. Another measure is the phenomenon of saturation, which is a characteristic feature of facilitated transport. There is a finite number of carriers or pores in a membrane, and each of them can handle only one ion at a time. Saturation will occur when all carriers are occupied (Figure 2). Equations (4) and (5) imply that the rate of passive diffusion increases linearly with the concentration difference because there are no sites to saturate. The carrier-facilitated transport can be described by:

$$V_0 = \frac{V_{max}[S]_{out}}{K_{tr} + [S]_{out}} \qquad (7)$$

Equation (7) looks familiar and, with some knowledge of enzyme kinetics, it is recognized as being similar to the Michaelis–Menten equation. V_0 represents the initial rate of transport into the membrane at an external concentration of $[S]_{out}$. V_{max} is the maximum transport rate of the substrate, and K_{tr} is analogous to the Michaelis constant K_m. This means that K_{tr} is the substrate concentration when the transporter is half-saturated. The transport rate approaches the maximum value at a high substrate concentration. In Figure 2, the straight line illustrates passive diffusion with, theoretically, no saturation.

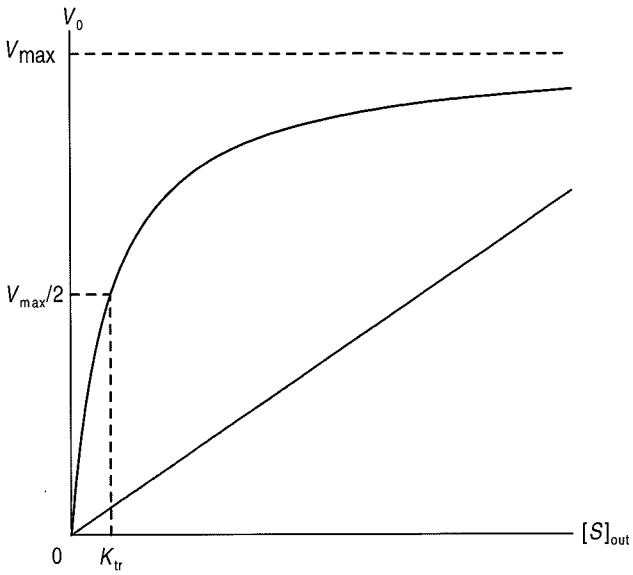

FIGURE 2 Facilitated and passive transport.

It still has to be remembered that passive and facilitated transport are diffusion processes, and as such they do not require energy. Pores are more effective because they offer open gates. Carriers increase the solubility in membranes by offering a hydrophobic outer surface. Irrespective of the kind of diffusion transport, the final free concentration on both sides of the membrane will be equal at equilibrium.

C. Three General Classes of Transport

With regard to transport across membranes, three different types have been identified, all of which depend on the number of substances and the direction in which each is transported. When only one substrate is transported, it is referred to as a *uniport transport process*—for example, the transport of glucose into red blood cells. The band 3 protein (the anion-exchange protein) is an example of an *antiport process*, in which one ion is transported out of and another into the cell. *Symport* is the transfer of two substrates in the same direction. Glucose and certain amino acids are transported via symport with Na^+. In this case, use is made of the gradient caused by the Na^+, K^+-ATPase in the plasma membrane. Figure 3 illustrates these general classes of transport. This characterization, however, takes into account only the direction of transport and does not show whether or not energy is required.

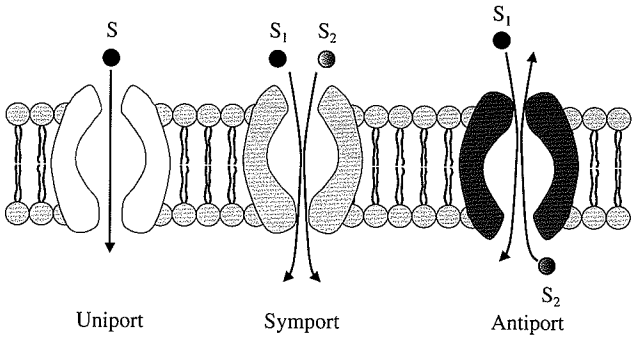

FIGURE 3 The three general classes of transport systems differ in the number of substrates transported and the direction in which each is transported. In uniport systems, only one substrate is transported. Symport and antiport systems are characterized by the transport of two substrates in the same and in opposite directions, respectively.

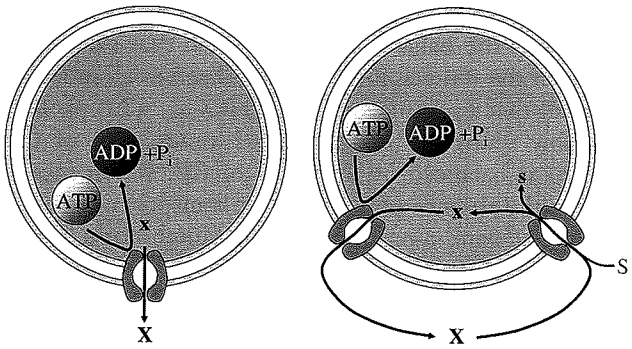

FIGURE 4 Active transport. In primary active transport (left), the energy released by ATP hydrolysis drives solute transport against an electrochemical gradient. In secondary active transport (right), a gradient of ion X (often Na^+) has been secured by primary active transport. Transport of X down its electrochemical gradient accordingly provides the energy to drive cotransport of a second solute (S) against its electrochemical gradient.

D. Active Transport: Transport Against a Concentration Gradient

In many situations, transport must be carried out even against concentration gradients. This requires a type of transport other than facilitated or passive diffusion. A calcium ion ratio of 30,000 must be maintained across membranes of the sarcoplasmic reticulum in muscle fibers. Using Eq. (1), such a ratio corresponds to $\Delta G = 25.6\,kJ\,mol^{-1}$, which indicates an insurmountable impediment. An active transport is necessary in such a scenario, but such a process is thermodynamically unfavorable and can only take place when coupled to a thermodynamically favorable process, such as absorption of light, an oxidation reaction, the breakdown of ATP, or an accompanying flow of some other chemical species down its electrochemical gradient. We can differentiate between *primary* and *secondary active transport* (Figure 4). In the former process, accumulation is coupled directly to a thermodynamically favorable chemical reaction, such as the conversion of ATP to ADP + P_i. When uphill transport of one solute is coupled to the downhill flow of a different solute that has originally been transported uphill by primary active transport, the process is secondary active transport.

The energy required to export one Ca^{2+} ion from the inside of the cell is 9.1 kJ. The energetic cost of moving an ion depends on the electrochemical potential or the sum of the chemical and electrical gradients (see, for example, Eq. (2)). Most cells maintain more than tenfold differences in ion concentrations across their plasma or intracellular membranes; therefore, for many cells and tissues active transport is a major energy-consuming process.

E. Types of Transport ATPases

The four known types of ATPases are P-type, V-type, F-type, and multidrug transporter. The last type will not be dealt with in this chapter because it is not involved in the transport of elements. P-type ATPases are the most versatile, at least from an elemental point of view. They all transport cations and are reversibly phosphorylated by ATP in the transport cycle. They are all integral proteins with multiple membrane-spanning regions, although they are only single polypeptides. This type of transporter is very widely distributed. The Na^+, K^+-ATPase, an antiporter for Na^+ and K^+, and the Ca^{2+}-ATPase, a uniporter for Ca^{2+}, are ubiquitous, well-understood P-type ATPases in animal tissues. They maintain disequilibrium in the ionic composition between the cytosol and the extracellular media. P-type ATPases are responsible for pumping H^+ and K^+ over the plasma membrane in parietal cells lining the mammalian stomach, thereby acidifying the contents. Bacteria use P-type ATPases to export toxic metal ions such as Cu^{2+}, Cd^{2+}, and Hg^{2+}. Table II provides a summary of the properties of the transport ATPases.

V-type ATPases act as proton pumps and are not structurally similar to the F-type ATPases. The name V-type derives from their role of keeping the pH of vacuoles of fungi and higher plants at 3 to 6. In addi-

TABLE II. Four Classes of ATPases

P-type ATPases	*Organism or Tissue*	*Type of Membrane*
Na^+, K^+	Animal tissues	Plasma
H^+, K^+	Acid-secreting (parietal) cells of mammals	Plasma
H^+	Fungi (*Neurospora*)	Plasma
H^+	Higher plants	Plasma
Ca^{2+}	Animal tissues	Plasma
Ca^{2+}	Myocytes of animals	Sarcoplasmic reticulum (ER)
Cd^{2+}, Hg^{2+}, Cu^{2+}	Bacteria	Plasma
V-type ATPases		
H^+	Animals	Lysosomal, endosomal, secretory vesicles
H^+	Higher plants	Vacuolar
H^+	Fungi	Vacuolar
F-type ATPases		
H^+	Eukaryotes	Inner mitochondrial
H^+	Higher plants	Thylakoid
H^+	Prokaryotes	Plasma

tion to acidification of vacuoles, the same occurs for lysosomes, endosomes, the Golgi complex, and secretory vesicles in animal cells. The complex structures of V-type ATPases are similar throughout the family and possess an integral transmembrane domain that comprises the proton channel and a peripheral domain containing the ATP-binding site and the ATPase activity.

The F-type ATPase is so called because it has been identified as an energy-coupling factor. F-type ATPases catalyze uphill as well as downhill transport of protons. The uphill process is propelled by ATP hydrolysis, whereas the downhill reaction drives ATP synthesis. In this case, we may call them ATP synthases rather than ATPases. The F-type ATPases are multi-subunit complexes. They provide a transmembrane pore for protons and a molecular machine using the energy release by downhill proton flow to form the phosphoanhydride bonds of ATP.

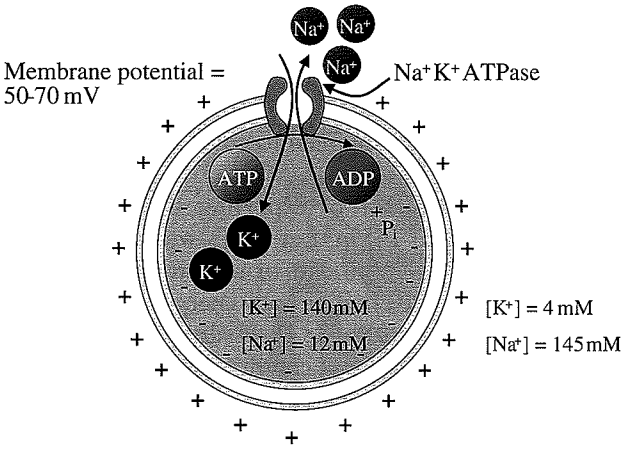

FIGURE 5 In animal cells, this active transport system is primarily responsible for establishing and maintaining the intracellular concentrations of Na^+ and K^+ and for generating the transmembrane electrical potential.

F. Ion Pumps

Anyone having been in the slightest contact with biochemistry or physiology has not been able to avoid the sodium–potassium pump (Figure 5). This remarkable ion pump maintains concentration gradients for sodium from the inside of cells to the outside of 12 and 145 m*M*, respectively. At the same time, the concentration of potassium is kept at 140 m*M* intracellularly, in contrast to 4 m*M* outside. This situation pertains to almost all animal cells and would not be possible to maintain based solely on passive diffusion. The motor of this pump is the Na^+, K^+-ATPase that couples ATP breakdown to the concomitant movement of both Na^+ and K^+ against their electrochemical gradients.

The sodium–potassium pump transports two K^+ ions into the cell and exports three Na^+ ions at the cost of one molecule of ATP converted into ADP and P_i. Na^+, K^+-ATPase is an integral protein comprised of two subunits of approximate molecular weight 50,000 and 110,000, both of which span the membrane. This transporter is a P-type ATPase. The mechanism seems simple; however, the import of two potassium ions and the simultaneous export of three sodium ions are still not fully understood. It is generally assumed, though, that the ATPase cycles between two forms, one of which is phosphorylated with a high affinity for K^+ and a low affinity for Na^+, as well as one that is dephosphorylated with a high affinity for Na^+ and low affinity for K^+. The breakdown of ATP to ADP and P_i by hydrolysis takes place in two steps catalyzed by the enzyme: formation

of the phosphoenzyme and hydrolysis of the phosphoenzyme with the overall net reaction:

$$ATP + H_2O \rightarrow ADP + P_i$$

In this way, energy is supplied to cover the expenditure of the pump.

Calculating the cost of exporting three moles of sodium from 12 to 145 m*M* at 37°C, we arrive at 39.5 kJ. Correspondingly, the cost of importing two moles of potassium is 4.8 kJ. The net energy necessary to perform the transport is 44.3 kJ. Hydrolyzing one mole of ATP under physiological conditions to ADP gives 31 kJ. According to this calculation, more than 44 kJ is required for the transport, which does not seem reasonable. The trick is that in most cells the concentration of ATP is much higher than the concentrations of ADP and P_i. The energy available in real life is thus enough to pay for the transport.

Free calcium ions in cytosol are usually kept at a concentration of about 100 n*M*, which is far below what is found outside cells; thus, it is a significant finding that the total concentration of calcium in cells is much higher. One reason is that inorganic phosphates such as P_i and PP_i occur at millimolar concentrations. The concentration of free calcium ions must be kept low because inorganic phosphates readily combine with calcium, and relatively insoluble calcium phosphate precipitates will form. Maintaining the concentration of free calcium ions requires effective pumping out of the cytosol. This is accomplished by a P-type ATPase, which is the plasma membrane Ca^{2+} pump. Another calcium ion pump of the P-type resides in the endoplasmic reticulum (ER) and moves Ca^{2+} into the ER lumen, which is separated from the cytosol. In myocytes (muscle cells), Ca^{2+} is usually sequestered in a specialized form of ER called the *sarcoplasmic reticulum*. These pumps are closely related in structure and mechanism and are collectively called *sarcoplasmic and endoplasmic reticulum calcium* (SERCA) pumps. In contrast to the plasma membrane Ca^{2+} pump, the SERCA pumps are inhibited by the tumor-promoting agent thapsigargin.

These different pumps—the plasma membrane Ca^{2+} pump and SERCA pumps—share similarities in that both are integral proteins cycling between two conformations in a mechanism not very different from that for Na^+, K^+-ATPase. The calcium-ion pump of the sarcoplasmic reticulum has been thoroughly characterized and has been identified as a prototype for Ca^{2+} pumps of the P-type. It is built from a single polypeptide ($M \approx 100{,}000$) spanning the membrane ten times. In a large cytosolic domain, there is a site for ATP binding, as well as an Asp residue undergoing reversible phosphorylation by ATP. This process favors a conformation with a high-affinity Ca^{2+}-binding site exposed at the cytosolic side, and the opposite process, dephosphorylation, favors a conformation with a low-affinity Ca^{2+}-binding site at the luminal side. One consequence of these cyclic changes of conformation is that the transporter binds Ca^{2+} on the side of the membrane where the calcium ion concentration is low and releases it on the side where the concentration is high. The energy released by hydrolysis of ATP to ADP and P_i during one cycle of phosphorylation and dephosphorylation drives Ca^{2+} across the membrane against the steep electrochemical gradient.

IV. UPTAKE AND REGULATION OF IRON

Iron is vital for all living organisms because it is essential for many metabolic processes, the most well known being oxygen transport; however, further examples include DNA synthesis and electron transport. Although iron is abundant in nature, the metal is most commonly found as the virtually insoluble Fe^{3+} hydroxide $Fe(OH)_3$. Thus, iron-uptake systems require strategies to solubilize Fe^{3+}. Many organisms use siderophores (low-molecular-weight molecules secreted by bacteria, some fungi, and plants), which can solubilize Fe^{3+} for uptake by siderophore-specific transport systems. Genetic and biochemical evidence has demonstrated the presence of multiple pathways for iron uptake by eukaryotic cells. In mammals, changes in iron absorption are the major control point for altering the iron content of the body and of individual cells.

The intestine is the major site of iron regulation with regard to controlling the uptake of dietary iron across the brush border and the release of absorbed iron across the basolateral membrane to the circulation. Cells responsive to iron uptake are born in the crypt of Lieberkühn, located in the duodenum and jejunum. These cells differentiate and move toward the absorptive surface of the villus, where they are referred to as *enterocytes*. Gradually, mature enterocytes move toward the tip of the villus and are sloughed into the intestinal lumen (Figure 6).

In the intestinal lumen, iron exists in the forms of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron salts. Because ferric iron becomes insoluble at pH values above 3, ferric ions must be reduced or chelated by amino acids or sugars to be efficiently absorbed. Most ferrous iron remains soluble even at pH 7, so absorption of ferrous iron salts

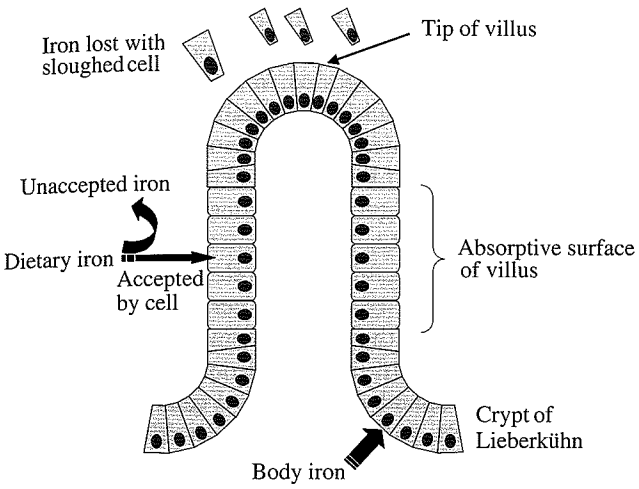

FIGURE 6 Iron is taken up into the enterocytes of the proximal small intestine from both the diet and blood plasma. The enterocytes are born in the crypts of Lieberkühn and move toward the villous tip to be discarded into the intestinal lumen at the end of a 2- to 3-day life span. (Adapted from Conrad and Umbreit, 2000.)

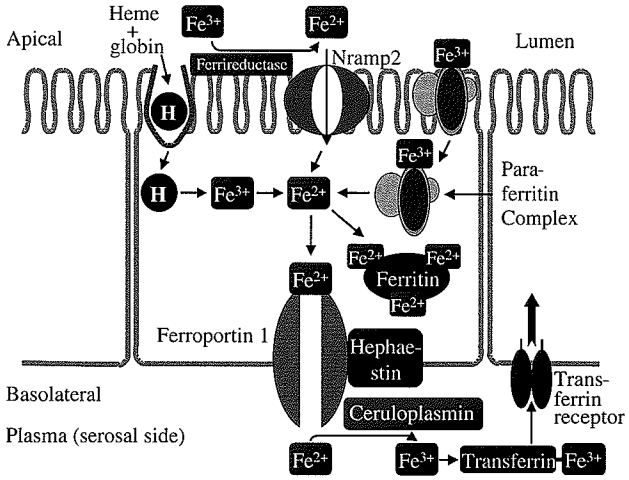

FIGURE 7 Pathways of heme and non-heme uptake of iron and transport in the intestine. The majority of the dietary ferric iron is reduced to ferrous iron or solubilized by mucin, ascorbic acid, or other reducing agents. The ferric iron in the lumen of the intestine is reduced by ferrireductase or in the cytoplasm by monooxygenase. An iron transporter, Nramp2, situated at the apical cell surface, transports most of the ferrous iron into the enterocyte. Yet another pathway for ferric and ferrous iron into the cell is the paraferritin complex, comprised of β integrin, mobilferrin, and flavin monooxygenase. Heme is taken up in the enterocytes as an intact metalloporphyrin (H). The uptake process is probably mediated by endocytosis. In the cytoplasm, heme is degraded by heme oxidase to release its inorganic iron. Two possibilities exist for the intracellular iron; either it is stored in ferritin or it is transported over the basolateral membrane by ferroportin I into plasma. Iron export seems to be facilitated by hephaestin concomitant with ferroportin I. Ferroportin I could also load iron onto transferrin assisted by the plasma ferroxidase, ceruloplasmin. (Adapted from Lieu *et al.*, 2001.)

is more efficient than absorption of ferric iron salts; however, most dietary inorganic iron is in the form of ferric iron. In most industrialized countries, two-thirds of the iron in the diet is present as ferric iron and one-third as heme iron (Carpenter & Mahorey, 1992). Reduction of ferric irons becomes necessary for efficient dietary iron absorption and is mediated by a mucosal ferrireductase that is present in the intestines. Inhibition of ferrireductase activity in intestinal cells reduces iron absorption, which demonstrates the importance of ferric iron reduction in dietary iron import. Alternatively, uptake of ferric irons might be mediated by the paraferritin pathway, though less efficiently. In addition to ferrireductase activity, the presence of dietary ascorbate provides a reduction of ferric iron to ferrous, whereby absorption is enhanced. Figure 7 illustrates the intestinal absorption and balance of iron.

A. Non-Heme Iron Uptake

Absorption of both heme and non-heme iron occurs predominantly in the crypt cells of the duodenum and jejunum (Wood & Han, 1998). Enterocytes, the specialized cells located on the intestinal villus, control the passage of dietary iron in the lumen of the intestine and the transfer of iron into the circulation of the body. To enter the circulation, dietary iron has to cross three cellular barriers: iron absorption over the apical membrane, intracellular iron transport through the cell, and iron export over the basolateral membrane and into the circulation. The enterocytes, however, have no transferrin receptors on the surface exposed to the lumen (Pietrangelo *et al.*, 1992); thus enterocytes differ from other nuclear-bearing cells. Consequently, there has to be a mechanism of absorption other than the usual transferrin–transferrin receptor pathway. Absorption of iron across the apical membrane of the enterocytes is mediated by a divalent cation transporter, which is called *Nramp2* or *divalent cation transporter 1* (DCT1) (Fleming *et al.*, 1997). Nramp is an acronym for *natural resistance-associated macrophage protein*; Nramp2 is highly homologous to Nramp1, a molecule that is important in host defense against pathogen infection. Evidence

speaks in favor of Nramp2 being responsible for iron transport from the duodenum lumen into the cytoplasm of enterocytes.

B. The Iron Importer: Nramp 2

The gene coding for Nramp2 in humans contains more than 36,000 bases. At least two different forms of mRNA are coded (Lee *et al.*, 1998), as this is the first step in the expression of the gene. One region of the Nramp2 isoform I is the 3′ untranslated region, which contains an iron-responsive element similar to the iron-responsive element present in the 3′ untranslated region of the mRNA transferrin receptor 1 (Tandy *et al.*, 2000). The isoform II, however, lacks the iron-responsive element. Expression of Nramp2 isoform I is, therefore, upregulated in iron-deficient animals and human intestinal cells, whereas expression of Nramp2 isoform II is not due to the lack of the iron-responsive element (Fleming *et al.*, 1999). The importance of the presence of a functional iron-responsive element in the Nramp2 isoform I is that its expression probably is controlled by intracellular iron concentration. Such a control should not be functional in the case of Nramp2 isoform II.

As might be expected, Nramp2 is highly expressed at the duodenum brush border, which corroborates its important role in intestinal iron absorption (Cannone-Hergeaux *et al.*, 1999), because enterocytes lack the transferrin receptor system at the absorptive surface. Nramp2 is located on the plasma membrane as well as on subcellular vesicular compartments. The Nramp2 protein is thought to consist of 12 transmembrane domains, and studies show that Nramp2 acts as a proton-coupled divalent cation transporter (Gunshin *et al.*, 1997). Nramp2 is, therefore, capable of transporting not only ferrous iron but also a number of divalent cations such as Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, and Pb^{2+} (Gunshin *et al.*, 1997). In addition, Nramp2 function is pH dependent, being optimal at a pH of <6 (Gunshin *et al.*, 1997). Nramp2 has been shown to be colocalized at the subcellular level with transferrin, and Nramp2 might be involved in transporting transferrin-bound iron across the membrane of endosomes into the cytoplasm (Gruenheid *et al.*, 1999).

C. Heme Iron Uptake

Hemoglobin iron from food is absorbed more efficiently than inorganic iron; therefore, absorption of iron from myoglobin and hemoglobin is different from the way in which inorganic iron is absorbed. Hemoglobin is enzymatically digested in the intestinal lumen, and the heme molecule is internalized by the enterocytes as an intact metalloporphyrin (Majuri & Grasbeck, 1987). It might be that the heme molecule enters the cell through a receptor-mediated process. Once inside the enterocyte, heme is metabolized by heme oxygenase, and inorganic iron is released. This is either stored as ferritin or transported across the basolateral membrane to enter the bloodstream (Figure 7). When the enterocyte ends its life cycle, iron in the form of ferritin will be sloughed with the aged cells and leave the body through the gastrointestinal tract. Humans have a limited means of eliminating iron; therefore, this process is an important mechanism of iron loss (Lieu *et al.*, 2001).

D. Paraferritin-Mediated Iron Uptake

Nramp2 is a much better transport agent for ferrous iron than ferric iron. In addition, ferrous and ferric iron can also be internalized by enterocytes in different pathways. Paraferritin is a membrane complex with a molecular weight of 520 kDa that contains β-integrin, mobilferrin (a homolog of calreticulin, which is a lectin-like chaperone promoting efficient folding of proteins in the ER), and flavin monooxygenase. It participates in the mucin-mediated iron uptake in the intestinal lumen (Figure 7) (Umbreit *et al.*, 1998). Experiments with erythroleukemia cells show that an anti-β_2-integrin monoclonal antibody blocks 90% of ferric citrate uptake. Little effect, however, was observed on the uptake of ferrous iron. Consequently, it seems that ferric iron is absorbed via the paraferritin-mediated pathway (Conrad *et al.*, 1999). A possible mechanism is that ferric iron is solubilized by mucin in the intestinal lumen, transferred to the mobilferrin- and β-integrin-containing paraferritin complexes, and then internalized (Conrad *et al.*, 1999). Having been internalized, flavin monooxygenase is associated with the complexes and ferric iron is reduced to ferrous iron in parallel with the activity of NADPH. The β-integrin- and mobilferrin-containing paraferritin complex also interacts with β_2 microglobulin. In addition, mobilferrin and β_2 microglobulin have been shown to play critical roles in the development of iron overload in hemochromatosis in animals (Rothenberg & Voland, 1996).

E. The Iron Exporter: Ferroportin1

A novel iron transporter gene, ferroportin1, has recently been identified (Donovan *et al.*, 2000). Sequence analysis of ferroportin1 shows that it has a stem-loop structure, typical of iron-responsive elements, in the 5′ untranslated region (Donovan *et al.*, 2000). It has been shown that the iron-responsive element binds to iron regulatory proteins 1 and 2. This indicates that expression of ferroportin1 is regulated by intracellular iron levels (McKie *et al.*, 2000). Studies of ferroportin1 demonstrate that ferroportin1 mediates iron efflux across membranes by a mechanism requiring an auxiliary ferroxidase activity (Donovan *et al.*, 2000; McKie *et al.*, 2000). Ferroportin1 is expressed highly in the placenta, liver, spleen, macrophages, and kidneys. In the cell, ferroportin1 is located on the basolateral membrane of duodenal enterocytes (McKie *et al.*, 2000). This suggests that ferroportin1 probably functions as an iron exporter in the enterocytes (Figure 7). Ferroportin1 is located on the basal surface of placental syncytiotrophoblasts, which probably suggests a role for ferroportin1 in iron transport into the embryonic circulation (Donovan *et al.*, 2000).

The function of ferroportin1 is believed to be in parallel with the membrane-resident ferroxidase hephaestin and serum ceruloplasmin (McKie *et al.*, 2000). Hephaestin has a high degree of similarity to ceruloplasmin, which is a multi-copper oxidase possessing ferroxidase activity, which is required for the release of iron into blood and the binding to transferrin. Hephaestin does not transport iron, which is similar to ceruloplasmin; however, it facilitates the transport of iron from enterocytes into the body's circulation (Harris *et al.*, 1998). Sex-linked anemic mice with defective hephaestin show normal dietary iron absorption into the enterocytes, but they suffer from a defect in the transport of iron from duodenum to the blood (Vulpe *et al.*, 1999). Unfortunately, the mechanism by which ferroportin1 mediates the transport of iron across the basolateral membrane and by which it interacts with hephaestin and ceruloplasmin is still unknown (Lieu *et al.*, 2001).

F. Regulation of Dietary Iron Absorption

The regulation of iron absorption by enterocytes is exercised in various ways. In the first place, it may be modulated by the amount of iron in recently consumed food. This mechanism is referred to as the *dietary regulator* (Andrews, 1999). Enterocytes are resistant to acquiring additional iron for several days after consumption, a phenomenon referred to as *mucosal block* (Andrews, 1999). A second regulatory mechanism, which monitors the iron levels stored in the body rather than the dietary iron status, is referred to as the *stores regulator*. When there is iron deficiency, the stores regulator can modify the amount of iron uptake by a factor of approximately two to three (Finch, 1994). Saturation of plasma transferrin with iron is also thought to influence the dietary absorption of iron, at least indirectly. A detailed mechanism of the stores regulator remains to be defined. The *erythropoietic regulator* is a third regulatory mechanism that has a greater capacity to increase iron absorption than the stores regulator. The erythropoietic regulator does not respond to the cellular iron levels; however, it does modulate iron absorption in response to the requirements for erythropoiesis. Further studies are required to increase our knowledge of the molecular mechanisms of intestinal iron absorption (Lieu *et al.*, 2001).

G. Transferrin Receptor-Mediated Iron Uptake

In the blood, iron is transported by the plasma glycoprotein transferrin, which has a molecular weight of about 80 kDa and a high affinity for ferric iron. Most cells in the body, except enterocytes in the intestine, get iron from transferrin. The uptake of iron in cells begins with the binding of transferrin to a receptor on the cell surface known as the *transferrin receptor*. It binds transferrin only when it carries iron. The maximum capacity of transferrin is two iron ions. The transferrin–receptor complex is then internalized through the endocytic pathway. Transferrin receptors do not interact directly with iron, yet they control iron uptake and storage by most cells in the organism. There are at least two types of transferrin receptors. Transferrin receptor 1 is a membrane-resident glycoprotein that is expressed in all cells, with the exception of mature erythrocytes. The other type, transferrin receptor 2, is a homolog of transferrin receptor 1. It is specifically expressed in the liver, particularly in the hepatocytes. Following internalization, the endosome is covered with clathrin. This is a protein complex of three large and three small polypeptide chains that is thought to help bend the membrane in the internalization process. The endosome is then uncoated by an uncoating ATPase. Then protons are pumped into the endosome, causing iron to be released from the transferrin. Iron then passes

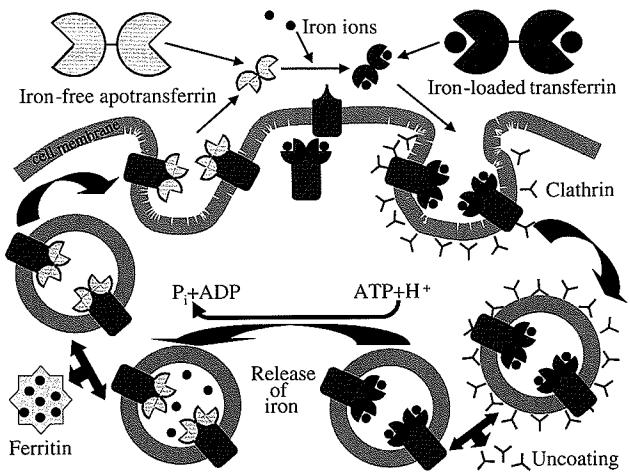

FIGURE 8 A schematic view of the extracellular binding of iron to apotransferrin, receptor-mediated endocytosis, release of iron into the endosome (driven by ATP), and the loading of iron into ferritin.

through the endosomal membrane and enters the intracellular labile pool. Intracellular iron in the labile pool can then be utilized for the synthesis of heme- and nonheme-containing proteins or stored within ferritin with a molecular weight of 474 kDa. The storage capacity of ferritin is as high as 4500 iron atoms. The receptor-bound transferrin is recycled back to the cell surface for reuse after completing a cycle of highly specific and efficient cellular iron uptake (Figure 8).

H. The Iron-Binding and Iron-Transport Protein: Transferrin

Transferrin is a single polypeptide chain of a glycoprotein that consists of two globular domains. Both domains offer a high-affinity binding site for one iron ion. Iron affinity of transferrin is pH dependent, and iron is released from transferrin when the pH is below 6.5. Transferrin might also be involved in the transport of a number of metals, such as aluminum, manganese, copper, and cadmium (Davidsson *et al.*, 1989; Moos *et al.*, 2000), but iron has the highest affinity to transferrin and will drive other metals out.

The liver is the primary site of synthesis of transferrin (Takeda *et al.*, 1998); however, it is synthesized in significant amounts in the brain, testis, lactating mammary gland, and some fetal tissues during development. The three different forms of transferrin, existing as a mixture, are iron-free (apotransferrin), one-iron (monoferric), and two-iron (transferrin diferric). The ratio between these forms depends on the concentration of iron and transferrin in blood plasma. Under normal conditions, most of the iron molecules in blood plasma are bound to transferrin (Lieu *et al.*, 2001). The main function of transferrin is to capture iron from plasma and to transport it to various cells and tissues in the organism.

1. The Transferrin-Binding and Transferrin-Transport Protein: Transferrin Receptor 1

Transferrin receptor 1 is a dimer comprised of two identical subunits and having a molecular weight of approximately 90 kDa. The receptor crosses the plasma membrane. The monomers are joined by two disulfide bonds and consist of three domains: a 61-residue amino-terminal domain, a 28-residue transmembrane region that helps to anchor the receptor into the membrane, and a large extracellular carboxyl terminus of 671 amino acid residues (McClelland *et al.*, 1984). As a type II membrane protein, the carboxyl terminal ectodomain of the transferrin receptor 1 is critical for transferrin binding. Indeed, replacement of the carboxyl-terminal, 192-amino-acid residues of the human transferrin receptor 1 with the corresponding region of the chicken transferrin receptor dramatically reduces or completely abolishes its binding affinity for transferrin. Because each ectodomain contains a binding site for the transferrin molecule, a homodimer of transferrin receptor 1 can bind up to two molecules of transferrin simultaneously.

Transferrin receptor 1 is synthesized intracellularly in the ER. Additionally, it undergoes a number of post-translational modifications. Its ectodomain is comprised of three nitrogen-linked glycosylation sites and one oxygen-linked glycosylation site (Kohgo *et al.*, 2002). Correct folding is strongly dependent on the nitrogen-linked glycosylation sites of transferrin receptor 1. If the oxygen-linked glycosylation at threonine 104 is eliminated, the cleavage of transferrin is enhanced, which, in turn, promotes the release of its ectodomain (Rutledge & Enns, 1996). The segment of transferrin receptor 1 that crosses the membrane consists of 18 hydrophobic amino acids and also undergoes posttranslational modifications. The hydrophobic membrane-crossing segment is covalently bound to fatty acids and is subjected to acylation with palmitate. This probably helps to fasten the receptor to the plasma membrane (Kohgo *et al.*, 2002).

The part of transferrin receptor 1 that is resident in the cytoplasm is important for the clustering of the receptor into the chlatrin-coated pits of the plasma

membrane and, subsequently, for endocytosis (Iacopetta *et al.*, 1988). A conserved internalization signal (YTRF; tyrosine, threonine, arginine, phenylalanine) within the 61-amino-acid residues in the cytoplasmic part of transferrin receptor 1 is critical for efficient endocytosis of the receptor (Collawn *et al.*, 1993).

Human transferrin receptor 1 is a tightly associated homodimer. Each transferrin receptor 1 monomer consists of three distinct globular domains (Rolfs & Hediger, 1999). The general form of the homodimer suggests that transferrin could bind to either side with no contact between the two transferrin molecules. The extracellular part of transferrin receptor 1 is separated from the membrane by a stalk, which presumably includes residues involved in disulfide bond formation and oxygen-linked glycosylation (Rolfs & Hediger, 1999; Lieu *et al.*, 2001).

Transferrin receptor 1 is the general mechanism for cellular uptake of iron from plasma transferrin. The current model of iron uptake from transferrin via receptor-mediated endocytosis in mammals is shown in Figure 8 and in greater detail in Figure 9. The first step, the binding of transferrin to transferrin receptor 1, is accomplished by a physical interaction that does not require an increase in temperature or energy (Conrad & Umbreit, 2000). Transferrin can bind one or two ferric ions, and the iron status of transferrin affects its affinity for its receptor. Diferric transferrin has the highest affinity, followed by monoferric transferrin; apotransferrin (without iron) has the lowest affinity. An estimated dissociation constant for diferric transferrin is about 2–7 n*M* (Lieu *et al.*, 2001). The plasma concentration of diferric transferrin is about 5 μ*M* under physiological conditions; consequently, most surface transferrin receptors become saturated with transferrin. Thus, the homodimeric transferrin receptor can mediate a maximum uptake of four atoms of iron at the same time.

The complexes consisting of transferrin receptor–transferrin–iron interact with adaptor proteins in the clathrin-coated pit and are then internalized by the cells through an endocytic pathway mediated by the receptor. The tyrosine internalization motifs located on the parts of the transferrin receptors that reside in the cytoplasm seem to be necessary for a high-affinity binding to the adaptor protein complexes on the plasma membrane. Importantly, but not surprisingly, this process is temperature and energy dependent (Lieu *et al.*, 2001). Inside the endosome, an ATPase proton pump causes acidification of the endosome and results in the release of iron from transferrin. The apotransferrins remain attached to the transferrin receptors and return to the cell surface, where they are released from the cells. The binding between transferrin and the transferrin receptor is dependent on the pH, which is critical to membrane uptake and the release of transferrin. The release of apotransferrin from its receptor occurs at neutral pH at the cell surface. Both the ligand and receptor, in this way, become available for recycling the absorption of iron. After its release from the transferrin, iron passes through the endosomal membrane into the cytoplasm via the iron transporter Nramp2 (also known as the DCT1). Iron that enters the cell can be utilized in the synthesis of heme or incorporated in iron-containing molecules. Intracellular iron can also be stored in the ferritin complexes or can modulate the activity of iron regulatory proteins (Lieu *et al.*, 2001).

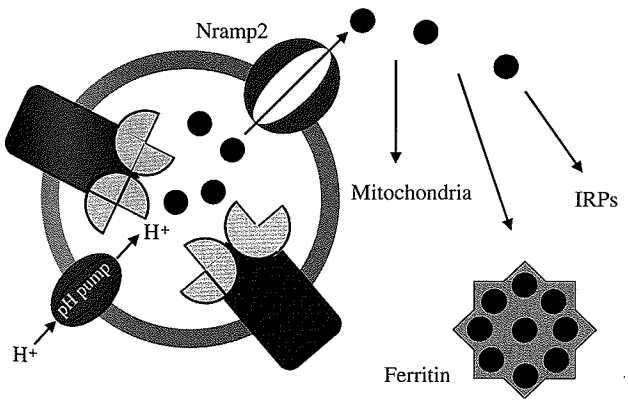

FIGURE 9 Release of iron from transferrin in the endosome and following events. A proton pump (energy-requiring) decreases the pH within the endosome with the consequence that iron is released from transferrin. The iron transporter Nramp2 carries out the subsequent transport of iron over the endosome membrane into the cytoplasm. IRP stands for iron-responsive protein.

2. Second Transferrin-Binding and Transferrin-Transport Protein: Transferrin Receptor 2

Transferrin receptor 2, which is a homolog of transferrin receptor 1, was recently identified. Its gene is located on chromosome 7q22 and gives rise to two transcripts approximately 2900 and 2500 bases in length. Like transferrin receptor 1, transferrin receptor 2 is a type II transmembrane glycoprotein, and it shares 66% similarity in its ectodomain with transferrin receptor 1. Although the cytoplasmic portion of transferrin receptor 2 is very different from transferrin receptor 1, transferrin receptor 2 also contains an internalization motif

(YQRV; tyrosine, glutamine, arginine, valine), which is similar to the YTRF motif in transferrin receptor 1. Transferrin receptor 2 does not possess iron-responsive elements. It seems that expression of transferrin receptor 2 is not regulated by an iron-regulatory, protein-mediated feedback regulatory mechanism in response to cellular iron status (Kawabata *et al.*, 1999).

In contrast to transferrin receptor 1, transferrin receptor 2 is primarily expressed in the liver. Like transferrin receptor 1, binding of transferrin receptor 2 to transferrin is also pH dependent. The binding of apotransferrin to transferrin receptors 1 and 2 only takes place at acidic pH. Expression levels of both transferrin receptors 1 and 2 correlate with stages of the cell cycle which, in turn, are related to requirements for iron during DNA synthesis. Nevertheless, transferrin receptor 2 differs from transferrin receptor 1 in its binding properties with transferrin and regulation of expression. Holotransferrin has a lower affinity for transferrin receptor 2 than for transferrin receptor 1.

Transferrin receptors 1 and 2 are likely not only to be regulated through distinct pathways but also to mediate iron uptake and storage by a different, yet unidentified, mechanism. Transferrin receptor 1 seems to play a general role in cellular iron uptake; however, transferrin receptor 2 appears to play a specific role in iron uptake and storage in the liver, due to its high expression in hepatocytes (Lieu *et al.*, 2001).

I. Control of Iron Metabolism

Not surprisingly, animal cells differ somewhat from plants and lower eukaryotes with regard to the way in which they control iron metabolism. Transcription is the preferred method by which plants and lower eukaryotes maintain iron homeostasis. In the yeast *Saccharomyces cerevisiae*, for example, the iron-regulated transcription factor AFT1 controls production of multiple gene products that are needed to make up the high-affinity iron transport systems. Similarly, in plants, the iron storage protein ferritin is transcriptionally regulated by iron, which differs from the way in which it is regulated in animal cells, as animal cells utilize post-transcriptional control of iron metabolism in most cell types in the body. Apparently, regulation of gene transcription has a more important role in cell-type-specific modulation of iron homeostasis. Tissue-specific regulation of the expression of H- and L-ferritin, an erythroid-specific isoform of 5-aminolevulinate synthase, is one example, as well as control of the relative expression of iron regulatory protein 1 (IRP1) and IRP2 between tissues. This means that mammalian iron homeostasis is maintained through integrated use of sensory and regulatory systems operating at multiple levels of gene regulation (Eisenstein, 2000).

Although iron is an essential trace element, it might be detrimental if it is available as a free ion; consequently, besides providing storage, it is also necessary to prevent toxicity. Alteration of ferritin gene transcription provides an important means by which the relative abundance of the ferritin subunits can be modified to meet the unique iron storage and/or detoxification needs of specific tissues. The ratio of the abundance of the heavy-chain (H) and light-chain (L) subunits varies among tissues, and this variation is probably due to tissue-specific differences in the rates by which the ferritin genes are transcribed (Tsuji *et al.*, 1999; Eisenstein, 2000). Gene transcription of ferritin can be modulated by both iron-dependent and iron-independent factors. Experiments show that an excess of iron can cause a selective increase in L-ferritin gene transcription in the liver. In other systems, though, H- and L-ferritin transcription is altered in parallel to iron. Transcription of the ferritin genes is also modulated by a number of iron-independent signaling pathways (Eisenstein, 2000). Synthesis of transferrin and transferrin receptor takes place in fewer tissues than is the case for the ferritins. Transcription thus seems to dictate their expression in specific tissues. Iron deficiency causes induction of the transcription of both genes (Testa *et al.*, 1989). In many cases, however, transferrin receptor expression in iron deficiency is controlled by the regulation of mRNA stability. Seemingly contradictory is the fact that transcription appears to be a more critical factor in the iron regulation of transferrin expression in a deficiency, although translational regulation may be important in reducing transferrin synthesis when there is excess iron (Eisenstein, 2000). The gene transcription of transferrin and transferrin receptor is enhanced during hypoxia to increase iron delivery to the erythron, which is necessary to advance erythropoiesis and increase oxygen-carrying capacity (Tacchini *et al.*, 1999; Eisenstein, 2000). During erythropoiesis, transcription plays a greater role in modulating transferrin receptor expression than in many non-erythroid cells. This makes increases in transferrin receptor expression possible without maximal induction of IRP activity and allows for simultaneous expression of the erythroid isoform of delta-aminolevulinate synthase (ALAS, eALAS). The growth state of cells also influences transferrin receptor gene transcription. Clearly, regulation of the transcription of the H- and L-ferritins, transferrin, and trans-

ferrin receptor genes contributes greatly to the maintenance of cell and organ iron homeostasis (Eisenstein, 2000).

J. Iron Regulatory Proteins and the Coordination of Iron Homeostasis

Iron regulatory proteins play a significant role in maintaining iron homeostasis by coordinating many of the mRNAs that encode proteins for the control of uptake or the metabolic fate of iron. Furthermore, changes in rates of transcription help establish the final level of these mRNAs. IRPs are considered central regulators of mammalian iron metabolism because they regulate the synthesis of proteins that is required for the uptake, storage, and use of iron by cells (Eisenstein, 2000). Important factors with regard to this regulation include the following:

- It is well accepted that IRPs are critical factors of the posttranscriptional regulation of transferrin receptor expression.
- IRPs play a major role in determining the iron storage capacity of cells by regulating translation of both H- and L-ferritin mRNA.
- Translation of the mRNA for the eALAS also seems to be regulated by IRPs.

In this role, IRPs may coordinate the formation of protoporphyrin IX with the availability of iron. IRPs may thus be important modulators of iron cycling in the body. In addition, the mRNA-encoding divalent metal transporter 1 (DMT1) and ferroportin1/iron-regulated gene 1 (IREG1) contain iron-responsive element (IRE)-like sequences. This suggests that IRPs might possibly affect the use of these mRNAs. DMT1 expression is iron regulated in some but not all situations; however, there are indications that the abundance of ferroportin1/IREG1 mRNA responds to changes in iron status. If the IREs in DMT1 and ferroportin1/IREG1 mRNA are functional, as is the case for transferrin receptor mRNA, then IRPs probably are major modulators of the transmembrane transport of transferrin and non-transferrin iron (Eisenstein, 2000).

V. UPTAKE AND REGULATION OF ZINC

A number of physiologic systems contribute to zinc homeostasis under different conditions. Central to the maintenance of zinc homeostasis, however, is the gastrointestinal systems, especially the small intestine, liver, and pancreas. Specifically, the processes of absorption of exogenous zinc and gastrointestinal secretion and excretion of endogenous zinc are critical to zinc homeostasis throughout the body. During evolution, cells developed efficient uptake systems to allow for the accumulation of zinc even when it is scarce. These uptake systems use integral membrane transport proteins to move zinc across the lipid bilayers of the plasma membrane. Once inside a eukaryotic cell, a portion of the zinc must be transported into intracellular organelles to serve as a cofactor for various zinc-dependent enzymes and processes present in those compartments; therefore, transporter proteins must be present in organelle membranes to facilitate this flux of zinc. Zinc can also be stored in certain intracellular compartments when supplies are high and used later if zinc deficiency ensues. Again, zinc transporters are required to facilitate this transport in and out of organelles.

A. Families of Zinc Transporters in Eukaryotes

Many types of transporters have been found to be involved in zinc transport. In prokaryotes, transporters of the ATPase binding cassette (ABC) family have been demonstrated to work in zinc uptake. The zinc ABC proteins of *Escherichia coli*, for example, are a major source of zinc incorporation for these cells. A family of P-type ATPases functions as zinc efflux transporters; the ZntA protein in *E. coli* is one such transporter. Interestingly, this protein is important for zinc detoxification by pumping the metal ion out of the cell when intracellular zinc levels get too high (Hantke, 2001).

Eukaryotes have been found not to use ABC transporters or P-type ATPases; instead, zinc transport apparently is accomplished by two other families of transporters. The uptake of zinc and transport from the extracellular space to the cytoplasm have been found to be associated with the ZIP (Zrt-, Irt-like proteins) family. Additionally, the mobilization and transport of stored zinc from an organelle to the cytoplasm have been shown to be carried out by ZIP transporters. The CDF (cation diffusion facilitator) family does the opposite of the ZIP proteins; namely, it pumps zinc from the cytoplasm out of the cell or into the lumen of an organelle. All of the known members of these families play roles in metal ion transport, and zinc is often the substrate; consequently, it might be that several other

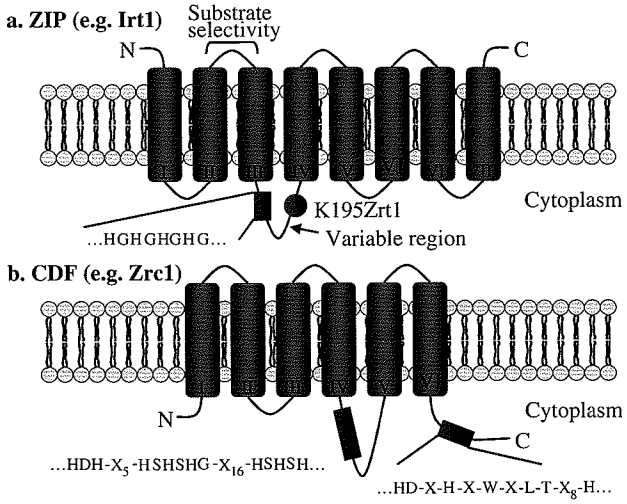

FIGURE 10 A sketch of the predicted membrane topology of ZIP and CDF proteins. (a) ZIP proteins as well as Irt1 are predicted to have eight membrane-crossing domains (I–VIII). Indicated as the variable region are the conserved and functionally important residues in domains VI and V, the ubiquitinated K195 in Zrt1, and the extracellular loop region affecting Irt1 substrate specificity. (b) The majority of CDF transporters as well as Zrc1 are predicted to have six membrane-crossing domains (I–VI). Conserved polar or charged residues within the membrane-crossing domains I, II, and V are indicated. H, histidine; G, glycine; D, aspartate; S, serine; L, lysine; T, threonine; W, tryptophan; and X, any amino acid. (Adapted from Gaither and Eide, 2001.)

members work in zinc transport (Gaither & Eide, 2001).

Two of the first members of the ZIP family to be discovered were Zrt1 of *Saccharomyces cerevisiae* and Irt1 of *Arabidopsis thaliana* (Gaither & Eide, 2001); thus the name ZIP transporters. Zrt1 is a zinc uptake transporter in yeast, and Irt1 is an iron transporter in plants. Currently, 86 ZIP members can be found in the protein sequence database at the National Center for Biotechnology Information (NCBI). This list includes proteins from eubacteria, archaea, fungi, protozoa, insects, plants, and mammals.

The degree of sequence conservation can be used to split the ZIP family into subfamilies. Most proteins in the ZIP family are predicted to have eight membrane-crossing domains; however, some may have as few as five. The majority of ZIP proteins share a similar predicted topology where the amino and carboxyl termini are located in the extracellular space (Figure 10a). Parts of this topology have been corroborated for some members of the family. Examples include the amino terminus of Zrt1 and the carboxyl terminus of hZip2, which have been shown to be on the outside surface of the plasma membrane. Many of the ZIP proteins have a long loop region located between membrane-crossing domains III and IV. This region is called the "variable region," because both its length and sequence show little conservation among the family members. Many of the ZIP proteins are characterized by the presence of many histidine residues. In Zrt1, this sequence is histidine–aspartate–histidine–threonine–histidine–aspartate–glutamate . . . , and in Irt1 the sequence is histidine–glutamate–histidine–glutamate–histidine–glutamate–histidine. . . . The function of this region is not known; however, it is acknowledged to be a potential metal binding domain. Consequently, its conservation in many of the ZIP proteins implies a role in metal ion transport or its regulation (Gaither & Eide, 2001).

The mechanism of transport used by the ZIP proteins has yet to be unveiled. Conspicuously, the zinc uptake by human hZip2 zinc transporter has been shown to be energy independent (Gaither & Eide, 2000); however, this finding does not correspond with studies of the yeast zinc transporters Zrt1 and Zrt2, which demonstrated strict energy dependence (Zhao & Eide, 1996). Fungal and human ZIP proteins may, consequently, use different mechanisms. Zinc uptake by hZip2 was stimulated by HCO_3^- but was not dependent on K^+ or Na^+ gradients (Gaither & Eide, 2000); it has been suggested that hZip2 functions *in vivo* by a Zn^{2+}–HCO_3^- symport mechanism. Another possibility is that zinc uptake by these proteins may be driven by the concentration gradient of the metal ion substrate. Although the total level of zinc in cells can be as high as several hundred micromoles (Mantzoros *et al.*, 1998), only small amounts of that zinc are present in a "free" or labile form. Estimates of the labile pool of zinc in cells are in the nanomolar range (Suhy & O'Halloran, 1995). A concentration gradient of labile zinc across the plasma membrane may thus be an important driving force for Zn^{2+} uptake. The negative-inside membrane potential existing in cells could also be a driving force for the uptake of zinc (Gaither & Eide, 2001; Zhang & Allen, 1995).

The CDF (cation diffusion facilitator) proteins were early recognized to often play roles in metal ion transport (Nies & Silver, 1995). They are similar to the ZIPs found in organisms at all phylogenetic levels. Many members of this family have been implicated specifically in the transport of zinc from the cytoplasm out of the cell or into organellar compartments (Gaither & Eide, 2001). The CDF family was recently said to comprise 13 members; however, more sequence data and better

tools have increased the number of members to 101 (Gaither & Eide, 2001).

The CDF family can be divided into three different subfamilies (I, II, and III) based on clusters or proteins with greater sequence similarities. CDF subfamily I is found mostly in prokaryota, including both eubacteria and archaea. Subfamilies II and III are comprised of about equal numbers of species of eukaryotic and prokaryotic origin. Six membrane-crossing domains seem to be common in a majority of the members of the CDF family. Their predicted membrane topology is similar to that shown for one such protein, Zrc1, from *Saccharomyces cerevisiae*, shown in Figure 10b (Gaither & Eide, 2001).

B. Zinc Transport and Its Regulation in Plants

Both ZIP and CDF family genes have been discovered in many plant species and have contributed to our increasing understanding of zinc transport and regulation in plants. The number of ZIP family members in plants is remarkable. The *Arabidopsis* genome contains 18 ZIP family genes from three of the four subclasses of ZIP proteins. ZIP subfamily II, however, does not have a plant representative. Plants and animals are multicellular organisms, as reflected by the high number of potential metal ion transport proteins, probably due to the greater diversity of tissue-specific roles played by these proteins (Gaither & Eide, 2001). The first ZIP protein to be discovered in any organism was Irt1 (iron-regulated transporter 1) (Eide *et al.*, 1996). Its gene (*IRT1*) was cloned because its expression in a yeast mutant with an impaired iron uptake suppressed the growth defect of this strain when growth media contained low amounts of iron. Irt1 expression indeed increased iron uptake in this yeast strain, as confirmed by biochemistry (Eide *et al.*, 1996). Later studies showed that Irt1 could also transport Zn^{2+}, Mn^{2+}, and Cd^{2+} (Korshunova *et al.*, 1999). Iron accumulation, rather than the transport of other metals such as zinc, seems to be the main function of Irt1 in plants. In addition, Irt1 is expressed only in the roots of plants for which iron access is restricted. If Irt1 takes part in the accumulation of metals other than iron, such as zinc, this probably occurs only under iron-limiting conditions (Gaither & Eide, 2001). It has, indeed, been observed that iron-limited plants accumulate higher levels of other metals such as zinc, manganese, and cadmium (Cohen *et al.*, 1998). These findings corroborate the prediction of Gaither and Eide (2001).

Zip1 through Zip4, ZIP transporters in *Arabidopsis*, may play roles in zinc transport. In *Saccharomyces cerevisiae*, the expression of Zip1, Zip2, or Zip3, each with distinct biochemical properties, results in increased zinc uptake. These proteins, consequently, most probably are zinc transporters. Zip4 expressed in yeast, however, does not result in increased zinc uptake. This may be due to poor expression or mislocalization of the protein in the yeast cell. *ZIP1* is expressed predominantly in roots while *ZIP3* and *ZIP4* mRNA could be found in both roots and shoots. The induction of *ZIP1*, *ZIP3*, and *ZIP4* mRNA takes place under zinc-limiting conditions. A role for these proteins in zinc transport is thus further confirmed. Neither subcellular localization of these proteins nor tissue-specific expression has been determined, so their exact roles cannot be assessed as yet. It is quite clear that some mechanism of regulation exists in plants because there is a zinc-responsive regulation of mRNA levels in response to zinc availability (Gaither & Eide, 2001).

If regulation of the expression of zinc transporters was altered in any way, zinc accumulation in plants would probably be greatly impacted. This presumption may in part explain the physiology of an unusual group of plants called *metal hyperaccumulators*. These are plants that take up large quantities of metal ions from the soil. They are of great interest because of their potential to remove metal pollutants from surface soils in a process called *phytoremediation* (Raskin, 1995; Gaither & Eide, 2001). A well-known hyperaccumulator is *Thlaspi caerulescens*, a member of the Brassicaceae family that also includes *Arabidopsis*. Certain ecotypes of *T. caerulescens* are capable of accumulating zinc in their shoots at levels up to as much as $30{,}000\,\mu g\,g^{-1}$ without evident toxic effects (Gaither & Eide, 2001). Plants that are not hyperaccumulators normally accumulate only 0.1% of that level. A salient ability to accumulate and detoxify metal ions should therefore be a significant property of hyperaccumulators. In studies of *T. caerulescens*, it was found that the maximum velocity, V_{max}, was elevated almost fivefold compared to a non-hyperaccumulating ecotype, *T. arvense*, although there was no difference in the Michaelis-Menten constant K_m (Lasat *et al.*, 2000). Expression of zinc uptake transporters should thus be higher in *T. caerulescens*. *ZNT1*, a ZIP family member, has been cloned from *T. caerulescens* and *T. arvense*. In *T. arvense*, Znt1 is expressed at a low level and regulated by zinc status. In *T. caerulescens* this gene is expressed at a much higher level and is unaffected by zinc availability. The increased zinc accumulation in this and perhaps other metal hyperaccumulating plant species can thus be explained by Znt1 expression.

Many members of the CDF family are also contained in the genomes of plant species. For example, *Arabidopsis* alone encodes ten CDF member genes. The proteins expressed by these genes are likely to function in subcellular zinc compartmentalization as well as in zinc efflux. The Zat protein of *Arabidopsis* is the only plant CDF member to have been studied. This protein seems to be a zinc transporter; however, *ZAT* mRNA expression is not zinc regulated. Transgenic plants overexpressing the *ZAT* gene demonstrate increased zinc resistance. In roots of these transgenic plants, the zinc content was also found to be increased, which suggests that Zat transports zinc into an intracellular compartment (*e.g.*, the vacuole or root cells). In any multicellular organism, zinc transporters are required for both cellular zinc uptake as well as efflux to allow utilization of the metal. Plants, for example, need a zinc efflux transporter to pass zinc from the root tissue into the xylem for distribution to aerial portions of the plant. CDF proteins such as Zat probably perform this function as well (Gaither & Eide, 2001).

C. Zinc Transporters and Their Regulation in Mammals

Both the ZIP and CDF families are represented by several zinc transporters found in mammalian organisms. Fourteen ZIP genes have been identified in humans, and three have been found in the mouse. Functional data are available for three of the human genes (*hZIP1*, *hZIP2*, and *hZIP4*). Recently, a subfamily of mouse zinc transporter genes was characterized (Dufner-Beattie *et al.*, 2003). The proteins hZip1 and hZip2 appear to play roles in zinc uptake across the plasma membrane. Expression of *hZIP2* mRNA has been detected in prostate and uterine tissue as well as monocytes, indicating restricted tissue specificity (Kambe *et al.*, 2004). Overexpressed *hZIP2* in cultured K562 erythroleukemia cells resulted in an increased accumulation of zinc compared to control cells. Furthermore, the hZip2 protein was localized to the plasma membrane of these cells (Kambe *et al.*, 2004). These results indicated that *hZIP2* might serve in zinc uptake in the few tissues where it is expressed (Gaither & Eide, 2001).

Endogenous uptake of zinc was shown to be biochemically different from uptake mediated by hZip2 in the K562 cell line in a number of ways. For example, HCO_3^- treatment stimulated zinc uptake mediated by hZip2, whereas the endogenous system did not react. Moreover, several other metal ions (*e.g.*, Co^{2+}, Fe^{2+}, and Mn^{2+}) significantly inhibited zinc uptake by hZip2; however, the endogenous uptake was far less sensitive. It has recently been demonstrated that another ZIP transporter, hZip1, represents the endogenous zinc uptake system in K562 cells (Gaither & Eide, 2001). Three important observations support this hypothesis. First, K562 cells express *hZIP1* mRNA, and the functional hZip1 protein is localized to the plasma membrane of these cells. Second, a twofold increase in zinc uptake activity was a consequence of a twofold overexpression of hZIP1 mRNA. It was not possible to distinguish, by biochemical means, the increased uptake of zinc in hZip1-overexpressing cells from the endogenous system. Last, but not least, antisense oligonucleotides targeted to inhibit *hZIP1* expression also inhibited the endogenous zinc uptake activity. The hypothesis that hZip1 is the endogenous transporter in K562 cells is thus strongly supported. The antisense *hZIP1* oligonucleotide treatment reduced zinc uptake to 10–20% of control levels, again corroborating the idea that hZip1 is the major pathway of zinc uptake in these cells (Gaither & Eide, 2001).

A wide variety of different cell types demonstrates expression of hZIP1, in sharp contrast to the *hZIP2* gene. The results of Gaither and Eide (2001), therefore, suggest that hZip1 is an important candidate for being the primary factor for zinc uptake in many human tissues. A recent study by Franklin et al. (2003) in which a correlation was found between *hZIP1* expression levels and zinc uptake in human malignant cell lines derived from the prostate provided significant support for this conclusion. Prostate cell lines LNCap and PC-3 possess high levels of zinc uptake activity that is stimulated by prolactin and testosterone. It was found that *hZIP1* is expressed in LNCap and PC-3 cells, and this expression is increased by prolactin and testosterone treatment (Franklin *et al.*, 2003). Expression of *hZIP1* was also repressed by adding zinc to the medium, which suggested that some regulation of zinc uptake occurs in response to cellular zinc status. Lioumi *et al.* (1999) recently reported a closely related ortholog of hZip1 obtained from the mouse; this protein was named Zirtl for zinc-iron regulated transporter-like protein. The *ZIRTL* gene is expressed in a wide variety of tissues as is *hZIP1*.

A conspicuous finding is that the transporters hZip1 and hZip2 have a surprisingly low affinity for their substrate. The K_m values of both proteins are about 3 μM for free Zn^{2+} ions. Additionally, zinc transporters in a wide variety of mammalian cells have K_m values of the same order. We are faced with an apparent paradox that arises when considering the free Zn^{2+} concentration in

mammalian serum. The total zinc concentration of serum is about 15 to 20 μM, and very little of that amount is present in an unbound form (Zhang & Allen, 1995). About 75% of Zn^{2+} is bound to albumin, and 20% is bound to α_2-macroglobulin. What does not exist in free form is complexed with amino acids such as histidine and cysteine. The serum has a high binding capacity for metals; thus, the free Zn^{2+} concentration in serum is estimated to be in the low nanomolar range. It is difficult to understand how such a low concentration of substrate would allow these transporters to contribute to zinc accumulation by mammalian cells under physiological conditions. The cellular requirements of zinc have to be considered and compared to transporter capacity. Recent studies showed that the capacity, expressed as V_{max}, for uptake is so high relative to the cellular demand for zinc that adequate levels can be obtained despite the apparent low affinity of the transporters (Gaither & Eide, 2001).

Curiously, a ferrous iron transporter, the DCT1/DMT1/Nramp2 Fe^{2+}, may be involved in zinc uptake. This transporter is a member of the Nramp family of transporters and is not related to ZIP or CDF proteins. Experiments with *Xenopus* oocytes suggested that cation influx currents could indicate Zn^{2+} movement across the membrane; however, more recent results have indicated that the currents recorded in these oocytes result from Zn^{2+}-induced proton fluxes rather than transport of the metal ion (Sacher *et al.*, 2001).

Export of zinc from the cell as well as transport into intracellular organelles is related to mammalian CDF family members. Seven CDF genes in humans and six in the mouse genome have been identified plus a small number of others from the rat and other mammals. Four of the mammalian genes, *ZnT-1*, *ZnT-2*, *ZnT-3*, and *ZnT-4*, have been functionally characterized to such an extent that their roles in zinc metabolism are not in doubt. ZnT-1 is a zinc export transporter in the plasma membrane of mammalian cells; consequently, ZnT-1 may play a role in the cellular detoxification of zinc by exporting unnecessary metal ions out of the cell. An observation that cells overexpressing this transporter show higher zinc resistance than control cells further corroborates this role. ZnT-1 may also be involved in the dietary absorption of zinc in the intestine as well as in the reabsorption of zinc from urine in the renal tubules of the kidney. The intestinal enterocytes of the duodenum and the jejunum (*i.e.*, the primary sites of zinc absorption) express ZnT-1, and the protein is found localized to the basolateral membrane. This indicates a role in transporting zinc out of the enterocyte and into the bloodstream. The protein is also found on the basolateral surface of renal tubule cells, where it would be expected to appear to be involved in transporting zinc that has been reabsorbed from the urine back into the circulation. It is well established that the loss of zinc in urine is very low because of an efficient renal reabsorption (Gaither & Eide, 2001).

Intracellular zinc sequestration and storage may be dependent on ZnT-2, a role similar to that proposed for Zrc1 and Cot1 in yeast. ZnT-2 is located in the membrane of the late endosome that accumulates zinc when cells are grown under high zinc conditions (Palmiter *et al.*, 1996).

The third CDF transporter, ZnT-3, has a role similar to that of ZnT-2. It also transports zinc into an intracellular compartment where the metal may play a role in neuronal signaling. Messenger RNA of *ZnT-3* has been detected only in the brain and testis and is most abundant in the neurons of the hippocampus and the cerebral cortex. The protein is localized in membranes of synaptic vesicles in these neurons, which suggests that the protein transports zinc into this compartment. This hypothesis is further supported by the fact that a subset of glutamatergic neurons contains histochemically reactive zinc in their synaptic vesicles. The ZnT-3 protein was colocalized with these zinc-containing vesicles. Furthermore, a mouse line lacking *ZnT-3* did not accumulate zinc in these vesicles; therefore, the protein ZnT-3 must be required for the transport of zinc into synaptic vesicles in some types of neurons where it may play a neuromodulatory role (Cousins & McMahon, 2000).

The protein ZnT-4 is expressed in the mammary gland, brain, and small intestine. In the mammary gland, it is responsible for zinc transport into milk. In fact, mutations in the *ZnT-4* gene produced a mutant mouse referred to as the lethal milk (lm) mouse. This mutant gene is the *lm* gene. Pups of any genotype suckled on *lm/lm* dams die before weaning, and the cause of death is zinc deficiency from an insufficient supply of zinc in the milk. In intestinal enterocyte ZnT-4 is localized in endosomal vesicles concentrated at the basolateral membrane. It seems that ZnT-4, in a manner similar to ZnT-1, may facilitate transport of zinc into the portal blood (Kambe *et al.*, 2004).

It is becoming increasingly clear that regulation of zinc export in many cell types is managed by zinc. It has been demonstrated that *ZnT-1* mRNA is upregulated during ischemia, which is known to cause zinc influx into neurons. Cultured neurons transiently increased *ZnT-1* mRNA when exposed to zinc, a finding that is in accordance with *ZnT-1* regulation as a result of zinc

influx (Gaither & Eide, 2001). Transcriptional control of *ZnT-1* could therefore contribute to zinc detoxification by stimulating its export. As *ZnT-1* is expressed in many cell types, this could be a general mechanism of cellular zinc homeostasis. Zinc absorption may also be dependent on the transcriptional control of *ZnT-1*. Messenger RNA levels were found to be increased in enterocytes following an oral dose of zinc (Cousins & McMahon, 2000). The location of the ZnT-1 protein on the basolateral membrane of these cells suggests that upregulation of ZnT-1 promotes zinc absorption by facilitating transport into the portal blood.

Regulation of zinc uptake transporters in mammals is less well known. Evidence suggests that the activity of these transporters is controlled by the levels of zinc. Zinc uptake in brush border membrane vesicles, for example, has been found to increase in zinc-deficient rats. Additionally, cultured endothelial cells grown under low-zinc conditions displayed a higher rate of zinc uptake than zinc-replete cells. Zinc deficiency may thus increase the expression or activity of zinc uptake transporters in some cell types. A hypothetic mechanism of this regulation could be similar to that described in yeast. This is supported by the finding that *hZIP1* mRNA levels in cultured malignant prostate cells were reduced when treated with zinc. This suggests a transcriptional control mechanism. Such a mechanism would play a critical role in mammalian zinc homeostasis (Gaither & Eide, 2001; Kambe *et al.*, 2004).

VI. UPTAKE AND REJECTION OF COPPER

The transport and cellular metabolism of copper depends on a series of membrane proteins and smaller soluble peptides that comprise a functionally integrated system for maintaining cellular copper homeostasis. Inward transport across the plasma membrane appears to be a function of integral membrane proteins that form the channels that select copper ions for passage. Two membrane-bound, copper-transporting ATPase enzymes—ATP7A and ATP7B (the products of Menkes' and Wilson's disease genes, respectively)—catalyze an ATP-dependent transfer of copper to intracellular compartments or expel copper from the cell. ATP7A and ATP7B work in concert with a series of smaller peptides, the copper chaperones, which exchange copper at the ATPase sites or incorporate the copper directly into the structure of copper-dependent enzymes such as cytochrome *c* oxidase and Cu–Zn superoxide dismutase (CuZnSOD). This enzyme is found in the cytoplasm and scavenges the superoxide anion, a reactive oxygen species. These mechanisms come into play in response to a high influx of copper or during the course of normal copper metabolism.

A. Accessing the Intracellular Pool

In a defined culture medium (an artificial environment), cells can get copper ions from a great number of suitable donors. The uptake process is usually rapid and, curiously enough, does not depend on the ATP status of the cell, so it seems that the uptake process is not energy demanding. It is tempting to conclude that a passive copper transport system exists in the membrane (Tong & McArdle, 1995). Plasma factors seem to have a similar capability to influence cellular access *in vivo*. The capacity of amino acids and chloride and bicarbonate ions to stimulate copper uptake gives some support for this idea (Harris, 2000). Inhibitors of protein biosynthesis seem to influence increased uptake of copper. This indicates that the transport system has a two-sided character and accounts for both import and export (Harris, 2000).

To work properly, a transport mechanism requires an easy exchange of copper. There has been some discussion about whether albumin- and ceruloplasmin-bound copper actually could be sources of copper for the tissues; however, a reducing environment, be it in plasma or in the membrane, can compromise binding strengths, making release of copper possible. These can be compromised by reducing systems in the membrane. Even amino acids could interact with the copper–protein complex (Harris, 2000).

B. Albumin as a Copper Transport Factor

Albumin is the most abundant protein in plasma; therefore, it is a good candidate for copper transport because it can bind copper in several sites on the protein (Masuoka & Saltman, 1994). The best candidates for transportation seem to be the sites at the extreme N terminal region or certain cysteine residues within the protein. At the N terminal, a histidine at position 3 may bind copper (Cu^{2+}); however, this site does not reach a rapid equilibrium with unbound copper. Histidine forms stable complexes with Cu^{2+}, and the histidine concentration in plasma is about 135 μM. These factors favor histidine as a transport ligand for copper. The

interaction is reflected by a ternary complex with albumin–Cu(II) and histidine. Furthermore, the interaction induces the protein to release copper as a histidine–Cu(II) complex. It seems, however, that the histidine ligand is not transported across the membrane (Hilton *et al.*, 1995). This would suggest that the role of histidine ends at the cell surface (Harris, 2000).

C. Ceruloplasmin as Copper Transporter

Ceruloplasmin was first isolated from plasma and characterized as a copper-containing protein by Holmberg and Laurell (1948). This protein is a member of a class of proteins known as multicopper oxidases, which are characterized by three distinct copper sites. Ceruloplasmin is also a ferroxidase and plays an important role in oxidizing Fe^{2+} to Fe^{3+} for incorporation in apotransferrin. As a copper transporter, it contains about 95% of the copper in plasma. Multicopper oxidases utilize the electron chemistry of bound copper ions to couple substrate oxidation with the four-electron reduction of dioxygen. Electrons pass from the substrate to the type I copper, then to the trinuclear copper cluster, and subsequently to the oxygen molecule bound at this site (Garrick *et al.*, 2003). Human ceruloplasmin is encoded in 20 exons encompassing about 65 kb of DNA localized to chromosome 3q23–q24. The human ceruloplasmin gene in hepatocytes is expressed as two transcripts of 3.7 and 4.2 kb. These come from use of alternative polyadenylation sites within the 3′ untranslated region. When these transcripts are expressed in the liver, the 1046-amino-acid protein ceruloplasmin is found in plasma (Bielli & Calabrese, 2002).

D. Membrane Transport of Copper

Some years ago, a major area of investigation was agents that transported copper in plasma. Lately, however, interest has been focused on interactions at the membrane surface and transport intracellularly. What brought about this change in focus was the identification of specific copper-transporting proteins in the membrane as well as within the cell. Yeast has both low- and high-affinity systems for copper uptake, and these mediate copper transport (Eide, 1998). In yeast, the copper transport 1 (*CTR1*) gene was the first to be identified. Surprisingly, this gene was not directly for copper transport but was essential for iron transport in *Saccharomyces cerevisiae*. In fact, the copper was required for Fet3. This is a multicopper ferroxidase that catalyzes the oxidation of Fe^{2+} to Fe^{3+} to make absorption by a ferric transport protein possible (Dancis *et al.*, 1994). The protein Ctrp1 is expressed by this gene and is a membrane-crossing protein. In addition, it is heavily glycosylated, with a serine- and methionine-rich composition in which the structural motif methionine–X–X–methionine is repeated 11 times. Ctrp1 transports Cu^{+} and not Cu^{2+} or any other metal ions. The identification of this protein establishes a mechanistic link between copper and iron uptake (Harris, 2000). Strains of *S. cerevisiae* are equipped with a second high-affinity transporter gene, *CTR3* (Knight *et al.*, 1996). The expressed protein, Ctrp3, restores copper-related functions in strains lacking the *CTR1* gene. A structurally similar transporter gene in *Arabidopsis thaliana*, *COPT1*, was later discovered as well as the human transporter *hCTR1*. In HeLa cells, the *hCTR1* gene is located on chromosome 9 (9q31/32, to be more precise) (Harris, 2000). The human hCtr1 protein is much smaller than the yeast Ctrp1 protein, based on cDNA sequence data. All these copper transport proteins recognize only Cu^{+}; thus, there must be a reductase in the membrane to reduce Cu^{2+} to Cu^{+} at the moment of membrane penetration. In yeast, the expression of *FRE1* and *FRE2* reduces both Cu^{2+} and Fe^{3+} for transport. Expression of a third gene, *FRE7*, increases when extracellular copper becomes very limiting (Martins *et al.*, 1998).

The first important factor to take into consideration is that the cytosolic environment is highly reducing. This means that reducing Cu^{2+} to Cu^{+} is a straightforward process. The nitrogen-containing molecule glutathione (γ-glutamylcysteinglycine, or GSH) is the most common intracellular thiol. It has a concentration in mammalian cells of 0.5 to 10 m*M* and is a reducing agent in many reactions. Glutathione was one of the earliest intracellular components identified with copper transport (Denke & Farburg, 1989). Glutathione may also play the role of a general transporter of copper ions by delivering copper to Ctr1 in the plasma membranes. Glutathione reduces and binds Cu^{+} and delivers it to metallothioneins and to some copper-dependent apoenzymes such as superoxide dismutase and hemocyanin (Tapiero *et al.*, 2003). Formation of Cu(I)–GSH is a spontaneous reaction apparently independent of enzyme involvement. When cellular GSH is low, cells are slower to take up copper from the medium and have a lower cellular concentration at steady state (Harris, 2000).

A decline in GSH levels in a cell impairs the subsequent binding of copper to apo-CuZnSOD or the

delivery of copper to the cytosolic enzymes; thus, Cu–GSH complexes have the capacity to mediate Cu(I) transfer to a variety of binding sites on macromolecules. The function of GSH, then, extends beyond that of preventing copper toxicity to playing an important role in internal copper metabolism (Harris, 2000).

A new family of soluble metal receptor proteins acting in the intracellular trafficking of metal ions is the metallochaperones. These metal receptors do not act as scavengers or detoxifiers. On the contrary, they act in a "chaperone-like" manner by guiding and protecting metal ions while facilitating appropriate partnerships (O'Halloran & Culotta, 2000). Copper chaperones are a family of cytosolic peptides that form transient complexes with Cu^{+}. An invariant methionine–X–cysteine–X–X-cysteine metal-binding motif in the N-terminal region is a structural feature of most chaperones. Their function is to guide copper ions in transit to specific proteins that require copper (Harris, 2000). Significant examples of metallochaperones are the gene products of the mercury resistance (*mer*) operon of the Gram-negative transposon Tn*21*, including MerP. MerP is a small, soluble periplasmic protein that transports Hg^{2+} to a membrane transporter and eventually to a reductase that reduces the Hg^{2+} to the volatile Hg^{0} as part of a detoxifying mechanism (Hamlett *et al.*, 1992). Chaperones for copper act in a similar manner, moving copper from one location in the cell to another, often crossing membrane boundaries. In contrast to nuclear activating factors, chaperones demonstrate no capacity to enter the nucleus or interact with DNA. ATX1 and copZ are copper chaperones in *Saccharomyces cerevisiae* and *Enterococcus hirae*, respectively, and both have ferredoxin-like folds with the $\beta\alpha\beta\beta\alpha\beta$ motif in the folded chains. Copper as Cu(I) is bound to two cysteine sulfur groups, forming a linear bidentate ligand. This structure probably allows for easy exchange of the bound copper to a structurally similar copper-binding site on the receiving protein (Harris, 2000). Of utmost significance is that other structural features allow the peptide to specify the target proteins with virtually no possibility for mismatch. The targets for these copper chaperones are cytochrome *c* oxidase in the mitochondria, ATP7B in the trans-Golgi, and the apo form of copper- and zinc-dependent superoxide dismutase (CuZnSOD) in either peroxisomes or the cell cytosol. The obvious advantage of a chaperone is that it selects the receiving molecule with high precision. This is not the case with Cu(I)–GSH, which has no target-specifying property. A conceptual disadvantage is that chaperones force a partitioning of copper into multiple pools in order to replenish copper enzymes (Harris, 2000). To date, three chaperones have been described in yeast, and all three are known to share structural features with mammalian and plant counterparts. The following is a brief description of known chaperones.

In yeast cells lacking superoxide dismutase activity (*sod1*Δ) and auxotrophic for lysine, *ATX1* (antioxidant 1) was identified as an antioxidant gene that suppressed oxygen toxicity (Lin *et al.*, 1997). The human homolog of *ATX1*, named *ATOX1*, was shown to complement yeast lacking *ATX1*. Like ATX1, the human ortholog contains one methionine–threonine–cysteine–X–glycine–cysteine copper-binding domain (Harris, 2000). The copper chaperone (CCH) in *Arabidopsis* has a 36% sequence identity with ATX1. Chaperones with the ATX1 structural domain target P-type ATPases. The ATPase in yeast is Ccc2p, a membrane-bound protein that mediates the transfer of copper to a late or post Golgi compartment. By the same reasoning, ATOX1 in mammals is thought to target ATP7B, which is a P-type ATPase that occurs in Wilson's disease. The ATPase ATP7B transfers copper to apo-ceruloplasmin or forces its extrusion into the bile, whereas Ccc2p transports copper to Fet3p, which is a multicopper oxidase that oxidizes Fe^{2+} to Fe^{3+} for incorporation into the ferric ion transporter and subsequent delivery (Harris, 2000).

Another copper chaperone, COX17, carries out the transport of copper to cytochrome oxidase in the mitochondria of the yeast *Saccharomyces cerevisiae*. A human homolog of COX17 has also been reported. Cox17, acting as a mitochondrial copper shuttle, is the only known chaperone that violates the glycine–methionine–X–cysteine–X–X-cysteine consensus motif. The copper binding sites on Cox17 are cysteine residues occurring in tandem (Cys14 and Cys16) and positioned near the N terminus. A unique property is the binding of Cu(I) to Cox17, which is a binuclear cluster that is similar to the copper cluster in metallothionein, with the exception that the Cox17–Cu(I) complex is more labile. In *S. cerevisiae* the delivery of copper to cytochrome oxidase apparently involves two inner mitochondrial membrane proteins, SCO1 and SCO2, which are penultimate receivers of the copper (Harris, 2000).

LYS7 is a 27-kDa copper chaperone that delivers copper to the apo-SOD1. There are mutants of yeast LYS7 mutants; they are defective in SOD1 (the gene encoding CuZnSOD) activity and are unable to incorporate copper into the protein. A single methionine–histidine–cysteine–X–X–cysteine consensus sequence is present in the N-terminal region of the protein. The copper chaperone for SOD (CCS) is a human counterpart of comparable size and 28% sequence identity (Harris, 2000).

The most significant finding with regard to copper transport in cells is without a doubt the discovery, cloning, and sequencing of the genes responsible for Menkes' and Wilson's diseases. These diseases have constituted important models of abnormal copper metabolism in humans. The etiology of these diseases was unknown for a long time. Classical Menkes' disease is an X-linked (*i.e.*, strikes mainly males) copper deficiency. The incidence is about 1 in 200,000. Boys with the disease usually do not survive past 10 years of age. Manifestations of the disease are a series of enzyme defects. Hypopigmented hair is caused by a deficiency of the tyrosinase required for melanin synthesis. Connective tissue abnormalities, including aortic aneurysms, loose skin, and fragile bones, result from reduced lysyl oxidase activity and consequent weak cross-links in collagen and elastin. Severe neurological defects are a predominant feature of the classical form of the disease and possibly result from the reduced activities of cytochrome oxidase; however, effects due to the reduced activity of superoxide dismutase, peptidylglycine-α-amidating monooxygenase, and dopamine-β-monooxygenase may also contribute to brain abnormalities (Suzuki & Gitlin, 1999). All of these enzymes are copper dependent. A diagnostic feature is the unusual steely or kinky hair caused by reduced keratin cross-linking, a process that also is copper dependent. The copper deficiency in Menkes' disease is caused by reduced uptake of copper across the small intestine (Figure 11) catalyzed by the Menkes' protein ATP7A, compounded by defective distribution of copper within the body wherever ATP7A is required for copper transport. ATP7A is involved in the transport of copper across the blood–brain barrier, which explains the marked brain copper deficiency and consequent severe neurological abnormalities in patients (Suzuki & Gitlin, 1999).

Wilson's disease, or hepatolenticular degeneration, is an autosomal recessive copper toxicosis condition with an incidence of 1 in 50,000 to 1 in 100,000, depending on the population. Very high concentrations of copper accumulate in the liver because of impaired biliary excretion of copper or failure to incorporate copper into ceruloplasmin, the major copper-binding protein in the circulation. This will ultimately cause the death of hepatocytes. The disease has a variable age of onset but is rarely observed in children younger than five and can present as a hepatic or neurologic disease. Copper may be released from damaged hepatocytes and accumulates in extrahepatic tissues, including the central nervous system. The diagnostic copper deposits that can sometimes be seen in the cornea of the eyes are known as the

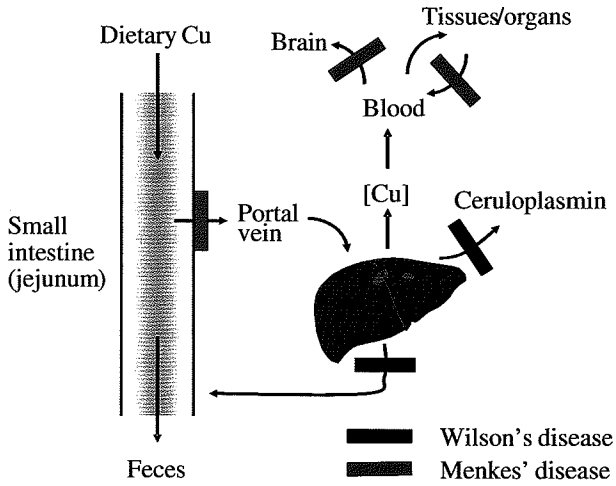

FIGURE 11 A schematic view of copper pathways and the blocks in Menkes' disease and Wilson's disease. Copper is taken up by enterocytes in the small intestine and exported over the basolateral membrane into the portal circulation. The export mechanism is defective in Menkes' disease; consequently, an overload of copper occurs in the enterocytes and subsequent copper deficiency in the organism. The liver normally takes up most of the copper in the portal circulation. If there is a copper overload, excess copper is excreted in the bile. This process is blocked in Wilson's disease. Furthermore, the delivery of copper to ceruloplasmin is also blocked. (Adapted from Mercer, 2001.)

Kayser-Fleischer rings disorder, which results from pathological accumulations of copper, predominantly in the liver and brain tissues. The dominant symptoms relate to a failure to release liver copper into bile. Both diseases have provided unprecedented molecular insights into genetic factors that regulate copper transport and bioavailability to organs and tissues (Suzuki & Gitlin, 1999).

Menkes' and Wilson's diseases are caused by mutations in genes on the X chromosome (Xq13) and chromosome 13 (13q14.3). The isolation and sequencing of these disease genes have revealed that both code for P-type Cu-ATPases. The Wilson's (ATP7A) and Menkes' (ATP7B) proteins are specific copper transporters; furthermore, ATP7B and ATP7A have a 57% sequence homology to one another. In addition, they have remarkable parallels to copper-binding proteins in bacteria. The gene for Menkes' disease spans about 150 kb. Its mRNA is 8.3 to 8.5 kb, encompassing 23 exons that range in size from 77 to 4120 bp, with a single open reading frame and an ATG start codon in the second exon. Exon 23 contains the TAA stop codon, 274 bp that are translated, and a 3.8-kb untranslated region that has the polyadenylation site. When analyzed as a cDNA,

ATP7A mRNA encodes a protein of exactly 1500 amino acids; however, it may have additional nucleotide sequences at the 5′ end. A 22-amino-acid presequence generated by an in-frame ATG site upstream occurs in some ATP7A transcripts (Harris, 2000). Strong expression of Menkes' disease mRNA is observed in muscle, kidney, lung, and brain. In placenta and pancreas, the expressions are weaker, and liver shows only traces. The Wilson's disease transcript is 7.5 kb and encodes a protein of 1411 amino acids. In contrast to the Menkes' disease gene, the Wilson's disease gene is strongly expressed in the liver and kidney (Harris, 2000).

The biological functions of ATP7A and ATP7B are different, although their structures are similar. The Menkes' disease protein (ATP7A) seems to be responsible for the regulation of copper-ion release at the outer membrane. Experiments with Chinese hamster ovary cells have shown that overexpression of ATP7A makes the cells tolerate highly toxic amounts of copper in their immediate environment (Camakaris *et al.*, 1995). Superior tolerance is manifested by forced expulsion that prevents copper accumulation. The similarity in overall appearance between ATP7A and ATP7B might lead to the conclusion that they are similarly distributed. ATP7B, in contrast to the membrane association of ATP7A, resides within an internal organelle of the cell, where it functions to incorporate copper into apo-ceruloplasmin. This process takes place in either the ER or a Golgi compartment. Additionally, the protein works to force the release of copper into the bile (Harris, 2000).

The structure of ATP7A contains a comparatively large, heavy-metal binding domain (Hmb). This domain is comprised of six metal-binding cysteine clusters within the structural motif glycine–methionine–threonine/histidine–cysteine–X–serine–cysteine which contain eight transmembrane (Tm) regions. The purpose of these Tm regions is to guarantee anchorage and orientation of the protein. The correct assembly defines the channel through which copper ions pass. ATP7A is a type II membrane protein and is thus defined because both the —NH_2 and the —COOH termini are on the cytosolic side of the membrane. Two flexible loops, one on a 135-residue chain and the second on a 235-residue chain, extend into the cytosol. The smaller one is between Tm 4 and 5 and the larger one is between Tm 6 and 7 (Harris, 2000). The bacterial CopA protein contains a smaller Hmb with a single glycine–methionine–threonine/histidine–cysteine–X–X–X motif (Solioz *et al.*, 1994). A yeast Cu-ATPase has the motif at most twice, which leads one to speculate whether the 650-residue Hmb region performs some

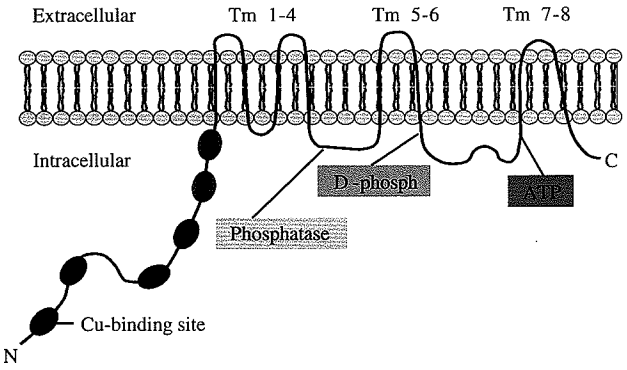

FIGURE 12 An illustration of the Menkes' and Wilson's Cu-ATPases. Eight membrane-crossing (Tm) domains are predicted for both proteins. Most of the protein is localized in the cytoplasm. In addition to the ATP-binding site, phosphatase domain and phosphorylated aspartic acid (D-phosph) is common to all P-type ATPases. The N terminal contains six copper-binding motifs that interact with copper chaperones. (Adapted from Mercer 2001.)

transport-related function other than binding copper for export. A cysteine–proline–cysteine motif in Tm 6 is thought to build the channel that allows copper in the cytosol to be transported across the membrane (Figure 12). Copper(I) is the preferred ion to bind to ATP7A and ATP7B. There is little or no affinity of the Hmb to bind to Fe(II), Fe(III), Ca(II), Mg(II), Mn(II), or Ni(II); however, Zn(II) shows some binding (Harris, 2000).

The perinuclear area within the region of the cell thought to represent the Golgi seems to be the localization of ATP7A. ATP7A-loaded vesicles have been postulated to continually be moving between the Golgi and plasma membrane. Experiments show that high concentrations of copper in the exterior of the cell induce movement of the marked vesicles to the cell boundary. The Wilson's disease ATP7B is also localized in the Golgi. A truncated homolog of ATP7B that lacks four of the eight membrane-spanning domains seems to reside in the cytosol. A structural analog of the Wilson's disease gene or Menkes' disease gene, the *CCC2* gene in yeast, also encodes a P-type ATPase that exports cytosolic copper to the extracytosolic domain of Fet3p, a copper oxidase required for iron uptake (Harris, 2000).

The mouse homolog of ATP7A, the Atp7a, is expressed in all tissues but is particularly strong in the choroid plexus of the brain. The localization in the brain, more specifically the blood–brain barrier, places the ATPase in a strategic position to control the flow of copper into the ventricles of the brain. An animal model of Menkes' disease is the macular mutant mouse. A

gradual erosion of cytochrome *c* oxidase activity in the brain has been observed in this model. The effect can be partially prevented by a single injection of copper in an early perinatal period (Megura *et al.*, 1991). Copper shows a propensity to accumulate in brain blood vessels and in astrocytes which apparently hinders its movement to neurons (Kodama, 1993); furthermore, embryonic mouse liver expresses Atp7a mRNA in contrast to adults. The rat homolog of ATP7B, Atp7b, is expressed early only in the central nervous system, heart, and liver. With development, Atp7b appears in intestine, thymus, and respiratory epithelia (Kuo *et al.*, 1997). Transfection assays in yeast have demonstrated that the two ATPases share biochemical functions. Atp7b in hepatocytes from (LEC) rats, a model for Wilson's disease, mimics the sequestration of ceruloplasmin in cotransfected cells. The data support a metabolic connection between the plasma copper protein and Wilson's disease ATPase (Cox & Moore, 2002). ATP7B may also be localized on the apical surface of hepatocytes, a location that allows the protein to expel copper ions into the bile (Fuentealba & Aburto, 2003).

SEE ALSO THE FOLLOWING CHAPTER

Chapter 6 (Biological Functions of the Elements)

FURTHER READING

Andrews, N. C. (1999). Disorders of Iron Metabolism, *N. Engl. J. Med.*, 341, 1986–1995.

Bielli, P., and Calabrese, L. (2002). Structure to Function Relationships in Ceruloplasmin: A "Moonlighting" Protein, *Cell. Mol. Life Sci.*, 59, 1413–1427.

Cannone-Hergeaux, F., Gruenheid, S., Ponka, P., and Gros, P. (1999). Cellular and Subcellular Localization of the Nramp2 Iron Transporter in the Intestinal Brush Border and Regulation by Dietary Iron, *Blood*, 93, 4406–4417.

Carpenter, C. E., and Mahorey, A. W. (1992). Contributions of Heme and Non-Heme Iron to Human Nutrition, *CRC Crit. Rev. Food Sci. Nutr.*, 31, 333–367.

Cohen, C. K., Fox, T. C., Garvin, D. F., and Kochian, L. V. (1998). The Role of Iron-Deficiency Stress Responses in Stimulating Heavy-Metal Transport in Plants, *Plant Physiol.*, 116, 1063–1072.

Collawn, J. F., Lai, A., Domingo, D., Fitch, M., Hatton, S., and Trowbridge, I. S. (1993). YTRF is the Conserved Internalisation Signal of the Transferrin Receptor, and a Second YTRF Signal at Position 31–34 Enhances Endocytosis, *J. Biol. Chem.*, 268, 21686–21692.

Camakaris, J., Petris, M. J., Bailey, L., Shen, P. Y., Lockhart, P., Glover, T. W., Barcroft, C., Patton, J., and Mercer, J. F. (1995). Gene Amplification of the Menkes (MNK: ATP7A) P-type ATPase Gene of CHO Cells is Associated with Copper Resistance and Enhanced Copper Eflux, *Hum. Mol. Genet.*, 4, 2117–2123.

Conrad, M. E., and Umbreit, J. N. (2000). Iron Absorption and Transport—An Update, *Am. J. Hematol.*, 64, 287–298.

Conrad, M. E., Umbreit, J. N., and Moore, E. G. (1999). Iron Absorption and Transport, *Am. J. Med. Sci.*, 318, 213–229.

Cousins, R. J., and McMahon, R. J. (2000). Integrative Aspects of Zinc Transporters, *J. Nutr.*, 130, 1384S–1387S.

Cox, D. W., and Moore, S. D. (2002). Copper Transporting P-Type ATPases and Human Disease, *J. Bioenerg. Biomembr.*, 34, 333–338.

Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1994). Molecular Characterization of a Copper Transport Protein in *S. cerevisiae*: An Unexpected Role for Copper In Iron Transport, *Cell*, 76, 393–402.

Davidsson, L., Lönnerdal, B., Sandström, B., Kunz, C., and Keen, C. L. (1989). Identification of Transferrin as the Major Plasma Carrier Protein for Manganese Introduced Orally or Intravenously or After *In Vitro* Addition in the Rat, *J. Nutr.*, 119, 1461–1464.

Denke, S. M., and Farburg, B. L. (1989). Regulation of Cellular Glutathione, *Am. J. Physiol.*, 257, L163–L173.

Donovan, A., Brownile, A., Zhou, Y., Shepard, J., Pratt, S. J., Moynihan, J., Paw, B. H., Drejer, A., Barut, B., Zapata, A., Law, T. C., Brugnarall, C., Lux, S. E., Pinkus, G. S., Pinkus, J. L., Kingsley, P. D., Pails, J., Fleming, M. D., Andrews, N. C., and Zon, L. I. (2000). Positional Cloning of Zebrafish Ferroportin Identifies a Conserved Vertebrate Iron Exporter, *Nature*, 403, 776–781.

Dufner-Beattie, J., Langmade, S. J., Wang, F., Eide, D., and Andrews, G. K. (2003). Structure, Function, and Regulation of a Subfamily of Mouse Zinc Transporter Genes, *J. Biol. Chem.*, 278, 50142–50150.

Eide, D. J. (1998). The Molecular Biology of Metal Ion Transport in Saccharomyces Cerevisiae, *Annu. Rev. Nutr.*, 18, 441–469.

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1966). A Novel Iron-Regulated Metal Transporter from Plants Identified by Functional Expression in Yeast, *Proc. Natl. Acad. Sci. U.S.A.*, 93, 5624–5628.

Eisenstein, R. S. (2000). Iron Regulatory Proteins and the Molecular Control of Mammalian Iron Metabolism, *Annu. Rev. Nutr.*, 20, 627–662.

Finch, C. (1994). Regulators of Iron Balance in Humans, *Blood*, 84, 1697–1702.

Fleming, M. D., Trenor, C. C., Su, M. A., Foernzler, D., Beier, D. R., Dietrich, W. F., and Andrews, N. C. (1997). Microcytic Anemia Mice Have a Mutation in Nramp2, a Candidate Iron Transporter Gene, *Nature Genet.*, 16, 383–386.

Fleming, R. E., Miqas, M. C., Zhou, X. Y., Jiang, J., Britton, R. S., Brunt, E. M., Tomatsu, S., Waheed, A., Bacon, B. R., and Sly, W. S. (1999). Mechanism of Increased Iron Absorption in Murine Model of Hereditary Hemochromatosis: Increased Duodenal Expression of the Iron Transporter DMT1, *Proc. Natl. Acad. Sci. U.S.A.*, 96, 3143–3148.

Franklin, R. B., Ma, J., Zou, J., Guan, Z., Kykoyi, B. I., Feng, P., and Costello, L. C. (2003). Human ZIP1 is a Major Zinc Uptake Transporter for the Accumulation of Zinc in Prostate Cells, *J. Inorg. Biochem.*, 96, 435–442.

Fuentealba, I. C., and Aburto, E. M. (2003). Animal Models of Copper-Associated Liver Disease, *Comp. Hepatol.*, 2, 5–16.

Gaither, L. A., and Eide, D. J. (2000). Functional Characterization of the Human hZIP2 Zinc Transporter, *J. Biol. Chem.*, 275, 5560–5564.

Gaither, L. A., and Eide, D. J. (2001). Eukaryotic Zinc Transporters and Their Regulation, *Biometals*, 14, 251–270.

Garrick, M. D., Nunez, M. T., Olivares, M., and Harris, E. D. (2003). Parallels and Contrasts Between Iron and Copper Metabolism, *Biometals*, 16, 1–8.

Gruenheid, S., Cannone-Hergeaux, F., Gauthier, S., Hackam, D. J., Grinstein, S., and Gros, P. (1999). The Iron Transport Protein NRAMP2 is an Integral Membrane Glycoprotein That Colocalizes with Transferrin in Recycling Endosomes, *J. Exp. Med.*, 189, 831–841.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., and Hediger, M. A. (1997). Cloning and Characterization of a Mammalian Proton-Coupled Metal-Ion Transporter, *Nature*, 388, 482–488.

Hamlett, N. V., Landale, E. C., Davis, B. H., and Summers, A. O. (1992). Roles of the *Tn21*, *merT*, *merP*, and *merC* Gene Products in Mercury Resistance and Mercury Binding, *J. Bacteriol.*, 174, 6377–6485.

Hantke, K. (2001). Bacterial Zinc Transporters and Regulators, *Biometals*, 14, 239–249.

Harris, E. D. (2000). Cellular Copper Transport and Metabolism, *Annu. Rev. Nutr.*, 20, 291–310.

Harris, Z. L., Klomb, L. W., and Gitlin, J. D. (1998). Aceruloplasminemia: An Inherited Neurodegenerative Disease with Impairment of Iron Homeostasis, *Am. J. Clin. Nutr.*, 96, 10812–10817.

Hilton, M., Spenser, D. C., Ross, P., Ramsey, A., and McArdle. H. J. (1995). Characterisation of the Copper Uptake Mechanism and Isolation of Ceruloplasmin Receptor/Copper Transporter in Human Placental Vesicles, *Biochim. Biophys. Acta*, 1245, 153–160.

Holmberg, C. G., and Laurell, C. B. (1948). Investigations in Serum Copper. II. Isolation of the Copper-Containing Protein and a Description of Some of Its Properties, *Acta Chem. Scand.*, 2, 550–556.

Iacopetta, B. J., Rothenberger, S., and Kuhn, L. C. (1988). A Role for the Cytoplasmic Domain in Transferrin Receptor Sorting and Coated Pit Formation During Endocytosis, *Cell*, 54, 485–489.

Kambe, T., Yamaguchi-Iwai, Y., Sasaki, R., and Nagao, M. (2004). Overview of Mammalian Zinc Transporters, *Cell. Mol. Life Sci.*, 61, 49–68.

Kawabata, H., Yang, R., Hirama, T., Vuong, P. T., Kawano, S., Gombart, A. F., and Koeffler, H. P. (1999). Molecular Cloning of Transferrin Receptor 2. A New Member of the Transferrin Receptor-Like Family, *J. Biol. Chem.*, 274, 20826–20832.

Knight, S. A. B., Labbé, S., Kwon, L. F., Kosman, D. J., and Thiele, D. J. (1996). A Widespread Transposable Element Marks Expression of a Yeast Copper Transport Gene, *Genes Dev.*, 10, 1917–1929.

Kodama, H. (1993). Recent Developments in Menkes' Disease, *J. Inherited Metab. Dis.*, 16, 791–799.

Kohgo, Y., Torimoto, Y., and Kato, J. (2002). Transferrin Receptor in Tissue and Serum: Updated Clinical Significance of Soluble Receptor, *Int. J. Hematol*, 76, 213–218.

Korshunova, Y. O., Eide, D., Clark, W. G., Guerinot, M. L., and Pakrasi, H. B. (1999). The IRT1 Protein from *Arabidopsis thaliana* Is a Metal Transporter with a Broad Substrate Range, *Plant Mol. Biol.*, 40, 37–44.

Kuo, Y. M., Gitschier, J., and Packman, S. (1997). Developmental Expression of the Mouse Mottled and Toxic Milk Genes Suggests Distinct Functions for the Menkes and Wilson Disease Copper Transporters, *Hum. Mol. Genet.*, 6, 1043–1049.

Lasat, M. M., Pence, N. S., Garvin, D. F., Ebbs, S. D., and Kochian, L. V. (2000). Molecular Physiology of Zinc Transport in the Zn Hyperaccumulator *Thlapsi caerulescens*, *J. Exp. Biol.*, 51, 71–79.

Lee, P. L., Gelbart, T., West, C., Halloran, C., and Beutler, E. (1998). The Human Nramp2 Gene Characterization of the Gene Structure, Alternative Splicing, Promoter Region and Polymorphisms, *Blood Cells Mol. Dis.*, 24, 199–215.

Lieu, P. T., Heiskala, M., Peterson, P. A., and Yang ,Y. (2001). The Roles of Iron in Health and Disease, *Mol. Aspects Med.*, 22, 1–87.

Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V., and Culotta, V. C. (1997). A Role for the *Saccharomyces cerevisiae*

ATX1 Gene in Copper Trafficking and Iron Transport, *J. Biol. Chem.*, 272, 9215–9220.

Lioumi, M., Ferguson, C. A., Sharpte, P. T., Freeman, T., Marenholz, I., Mischke, D., Heizmann, C., and Ragoussis, J. (1999). Isolation and Characterization of Human and Mouse ZIRTL, a Member of the IRT1 Family of Transporters, Mapping Within the Epidermal Differentiation Complex, *Genomics*, 62, 272–280.

Majuri, R., and Grasbeck, R. (1987). A Rosette Receptor Assay with Haem-Microbeads. Demonstration of a Haem Receptor on K562 Cells, *Eur. J. Haematol*, 38, 21–25.

Mantzoros, C. S., Prasad, A. S., Beck, F. W. J., Grabowski, S., Kaplan, J., Adair, C., and Brewer, G. J. (1998). Zinc May Regulate Serum Leptin Concentrations in Humans, *J. Am. Coll. Nutr.*, 17, 270–275.

Martins, L. J., Jensen, T. L., Simons, J. R., Keller, G. L., and Winge, D. R. (1998). Metalloregulation of *FRE*1 and *FRE*2 Homologs in Saccharomyces Cerevisiae, *J. Biol. Chem.*, 273, 23716–23721.

Masuoka, J., and Saltman, P. (1994). Zinc(II) and Copper(II) Binding to Serum Albumin, *J. Biol. Chem.*, 269, 25557–25561.

McClelland, A., Kuehn, L. C., and Ruddle, F. H. (1984). The Human Transferrin Receptor Gene: Genomic Organization, and the Complete Primary Structure of the Receptor Deduced from a cDNA Sequence, *Cell*, 39, 267–274.

McKie, A. T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T. J., Farzaneh, F., Hediger, M. A., Hentze, M. W., and Simpson, R. J. (2000). A Novel Duodenal Iron-Regulated Transporter, IREG1, Implicated in the Basolateral Transfer of Iron to the Circulation, *Mol. Cell*, 5, 299–309.

Meguro, Y., Kodama, H., Abe, T., Kobayashi, S., Kodama, Y., and Nishimura, M. (1991). Changes of Copper Level and Cytochrome *c* Oxidase Activity in the Macular Mouse with Age, *Brain Dev.*, 13, 184–186.

Mercer, J. F. B. (2001). The Molecular Basis of Copper-Transport Diseases, *Trends Mol. Med.*, 7, 64–69.

Moos, T., Trinder, D., and Morgan, E. H. (2000). Cellular Distribution of Ferric Iron, Ferritin, Transferrin and Divalent Metal Transporter 1 DMT1 in Substantia Nigra and Basal Ganglia of Normal and Beta 2-Microglobulin Deficient Mouse Brain, *Cell. Mol. Biol.*, 46, 549–561.

Nies, D. H., and Silver, S. (1995). Ion Efflux Systems Involved in Bacterial Metal Resistance, *J. Ind. Microbiol.*, 14, 186–199.

O'Halloran, T. V., and Culotta, C. V. (2000). Metallochaperones: An Intracellular Shuttle Service for Metal Ions, *J. Biol. Chem.*, 275, 25057–25060.

Palmiter, R. D., Cole, T. B., and Findley, S. D. (1996). ZnT-2, a Mammalian Protein that Confers Resistance to Zinc by Facilitating Vesicular Sequestration, *EMBO J.*, 15, 1784–1791.

Pietrangelo, A., Rocchi, E., Casalgrandi, G., Rigo, G., Ferrari, A., Perini, M., Ventura, E., and Cairo, G. (1992). Regulation of Transferring, Transferrin Receptor, and Ferritin Genes in Human Duodenum, *Gastroenterology*, 102, 802–809.

Raskin, I. (1995). Plant Genetic Engineering May Help with Environmental Cleanup, *Proc. Natl. Acad. Sci. U.S.A.*, 93, 3164–3166.

Rolfs, A., and Hediger, M. A. (1999). Metal Ion Transporters in Mammals: Structure, Function and Pathological Implications, *J. Physiol.*, 518, 1–12.

Rothenberg, B. E., and Voland, J. R. (1996). Beta2 Knockout Mice Develop Parenchymal Iron Overload: A Putative Role for Class I Genes of the Major Histocompatibility Complex in Iron Metabolism, *Proc. Natl. Acad. Sci. U.S.A.*, 93, 1529–1534.

Rutledge, E. A., and Enns, C. A. (1996). Cleavage of the Transferrin Receptor Is Influenced by the Composition of the O-Linked Carbohydrate at Position 104, *J. Cell. Physiol.*, 168, 284–293.

Sacher, A., Cohen, A., and Nelson, N. (2001). Properties of the Mammalian and Yeast Metal-Ion Transporters DCT1 and Smf1p Expressed in *Xenopus laevis* Oocytes, *J. Exp. Biol.*, 204, 1053–1061.

Solioz, M., Odermatt, A., and Krapf, R. (1994). Copper Pumping ATPases: Common Concepts in Bacteria and Man, *Fed. Eur. Biochem. Soc. Lett.*, 346, 44–47.

Suhy, D., and O'Halloran, T. V. (1995). Metal Responsive Gene Regulation and the Zinc Metalloregulatory Model. In *Metal Ions in Biological Systems* (H. Siegel, Ed.), Marcel Dekker, New York, Vol. 32, pp. 557–558.

Suzuki, M., and Gitlin, J. D. (1999). Intracellular Localisation of the Menkes' and Wilson's Disease Proteins and Their Role in Intracellular Copper Transport, *Pediatr. Int.*, 41, 436–442.

Tacchini, L., Bianchi, L., Bernelli-Zazzera, A., and Cairo, G. (1999). Transferrin Receptor Induction by Hypoxia: HIF-1-Mediated Transcriptional Activation and Cell-Specific Post-Transcriptional Regulation, *J. Biol. Chem.*, 274, 24142–24146.

Takeda, A., Devenyi, A., and Connor, J. R. (1998). Evidence for Non-Transferrin-Mediated Uptake and Release of Iron and Manganese in Glial Cell Cultures from Hypotransferrinemic Mice, *J. Neurosci. Res.*, 51, 454–462.

Tandy, S., Williams, M., Leggett, A., Lopez-Jimenez, M., Dedes, M., Ramesh, B., Srai, S. K., and Sharp, P. (2000). Nramp2 Expression Is Associated with pH-Dependent Iron Uptake by the Brain: Effects of Altered Iron Status, *J. Biol. Chem.*, 275, 1023–1029.

Tapiero, H., Townsend, D. M., and Tew, K. D. (2003). Trace Elements in Human Physiology and Pathology: Copper. *Biomed. Pharmacother.*, 57, 386–398.

Testa, U., Petrini, M., Quaranta, M. T., Pelosi-Testa, E., Mastroberardino, G., Camagna, A., Boccoli, G., Sargiacomo, M., Isacchi, G., Cozzi, A., Arosio, P., and Peschle, C. (1989). Iron Up-Modulates the Expression of TfRs During Monocyte–Macrophage Maturation, *J. Biol. Chem.*, 264, 13181–13197.

Tong, K. K., and McArdle, H. J. (1995). Copper Uptake by Cultured Trophoblast Cells Isolated from Human Term Placenta, *Biochim. Biophys. Acta*, 1269, 233–236.

Tsuji, Y., Moran, E., Torti, S. V., and Torti, F. M. (1999). Transcriptional Regulation of the Mouse Ferritin H Gene: Involvement of p300/CBP Adaptor Proteins in FER-1 Enhancer Activity, *J. Biol. Chem.*, 274, 7501–7507.

Umbreit, J. N., Conrad, M. E., Moore, E. G., and Latour, L. F. (1998). Iron Absorption and Cellular Transport: The Mobilferrin/Paraferritin Paradigm, *Sem. Hematol.*, 35, 13–26.

Vulpe, C. D., Kuo, Y.-M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., Gischier, J., and Anderson, G. J. (1999). Hephaestin, a Ceruloplasmin Homolog Implicated in Intestinal Iron Transport, Is Defective in the sla Mouse, *Nature, Genet.*, 21, 195–199.

Wood, R. J., and Han, O. (1998). Recently Identified Molecular Aspects of Intestinal Iron Absorption, *J. Nutr.*, 128, 17648–17654.

Zhang, P., and Allen, J. C. (1995). A Novel Dialysis Procedure Measuring Free Zn^{2+} in Bovine Milk and Plasma, *J. Nutr.*, 125, 1904–1910.

Zhao, H., and Eide, D. (1996). The Yeast ZRT1 Gene Encodes the Zinc Transporter of a High Affinity Uptake System Induced by Zinc Limitation, *Proc. Natl. Acad. Sci. U.S.A.*, 93, 2454–2458.