Comparison of the Tyrosine Aminotransferase cDNA and Genomic DNA Sequences of Normal Mink and Mink Affected with Tyrosinemia Type II

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Abstract

Type II tyrosinemia, designated Richner-Hanhart syndrome in humans, is a hereditary metabolic disorder with autosomal recessive inheritance characterized by a deficiency of tyrosine aminotransferase activity. Mutations occur in the human tyrosine aminotransferase gene, resulting in high levels of tyrosine and disease. Type II tyrosinemia occurs in mink, and our hypothesis was that it would also be associated with mutation(s) in the tyrosine aminotransferase gene. Therefore, the transcribed cDNA and the genomic tyrosine aminotransferase gene were sequenced from normal and affected mink. The gene extended over 11.9 kb and had 12 exons coding for a predicted 454-amino-acid protein with 93% homology with human tyrosine aminotransferase. FISH analysis mapped the gene to chromosome 8 using the Mandahl and Fredga (1975) nomenclature and chromosome 5 using the Christensen et al. (1996) nomenclature. The hypothesis was rejected because sequence analysis disclosed no mutations in either cDNA or introns that were associated with affected mink. This suggests that an unlinked gene regulatory mutation may be the cause of tyrosinemia in mink.

Introduction

A recent updating of the compilation of inherited human metabolic diseases lists 1734 total genetic loci associated with clinical diseases (Amberger et al. 2001; Scriver et al. 2001). One of these human inherited metabolic diseases is tyrosinemia type II, also called Richner-Hanhart syndrome (Mitchell et al. 2001). Tyrosinemia type II has been reported in American mink (Mustela vison) of the dark color phase (Christensen et al. 1979; Goldsmith et al. 1981). Two apparently allelic forms of tyrosinemia type II mink have been described, the late onset form called “fall form” and the early onset form known as “spring form” (Christensen et al. 1986). Because all mink are born at approximately the same time in the spring, rather than at all times throughout the year, the spring form is a more acute early-onset form than the fall form (Christensen et al. 1986). The fall form is substantially more prevalent and is widespread in commercial dark mink farms and ranches throughout the northern latitudes (personal communication from J. R. Gorham, USDA Animal Disease Research Unit, Washington State University, 1994).

Tyrosinemia type II in mink is a deleterious disease that leads to the death of affected kits (Christensen et al. 1979, 1986; Goldsmith et al. 1981). Because it is an autosomal recessive disease and because homozygous affected mink are...
removed from the breeding pool because of death prior to the onset of breeding age, the disease is maintained by the production and breeding of heterozygous mink that do not manifest disease. To eliminate breeding mink heterozygous for tyrosinemia, a method to identify heterozygotes is needed. A previous study indicated that assay of tyrosine aminotransferase in tissues that are easily obtained could not be used to identify heterozygotes (Prieur et al. 2001). Therefore, a DNA-based diagnostic method may be useful to identify heterozygous mink.

The similarity of the clinical signs in all mink with the fall form of tyrosinemia as well as among those with the spring form, suggest that the fall and likely the spring forms are due to single point mutations for each allelic form. The practice of selling male breeding mink internationally, coupled with the recognition and almost simultaneous emergence of the fall form of tyrosinemia in Europe and North America, further suggest a founder effect of specific genetic mutations similar to that described among human populations (Mange and Mange 1994). Thus, identification of the specific mutation in each allelic form would be required to develop genetic-based diagnostic tests to identify heterozygous mink.

The purpose of this study was to clone and sequence the tyrosine aminotransferase gene from normal mink and mink with spring and fall forms of tyrosinemia type II. Identification of mutations in affected mink would provide genetic-based tests to identify heterozygous mink for removal from the breeding pool. Such techniques have been used in programs to eliminate other genetic diseases of animals (Henthorn et al. 2000; Leipprandt et al. 1999; Shin et al. 1997; Shuster et al. 1992).

Materials and Methods

Experimental Mink

The source for the normal mink tyrosine aminotransferase gene (tat) was an adult blue mink homozygous for the Aleutian gene (aα). Tyrosinemia has never been observed in mink of this color phase but is widespread in American dark mink (Prieur et al. 2001). A kit affected with the fall form of tyrosinemia was selected from offspring of a breeding group of carriers established at Washington State University (Prieur et al. 2001). A mink ranch in Utah provided a kit exhibiting the classical symptoms of spring form tyrosinemia. Serum from affected animals was tested for elevated levels of tyrosine using high-performance liquid chromatography (HPLC) (Christensen et al. 1986). The serum tyrosine concentration was 1.6 mM and 2.5 mM for the fall form and spring form animals, respectively, and are typical serum tyrosine concentrations for mink with tyrosinemia type II. The normal range for serum tyrosine in mink is 0.02 mM to 0.19 mM (Christensen et al. 1986).

Primers for 3’ and 5’ RACE

Primers for 3’ rapid amplification of cDNA ends (RACE) were selected by comparing published sequences for rat and human tyrosine aminotransferase (Oddos et al. 1989; Rettenmeier et al. 1990; Scherer et al. 1982; Shinomiya et al. 1984) in the GenBank database using the Bestfit program from the Genetics Computer Group (Bioloisky and Burks 1988; Devereux et al. 1984). Sense primers for 3’ RACE were chosen from conserved areas between the rat and human tat genes corresponding to nucleotides 333 to 349 (5’ GGA CCC TAC TGT GTT TG 3’) of the human tat gene (GenBank accession numbers X52520, M18340, and K01265). The antisense primer was provided in a 3’ RACE System (Gibco/BRL Life Technologies) as a Universal Amplification Primer (UAP) with three restriction endonuclease sites and a Not I half-site that binds to that portion of the poly-dT containing Adapter Primer (AP) used for first strand synthesis from mRNA. A 5’ extension of the UAP is a UD (uracil DNA glycosylase) cloning region (5’ CUA CUA CUA CUA 3’) containing dUMP residues that are used for inserting the cDNA into the pAMP1 plasmid provided in the kit (Nisson et al. 1991). These dUMP residues were also added to the gene specific 5’ primer as 5’CAU CAU CAU CAU CAU CAU 3’ for directional cloning.

3’ RACE nested antisense primers were selected from the gene-specific sequence (Gsp) derived from the cDNA clone isolated from the 3’ RACE, Gsp1 (5’ GAT TTC CAA GAC TTC TGT 3’), and Gsp2 (5’ GTA GAG TTT GAC CTC AAT TCC 3’). The sense primer was provided in the 5’ RACE System (Gibco/BRL Life Technologies) as an Abridged Anchor Primer containing a poly-dG tail with selective placement of deoxynosine residues, three restriction enzyme sites, and a Not I half-site.

3’ and 5’ RACE

Mink were injected IM with 1 mg dexamethasone/kg to induce transcription of tat (Scherer et al. 1982) then euthanized 4.5 h later, at which time liver tissue was flash-frozen in liquid nitrogen to preserve mRNA and then stored at −86°C. Total RNA was isolated from the liver tissue using TRIzol reagent, and 3’ RACE was performed with a 3’ RACE System (Gibco/BRL Life Technologies) according to the manufacturer’s recommendations (Frohman et al. 1988; Frohman 1994). Briefly, first-strand synthesis was primed via the poly-A tail of mRNA using the poly-dT-containing Adapter Primer and reverse transcriptase. The first-strand synthesis product was then used as a template for polymerase chain reaction (PCR) with the gene-specific upstream primer and the Universal Amplification Primer. The resulting cDNA was digested with UDG and ligated into pAMP1 plasmid vector prepared to have compatible ends for the uracil-containing primers (Gibco/BRL Life Technologies). The ligation was then used to transform Escherichia coli. Transformants were screened for inserts by digestion of DNA minipreps with restriction endonucleases and inserts were sequenced.

5’ RACE was done according to the manufacturer’s instructions with the same mRNA using Gsp1 primer for first-strand synthesis with reverse transcriptase. A homopolymeric tail was added at the 5’ end with dCTP and terminal
deoxytransferase followed by PCR with Gsp2 primer and the Abridged Anchor Primer. The product was then digested with Sal I, specific for the Abridged Anchor Primer, and Cla I, specific for the mink tat gene. The digested fragment was ligated to pSPORT-1 (Gibco/BRL Life Technologies) and the ligation used to transform E. coli. Transformants were screened by restriction endonuclease digestion of DNA minipreps for inserts and inserts were sequenced.

Genomic Library Construction

A library representing $1.59 \times 10^6$ recombinants was made by partially digesting genomic DNA from a mink with fall form type II tyrosinemia with EcoRI and size fractionating in an agarose gel. Fragments in a range from 15 to 21 kb in length were excised, isolated, and ligated into a previously EcoRI-digested and dephosphorylated Lambda DASH II vector then packaged with Gigapack II Gold packaging extract (Stratagene). XL-1 blue E. coli was used to amplify and titer the library (Sambrook et al. 1989).

Library Screening

The amplified genomic library was screened for tat-specific clones by using a gene-specific probe made from the isolated cDNA clones and plaque lift hybridization (Sambrook et al. 1989). Plaques with positive signals were plaque purified three times and the mink tat specificity confirmed by sequencing.

Sequencing

Sequencing was done at the Laboratory for Biotechnology and Bioanalysis at Washington State University using dye-labeled deoxy nucleotide cycle sequencing and the ABI 377 automated sequencer (Rao 1994; Trower et al. 1995). Templates used were from the cDNA clones, the Lambda genomic clone, and PCR fragments generated from genomic DNA by “genome walking” using primers derived from cDNA sequence (Parker et al. 1991). Both strands of the DNA were sequenced for confirmation and possible PCR artifacts eliminated by cross-checking with all three template types. Computer-assisted sequence analysis was done using the bestfit and pileup programs included in the GCG 7.0 (Genetics Computer Group version 7.0) analysis package.

FISH Analysis

FISH (fluorescence in situ hybridization) was done by the Cell Culture Laboratory at the Children’s Hospital of Michigan, Detroit, using the tat-specific sequence from our genomic Lambda clone as a probe. The probe was labeled with Spectrum Orange dUTP by standard Nick Translation protocol provided by the manufacturer (Vysis). Mink lung cells, ATCC mink lung cell line MV1Lu, were incubated with colcemid, and unbanded chromosome preparations were hybridized for 24 h and then counterstained with DAPI, inducing G-banding. Slides were examined using a Zeiss Axiopt phot epifluorescence microscope equipped with appropriate filter combinations or the images were acquired and digitized with a fluorescence microscope/CCD camera interfaced to a computer workstation. At least 20 metaphases were analyzed.

Results and Discussion

Sequence of Mink tat Gene

cDNA clones of mink tat were constructed using PCR-based RACE and then sequenced using primers listed in Table 1. In addition, tat-containing genomic clones from a mink lambda genomic library and genomic PCR fragments were sequenced for further confirmation. The mRNA transcript for mink tat, determined by sequencing cDNA and genomic clones, was 2274 nucleotides in length with 97 nucleotides of 5’ flanking noncoding sequence, 815 nucleotides of 3’ flanking noncoding sequence, and 1362 nucleotides of coding sequence resulting in a predicted protein of 454 amino acids (Figure 1). Sequence data for the M. vison tyrosine aminotransferase gene has been deposited with GenBank under accession number AF163863. The gene extended over 11.9 kb of genomic DNA and had 12 exons and 11 introns (Figure 2). The similarity of the nucleotide sequence of mink tat to human tat nucleotide sequence was 84.4% with the 3’ flanking area, 90% without the 3’ flanking area, and 93% at the amino acid level.

tat Chromosome Assignment

FISH analysis of a mink lung cell line MV1Lu, ATCC CCL-64, using a tat genomic clone for hybridization was analyzed (Figure 3). Two medium metacentrics showed a definite red glow in the middle of one arm when observed under triple-pass filter. These chromosomes were compared to mink chromosomes as described by Mandahl and Fredga (1975). When observed under DAPI filter, this pair was determined to be chromosome 8, and the region showing hybridization

Table 1. Primer sequences used for cDNA clone sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ end sequence</th>
<th>3’ end sequence</th>
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<tbody>
<tr>
<td>MTAT-F2</td>
<td>GCT GAG TCA GCG GAT CCT G 3’</td>
<td>CTA ACA GAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT-F4</td>
<td>GAG TGA TCC TGC GAT CCC 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT-F6</td>
<td>CGA ATT TCT TCC GAG TGG T 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT-F8</td>
<td>AAG TGG AGA CTA GAA AAA 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
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<tr>
<td>MTAT-F10</td>
<td>CAC CGA ATC CCA GAA TCT C 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT-F13</td>
<td>CAA GCC ATG AAA GAT GCG 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT-F15</td>
<td>TAC CAT TGT GCT GAA GGG AGC 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT-F6</td>
<td>CAA ATG CAT GTC AAC ATT G 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
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<td>MTAT-R1</td>
<td>CAG GAT CCG CTG ACT CAG C 3’</td>
<td>ACC ACT CGG AAG AAA TCC G 5’</td>
</tr>
<tr>
<td>MTAT-R3</td>
<td>ACC ACT CGG AAG AAA TCC G 3’</td>
<td>TCT TCC TAG TCT CCA CTT 5’</td>
</tr>
<tr>
<td>MTAT-R5</td>
<td>ACC ACT CGG AAG AAA TCC G 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
</tr>
<tr>
<td>MTAT-R7</td>
<td>CTT TCC TAG TCT CCA CTT 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<td>MTAT-R9</td>
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<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<td>CTA ATG CAT GTC AAC ATT G 5’</td>
</tr>
<tr>
<td>MTAT-R12</td>
<td>CTA ATG CAT GTC AAC ATT G 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<tr>
<td>MTAT-R14</td>
<td>GTA TCG CTA GCA ACT AAC CG 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<tr>
<td>MTAT2.2-F1</td>
<td>GTA ATG CAT GTC AAC ATT G 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
</tr>
<tr>
<td>MTAT10-F1</td>
<td>CAA CAA GAA ATG TCT GGT AAG 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<td>MTAT10-F3</td>
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<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<td>CAA CAA GAA ATG TCT GGT AAG 3’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
</tr>
<tr>
<td>MTAT10-R2</td>
<td>MTAT-F2 5’ CTA ACA GAA ACT TTC TGG 3’</td>
<td>CTA AGT CAA ACT TTC TGG 5’</td>
</tr>
<tr>
<td>MTAT2.2-R1</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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<td>MTAT2.2-R2</td>
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<td>CTA ATG CAT GTC AAC ATT G 5’</td>
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</table>

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Figure 1. Sequence of exons A to L and flanking sequences of the mink tyrosine aminotransferase gene. Nucleotide position +1 is the transcription start site. Nucleotides 5′ to nucleotide +1 have negative numbers. The ends of the introns are in lowercase and lengths are given as determined by sequencing. The predicted amino acid sequence is shown above the nucleotide sequence and amino acids are numbered beginning with 1 at the initiation methionine and at the beginning of each exon. A dot is over the nucleotide followed by that nucleotide number. The TATA- and CAAT-like sequences are underscored and the polyadenylation signal is overscored.
with the probe was near a secondary constriction area in the p arm at 8p21. The distinctive secondary constriction on the p arm of chromosome 8 is more evident in Figure 4. This characteristic of chromosome 8 was described originally in 1961 (Fredga 1961) and subsequently verified (Mandahl and Fredga 1975; Nes 1962). There were random signals observed on other chromosomes, but these had no consistent distribution. In 1996 a new nomenclature for mink chromosomes was published (Christensen et al. 1996), and with this system the midsized metacentric with the distinctive secondary constriction was designated chromosome 5. A zoo-FISH painting study reported synteny between human chromosome 13 and the p arm of this distinctive mink chromosome, chromosome 5 using the nomenclature of Christensen et al. (1996) or chromosome 8 using the Mandahl and Fredga (1975) nomenclature (Hameister et al. 1997). However, the human tat gene was mapped to 16q22 (Barton et al. 1986; Natt et al. 1987). The p arm of mink chromosome 7 has been shown to be syntenic with human 16q (Hameister et al. 1997; Kuznetsov et al. 2003). This discrepancy (with either nomenclature system) with predicted mink/human synteny was unexpected, but not surprising because discrepancies in synteny have been observed for other mink genes (Kuznetsov et al. 2003) and with predicted human/mouse synteny (Thomas et al. 2003; Waterston et al. 2002). Until the mink tat gene can be anchored to a specific chromosome by comparative sequencing and genetic map alignment, the 8p21 chromosome assignment for mink tat should be considered provisional.

Comparison of tat cDNA from Normal and Mink with Tyrosinemia

Mutations that might be responsible for type II tyrosinemia in mink were investigated by comparing cDNA and genomic DNA from normal mink with that from fall form and spring form affected mink. In human tyrosinemia type II, 15 different tat mutations have been found (Huh et al. 1998; Natt et al. 1992). Sequences from cDNA clones from normal mink, fall form, and spring form tyrosinemic mink were compared from the 5' untranslated region (UTR) through the 3' UTR. Quite surprisingly, no nucleotide differences were found. On the initiation of this project we hypothesized that the spring and fall forms of mink tyrosinemia were due to allelic mutations of the tat gene. This is not the case. Past research (Christensen et al. 1986) provided evidence that the mutations were allelic and that the crossing of mink carriers of the spring and fall forms resulted in an intermediate form. Thus these two conditions are assumed to be the result of mutations of the same gene.

Figure 2. Map of the mink tyrosine aminotransferase gene. Exons A through L are shown in solid blocks and introns 1 through 11 as dashed lines. Numbers above the exons and below the introns refer to the size of either in nucleotides.

Figure 3. Simultaneous FISH for chromosomal location assignment of mink tyrosine aminotransferase to 8p21. Arrows indicate probe binding site.

Figure 4. Black and white image of simultaneous FISH for chromosomal location assignment of mink tyrosine aminotransferase to 8p21. Arrows indicate probe binding site.
Intron 9–10

Introns 4–5

Introns 2–3

Intron 1

MTAT1–2F
5’ GTA ACA CAG AGC ATT CCA G 3’

MTAT1–378R
5’ TGA CAT GCA TAT CCA GAA C 3’

MTAT3–111F
5’ GTA TCA GAT GAA TGG CCA C 3’

MTAT3–469R
5’ AAG AAG CAA TCT CCT CCC 3’

MTAT4–458
5’ GGA GGA GAT TGC TTC TTA C 3’

MTAT4–787
5’ GGA GAT GAC TTC TAC TGA ACAC 3’

MTAT-738F
5’ TAA CAA CCC ATC AAA CCC 3’

MTAT-1092R
5’ ATG CAG GAT GCT TTT CAG 3’

MTAT-1102F
5’ CAG GAG TTC TAC CAC AAC AC 3’

MTAT-1297R
5’ ATT GCT CAG CAA CTA ACC 3’

MTAT-1236F
5’ GGA ACA TTT CCC AGA ATT TG 3’

MTAT-1383R
5’ CTC CAG CAT CAC TTT G 3’

MTAT-10595F
5’ CCC CCT GAC ATA AGT GAT CC 3’

MTAT-12362R
5’ GGG CAT AAG AGA ATT TGA AGG 3’

### Table 2. Primer sequences for PCR of introns and untranslated regions

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<tr>
<th>Region</th>
<th>Primer Forward</th>
<th>Primer Reverse</th>
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<tbody>
<tr>
<td>5’ UTR</td>
<td>MTAT-311F</td>
<td>5’ CCA AAT CAT TAG CAC TCC 3’</td>
</tr>
<tr>
<td></td>
<td>MTAT-1265R</td>
<td>5’ GAC CTG CTG AGA TAG AAG 3’</td>
</tr>
<tr>
<td>Intron 1</td>
<td>MTAT1–2F</td>
<td>5’ GTA ACA CAG AGC ATT CCA G 3’</td>
</tr>
<tr>
<td></td>
<td>MTAT1–378R</td>
<td>5’ TGA CAT GCA TAT CCA GAA C 3’</td>
</tr>
<tr>
<td>Intron 2–3</td>
<td>MTAT3–111F</td>
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<tr>
<td></td>
<td>MTAT3–469R</td>
<td>5’ AAG AAG CAA TCT CCT CCC 3’</td>
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<td>Intron 4–5</td>
<td>MTAT4–458</td>
<td>5’ GGA GGA GAT TGC TTC TTA C 3’</td>
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<td>5’ GGA GAT GAC TTC TAC TGA ACAC 3’</td>
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<tr>
<td>Intron 6–7–8</td>
<td>MTAT-738F</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>MTAT-1297R</td>
<td>5’ ATT GCT CAG CAA CTA ACC 3’</td>
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<td>Intron 11</td>
<td>MTAT-1236F</td>
<td>5’ GGA ACA TTT CCC AGA ATT TG 3’</td>
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<td>3’ UTR</td>
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<td>5’ CCC CCT GAC ATA AGT GAT CC 3’</td>
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<tr>
<td></td>
<td>MTAT-12362R</td>
<td>5’ GGG CAT AAG AGA ATT TGA AGG 3’</td>
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### Table 3. Number of thymidines beginning at nucleotide position 4725

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<th>Mink phenotype</th>
<th>Mink identity</th>
<th>Number of thymidines</th>
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<td>Normal</td>
<td>#85</td>
<td>12</td>
</tr>
<tr>
<td>Normal</td>
<td>#289</td>
<td>13</td>
</tr>
<tr>
<td>Late onset form</td>
<td>#582</td>
<td>13</td>
</tr>
<tr>
<td>Late onset form</td>
<td>#580</td>
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<tr>
<td>Early onset form</td>
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<td>Early onset form</td>
<td>#H3–5</td>
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<tr>
<td>Early onset form</td>
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<td>11</td>
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<tr>
<td>Early onset form</td>
<td>#23–133A</td>
<td>11</td>
</tr>
</tbody>
</table>

Beginning with nucleotide position +4725, a varying length of thymidines was present. The length was 13 thymidines long in the early-onset mink, 13 thymidines long in the late-onset mink, and 12 thymidines long in the control mink. These lengths of thymidines were observed to be homozygous in each mink. In an effort to determine the significance of this variation, PCR products of additional mink of all three phenotypes were analyzed using primers MTAT4847F and MTAT5204R. The additional mink analyzed included four early-onset mink, two late-onset mink, and two control mink. Table 3 lists the results observed. As is evident, there is variation in the length of this string of thymidines, and the length is unrelated to tyrosinemia status of the mink. Thus it is concluded that the variations seen in this intron are not related to the expression or nonexpression of the tyrosinemia trait.

### Interpretation

Tyrosine is catabolized by a series of enzymatic reactions. A deficiency of any one of the catabolic enzymes involved will lead to an accumulation of tyrosine and tyrosinemia. In humans, three distinct genetic diseases of tyrosine catabolism have been identified, tyrosinemia I, tyrosinemia II, and tyrosinemia III (Mitchell et al. 2001). Tyrosinemia I is due to a deficiency of fumarylacetoacetate hydrolase, tyrosinemia II is due to a deficiency of tyrosine aminotransferase, and tyrosinemia III is due to a deficiency of 4-hydroxyphenylpyruvate dioxygenase. In both tyrosinemia I and III, tyrosinemia is present but levels of tyrosine aminotransferase, the first enzyme involved in the catabolic pathway of tyrosine, are not diminished.

Inherited tyrosinemia of mink has been attributed to tyrosine aminotransferase deficiency and has thus been proposed as a model for human tyrosinemia II (Goldsmith et al., 1981). Goldsmith and coauthors (1981) demonstrated that affected mink have no tyrosine aminotransferase activity as measured immunologically and biochemically and Christensen and colleagues (1986) found that levels of tyrosine aminotransferase in livers of mink with the early onset form of tyrosinemia were zero compared to levels of about 10 U/g of liver extract in normal mink. Based on these observations and the similarities of lesions in mink compared to those in humans with tyrosinemia type II, it has been the hypothesis by scientists working with tyrosinemia mink that it is a disorder due to an inherited deficiency of tyrosine aminotransferase (Christensen et al. 1979, 1986; Goldsmith et al. 1981). We have also operated under this assumption.

### Comparison of Noncoding Sequences

It has been reported that defects in RNA splicing can result in exon skipping, activation of cryptic splice sites, inclusion of the intron in a flanking exon, or influencing nonadjacent splice sites (Huhn et al. 1998; Maquat 1996). The possibility that there is a mutation at a splice donor or acceptor site affecting exon splicing, as in human type II tyrosinemia (Natt et al. 1992), was investigated by completely sequencing all of the introns of genomic clones or PCR products from genomic DNA of normal and affected mink made by using DNA-specific primers flanking intronic borders (Table 2). No differences were found at the splice donor or acceptor sites or in any intron sequences that would explain the lack of tyrosine aminotransferase activity in the affected mink. Mutations in or near the polyadenylation signal or in the tyrosine aminotransferase promoter (nt –350 to +1) could also diminish or extinguish expression (Proudfoot 1991; Schweizer-Groger et al. 1994). Again, there were no differences in the promoter or polyadenylation signal sequences between normal and affected mink.

### Analysis of Intron 4 Differences

Although no differences were detected in coding regions of the tyrosine aminotransferase gene or in the splice sites in the three types of mink, one difference was detected in intron 4.
A total of 15 different mutations in the human tyrosinemia aminotransferase gene have been documented as causes of human tyrosinemia II, and all of these mutations are in the tyrosine aminotransferase structural gene (Huhn et al. 1998; Michell et al. 2001; Natt et al. 1986, 1992). No references to human tyrosinemia II could be found in the literature in which it was not the tyrosine aminotransferase gene that was mutated. Previous studies have documented that the inherited disease known as mink tyrosinemia II is associated with reduced levels of tyrosine aminotransferase, but the studies reported herein demonstrated that the disease is not due to a mutation of the tyrosine aminotransferase gene. This leaves the possibility that there are human inherited disorders of human tyrosinemia II that are due to mutations at loci that control expression of the tyrosine aminotransferase gene but are not linked to the tyrosine aminotransferase gene. The demonstration of a variable length of thymidines in intron 4 in mink was not constant and was not associated with the mink tyrosinemia disease. The absence of a causal mutation in the coding and intronic regions of the gene and in the upstream and downstream flanking regions raise the probability that there are genetic elements controlling the expression of the gene at sites other than those closely linked to it. Furthermore, these observations suggest that mutations at one of these sites are responsible for the observed forms of mink tyrosinemia II. These observations also suggest that a similar situation may be found, in the future, relating to tyrosinemia II in humans.

Areas for Future Study

The most obvious focus of research to determine the cause of a genetic disease is to isolate the suspect gene from an affected individual and compare the sequence to the normal gene. In some cases a normal gene may still not function properly due to mutations in cis-acting enhancer elements or trans-acting factors, such as with familial hypercholesterolemia, maturity-onset diabetes of the young, and hereditary 1,25-dihydroxyvitamin D-resistant rickets (Hansen et al. 2002; Kovisto et al. 1994; Malloy et al. 2002).

Studies have shown that upstream cis-acting enhancer elements in combination with intracellular liver-specific and ubiquitous trans-acting factors play a critical role in induction of TAT (Nitsch et al. 1993; Schweizer-Groyer et al. 1994). Mapping of DNase I hypersensitive sites found 7 such sites within 11 kb of the 5′-flanking sequence of tat (Nitsch and Schutz 1993). A glucocorticoid response element (GRE) at −2.5 kb; a glucocorticoid modulatory (GME) element near −3.6 kb; a negative element blocking GME at −3105 bp; four tissue-specific elements at −11, −5.5, −3.6, and −2.6 to −2.3 kb; and a tissue nonspecific element between −3.0 to −2.6 kb have been identified (Collier et al. 1996). These elements are involved in multifactorial processes that when interrupted can lead to posttranslational modifications or extinction of essential transcriptional activators for tat expression. The enhancing elements, DNase hypersensitive sites, and GRE in the flanking regions are linked to tat and thus mutations of these cannot be the cause of tyrosinemia in mink. However, the factors and elements that bind to them are not linked and should be investigated as possible causes of tyrosinemia type II in mink.

The purpose of this study was to clone and sequence the normal mink tyrosine aminotransferase gene as well as the genes in mink with spring and fall forms of tyrosinemia type II in an effort to develop a DNA sequence-based test to screen heterozygous mink and remove them from the breeding pool. However, no mRNA or genomic DNA tat sequence differences were found between normal and affected mink. This result will help focus future research to define the genetic change resulting in the mink disease.

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