

Tandem Duplication Via Light-Strand Synthesis May Provide a Precursor for Mitochondrial Genomic Rearrangement

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A tandem duplication of the mitochondrial tRNA^{Thr} and tRNA^{Pro} genes in the amphisbaenian reptile *Bipes biporus* is the first case reported of a tandem duplication restricted to a single pair of tRNA genes in a vertebrate mitochondrial genome. Such duplications have been predicted, however, as intermediate steps in the evolution of observed mitochondrial genomic rearrangements through errors in light-strand replication. The tandem duplication reported here is evolutionarily associated with displacement of the origin for light-strand replication from its typical location in vertebrate mitochondrial genomes and loss of the dihydrouridine stem from the tRNA^{Cys} gene; these factors implicate light-strand replicational errors in the tandem duplication of genic regions. Pseudogene formation in tandemly duplicated sequences appears to be an intermediate step in genomic rearrangement. However, formation of pseudogenes in the *Bipes* mitochondrial genome occurs in a pattern that precludes subsequent genomic rearrangement. Functional constraints placed on cleavage of mitochondrial transcripts by tRNA genes also may prevent mitochondrial genomic rearrangement.

Introduction

Although most vertebrates share an evolutionarily conserved arrangement of genes in the mitochondrial genome, recent studies have revealed numerous independently evolved rearrangements of the mitochondrial genome (for a review see Macey et al. 1997a; also see Kumazawa et al. 1996). We proposed a model for mitochondrial genomic rearrangement that can explain most but not all of the observed rearrangements (Macey et al. 1997a). This model features errors in replication of the light strand and suggests that homoplasmy (parallelisms, reversals, and convergences) is unlikely for mitochondrial genomic rearrangements. The few mitochondrial genomic rearrangements that cannot be explained by errors in light-strand replication appear to involve either transposition (Macey et al. 1997a) or duplication and concerted evolution associated with replication of the heavy strand (Kumazawa et al. 1996).

All mitochondrial genomic rearrangements reported among vertebrates involve tRNA genes (often with other genes), which average 70 bp in length. Rearrangements of tRNA genes among vertebrates often constitute simple switches in the relative positions of two genes (Macey et al. 1997a). Genomic rearrangements involving replicational errors on the light strand are predicted to go through an intermediate step featuring tandem duplications involving tRNA genes (Macey et al. 1997a). Our hypothesis further predicts that vertebrates exhibiting tandemly duplicated genes should show displacement of the origin for light-strand replication (O_L) (Macey et al. 1997a). In lepidosaurian reptiles, loss of the dihydrouridine (D) stem of the tRNA^{Cys} gene may lead to displacement of the O_L and gene rearrangement in an evolutionary cascade (Macey et al. 1997c), as observed for the tuatara (*Sphenodon punctatus*; Seutin et al. 1994;

Quinn and Mindell 1996) and acrodont lizards (Agamidae and Chamaeleonidae; Macey et al. 1997a, 1997b, 1997c).

We report here the first observation of tandem duplication of a pair of tRNA genes as predicted by our model for genomic rearrangement. All previously reported tandem duplications in vertebrate mitochondrial genomes range in size from ~1 to ~10 kb (Moritz and Brown 1986, 1987; Wallis 1987; Moritz 1991; Stanton et al. 1994) and are too large to qualify as potential intermediate states for simple rearrangements of two tRNA genes. The duplication reported here occurs between the cytochrome *b* gene and the control region in the mitochondrial genome of the amphisbaenian reptile *Bipes biporus*. As predicted by Macey et al. (1997c), this tandem duplication is evolutionarily associated with displacement of O_L and loss of the D-stem from the tRNA^{Cys} gene (Macey et al. 1997a, 1997b).

Methods

Laboratory protocols follow the procedures described in Macey et al. (1997a, 1997b, 1997c) except that annealing temperatures for amplifications of genomic DNA were 50–53°C and those for sequencing reactions were 53–58°C.

Amplifications from genomic DNA were done in two ways and the products were completely sequenced independently. Sequences acquired from overlapping amplifications were identical. Initial amplifications were done with L15810 and H16407, and both strands were subsequently sequenced using these primers. A second amplification was done with L15221 and H16407, with both strands sequenced using the following primers (numbers refer to the 3' end of the human mitochondrial genome [Anderson et al. 1981], where L and H correspond to light and heavy strands): L15221, 5'-TGAGGCCAAATATCATTCTGAGG-3' (modified from Smith and Patton 1991); L15643, 5'-GCCTATGCCATCTTACGCTC-3'; H15624, 5'-GAGCGTAA-GATGGCATAGGC-3'; L15810, 5'-ATCTTAATCGGC-CAAATAGCMTC-3' (M = C or A); and H16407, 5'-

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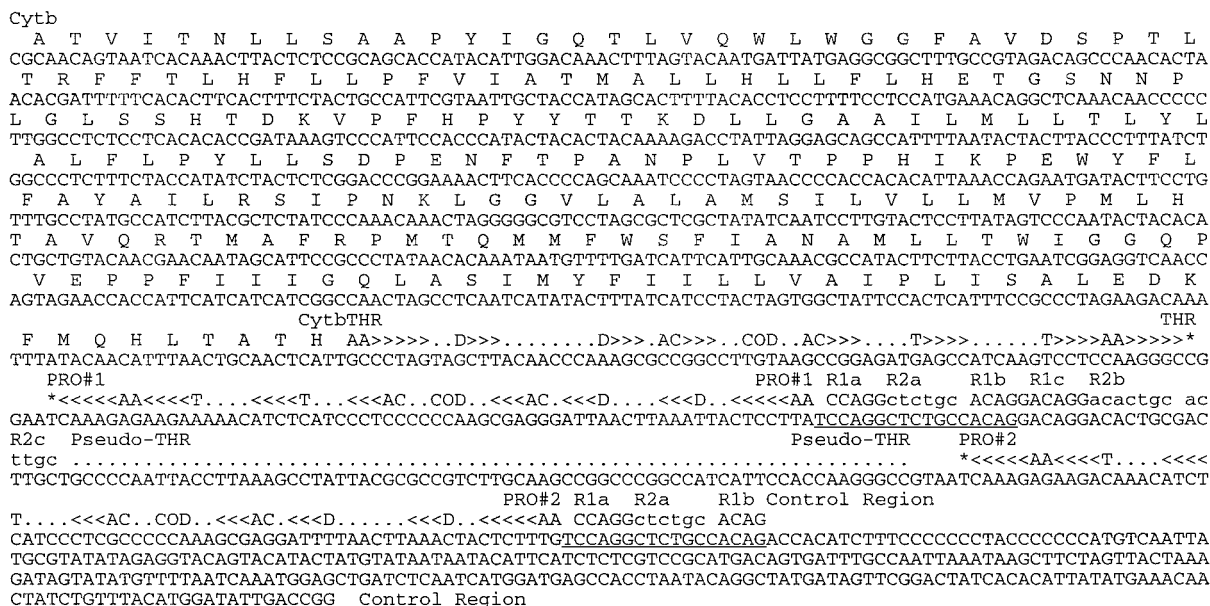


FIG. 1.—Gene arrangement in a segment of mitochondrial DNA from the amphisbaenian reptile *Bipes biporus*. Sequences are shown in 100-base blocks presented as light-strand sequence from 5' to 3'. The 242 amino acids are designated by their single-letter codes over the second codon position in the gene encoding cytochrome *b*, and tRNA secondary structure is designated above the sequence. Stems are indicated by arrows in the direction encoded: AA = amino acid acceptor stem; D = dihydrouridine stem; AC = anticodon stem, T = TΨC stem. The positions of anticodons in tRNA genes are designated COD. Asterisks indicate the unpaired 3' tRNA position 73. Periods denote nonstem bases. No secondary structure is shown for the second tRNA^{Thr} gene because it appears not to encode a tRNA having a stable secondary structure. Note that the first base shown for cytochrome *b* is a third-position base. The cytochrome *b* gene ends with a T (position 728) that is probably polyadenylated posttranscriptionally during processing of the primary transcript to produce a stop codon, TAA (Anderson et al. 1981; Ojala, Montoya, and Attardi 1981; Macey et al. 1997c). Between the tandemly duplicated pairs of tRNA genes are directly repeated sequences of two types (R1 and R2), which are depicted above the sequence, with R1 repeats shown in capitals and R2 repeats shown in lower case. A 17-base exact repeat following each tRNA^{Pro} gene is singly underlined and consists of R1a, R2a, and R1b repeats.

CGGGTTGCTGATTTACGTGAG-3' (Quinn and Mindell 1996). The specimen of *Bipes biporus* is deposited in the Museum of Vertebrate Zoology, University of California at Berkeley (MVZ 137543), and is from Baja California Sur, Mexico. The GenBank accession number for the sequence reported in this paper is AF013251 and the sequence from ND1 to COI from the same specimen is U71335 (Macey et al. 1997a, 1997b).

Results

Authentic Mitochondrial DNA

The sequence reported here shows strong strand bias against guanine on the light strand (G = 14%, T = 26%, A = 30%, and C = 30%; see Macey et al. 1997a, 1997c for similar strand bias in reptiles), which is characteristic of the mitochondrial genome but not the nuclear genome. At least one copy of each duplicated tRNA gene appears to encode sequences that form stable secondary structures, and the protein-coding gene, cytochrome *b*, has no premature stop codons. Therefore, we interpret this sequence as authentic mitochondrial DNA.

Tandem Duplication

The sequence acquired from the amphisbaenian *Bipes biporus* contained a tandem duplication of the tRNA^{Thr} and tRNA^{Pro} genes situated between the cytochrome *b* gene and the control region (fig. 1). The second copy of the tRNA^{Thr} gene (fig. 1) appears to be

nonfunctional, because it does not encode a transfer RNA that can form a secondary structure standardly observed for mitochondrial tRNAs (Kumazawa and Nishida 1993). The first copy of the tRNA^{Pro} gene (fig. 2B) is at least under relaxed selection because it specifies a tRNA secondary structure that deviates from those of other reptiles and shows unexpected bases at sites otherwise invariant among reptiles (fig. 3). This copy may be nonfunctional. The first tRNA^{Thr} gene (fig. 2A) and the second tRNA^{Pro} gene (fig. 2C) appear to encode tRNAs with stable secondary structures that show considerable resemblance to those observed in other reptiles (figs. 2 and 3).

Between the tandemly duplicated pairs of tRNA genes are directly repeated sequences of two types (R1 and R2) (fig. 1). These repeats are distributed as follows: R(repeat)1, R2, R1, R1, R2, R2. The repeats are imperfect, with the first R1 repeat consisting of CCAGG (R1a) and the other two R1 repeats consisting of ACAGG (R1b,c). The R2 repeats also are imperfect, with the first one represented by CTCTGC (R2a), the second one by CACTGC (R2b), and the third one by CT-TGC (R2c). Note that R2c requires a deletion of the third base. A 17-base exact repeat (TCCAGGCTCTGCCACAG) following each tRNA^{Pro} gene is singly underlined in figure 1 and consists of R1a, R2a, and R1b repeats. This observation suggests that the original tandem duplication included at least the first 17 bases of the control region. It is interesting to note that all ac-

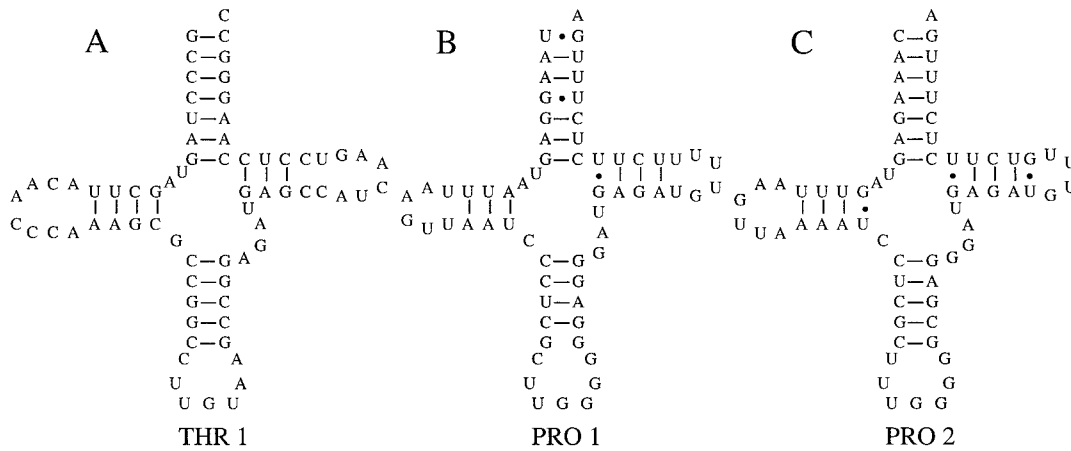


FIG. 2.—A, Secondary structure of the presumably functional first copy of the tRNA^{Thr} gene. B, Secondary structure corresponding to the possibly nonfunctional first copy of the tRNA^{Pro} gene. C, Secondary structure corresponding to the presumably functional second copy of the tRNA^{Pro} gene.

rodent reptiles that have the tRNA^{Ile} and tRNA^{Gln} genes rearranged also have short noncoding sequences located between the rearranged genes (Macey et al. 1997a, 1997c). Kumazawa and Nishida (1995) also report a large noncoding region adjacent to a set of rearranged tRNA genes.

Discussion
Mechanistic Implications

All known rearrangements of the vertebrate mitochondrial genome include tRNA genes, and many rearrangements are simple switches of two tRNA genes (Macey et al. 1997a). The tandem duplication reported here is the first case of mitochondrial genomic duplication among vertebrates consisting entirely of two tRNA genes. All other tandemly duplicated gene regions of the vertebrate mitochondrial genome involve multi-genic regions at least 1 kb in length (Moritz and Brown 1986, 1987; Wallis 1987; Moritz 1991; Stanton et al. 1994). Mitochondrial genomic reorganization under a model of tandem duplication should show intermediate states involving duplicated tRNA genes (Macey et al. 1997a). *Bipes bipes* represents a unique example among vertebrates where duplicate copies of two tRNA genes are either under relaxed selection or nonfunction-

al. A duplication of genes may lead to a mitochondrial genomic rearrangement (Moritz, Dowling, and Brown 1987) if two or more nonadjacent gene regions undergo separate deletion events (Macey et al. 1997a). If contiguous genes get deleted, no rearrangement will occur (Macey et al. 1997a, fig 7) (table 1).

In *Bipes bipes*, the first copy of the tRNA^{Thr} gene, located at one end of the duplicated segment, appears to remain functional, whereas the second copy, located in the middle of the segment, appears to have become a pseudogene (fig. 1). Deletions of the tRNA^{Thr} pseudogene and one of the adjacent tRNA^{Pro} genes constitute the only deletions that permit retention of all necessary genic functions in the mitochondrial genome of *Bipes bipes*. Such deletions would restore the original order of genes and would not produce a rearrangement (table 1). If pseudogene formation had affected the nonadjacent tRNA^{Thr} and tRNA^{Pro} genes located at opposite ends of the cluster, subsequent deletion of the pseudogenes would produce a genomic rearrangement. The tandem duplication observed in *Bipes bipes* helps to explain why rearrangement of genes by this mechanism is uncommon and therefore extremely unlikely to show homoplasmy. Even when the required intermediate state of tandem duplication occurs, rearrangement of the

tRNA ^{Pro}		*<<<<<AA<<<<T<<<<T<<<AC . . COD . . <<<AC . -<<<D <<<D . . <<<<<AA
Gene	Turtle	TCAAAAGAGAAGGACTTAAACCTTCATCCCGGTCGCCAAAACCGGA-ATCCTACAA--TTAAACTATCCTTTG
	Bird	TCAGAAAAGGAGGGCTCAAACCTCCATCTCCAGCTCCCAAAGCTGGT-ATTTTCAA---ATAAACTACTCTCTG
	Crocodile	TCAAGAAGATAGACATGAC-TCTACTCTTCCGGCCCCCAAAGCCGAC-ATTTCTTA---TTAAACTACTTCTTG
	Tuatara	TCAAGAAAAGAGGATCTTT-CCCCTATCCCCGGTCCCGAACCAGG-ATTTTAA---ATAAACTATTTCTTG
	Snake	TCAAAGAGGG---TATC-----CCATCTCTGGCCCCCAAAGCCAGC-ATTTTACTAC-TTAAACTACTCTCTG
	Lizard	TCAAAGAGAGGGCCCC---CCCCATCTCCGGTCCCAAACCGAA-ATTTTATCTTTTAAACTATCTCTG
	<i>Bipes Pro 2</i>	TCAAAGAGAAGACAAAC---ATCTCATCCCTCGCCCCCAAAGCGAGG-ATTTTAAAC---TTAAACTACTCTTTG
	<i>Bipes Pro 1</i>	TCAAAGAGAAGAA <u>AAAC</u> ---ATCTCATCCCTC-CCCCAA-GCGAGGGATT--AAC---TTAA <u>AA</u> TACTCTCTT <u>A</u>

FIG. 3.—Aligned light-strand DNA sequences of the tRNA^{Pro} gene from reptiles. Taxa shown are a turtle (*Chelonia mydas*; Quinn and Mindell 1996), bird (*Gallus gallus*; Desjardins and Morais 1990), crocodile (*Crocodylus porosus*; Quinn and Mindell 1996), tuatara (*Sphenodon punctatus*; Quinn and Mindell 1996), snake (*Ovophis okinavensis*; Kumazawa et al. 1996), lizard (*Cnemidophorus uniparens*; Stanton et al. 1994), and two duplicated genes from *Bipes bipes*. Bases that differ between the two tRNA^{Pro} gene copies in *Bipes bipes* are doubly underlined in the tRNA^{Pro#1} gene. Note that the *Bipes bipes* tRNA^{Pro#1} gene is aligned to the secondary structure expected from the tRNA^{Pro#2} gene and other reptilian tRNA^{Pro} genes. This alignment requires length changes in tRNA^{Pro#1} in regions that normally do not accept deviations in length (Kumazawa and Nishida 1993). Also note the change in the position normally conserved as C in the D-stem of reptiles to a T in tRNA^{Pro#1} and the change in the position normally conserved as G in the AA-stem of reptiles to A in tRNA^{Pro#1}.

Table 1
Genomic Reorganization Under a Model of Tandem Duplication of Genes in *Bipes biporus*

	No. of Regions Deleted	Duplication		Result	
<i>Bipes</i>	1	T₁P₁T₂P₂	→	T ₂ P ₂	No gene rearrangement
<i>Bipes</i>	1	T ₁ P₁T₂P₂	→	T ₁ P ₁	No gene rearrangement
<i>Bipes</i>	1	T ₁ P ₁ T₂P₂	→	T ₁ P ₂	No gene rearrangement
<i>Bipes</i>	2	T₁P₁T₂P₂	→	P ₁ T ₂	Gene rearrangement

NOTE.—Genes to be deleted in each hypothetical example are depicted in bold type and copy number is subscripted. Because tRNA^{Thr#2} (T₂) is a pseudogene, it is the only copy of tRNA^{Thr} subject to deletion without destroying an essential genic function. Because T₂ is adjacent to both tRNA^{Pro#1} (P₁) and tRNA^{Pro#2} (P₂), all feasible deletions of two duplicate genes would affect contiguous sequences and would not change gene order. Deletion of the more divergent P₁ gene is indicated with a double underline and illustrates the case where tandem duplication has led to a nonfunctional pair of genes located in the center of a duplicated tRNA gene cluster.

genes will not occur if adjacent copies of the duplicated genes become pseudogenes (Macey et al. 1997a).

Constraints on Genomic Rearrangement

When the tRNA^{Thr} gene is in the position typical for vertebrates, it directly follows the cytochrome *b* gene, and because both of these genes are encoded on the heavy strand, processing of the primary heavy-strand transcript does not allow these two genes to overlap (Ojala, Montoya, and Attardi 1981). In this case, either the stop codon for cytochrome *b* must be encoded within the cytochrome *b* gene or, following cleavage of the primary transcript, a 3'-terminal T or TA on the cytochrome *b* mRNA must get polyadenylated to form the stop codon, TAA (Anderson et al. 1981; Ojala, Montoya, and Attardi 1981). In *Bipes biporus*, cytochrome *b* ends with a T that is probably converted to a stop codon by polyadenylation following cleavage of the primary heavy-strand transcript (Anderson et al. 1981; Ojala, Montoya, and Attardi 1981). If the first tRNA^{Thr} gene were removed and the cytochrome *b* gene were followed by the light-strand encoded tRNA^{Pro} gene, the heavy-strand transcript would be cleaved at the end of the cytochrome *b* mRNA only if the noncoding strand of the tRNA^{Pro} gene could signal precise processing (Attardi 1985).

Four genomic rearrangements among vertebrates have placed light-strand encoded tRNA genes after protein-coding genes transcribed on the heavy strand. These protein-coding genes typically are followed by heavy-strand encoded tRNA genes that signal proper processing of the primary transcript (Ojala, Montoya, and Attardi 1981). In acrodont lizards, the heavy-strand encoded gene for NADH dehydrogenase subunit 1 (ND1) is followed by the light-strand encoded tRNA^{Gln} gene, and a conserved sequence in the amino acid acceptor stem of the tRNA^{Gln} gene is presumed to double as a stop codon for ND1 (Macey et al. 1997c). Interestingly, the overlap of genes in which a conserved tRNA-encoding sequence provides the stop codon for an adjacent protein-coding gene occurs also in marsupials to terminate the coding region of the ND2 gene. In this case, a sequence conserved across vertebrates (see Kumazawa and Nishida 1993) in the first three bases of the light-

strand encoded tRNA^{Ala} gene appears to double as the stop codon, TAA, for the heavy-strand encoded ND2 gene (see Pääbo et al. 1991; Janke et al. 1994). Both the acrodont ND1 and the marsupial ND2 genes lack a terminal T or TA that could serve as a stop codon if polyadenylated during processing of the heavy-strand transcript. In two different taxa, lamprey (*Petromyzon marinus*; Lee and Kocher 1995) and tuatara (*Sphenodon punctatus*; Quinn and Mindell 1996), genomic rearrangement has produced a gene junction of cytochrome *b* and tRNA^{Pro}. In both instances, the heavy-strand encoded cytochrome *b* gene contains a complete stop codon and does not overlap the light-strand encoded tRNA^{Pro} gene.

In *Bipes biporus*, if the first tRNA^{Pro} gene were to follow the cytochrome *b* gene directly, an additional 17 amino acids would result before a TAA sequence in the D-loop/stem could serve as a stop codon. This stop codon could be disrupted by length variation in the TΨC, variable, or D-loops of the tRNA^{Pro} gene. In addition, if the first tRNA^{Thr} sequence were to become nonfunctional and fail to be processed out of the primary transcript, cytochrome *b* would have to be extended by 12 amino acids to utilize a TAA sequence in the anticodon loop. Hence, genomic rearrangements placing the cytochrome *b* and tRNA^{Pro} genes adjacent may be precluded by functional constraints in *Bipes biporus*.

Light-Strand Synthesis

Sequences between the tRNA^{Asn} and tRNA^{Cys} genes, where the origin for light-strand replication in vertebrates normally is situated, are abnormal and presumably nonfunctional in *Bipes biporus* (Macey et al. 1997a). Although a stem-and-loop structure can be formed (see Macey et al. 1997a, fig. 3C), it lacks functional characteristics known to be required for replication in mammals. This result, combined with the observation that most vertebrate mitochondrial genomic rearrangements are associated with the apparent absence of a light-strand replication origin between the tRNA^{Asn} and tRNA^{Cys} genes, suggests displacements of the origin for light-strand synthesis in *Bipes biporus*. In addition, selective constraints on bases that overlap the O_L and those that encode tRNA^{Cys} may interfere with light-

strand synthesis (Macey et al. 1997c). The tRNA^{Cys} gene lacks a sequence encoding a D-stem in many lepidosau- rian reptiles (Seutin et al. 1994; Macey et al. 1997b), possibly destroying function of O_L (Macey et al. 1997c). Three independent lineages of reptiles, the tuatara (*Sphenodon punctatus*; Seutin et al. 1994; Quinn and Mindell 1996), acrodont lizards (Agamidae and Chamaeleonidae; Macey et al. 1997a, 1997b, 1997c), and the amphisbaenian *Bipes biporus*, are now known to have separately derived states for three mitochondrial structural features: (1) a gene order atypical for vertebrates, (2) a probable movement of the O_L, and (3) no D-stem encoded in the tRNA^{Cys} gene.

Additional studies of mitochondrial structural fea- tures among amphisbaenian reptiles can test further the predictions of our model for mitochondrial genomic re- arrangement (Macey et al. 1997a) and its evolutionary association with displacement of the O_L and absence of the D-stem in the tRNA^{Cys} gene (Macey et al. 1997c).

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