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MOLECULAR MECHANISMS OF VITAMIN E TRANSPORT

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ABSTRACT

If the function of vitamin E is that of an antioxidant and the various forms of vitamin E have similar antioxidant activities, then why does *RRR*-α-tocopherol have the highest biologic activity? This chapter describes how interactions by investigators from various scientific disciplines using stable isotopes, molecular biology tools, and sophisticated genetic studies of humans with vitamin E deficiency have led to an understanding of this problem. This chapter provides an overview of (*a*) studies using deuterated tocopherols that demonstrated that the plasma preference for α-tocopherol is dependent on metabolic processes in the liver; (*b*) the isolation, molecular biology, and function of the α-tocopherol transfer protein; and (*c*) studies that demonstrated that patients who were vitamin E deficient as a result of no known cause had defective α-tocopherol transfer protein genes. Finally, we focus on the future—what remains to be learned about the regulation of vitamin E in tissues.

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INTRODUCTION

Vitamin E has been an enigma in nutrition research for over 60 years. In 1937, Emerson et al (15) described the existence of various vitamin E homologues that had different abilities to prevent vitamin E deficiency. This fat-soluble vitamin, which was required for fertility, was difficult to deplete from tissues and required elaborate manipulations to cause deficiency symptoms to occur in experiment animals; human vitamin E deficiency was virtually unknown. Unlike the water-soluble vitamins that frequently are cofactors in essential enzymatic reactions, for decades vitamin E could not be shown to associate with any proteins. The only demonstrable vitamin E activity was that of a chain-breaking, lipid-soluble antioxidant (8).

In part, confusion about vitamin E arises from its various chemical forms. In nature, vitamin E occurs in eight different forms with varying biologic activities and varying degrees of efficacy to alleviate deficiency symptoms. Additionally, the synthetic form of vitamin E, *all rac* α-tocopherol, is not identical to the naturally occurring α-tocopherol because the synthetic material has eight different stereoisomers arising from the three chiral centers in the vitamin E tail. Although the various naturally occurring forms of vitamin E have somewhat different antioxidant activities, the various synthetic α-tocopherol forms have identical antioxidant activities but different biologic activities. This too created confusion. If the function of vitamin E is that of an antioxidant and the various forms of vitamin E have similar antioxidant activities, then why does *RRR*-α-tocopherol have the highest biologic activity?

The use of stable isotopes, molecular biology tools, and sophisticated genetic studies, along with scientific collaborations crossing international boundaries, have begun to unravel the mystery of vitamin E. This chapter discloses how these scientific interactions by investigators from various disciplines has led to an understanding of the basis for the biologic activity of vitamin E. Ultimately these studies all center on the α-tocopherol transfer protein (α-TTP) and its ability to preferentially bind and transfer *RRR*-α-tocopherol.

VITAMIN E HOMOLOGUES

Vitamin E occurs in nature in eight different forms: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols (21). Tocotrienols differ from tocopherols in that tocotrienols have an unsaturated side chain, whereas tocopherols have a phytyl tail with three chiral centers. Chemically synthesized α-tocopherol contains eight different stereoisomers, only one of which, *RRR*-α-tocopherol, occurs naturally. Commercially available vitamin E supplements contain either the natural form (*RRR*-α-tocopherol) or the synthetic form (*all racemic* α-tocopherol), or their esters.

Currently, an important area of study is defining the roles of the various forms of vitamin E. For example, despite as much as 10-fold higher dietary γ-tocopherol intakes (6), α-tocopherol represents the majority of plasma vitamin E; γ-tocopherol represents only about 10%–20% (38). Nonetheless, γ-tocopherol may be important for protection from reactive nitrogen species because the absence of one methyl group on the chromanol ring allows it, but not α-tocopherol, to be nitrated (13).

PLASMA TRANSPORT OF VITAMIN E

Studies examining mechanisms for the higher plasma α-tocopherol despite higher dietary γ-tocopherol intakes led to the recognition that the liver controls plasma vitamin E concentrations, as reviewed by Traber & Sies (40). When *RRR*-α- and γ-tocopherols and the synthetic stereoisomer *SRR*-α-tocopherol, labeled with different amounts of deuterium, were fed in a single dose to normal humans, the plasma initially contained equal concentrations of these three forms, but by 24 h it was enriched with *RRR*-α-tocopherol (representative subject, Figure 1) (36). Traber et al (36, 37) found that there was no discrimination between the deuterated tocopherols during absorption and chylomicron secretion by the intestine, but during very-low-density-lipoprotein (VLDL) secretion by the liver, a preference for *RRR*-α-tocopherol was observed. Based on findings with deuterated vitamin E, Traber et al (36, 37, 39) suggested that the liver contains a mechanism for the preferential secretion of *RRR*-α-tocopherol in nascent VLDL, and this could be the function of α-TTP.

LIVER α-TOCOPHEROL TRANSFER PROTEIN

Vitamin E is a highly lipophilic substance that travels in the circulation in association with plasma lipoproteins. In the liver, the most important organ in the processing of dietary α- and γ-tocopherols, transfer between intrahepatic compartments is believed to involve specific cytosolic proteins that bind and transfer α-tocopherol. In 1975, Catignani (9) identified a cytosolic protein with a

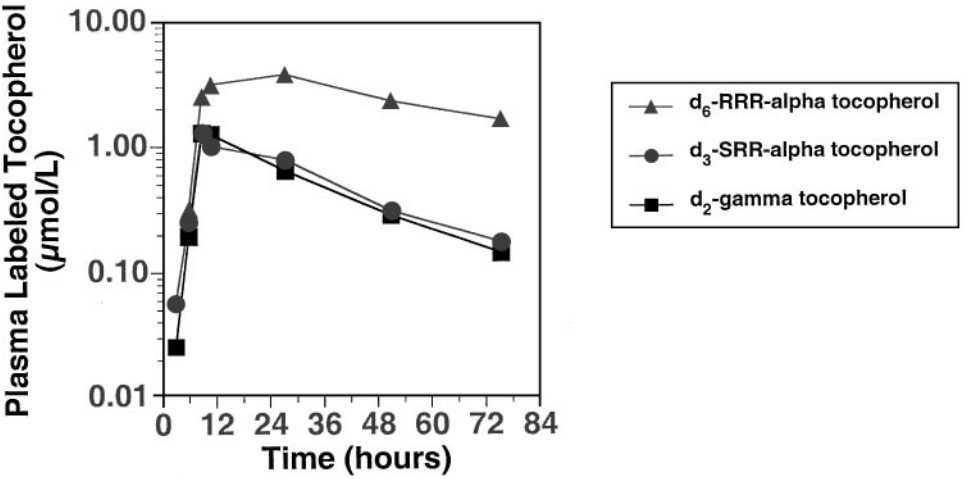

Figure 1 Typical plasma response to deuterated vitamin E intakes. Shown are the plasma concentrations of deuterated tocopherols following a single oral dose containing 50 mg each of *RRR*-α-, *SRR*-α-, and γ-tocopherols labeled with different amounts of deuterium.(Adapted from Reference 36.)

molecular weight of 31,000 that binds α-tocopherol from rat liver. Subsequently, Catignani & Bieri (10) reported key features of this protein. Only α-tocopherol and, to a lesser extent, α-tocotrienol and γ-tocopherol exhibited competition against the binding of α-tocopherol to this protein, and α-tocopheryl acetate and α-tocopherol quinone had no effect on binding. Moreover, the binding protein was identified only in the liver.

In 1981, using in vitro assays, Mowri et al (25) and Murphy & Mavis (26, 27) independently demonstrated that a liver cytosolic protein similar to the α-tocopherol binding protein observed by Catignani is also capable of transferring α-tocopherol between liposomes and mitochondria or between liposomes and microsomes. In 1982, Behrens & Madere (3) showed that labeled α-tocopherol was associated with a 32,000-molecular-weight fraction in gel filtration of hepatic cytosol after oral dosing in vivo with labeled α-tocopherol. They partially purified the fraction from rat liver, using ammonium sulfate fractionation, gel filtration, and ion exchange chromatography, and showed transfer of labeled tocopherol from the binding protein to microsomes. This suggests that α-tocopherol binding and transfer activities were expressed by the same protein.

Purification and cDNA Cloning

The hepatic α-tocopherol transfer protein was purified to homogeneity from rat liver by Sato et al in 1991 (31). The procedure included pH 5.1 acid treatment

of a 15,000 × *g* supernatant fraction, ammonium sulfate fractionation, gel filtration, ion exchange, hydroxylapatite, chromatofocusing, and Blue Sepharose chromatographies. α-Tocopherol transfer activity was determined after chromatofocusing into two peaks with isoelectric points of 5.0 and 5.1. Both isomers have similar α-tocopherol–binding and transfer activities and are likely generated from a single gene, possibly by a posttranslational modification. However, the biological significance of the presence of charge isomers remains unknown. Overall purification of both isomers was approximately 1000-fold. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of both isomers revealed a single band with a molecular weight of 30,500. Similar results were obtained by Yoshida et al using α-tocopherol binding as an assay (45). Antiserum raised against α-tocopherol binding protein detected an approximately 30-kDa band in the liver but not in the cytosol of other organs, such as the heart, spleen, testis, and lung, nor in the lysate of fractionated Ito cells, endothelial cells, or Kupffer cells isolated from rat liver. Semi-quantitative ELISA demonstrated that rat liver cytosol contained approximately 2 mg of tocopherol binding/transfer protein per g of cytosol protein.

Sato et al (30) succeeded in isolating a cDNA clone encoding α-TTP from rat liver. The cDNA contained an open reading frame encoding 278 amino acids with a calculated molecular weight of 31,845. Subsequently, Arita et al (2) isolated a cDNA for the human α-TTP consisting of 278 amino acids and exhibiting 94% identity with rat α-TTP. Both Southern-blot hybridization of human-hamster somatic cell hybrid lines and fluorescence in situ hybridization revealed a single α-TTP gene corresponding to the 8q13.1–13.3 region of chromosome 8.

Ligand Specificity

It has been repeatedly demonstrated that α-TTP selectively recognizes α-tocopherol, the most biologically active form of vitamin E, among tocopherol analogues. Hosomi et al (19) studied in detail the structural characteristics of vitamin E analogs required for α-TTP recognition. Ligand specificity was assessed by evaluating the competition of nonlabeled vitamin E analogs and α-[^{3}H]tocopherol for transfer from liposomes to crude rat liver mitochondria in vitro. According to their data, relative affinities calculated from the degree of competition were *RRR*-α-tocopherol, 100%; β-tocopherol, 38%; γ-tocopherol, 9%; δ-tocopherol, 2%; α-tocopheryl acetate, 2%; α-tocopherol quinone, 2%; *SRR*-α-tocopherol, 11%; α-tocotrienol, 12%; and Trolox, 9%. Three important facts concerning ligand specificity can be drawn from these data: (*a*) All three methyl groups on the chromanol ring are important for recognition by α-TTP, but the methyl group at position 5 is especially critical in light of the difference in affinity between β- and γ-tocopherols; (*b*) the hydroxyl group on

the chromanol ring is essential for α-TTP recognition; and (*c*) α-TTP also recognizes the phytyl chain structure and its orientation, but a tocopherol analog without a side chain still possesses 10% affinity for α-TTP compared with one containing the phytyl chain.

Tissue Distribution

By both Northern (RNA) blot and Western blot (immunoblot) analyses α-TTP was demonstrated to be exclusively expressed in the liver in both rats and humans (2, 30, 45). As mentioned above, in the liver most parenchymal hepatocytes express α-TTP, but other types of cells do not. However, recent studies by Hosomi et al (20) indicated that the message for α-TTP was also detectable at extremely low levels in some rat tissues, including brain, spleen, lung, and kidney. In the brain, the α-TTP transcript was detected predominantly in the Purkinje cell layer of the cerebellar cortex by in situ hybridization histochemistry (20). They speculated that α-TTP present in such a region may be involved in the transport of α-tocopherol from the general circulation into the spinocerebral space.

It has been known for nearly 75 years that vitamin E is required for rat fetal development (16)—the classic test for effectiveness of vitamin E supplements fed to vitamin E–deficient rat mothers is to measure fetal resorption (17, 23). Furthermore, exencephaly has been reported as a symptom of vitamin E deficiency in fetal rats (12, 43). Thus, vitamin E, delivered by VLDL to the mouse fetus, could be necessary for successful fetal growth and development.

Sequence Homology

Search of protein sequences revealed that the primary structure of α-TTP showed notable similarity with those of the cellular retinaldehyde-binding protein (CRALBP) and yeast SEC14 protein. CRALBP is abundantly expressed in the retinal pigment epithelium and Muller cells of the neuroretina, where it carries 11-*cis*-retinol and 11-*cis*-retinaldehyde among various forms of retinoids (7, 14, 29). SEC14 is a protein that exhibits phosphatidylinositol and phosphatidylcholine transfer activity (22). Human α-TTP and CRALBP share 47.4% similarity, and α-TTP and SEC14 share 37.4% similarity. However, these three proteins do not belong to the superfamily of lipophile-binding proteins that include cellular and plasma retinol-binding protein or fatty acid-binding proteins. They may form a novel family of proteins that binds a certain class of hydrophobic substances and transport these ligands within cells. CRALBP is thought to play a role in the visual cycle, the process by which all-*trans*-retinaldehyde, released during the breakdown of activated visual pigment in photoreceptors, is enzymatically processed into 11-*cis*-retinaldehyde and returned to the photoreceptors for visual pigment regeneration. Recently it was

demonstrated that patients affected with autosomal recessive retinitis pigmentosa have a mutation in the CRALBP gene (24). Yeast SEC14 gene was originally isolated as a gene essential for export of secretory proteins from the Golgi complex. The biologic function of SEC14 was studied in detail by Kearns et al (22). SEC14 may employ its phosphatidylcholine and phosphatidylinositol bound to preserve the Golgi diacylglycerol level, which is assumed to be the key modulator of the Golgi-mediated secretory pathway, by regulating phosphatidylcholine biosynthesis or phosphatidylinositol hydrolysis. Although the tertiary structure of α-TTP has not been resolved, SEC14 crystal structure has been studied at 2.5-Å resolution (32). SEC14 consists of 12 α-helices, 6 ß-strands, and 8 310-helices (tightly wound helices that contain three amino acid residues per turn of α-helices) that cooperate to form a single large hydrophobic pocket capable of accommodating only one phospholipid molecule.

Function of Alpha-TTP in the Liver Cell

It has been hypothesized that α-TTP mediates the secretion of α-tocopherol taken up by the liver cells into the circulation. Arita et al (1) studied in detail α-TTP function using a cultured liver cell line. They devised an assay system using α-[^{14}C]tocopheryl acetate and α-TTP–expressing McARH7777 cells. α-TTP does not recognize α-[^{14}C]tocopheryl acetate itself; it recognizes it only after the acetate group has been hydrolyzed intracellularly. When α-[^{14}C]tocopheryl acetate was added to the cell cultures, it was taken up by the cells, hydrolyzed to produce α-[^{14}C]tocopherol in the cells, and resecreted into the culture media by the action of α-TTP. It was thus possible to quantitate the activity of α-TTP in the living cells by measuring the amount of α-[^{14}C]tocopherol appearing in the medium even in the presence of excess amounts of added α-[^{14}C]tocopheryl acetate in them. They proved with this system that the secretion of α-tocopherol was greatly stimulated in cells expressing α-TTP compared with the control cells lacking α-TTP. Unexpectedly, however, α-TTP–catalyzed α-tocopherol secretion was not coupled to VLDL secretion, as brefeldin A, which inhibits VLDL secretion, had no effect on α-tocopherol secretion. They speculated from these data that α-tocopherol is not assembled into VLDL in the liver cell but rather becomes associated with VLDL after its secretion, possibly in the liver sinusoidal spaces. Moreover, they found that α-tocopherol secretion was inhibited significantly by 25-hydroxycholesterol, a potent modulator of cholesterol metabolism. These data suggest that α-TTP-catalyzed α-tocopherol secretion utilizes a novel non-Golgi–mediated pathway that may be linked to cellular cholesterol metabolism and/or transport in the liver cells. More studies are definitely needed for understanding the mechanism of α-tocopherol secretion from the liver cells.

α-TTP GENE DEFECTS

Genetic defects in α-TTP are associated with a characteristic syndrome, ataxia with vitamin E deficiency, (AVED), previously called familial isolated vitamin E (FIVE) deficiency. AVED patients have neurologic abnormalities, which are similar to those of Friedreich's ataxia (4, 5) and are characteristic of neurologic abnormalities associated with human vitamin E deficiency (33). The neurologic symptoms are characterized by a progressive peripheral neuropathy with a specific "dying back" of the large caliber axons of the sensory neurons, which results in ataxia (34).

Studies Using Deuterated Tocopherols in AVED Patients

AVED patients are responsive to oral vitamin E supplements. A dose of 800–1200 mg/day is usually sufficient to prevent further deterioration of neurologic function and in some cases improvements have been noted, as reviewed by Sokol (33). Untreated patients have extraordinarily low plasma vitamin E concentrations (as low as 1/100 of normal), but when they are given vitamin E supplements, plasma concentrations reach normal within hours (34). However, when supplementation is halted, plasma vitamin E concentrations fall within days to deficient levels.

To test if they could absorb dietary amounts of vitamin E, Traber et al (41) administered a small dose (20 mg) of deuterated *RRR*-α-tocopheryl acetate. The patients absorbed and transported vitamin E in chylomicrons normally, but transport in VLDL was defective (41). Therefore, it was suggested the patients either lack, or have a defective, hepatic α-TTP (41).

The presumed function of the hepatic α-TTP is to select *RRR*-α-tocopherol from the various forms of vitamin E delivered to the liver and to facilitate its secretion in nascent VLDL. If patients with AVED lack this protein, then they should not be able to discriminate between stereoisomers of α-tocopherol. This hypothesis was tested in 8 of 11 known patients by administering a dose containing 20 mg each of natural and synthetic *RRR*- and *SRR*-α-tocopherols (labeled with different amounts of deuterium) and tracking lipoprotein deuterated tocopherols for 3 days (42).

The patients segregated into two groups: Four did not discriminate between the two tocopherols (nondiscriminators) and four did discriminate (discriminators). Major differences between the groups were observed in the transport of deuterated *RRR*-α-tocopherol. In control subjects, the areas under the plasma curves (AUC) for deuterated *RRR*-α-tocopherol were about threefold greater than for deuterated *SRR*-α-tocopherol, whereas in nondiscriminator patients, the plasma AUCs of the two isotopes were similar. In discriminators, *RRR*-α-

tocopherol AUCs were intermediate of those in the control and nondiscriminator groups. It is important to note that deuterated *SRR*-α-tocopherol AUCs were similar in all three groups, demonstrating that deuterated *SRR*-α-tocopherol can be used as a tracer of the nonspecific plasma vitamin E transport.

Disappearance rates were also calculated for each of the isotopes. The fractional disappearance rates in the nondiscriminator patients were 1.41 ± 0.58 pools/day for *RRR*-α-tocopherol and 1.31 ± 0.31 for *SRR*. This yields a half-life of approximately 13 h for both *RRR*- and *SRR*-α-tocopherols in these patients and documents their rapid plasma disappearance. In contrast to nondiscriminators, the control subjects demonstrated significant differences in the fractional disappearance rates between *RRR*- and *SRR*-α-tocopherols (0.40 ± 0.15 and 1.23 ± 0.62 pools/day; difference = 0.82 ± 0.58; $P < 0.01$). The apparent half-life of *RRR*-α-tocopherol in control subjects is approximately 48 h, consistent with the slow disappearance of α-tocopherol from the plasma. This slow disappearance of deuterated *RRR*-α-tocopherol results from a constant reincorporation of α-tocopherol as a result of α-TTP function.

Based on this isotopic data, Traber et al (41, 42) suggested the following: (*a*) that an hepatic α-TTP, which preferentially incorporates *RRR*-α-tocopherol into VLDL, is required to maintain plasma *RRR*-α-tocopherol concentrations via secretion in nascent VLDL by hepatocytes; (*b*) that nondiscriminators either lack this protein or have a marked defect in the *RRR*-α-tocopherol binding region of the protein; and (*c*) that patients who discriminate, but have difficulty maintaining plasma *RRR*-α-tocopherol concentrations, have a less severe defect, perhaps a defect in transfer function.

AVED and α-TTP Gene Defects

Studies of patients with AVED led to the characterization of genetic defects in α-TTP. In 1993, Ben Hamida et al (4) reported that autosomal recessive AVED is associated with a Friedreich ataxia-like phenotype in six Mediterranean families (four of them from Tunisia). These patients had extraordinarily low plasma vitamin E concentrations (<5 μg/ml) and had an onset between 4 and 18 years, with progressive development of cerebellar ataxia, dysarthria, absence of deep tendon reflexes, vibratory and proprioceptive sensory loss, and positive Babinski sign. They used linkage analysis, homozygosity mapping, and linkage-disequilibrium analysis in three large consanguineous Tunisian families to map the defective locus to chromosome 8q. Almost at the same time, Arita et al (2) identified independently the cDNA coding for human α-TTP and assigned the corresponding gene to chromosome 8q13.1–13.3 by fluorescence in situ hybridization analysis. Under this concerted series of investigations, α-TTP proved to be the strongest candidate for AVED.

In order to analyze the α-TTP gene in AVED patients, the genomic organization was determined and the transcript was found to be encoded by the five exons. Ouahchi et al (28) searched the mutations on each exon by single-stranded conformational polymorphism analysis. Patients of 17 unrelated AVED families were analyzed for DNA change, and in 15 families patients were found to be homozygous for the linkage haplotype. The most abundant cause was a 1-bp deletion at position 744 (744delA), which results in the replacement of the last 30 amino acids by an aberrant 14–amino acid peptide, in 12 families. A complex 4-bp insertion at positions 530 and 532 (530AG $\rightarrow$ GTAAGT) and a heterozygous 2-bp insertion at position 513 (513insTG) were also identified.

Gotoda et al (18) studied an AVED patient from an isolated Japanese island who began to have ataxia, dysarthria, and sensory disturbances in the sixth decade of life. This patient was homozygous for a point mutation that replaces histidine (CAT) with glutamine (CAG) at position 101 (H101Q). This mutation was associated with a mild phenotype, very late onset, and retinitis pigmentosa (18, 44). When expressed in COS-7 cells, the missense mutation produced a functionally defective α-TTP with approximately 11% of the transfer activity of the wild-type protein. Of the 801 island inhabitants examined, 21 were heterozygous for the H101Q mutation. Further investigation of this population would provide an opportunity to examine the possible association between serum vitamin E concentrations and the incidence of certain diseases.

Other subjects with mutations were examined; a total of 13 mutations in 27 families have been identified to date (11) (Figure 2). Two mutations, one nonsense (R134X) and one acceptor-splice-site mutation (205-1G $\rightarrow$ C), predict a major disruption of α-TTP synthesis. Missense mutations (R59W, E141K, R221W, R192H, and A120T) have been found; the former (R59W, E141K, and R221W) are associated with early onset of the AVED syndrome (10 $\pm$ 3 years), and the latter (R192H, A120T) are associated with late onset (29 $\pm$ 15 years).

Correlation Between the Severity of the Disease and the Type of Mutation

Cavalier et al (11) studied the correlation between the severity of the disease and the type of mutation from the data of a large number of patients. The severity of the disease can be modulated by nongenetic factors, including the amount of vitamin E in the daily diet and the time of initiation and dosage of vitamin E supplement treatment. Substitutions of amino acids that are not conserved in CRALBP and SEC14 (semiconservative missense mutations; R192H, A120T, and H101Q) cause milder changes in phenotypes than those seen in the majority of cases, which suggests that these missense mutations allow the production of partially functional proteins. Patients with these mutations may be able to

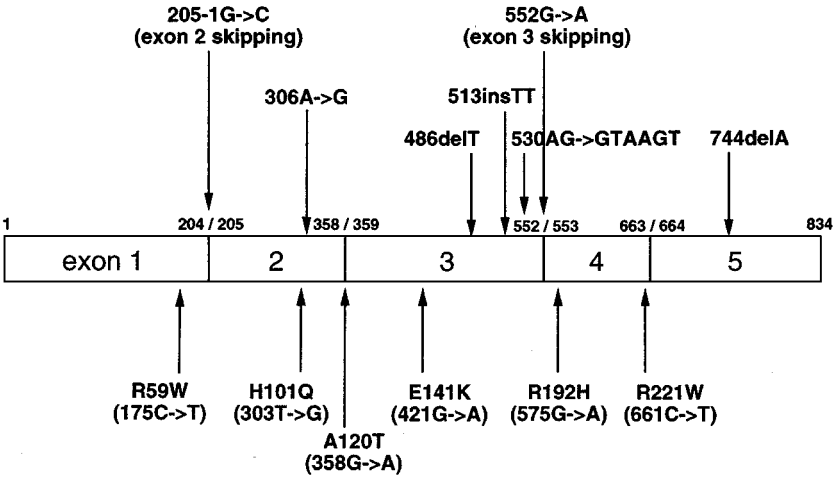

Figure 2 Location of known genetic defects in patients with ataxia with vitamin E deficiency.

preferentially incorporate the natural *RRR* stereoisomer into plasma, but to a lesser extent than do normal subjects.

These patients contrast with those who have a complete loss of the capability to preferentially incorporate the natural α-tocopherol stereoisomer into plasma. These latter patients are homozygous for severe truncating mutations (530AG → GTAAGT, 744delA, 486delT, and R134X). They all are associated with a severe, early onset form of the disease. All other truncating mutations and the substitutions of amino acids that are highly conserved in CRALBP and SEC14 (nonconservative missense mutations; R59W, E141K, R221W) also seem to be associated with the severe form of the disease, which suggests the important role of amino acids R59, E141, and R221.

FUTURE DIRECTIONS

Studies examining function of α-TTP have done much to explain the curious behavior of vitamin E. The plasma preference for *RRR*-α-tocopherol is readily explained by the preferential hepatic α-TTP binding and transfer of *RRR*-α-tocopherol. Studies are needed to trace the pathway by which *RRR*-α-tocopherol is transferred from the liver to plasma. Moreover, the specific portions of α-TTP necessary for *RRR*-α-tocopherol binding and transfer need to be characterized. The high degree of lipophilicity of vitamin E suggests that other tissues may require a vitamin E binding/transfer protein for regulation of tissue α-tocopherol concentrations.

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