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Genetics

cDNA sequence of a human skeletal muscle ADP/ATP translocator: Lack of a leader peptide, divergence from a fibroblast translocator cDNA, and coevolution with mitochondrial DNA genes

(human adenine nucleotide translocator/differential gene expression/evolution of oxidative phosphorylation genes)

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ABSTRACT **We have characterized a 1400-nucleotide cDNA for the human skeletal muscle ADP/ATP translocator. The deduced amino acid sequence is 94% homologous to the beef heart ADP/ATP translocator protein and contains only a single additional amino-terminal methionine. This implies that the human translocator lacks an amino-terminal targeting peptide, a conclusion substantiated by measuring the molecular weight of the protein synthesized *in vitro*. A 1400-nucleotide transcript encoding the skeletal muscle translocator was detected on blots of total RNA from human heart, kidney, skeletal muscle, and HeLa cells by hybridization with oligonucleotide probes homologous to the coding region and 3′ noncoding region of the cDNA. However, the level of this mRNA varied substantially among tissues. Comparison of our skeletal muscle translocator sequence with that of a recently published human fibroblast translocator cognate revealed that the two proteins are 88% identical and diverged about 275 million years ago. Hence, tissues vary both in the level of expression of individual translocator genes and in differential expression of cognate translocator genes. Comparison of the base substitution rates of the ADP/ATP translocator and the oxidative phosphorylation genes encoded by mitochondrial DNA revealed that the mitochondrial DNA genes fix 10 times more synonymous substitutions and 12 times more replacement substitutions; yet, these nuclear and cytoplasmic respiration genes experience comparable evolutionary constraints. This suggests that the mitochondrial DNA genes are highly prone to deleterious mutations.**

The ADP/ATP translocator, or adenine nucleotide translocator (ANT), is the most abundant mitochondrial protein (1). In its functional state it forms a dimer consisting of two identical 30-kDa subunits embedded asymmetrically in the inner mitochondrial membrane (2). The dimer forms a gated pore through which ADP is moved across the inner membrane into the mitochondrial matrix and ATP is moved from the matrix into the cytoplasm (2).

Mitochondrial energy production varies greatly in importance between human tissues (3). Because the ANT determines the rate of ADP/ATP flux between the mitochondrion and the cytosol, the ANT would be a logical site for regulating cellular dependence on oxidative energy metabolism. Such regulation could be accomplished by producing varying amounts of the ANT or by elaborating tissue-specific ANT isoforms with different kinetic properties. Although *Neurospora crassa* has only one ANT gene (4), antigenic and electrophoretic mobility differences among bovine heart, kidney, and liver ANTs (5, 6) suggest that mammals may have multiple ANT genes that are expressed in a tissue-specific manner. Tissue-specific expression of functionally similar genes encoding proteins involved in oxidative phosphorylation (Ox/Phos) has been reported for the bovine ATP synthase proteolipid (7).

The ANT and most other Ox/Phos genes are encoded in the nucleus, but 13 essential Ox/Phos polypeptides are encoded in the maternally inherited mitochondrial DNA (mtDNA) (3). The high interdependence of these proteins suggests that they may coevolve. However, previous studies have indicated that the mtDNA genes evolve 6–10 times more rapidly than the average nuclear DNA gene (8–10), and we have recently shown that mtDNA genes fix synonymous nucleotide substitutions 17 times faster than the nuclear-encoded β subunit of the F_1 ATP synthase [H^+-transporting ATP synthase; EC 3.6.1.34] (ATPSyn-β) (10). Because the ANT is functionally related to the ATP synthase, but is not a component of the same complex, we wanted to determine whether this nuclear gene showed a similar evolutionary disparity relative to the mtDNA genes.

In this paper we describe the characterization of a full-length cDNA encoding the human skeletal muscle ANT. We have compared our sequence‖ with a recently published cDNA sequence for a human fibroblast ANT cognate (11) and with a partial cDNA sequence of the bovine heart ANT (12). These data, together with mRNA analysis, demonstrate that there are two distinct ANTs that diverged about 275 million years before present (MYBP) and that the skeletal muscle ANT is expressed in heart, kidney, liver, skeletal muscle, and HeLa cells. Finally, by comparing the rate of evolution of the skeletal muscle ANT with that of mtDNA Ox/Phos genes, we found that the mtDNA genes are evolving 10–12 times faster than the ANT. Thus, nuclear Ox/Phos genes can evolve at different rates, but all nuclear Ox/Phos genes examined evolve more slowly than the mtDNA genes.

MATERIALS AND METHODS

Cloning, Clone Identification, and Sequencing. A full-length cDNA clone encoding the human skeletal muscle ANT was isolated from an Okayama–Berg cDNA library of lower leg muscle mRNA (13). This clone, previously designated as

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Abbreviations: ANT, adenine nucleotide translocator; ANT-H1, ANT deduced from human skeletal muscle cDNA; ANT-B1, ANT of bovine heart muscle; ANT-H2, ANT deduced from human fibroblast cDNA; Ox/Phos genes, genes encoding proteins involved in oxidative phosphorylation; ATPSyn-β, ATP synthase β subunit; MYBP, million years before present; λ_a, replacement substitution rate; λ_s, synonymous substitution rate; SSY, substitutions per site per year; mtDNA, mitochonrial DNA.

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‖This sequence of an adenine nucleotide translocator in human skeletal muscle is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02966).

clone H20d (kindly provided by Peter Gunning, Children's Medical Research Foundation, Camperdown, New South Wales, Australia) (14), was renamed pHMANT. Restriction fragments of the insert of this clone were sequenced by the chemical-cleavage procedure (15) or subcloned into M13 vectors and sequenced by the dideoxy chain-termination method (16). The derived amino acid sequence matched the peptide sequence of the bovine heart ANT (17).

The *Hin*dIII–*Hin*cII restriction fragment of the pHMANT insert was isolated and used to generate a radioactive probe (18). The probe was then used to screen a human fetal liver cDNA library cloned in bacteriophage λgt10 as previously described (10).

Computer Analysis. DNA sequences were analyzed with the MicroGenie programs (Beckman) (19). Analysis of the evolutionary divergence of human and bovine cDNA sequences was facilitated by using the programs of Li *et al.* (20), a Fortran compiler, and an IBM PC AT computer (10). Densitometric determinations were made using a video camera, a Microworks Digisector D5-65 interface card, and the SCAN program (21).

Oligonucleotide Probes Used for RNA Blot Hybridizations. Two 40- to 41-base oligonucleotides complementary to the cDNA-derived RNA sequence were made using a DNA synthesizer (model 380B, Applied Biosystems, Foster City, CA). The crude oligonucleotide mixtures were purified by fractionation on a 12% polyacrylamide/7 M urea gel. Bands corresponding to 40- to 41-nucleotide (nt) polymers were excised from the gel and electroblotted onto Whatman type DE-81 paper. DNA attached to the paper was eluted with 1 ml of 2 M triethylamine bicarbonate (pH 7.6) and lyophilized. Radioactive probes were made by 5′ end-labeling 5 pmol of oligonucleotide DNA with excess [γ-^{32}P]ATP (Amersham) in the presence of T4 polynucleotide kinase (Bethesda Research Laboratories).

RNA Isolation and RNA Blot Analysis. RNA was isolated by guanidine thiocyanate–cesium chloride density gradient centrifugation (22, 23). Total RNA (4–8 μg) and RNA standards (Bethesda Research Laboratories) were fractionated in 1.2% agarose gels containing 6.29% formaldehyde in a 20 mM Hepes buffer (pH 7.2) system. RNA blots on grade BA85 nitrocellulose (Schleicher & Schuell) were prehybridized at 42°C overnight in 5× standard saline citrate (SSC)/20% formamide/10× Denhardt's solution (23)/0.05 M sodium phosphate, pH 6.7, containing 500 μg of sonicated salmon sperm DNA per ml. They were then hybridized at 42°C in 3 ml of 5× SSC/20% formamide/1× Denhardt's solution/0.02 M sodium phosphate, pH 6.7, containing 500 μg of sonicated sperm DNA per ml and 6×10^6 cpm of the probe. Filters were washed at 65°C with three 10-min washes. Probe A was washed with 0.5× SSC, and probe B was washed with 1.0× SSC. All wash solutions contained 0.1% $NaDodSO_4$ and were prewarmed to 65°C but cooled to 55°C during the wash. Blots were autoradiographed using intensifying screens at −80°C for up to 10 days.

***In Vitro* Transcription and Translation of the pHMANT cDNA.** The pHMANT insert was recloned into the *Hin*dIII and *Hin*cII sites of M13mp8. The insert from this construct was recloned into the *Eco*RI and *Hin*dIII sites of pTZ19R (Pharmacia); one of these recombinant plasmids (pTZHANT11) was restricted with *Eco*RI and used as a template for *in vitro* transcription by T7 RNA polymerase (United States Biochemical, Cleveland). After removal of template DNA with RQ1 DNase (Promega Biotec, Madison, WI), the synthetic RNA was extracted with phenol/chloroform and with chloroform and stored at −80°C. This RNA is complementary to the oligonucleotide probes and was used as a control in the RNA blot analyses; it was also used as a template for *in vitro* translation.

The synthetic pTZHANT11 transcript was translated *in vitro* in a rabbit reticulocyte translation system (Promega Biotec) containing [^{35}S]methionine (Amersham). Ten microliters of the 60-μl translation mix were solubilized with 40 μl of 4% $NaDodSO_4$/5% 2-mercaptoethanol loading buffer. The proteins were separated by M_r using a 30-cm 0.1% $NaDodSO_4$/15% polyacrylamide gel in a Laemmli buffer system (24). Gels were fixed, stained with Coomassie blue, and impregnated with EN^{3}HANCE (New England Nuclear), and the translation product was detected by fluorography.

RESULTS

The cDNA sequence of the human ANT contains ≈1400 nt. The restriction map and sequencing strategy are presented in Fig. 1, and the complete sequence is shown in Fig. 2. The first methionine residue is located 104 nt downstream from the 5′ end of the sequence in an *Nco* I site, which is frequently found at the beginning of eukaryotic structural genes. A continuous open reading frame extends from the first methionine residue for 891 nt. An additional 299 nt are found 3′ to the termination codon, followed by a poly(dA) tail of ≈100 nt. An alignment of the human and the bovine amino acid sequences (17) shows that the two polypeptides are 94% homologous (Fig. 2). The only major difference between the human and bovine polypeptides is that the human peptide lacks an alanine residue corresponding to amino acid-149 of the bovine sequence.

Two partial cDNAs were isolated from a human fetal liver cDNA library and sequenced by the dideoxy chain-termination procedure. Clone HLANT1 encompasses nt 279–680 of the skeletal muscle sequence, whereas clone HLANT2

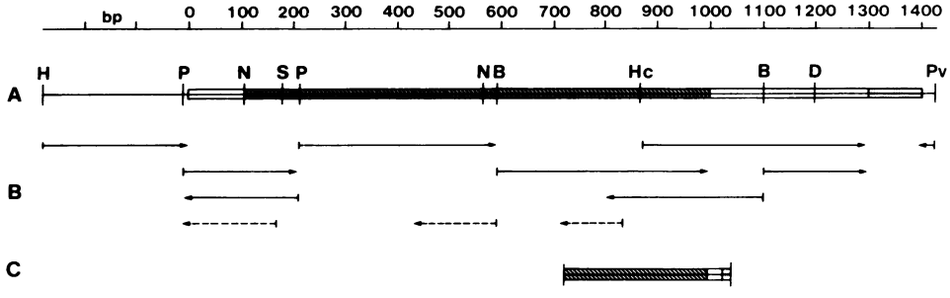

FIG. 1. Restriction map and sequencing strategy of pHMANT cDNA. (*A*) Restriction map of the pHMANT insert. Restriction sites: B, *Bgl* II; D, *Dra* I; H, *Hin*dIII; Hc, *Hin*cII; N, *Nco* I; P, *Pst* I; Pv, *Pvu* II; and S, *Sac* II. Lines represent SV40 linker regions of the Okayama–Berg vector, clear blocks represent 5′ and 3′ noncoding regions, hatched blocks indicate coding regions, and stippled blocks denote poly(dA) tails. (*B*) ⟶ and ⟵ are M13 clones sequenced by the dideoxy chain-termination method (16). ⟶ and ⟵ are restriction fragments sequenced by the chemical-cleavage procedure (15). (*C*) Alignment of the bovine cDNA sequenced previously (12); blocks marked are the same as in *A*.

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       10        20        30        40        50        60        70        80        90       100       110       120
CCCCCTAGCGTCGCGCAGGGTCGGGGACTGCGCGCGGTGCCAGGCCGGGCGTGGGCGAGAGCACGAACGGGCTGCTGCGGGCTGAGAGCGTCGAGCTGTCACCATGGGTGATCACGCTTG
                                                                                                  M  G  D  H  A  W
                                                                                                  S     Q     L
      130       140       150       160       170       180       190       200       210       220       230       240
GAGCTTCCTAAAGGACTTCCTGGCCGGGGCGGTCGCCGCTGCCGTCTCCAAGACCGCGGTCGCCCCCATCGAGAGGGTCAAACTGCTGCTGCAGGTCCAGCATGCCAGCAAACAGATCAG
  S  F  L  K  D  F  L  A  G  A  V  A  A  A  V  S  K  T  A  V  A  P  I  E  R  V  K  L  L  L  Q  V  Q  H  A  S  K  Q  I  S
                           G                 I
      250       260       270       280       290       300       310       320       330       340       350       360
TGCTGAGAAGCAGTACAAAGGGATCATTGATTGTGTGGTGAGAATCCCTAAGGAGCAGGGCTTCCTCTCCTTCTGGAGGGGTAACCTGGCCAACGTGATCCGTTACTTCCCCACCCAAGC
  A  E  K  Q  Y  K  G  I  I  D  C  V  V  R  I  P  K  E  Q  G  F  L  S  F  W  R  G  N  L  A  N  V  I  R  Y  F  P  T  Q  A

      370       380       390       400       410       420       430       440       450       460       470       480
TCTCAACTTCGCCTTCAAGGACAAGTACAAGCAGCTCTTCTTAGGGGGTGTGGATCGGCATAAGCAGTTCTGGCGCTACTTTGCTGGTAACCTGGCGTCCGGTGGGGCCGCTGGGGCCAC
  L  N  F  A  F  K  D  K  Y  K  Q  L  F  L  G  G  V  D  R  H  K  Q  F  W  R  Y  F  A  G  N  L  A  S  G  G  A  A  G  A  T
                                    I
      490       500       510       520       530       540       550       560       570       580       590       600
CTCCCTTTGCTTTGTCTACCCGCTGGACTTTGCTAGGACCAGGTTGGCTGCTGATGTGGGCAGGCGCGCCCAGCGTGAGTTCCATGGTCTGGGCGACTGTATCATCAAGATCTTCAAGTC
  S  L  C  F  V  Y  P  L  D  F  A  R  T  R  L  A  A  D  V  G  R  R  A  Q  R  E  F  H  G  L  G  D  C  I  I  K  I  F  K  S
                                                                  K  G  A           T           N           T
      610       620       630       640       650       660       670       680       690       700       710       720
TGATGGCCTGAGGGGGCTCTACCAGGGTTTCAACGTCTCTGTCCAAGGCATCATTATCTATAGAGCTGCCTACTTCGGAGTCTATGATACTGCCAAGGGGATGCTGCCTGACCCCAAGAA
  D  G  L  R  G  L  Y  Q  G  F  N  V  S  V  Q  G  I  I  I  Y  R  A  A  Y  F  G  V  Y  D  T  A  K  G  M  L  P  D  P  K  N

      730       740       750       760       770       780       790       800       810       820       830       840
CGTGCACATTTTTGTGAGCTGGATGATTGCCCAGAGTGTGACGGCAGTCGCAGGGCTGCTGTCCTACCCCTTTGACACTGTTCGTCGTAGAATGATGATGCAGTCCGGCCGGAAAGGGGC
  V  H  I  F  V  S  W  M  I  A  Q  S  V  T  A  V  A  G  L  L  S  Y  P  F  D  T  V  R  R  R  M  M  M  Q  S  G  R  K  G  A
        I                          T                       V
      850       860       870       880       890       900       910       920       930       940       950       960
CGATATTATGTACACGGGGACAGTTGACTGCTGGAGGAAGATTGCAAAAGACGAAGGAGCCAAGGCCTTCTTCAAAGGTGCCTGGTCCAATGTGCTGAGAGGCATGGGCGGTGCTTTTGT
  D  I  M  Y  T  G  T  V  D  C  W  R  K  I  A  K  D  E  G  A  K  A  F  F  K  G  A  W  S  N  V  L  R  G  M  G  G  A  F  V
                                                            P
      970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
ATTGGTGTTGTATGATGAGATCAAAAAATATGTCTAATGTAATTAAAACACAAGTTCACAGATTTACATGAACTTGATCTACAAGTTCACAGATCCATTGTGTGGTTTAATAGACTATTC
  L  V  L  Y  D  E  I  K  K  Y  V ***
                                 F
     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CTAGGGGAAGTAAAAAGATCTGGGATAAAACCAGACTGAAAGGAATACCTCAGAAGAGATGCTTCATTGAGTGTTCATTAAACCACACATGTATTTTGTATTTATTTTACATTTAAATTC

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
CCACAGCAAATAGAAATAATTTATCATACTTGTACAATTAACTGAAGAATTGATAATAACTGAATGTGAAACATCAATAAAGACCACTTAATGCACAAAAAAAAAAAAAAAAAAAAAAAA
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FIG. 2. Nucleotide sequence of the human skeletal muscle ANT (ANT-H1) from pHMANT. Amino acid sequence of the single open reading frame of 891 nt (297 codons) is shown beneath. Note the *Nco* I site containing the codon for the initial methionine residue and the poly(dA) tail. Bovine ANT amino acid sequence (17) is aligned underneath, and only variant amino acids are indicated. Two liver cDNA clones were colinear with nt 279–1206 (HLANT1 and HLANT2). Though dideoxy chain-termination sequencing ambiguities were consistently seen in the region 542–550, these ambiguities were resolved for the skeletal muscle sequence by Maxam–Gilbert sequencing (15).

covers nt 352–1206. Both of these liver cDNAs are colinear with the skeletal muscle sequence (Fig. 2).

The size of the human skeletal muscle ANT mRNA was determined in several human tissues by hybridizing RNA blots with two specific oligonucleotide probes, which are complementary to a portion of the coding region and the 3′-noncoding region of the muscle mRNA. The sequences of the probes are:

probe A (middle, coding region; nt 611–650)
5′-TGCCTTGGACAGAGACGTTGAAACCCTGGTAGAGCCCCCT-3′

probe B (3′ end, noncoding region; nt 1133–1173)
5′-TACATGTGTGGTTTAATGAACACTCAATGAAGCATCTCTTC-3′

Hybridization of these oligonucleotide probes to ANT RNA synthesized *in vitro* from pTZHANT11 (see *Materials and Methods*) demonstrated that both probes hybridized to this RNA (Fig. 3*A* for probe A) but that probe A bound to the target RNA about 10 times more efficiently than did probe B (data not shown). When these two probes were hybridized to RNA blots of total RNA from human heart, kidney, and HeLa cells (Fig. 3*B* for probe A), both probes hybridized to a transcript of the same size. When probe A was hybridized to skeletal muscle RNA, similar results were obtained (Fig. 3*C*). Further analysis of the kidney RNA revealed that the ANT transcript is ≈1430 nt long. Hence, it appears that all of these tissues have ANT mRNAs comparable in length and sequence with the skeletal muscle cDNA.

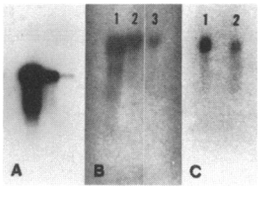

FIG. 3. Autoradiographs of RNA blots of synthetic ANT translocator RNA (*A*) and total human RNA (*B* and *C*). (*B*) Lanes: 1, human heart (ventricle); 2, kidney; and 3, HeLa cells. (*C*) Lanes: 1, kidney; 2, skeletal muscle (neck). All three filters were hybridized to oligonucleotide probe A. *A* was exposed overnight at room temperature. Transcript size is 1710 nt. *B* and *C* were exposed for 10 days and 40 hr, respectively, at −80°C with two intensifying screens. Transcript size is ≈1400 nt.

The relative proportions of ANT mRNAs in heart, kidney, and HeLa cell RNAs were estimated by normalizing probe A-ANT hybridization against that seen with the 1.8-kb *Eco*RI–*Sal* I fragment of the mouse 18S ribosomal RNA cistron (25). Densitometric comparison of labeling of ANT and ribosomal RNA transcripts indicated that heart and kidney have approximately the same amount of ANT mRNA but that, under these conditions, HeLa cells have less than a third as much ANT mRNA.

To determine whether the skeletal muscle cDNA has a leader peptide, we analyzed the deduced amino acid sequence upstream from the bovine sequence (17). Seven additional amino acid codons were found, but the only methionine residue in this region was at the *Nco* I site (positions 104–106), one amino acid 5′ to the bovine sequence. To determine whether this was the initiating methionine we transcribed synthetic mRNA *in vitro* from pTZHANT11 (Fig. 4*A*) and used it to synthesize the skeletal muscle ANT protein with an *in vitro* translation system (Fig. 4*B*). The resulting [^{35}S]methionine-labeled polypeptide was fractionated on a $NaDodSO_4$/polyacrylamide gel. The only radioactive translation product generated was a 30-kDa polypeptide, as shown in Fig. 4*B*; the size of this polypeptide is the same

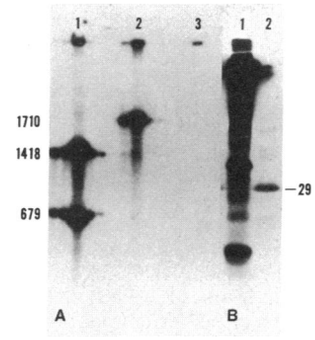

FIG. 4. Autoradiographs of *in vitro* transcription and translation products of the human skeletal muscle ANT (ANT-H1). *A* shows the autoradiograph of ^{32}P-labeled synthetic RNA transcripts separated on a 6% polyacrylamide gel containing 8 M urea; sizes of the transcripts (in nt) are indicated. Lanes: 1, Promega standards; 2, ANT transcript from plasmid pTZHANT11 cut 3′ to the cDNA with *Eco*RI; 3, ANT transcripts from plasmid pTZHANT11 cut 5′ to the cDNA with *Hin*dIII (the abortive transcripts migrated off the gel). *B* shows the fluorograph of ^{35}S-labeled synthetic proteins translated *in vitro* and resolved on a 0.1% $NaDodSO_4$/15% polyacrylamide gel. Lanes: 1, translation products of mixed Brome mosaic virus (BMV) RNAs (Promega); 2, translation product of pTZHANT11 mRNA. The position of the 29-kDa standard (Pharmacia) is indicated; protein standards were detected by Coomassie blue staining.

as that of the mature ANT isolated from beef heart mitochondria (2, 17). We can therefore conclude that our cDNA was full length and that the primary translation product contained only one additional amino acid relative to the mature protein.

DISCUSSION

Features of the cDNA and the Peptide Sequence. The human skeletal muscle ANT was found to be synthesized without an amino-terminal targeting sequence. This clearly sets it apart from many other nuclear-encoded mitochondrial oxidative phosphorylation proteins (26) but places it in the same class as the human fibroblast ANT cognate (11), the ANTs from other species (1, 4, 27, 28), the uncoupling protein (29–30), cytochrome *c* (28, 31), and cytochrome *c* oxidase subunit VIIa (32).

A Pennsylvanian Duplication of ANT Genes. The skeletal muscle ANT gene appears to be expressed in a wide variety of tissues. Our coding region and the 3′ noncoding region oligonucleotide probes hybridized to 1.43-kb mRNAs from human heart, kidney, skeletal muscle, and HeLa cells; our skeletal muscle and liver cDNA sequences were almost identical; and our skeletal muscle cDNA sequence is highly homologous to both the beef heart amino acid sequence (17) and the partial beef heart ANT cDNA sequence encoding a 91-amino acid peptide (12). The similarity between the human skeletal muscle ANT and the bovine heart ANT cDNAs is further supported by the close proximity of one of two ATTAAA poly(A)-addition signals four nucleotides downstream from the termination codon.

At the amino acid level, our human skeletal muscle ANT (ANT-H1) is 94% identical with the bovine heart ANT (ANT-B1). By contrast, both ANT-H1 and ANT-B1 are only 88–89% identical with the recently published ANT cognate deduced from a fibroblast cDNA (ANT-H2) (11). The amino acid differences between ANT-H1, ANT-B1, and ANT-H2 are distributed throughout the peptide sequences. Hence, ANT-H1 and ANT-H2 diverged long before ANT-H1 and ANT-B1 and thus must represent two functionally distinct genes.

Estimates of replacement substitutions (K_a) and synonymous substitutions (K_s) were obtained by computer analysis (see *Materials and Methods*) and confirm that ANT-H1 and ANT-B1 are more closely related to each other than either one is to ANT-H2 (Table 1). Assuming that the human and bovine lineages diverged 80 million years before the present (MYBP) (20), the ANT-H1/ANT-B1 replacement (λ_a) and synonymous (λ_s) substitution rates were estimated to be 0.148×10^{-9} and 3.18×10^{-9} substitutions per site per year (SSY), respectively (Table 1). These values and the λ_a and λ_s values obtained by comparing ANT-H1 and ANT-H2 were then used to estimate the time of divergence of these cognate ANT genes. This analysis suggests that the skeletal muscle and fibroblast ANT genes were duplicated and diverged about 275 MYBP during the Pennsylvanian period (310–270 MYBP) or, at the latest, during the Permian period (270–225 MYBP).

Tissue-Specific Expression of ANT Genes and ANT Antigenicity. Our oligonucleotide studies demonstrate that ANT-H1 is expressed in a variety of tissues but that the amounts of this transcript can vary 3-fold. Because of the extensive

Table 1. Sequence divergence of nuclear and cytoplasmic Ox/Phos genes

Gene	Sequences compared	Amino acids	Parameters				
			K_a	λ_a, ($\times 10^{-9}$ SSY)	K_s	λ_s, ($\times 10^{-9}$ SSY)	*k*C
ANT	H1/B1	91	0.0237	0.148	0.509	3.18	3.07
ANT	H1/H2	297	0.0705	ND	1.922	ND	ND
ANT	H2/B1	91	0.0761	ND	1.909	ND	ND
ATPSyn-β	H/B	357	0.0061	0.0381	0.309	1.93	3.93
Mean nDNA*			0.141	0.88	0.744	4.65	
COI	M/R	513	0.0225	0.662	1.173	34.5	3.95
ATPase 6	M/R	226	0.0332	0.976	0.819	24.1	3.21
ATPase 8	M/R	67	0.127	3.74	0.755	22.2	1.78
Mean mtDNA*	M/R		0.0626	1.84	1.098	32.3	2.87

H/B, human versus bovine sequence comparison; M/R, mouse versus rat sequence comparison; H1, B1, and H2, different ANT sequences compared as defined; ND, not determined; K_a, K_s, λ_a, and λ_s values were calculated as described (K_a and K_s in substitutions per site) (10, 20). ANT and ATPSyn-β values were calculated from H/B comparisons. For mtDNA genes, M/R comparisons were used in lieu of H/B comparisons because H/B comparisons yielded λ_a and λ_s and *k*C values that often were lower than those obtained from M/R comparisons. (COI is cytochrome *c* oxidase; EC 1.9.3.1; subunit 1. ATPase 6 and ATPase 8 are subunits 6 and 8 of the H^+-transporting ATP-synthase; EC 3.6.1.24.) Although this could be attributed to nonlinear mutation rates in mammalian mitochondrial genomes (33), we believe these unexpectedly low values to result from mutational saturation in the more divergent species (8–10).

*The mean nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) values were calculated previously (10, 20).

nucleotide divergence between ANT-H1 and ANT-H2, our oligonucleotide probes would not have hybridized to ANT-H2 mRNA. Hence, it remains unclear whether ANT-H2 is expressed in the same tissues as ANT-H1. Differences in expression of distinct ANT genes, such as ANT-H1 and ANT-H2, could explain the varying antigenicity of ANTs detected by Schultheiss and Klingenberg in heart, kidney, and liver (5, 6).

Coevolution of ANT-1 and the mtDNA Ox/Phos Genes. Having established that the ANT-H1 and ANT-B1 genes are the same, we compared the evolutionary rate of the ANT-1 genes with that of genes encoded by mtDNA. Comparison of synonymous and replacement substitution rates of ANT-H1 and ANT-B1 with the mtDNA rates of mouse and rat (10) (see Table 1 for explanation) revealed that the mtDNA genes fix synonymous substitutions 10 times faster than the ANT genes (32.3×10^{-9} SSY versus 3.18×10^{-9} SSY) and replacement substitutions 12 times faster than the ANT genes (1.84×10^{-9} SSY versus 0.148×10^{-9} SSY) (Table 1). The comparable values for ATPSyn-β are 17 times and 48 times, respectively. Hence, both the ANT and ATPSyn-β evolve much more slowly than the mtDNA Ox/Phos genes. However, it is also clear that the ATPSyn-β gene evolves more slowly than the ANT gene, with the ANT gene fixing 1.6 times more synonymous substitutions and 3.9 times more replacement substitutions than the ATPSyn-β gene. Thus, not only are the mtDNA Ox/Phos genes evolving more rapidly than nuclear Ox/Phos genes, but different nuclear Ox/Phos genes are also evolving at different rates.

To understand the significance of the differential mutation rates of Ox/Phos genes, we calculated the relative "selective constraints" acting on the various oxidative phosphorylation proteins. "Selective constraint" was calculated from our previously derived expression, $kC = -\ln(\lambda_a/\lambda_s) = -\ln(K_a/K_s)$ (10). Calculation of the kC value for ANT-H1 and ANT-B1 confirms that this is a highly constrained protein ($kC = 3.07$). Surprisingly, however, it is only slightly more constrained than the average mtDNA-encoded protein ($kC = 2.87$). Hence, mtDNA substitutions are as likely to be deleterious to oxidative phosphorylation proteins as nuclear DNA substitutions. Therefore, the high mtDNA mutation rate should result in a disproportionately high frequency of maternally inherited defects, which is consistent with the recent observation that certain neuromuscular diseases show a matrilineal pattern of inheritance (3).

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