Three mitochondrial DNA restriction polymorphisms in elite endurance athletes and sedentary controls

MIGUEL A. RIVERA, BERND WOLFARTH, FRANCE T. DIONNE, MONIQUE CHAGNON, JEAN-AIMÉ SIMONEAU, MARCEL R. BOULAY, THOMAS M. K. SONG, LOUIS PERUSSE, JACQUES GAGNON, ARTHUR S. LEON, D. C. RAO, JAMES S. SKINNER, JACK H. WILMORE, JOSEPH KEUL, AND CLAUDE BOUCHARD

Physical Activity Sciences Laboratory, Laval University, Québec, G1K 7P4, CANADA; Department of Physical Medicine, Rehabilitation and Sports Medicine, University of Puerto Rico Medical School, San Juan, P. R. 00936; Department of Rehabilitation and Preventive Sports Medicine, Freiburg University, Freiburg, GERMANY; Human Performance Laboratory, Lakehead University, Thunder Bay, Ontario, P7B 5E1, CANADA; School of Kinesiology and Leisure Studies, University of Minnesota, Minneapolis, MN 55455; Division of Biostatistics and Department of Genetics and Psychiatry, Washington University Medical School, St. Louis, MO 63110; Department of Kinesiology, Indiana University, Bloomington, IN 47405; and Department of Kinesiology and Health Education, The University of Texas at Austin, Texas, TX 78712

ABSTRACT

RIVERA, M. A., B. WOLFARTH, F. T. DIONNE, M. CHAGNON, J.-A. SIMONEAU, M. R. BOULAY, T. M. K. SONG, L. PÉRUSSE, J. GAGNON, A. S. LEON, D. C. RAO, J. S. SKINNER, J. H. WILMORE, J. KEUL, and C. BOUCHARD. Three mitochondrial DNA restriction polymorphisms in elite endurance athletes and sedentary controls. *Med. Sci. Sports Exerc.*, Vol. 30, No. 5, pp. 687–690, 1998. This study examined the associations between elite endurance athlete (EEA) status and three mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) in the subunit 5 of the NADH dehydrogenase (MTND5) locus and one in the D-loop region. A group of 125 Caucasian male EEA well endowed with the phenotypic expression of $\dot{V}O_{2max}$ (78.9 ± 3.8 mL·kg^{-1}·min^{-1}, mean ± SD) and 65 sedentary controls (SCON: $\dot{V}O_{2max}$ = 39.8 ± 8.2 mL·kg^{-1}·min^{-1}) participated in the study. $\dot{V}O_{2max}$ was determined during an incremental exercise test on a cycle ergometer or a motor-driven treadmill. mtDNA was extracted from white blood cells or lymphoblastoid cell lines and specific regions were amplified by the polymerase chain reaction. The Pearson Chi-square statistic test and Fisher exact test revealed no significant association ($P > 0.05$) between any of the three mtDNA RFLPs and EEA status. The MTND5-*BamHI* RFLP at bp 13,470 (morph 3) was found in 12.8% of the EEA and 12.3% of the SCON ($\chi^2 = 0.009$, $P = 0.92$). The prevalence of the MTND5-*NciI* RFLP at bp 13,364 (morph 2) was 12.9% and 14% for the EEA and SCON, respectively ($\chi^2 = 0.043$, $P = 0.83$). The D-loop-*KpnI* RFLP at bp 16,133 (morph 1) was found in 5.8% of the EEA and in 1.6% of the SCON (Fisher exact test = 1.80, $P = 0.18$). The MTND5-HincII RFLP at bp 12,406 (morph 1) was not present in this study sample. These results indicate no evidence for a difference in the frequency of two polymorphic restriction sites in the subunit 5 of the NADH dehydrogenase gene of mtDNA and one in the D-loop region between elite endurance athletes and sedentary controls. **Key Words:** MAXIMAL OXYGEN UPTAKE, POLYMERASE CHAIN REACTION, GENETIC VARIATION, RFLP, RESTRICTION ENZYMES, D-LOOP, NADH DEHYDROGENASE

A small body of research has revealed that the genetic heritability of maximal aerobic power ($\dot{V}O_{2max}$) and several determinants of endurance performance were statistically significant (5) and that there were large individual differences in the response of $\dot{V}O_{2max}$ to endurance training (16), but that much of the heterogeneity in response was likely accounted for by genetic differences in trainability (18). Interestingly, one study has suggested that inheritance of $\dot{V}O_{2max}$ could also be characterized by a maternal effect (15). A recent review of the genetic literature on aerobic performance proposed strategies and techniques to be used in the study of the genetic basis of aerobic performance and its response to training (5). With the development of gene probes and the restriction fragment length polymorphism (RFLP) technology (19), it is possible to screen for DNA polymorphisms among individuals and to look for associations between DNA variants and a phenotype of interest. An RFLP results from individual differences in DNA fragment sizes produced in a region of the nuclear or mitochondrial genome by an enzyme that cuts DNA at a specific nucleotide sequence (3). The RFLPs are caused by mutation at a restriction site and occur in both coding and noncoding regions of the genes.

0195-9131/98/3005-0687$3.00/0
MEDICINE & SCIENCE IN SPORTS & EXERCISE®

Submitted for publication January 1997.
Accepted for publication July 1997.

TABLE 1. Characteristics of elite endurance male athletes and male controls.

	Athletes (N = 125)		Controls (N = 65)	
	Mean ± SD	Range	Mean ± SD	Range
Age (yr)	27.4 ± 7.6	17–53	25.7 ± 6.2	17–39
Height (cm)	177.9 ± 5.8	160–195	178.1 ± 6.4	161–197
Body mass (kg)	69.8 ± 7.2	50–90	82.6 ± 17.3	45–140
BMI ($kg \cdot m^{-2}$)	22.0 ± 1.7	19–28	26.4 ± 5.2	19–44
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	78.9 ± 3.8	73–93	39.8 ± 8.2	22–50

Values are means ± SD.
BMI, Body mass index.

The human mitochondrial DNA (mtDNA) is a 16,569 base pairs (bp) circular duplex molecule located within the matrix of the double-membrane mitochondrion. mtDNA is inherited from the maternal oocyte, does not recombine, and is self replicative (1,22). It contains no introns. mtDNA codes for: 1) seven subunits of NADH dehydrogenase complex (MTND1, MTND2, MTND3, MTND4, MTND4L, MTND5, and MTND6), which transfer electrons from NADH to ubiquinone and is one of the three major respiratory enzyme complexes in the pathway from NADH to oxygen; 2) subunits 6 and 8 of the ATPase synthase which synthesizes ATP from ADP and P_i in the mitochondrial matrix; 3) a subunit of the cytochrome bc_1-complex, the second of the three major respiratory enzyme complexes, which transfers electrons from ubiquinone to cytochrome c; and 4) subunits I, II, and III of cytochrome c oxidase complex which is the third of the three major respiratory enzyme complexes in the pathway from NADH to oxygen. Moreover, mtDNA encodes two rRNAs and 22 tRNAs. The D-loop region is a noncoding segment that contains the promoters for transcription of the heavy and light mtDNA strands, the origin of replication of the heavy strand, and conserved sequences essential for mtDNA expression (7,12). Since this is a replication region, there is the possibility that mutations in this region could induce alterations in protein content by affecting transcription (10). Inasmuch as the mtDNA is transcribed from a single promoter region and that tRNAs, rRNAs, and mRNAs are released from one polycistronic transcript, any event affecting transcription of mtDNA may have a coordinated effect on the expression level of selected peptides of the aerobic-oxidative process. The traditional concept of individual variation in endurance performance considers that one of its major determinants is the skeletal muscle metabolic properties, particularly its oxidative potential (11,13,23). Therefore, the mitochondrial genome provides a few candidate genes for the study of elite endurance athlete status (EEA).

The concept of a genetic effect implies the existence of variations in DNA sequences that may affect gene products or gene expression. Along these lines, one study suggested an association between mitochondrial DNA (mtDNA) RFLPs, and $\dot{V}O_{2max}$ in the sedentary state and its response to endurance training (9). Carriers of three RFLPs in mtDNA, one caused by a base change in the tRNA for threonine at bp 15,925 and two others caused by base substitutions at bp 13,364 and bp 13,470 located in the gene coding for subunit 5 of the NADH dehydrogenase (MTND5), had a body-mass adjusted $\dot{V}O_{2max}$ in the sedentary (untrained) state significantly higher than noncarriers. A low response of $\dot{V}O_{2max}$ to endurance training was also observed for three carriers of a *HincII* RFLP at bp 12,406 (morph 1) also in MTND5. Moreover, carriers of a mtDNA D-loop *KpnI* RFLP at bp 16,133 presented a statistically significant higher $\dot{V}O_{2max}$ response to endurance training.

It has been postulated that the MTND5-NciI RFLP at bp 13,364 (morph 2) is created by the loss of a restriction site which can be a result of a mutation at any of the bases of the recognition sequence (9). The MTND5-BamHI RFLP at bp 13,470 (morph 3) could result from a base substitution (G-C) (9). The MTND5-HincII RFLP at bp 12,406 (morph 1) is present when a valine codon is substituted for an isoleusine. The D-loop KpnI RFLP at bp 16,133 (morph 1) is caused by the loss of a restriction site.

The aim of the present study was to determine whether some of the mtDNA RFLPs previously reported (9) to be associated with an elevated $\dot{V}O_{2max}$ in the untrained state and its response to endurance training were also associated with an EEA status.

METHODS

Subjects. A group of 125 unrelated Caucasian male EEA met the study acceptance criterion of a $\dot{V}O_{2max}$ of at least 73 $mL \cdot kg^{-1} \cdot min^{-1}$ (78.9 ± 3.8; mean ± SD). They were Americans (N = 10), Canadians (N = 42), Germans (N = 62), other Western Europeans (N = 7), and South Africans (N = 4). All these subjects had been engaged in endurance training and national/world class competitions for several years. They were specialists in biathlon, road cycling, triathlon, cross-country skiing, race walking, and track and long-distance road running. A second group of 65 unrelated sedentary Caucasian males from the HERITAGE Family Study (6) served as controls (SCON) and their

TABLE 2. Mitochondrial DNA restriction fragment length polymorphism (RFLP) location, restriction enzyme, and digested polymerase chain reation (PCR) products.

RFLP				Digested PCR Product (bp)	
Site (bp)	Enzyme	Gene Locus	Primers	Wild	Mutant
13,364	NciI	MTND5	Sense: 5′-AGGCGCTATCACCACTCTGTTCG-3′ Antisense: 5′-GAATTCCTGCGAATAGGCTTCCGGCTGCC-3′	364, 169	533
13,470	BamHI	MTND5	Sense: 5′-AGGCGCTATCACCACTCTGTTCG-3′ Antisense: 5′-GAATTCCTGCGAATAGGCTTCCGGCTGCC-3′	533	294, 259
12,406	HincII	MTND5	Sense: 5′-CAAATATCACTCTCCTACTTACAG-3′ Antisense: 5′-TGTAACGAACAATGCTACAG-3′	484, 218	702
16,133	KpnI	D-loop	Sense: 5′-TCAAAGCTTACACCAGTCTTGTAA-3′ Antisense: 5′-CCTGAAGTAGGAACCAGATG-3′	367, 124, 81	448, 124

MTND5, subunit 5 of NADH dehydrogenase.

$\dot{V}O_{2max}$ was 39.8 ± 8.2 mL·kg^{-1}·min^{-1}. The reported $\dot{V}O_{2max}$ for the SCON is within the range reported for sedentary young adults (17). The subjects in the SCON group were Americans and Canadians. Informed written consent was obtained from all subjects prior to any testing procedures. The subjects characteristics are presented in Table 1.

Maximal oxygen uptake ($\dot{V}O_{2max}$) measurements. The $\dot{V}O_{2max}$ of the athletes was determined when they were at their peak or from previous laboratory assessments of their training status by using incremental exercise tests on cycle ergometers or motor-driven treadmills which varied from laboratory to laboratory (20). The $\dot{V}O_{2max}$ of the SCON subjects was determined on a cycle ergometer (6).

DNA extraction and purification. Total DNA (nuclear and mitochondrial) was extracted from white blood cells or lymphoblastoid cell lines as previously described (9).

Polymerase chain reaction (PCR) amplification. Four RFLPs (Table 2) of the mtDNA molecule were amplified by PCR (2). PCR was performed in a DNA thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer Cetus, Norwalk, CT) using a 30-μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dATP, dGTP, dCTP, and dTTP, 0.3 μM of each forward and backward primers, 0.75 units of *Taq* polymerase (Perkin-Elmer Cetus), and 5 ng of total DNA. The amplification protocol was: 1) one cycle of denaturation at 95°C for 3 min, annealing at 55°C for 0.25 min, and extension at 70°C for 1.03 min; 2) 29 cycles of denaturation at 95°C for 0.25 min, annealing at 55°C for 0.25 min, and extension at 70°C for 1.03 min; and 3) one final 10-min elongation cycle at 72°C. Preventive contamination measures were taken by the inclusion of PCR reaction mixture without DNA (negative control) in every run of amplification. The primers used are identified in Table 2.

RFLP analysis. After each amplification 10 units of the enzyme generating the polymorphic site were added to the PCR product. Restriction digest conditions were those recommended by the enzyme manufacturer (New England Biolabs, Mississauga, Ontario, Canada). The digested fragments were separated by electrophoresis on 8% polyacrylamide gels. The gels were stained with ethidium bromide and photographed under ultraviolet light. mtDNA fragment sizes were determined relative to reference standards.

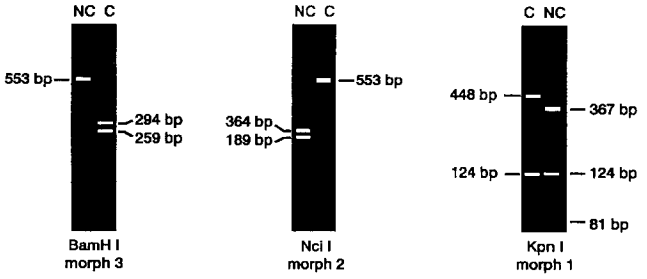

Figure 1—Restriction fragment patterns for carriers (C) and noncarriers (NC) of MTND5-*BamHI* RFLP at bp 13,470 (morph 3), MTND5-*NciI* RFLP at bp 13,364 (morph 2), and D-loop *KpnI* RFLP at bp 16,133 (morph 1). mtDNA fragment sizes are given in base pairs (bp).

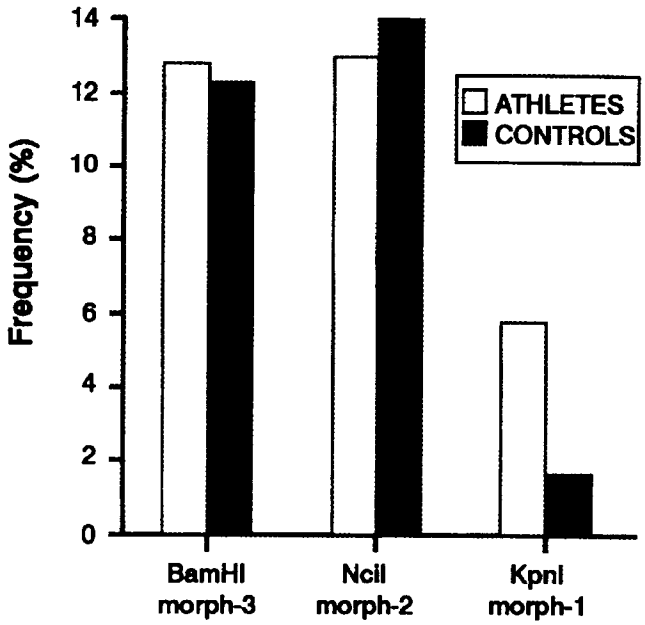

Figure 2—Relative frequencies (%) of three mtDNA sequence polymorphisms in elite endurance athletes and sedentary controls. No significant differences between athletes and controls ($P \geq 0.05$).

Statistical analysis. The Chi-square statistic test was used to compare the frequencies of each mtDNA morph between the EEA and SCON. When the expected frequency for a cell of the 2 × 2 contingency table was less than 5, the data were analyzed with the Fisher exact test (FET). The statistical power of each Chi-square test was estimated according to Cohen (8). For alpha = 0.05, the power of all tests was > 0.80. Statistical analyses were performed with SAS for Windows 6.08 package. P values < 0.05 were considered statistically significant.

RESULTS

Figure 1 illustrates the mtDNA restriction fragment patterns, also designated as morphs, which are of interest to this study. The prevalence of the mtDNA morphs in EEA and SCON is shown in Figure 2. No significant association ($P > 0.05$) was found between EEA and SCON for any of the RFLPs of the mtDNA molecule. The MTND5-NcI RFLP at bp 13,364 (morph 2) was found in 12.9% (16/124) of the EEA and 14% (8/57) of the SCON ($\chi^2 = 0.043$, $p = 0.83$). A similar result was observed for the MTND5-BamHI RFLP at bp 13,470 (morph 3), where 12.8% (16/125) of the EEA and 12.3% (8/65) of the SCON were carriers of the mutation ($\chi^2 = 0.009$, $p = 0.92$). The D-loop KpnI RFLP at bp 16,133 (morph 1) was found in 5.8% (7/121) of the EEA and in 1.6% (1/64) of the SCON (FET = 1.80, $p = 0.18$). These three RFLPs were not simultaneously observed in any of the EEA or SCON subjects. None of the EEA or SCON were carriers of the MTND5-HincII RFLP at bp 12,406 (morph 1). Previous work from our laboratory had detected this latter RFLP in Caucasians, but the prevalence was only 6% (9).

DISCUSSION

In the present investigation, three mtDNA polymorphisms were assessed in male EEA well endowed with the phenotypic expression of $\dot{V}O_{2max}$ and a group of SCON. This is the first genetic study that gathers a robust sample size of EEA (N = 125; $\dot{V}O_{2max}$ = 78.9 ± 3.8 $mL \cdot kg^{-1} \cdot min^{-1}$) and a SCON group ($\dot{V}O_{2max}$ = 39.8 ± 8.2 $mL \cdot kg^{-1} \cdot min^{-1}$) of male subjects to investigate the contribution of specific DNA sequence variants to EEA status. The two groups were strikingly different in terms of $\dot{V}O_{2max}$, which ranged from 22 to 50 $mL \cdot kg^{-1} \cdot min^{-1}$ in the SCON group and 73 to 93 $mL \cdot kg^{-1} \cdot min^{-1}$ in the EEA group. The finding of the present study was that the three RFLPs in mtDNA were not associated to EEA status. Therefore, these mutations are not likely to be important determinants of EEA status. Furthermore, the ancestral origin of the subjects in this study is not likely to be a confounding factor since the mtDNA RFLP frequencies were well within the range of those previously reported for Caucasians (4,9,14,21).

Considering that several major determinants such as O_2 delivery, O_2 transfer, and O_2 utilization are contributing to the interindividual differences in $\dot{V}O_{2max}$ (5), it cannot be excluded that some MTND5 variants may have functional consequences in sedentary subjects that may no longer be important for EEA. For instance, the mitochondrial content of skeletal muscle may play a major role among sedentary subjects (i.e., those with a high content could have a higher $\dot{V}O_{2max}$), whereas it is most likely that O_2 delivery is contributing even more to the differences in $\dot{V}O_{2max}$ between EEA and SCON.

In conclusion, there is no evidence for a difference in the frequency of the two RFLPs in the MTND5 gene and one in the D-loop region of mtDNA in elite endurance athletes versus sedentary controls. Because of the potential mtDNA contribution to aerobic-oxidative metabolism, other mtDNA mutations should be evaluated with respect to $\dot{V}O_{2max}$ and its response to endurance training. Along these lines, further studies should examine whether other mtDNA polymorphisms are associated with EEA status.

Thanks are expressed to Anne Marie Bricault, Manon Belair, Sonia Roy, and François Michaud for their technical assistance during the investigation. We are also grateful to Drs. N. Gledhill, G. Huber, E. Jakob, T. Noakes, G. Phillips, K. W. Rundell, and H. K. Rusko who kindly provided some of the DNA samples of athletes.

This work was supported by FCAR grant ER-2449, Natural Sciences and Engineering Research Council of Canada grant OPG 0042791, and National Heart, Lung and Blood Institute (HERITAGE Family Study) grants: HL45670, HL47323, HL47317, HL47327, and HL47321.

Miguel A. Rivera was supported by a Faculty Development program award of the University of Puerto Rico Medical School.

Address for correspondence: Claude Bouchard, Ph.D., Physical Activity Sciences Laboratory, PEPS, Laval University, Ste-Foy, Quebec, G1K 7P4, Canada. E-mail: claude.bouchard@edp.ulaval.ca.

REFERENCES

1. Anderson, S., A. T. Bankier, B. G. Barrell, et al. Sequence and organization of the human mitochondrial genome. *Nature* 290: 457–465, 1981.
2. Arnheim, N. and H. Erlich. Polymerase chain reaction strategy. *Annu. Rev. Biochem.* 61:131–156, 1992.
3. Berg, P. and M. Singer. *Dealing with Genes: The Language of Heredity*. Mill City, CA: University Science Books, 1992, p. 249.
4. Blanc, H., K.-H. Chen, M. A. D'Amore, and D. C. Wallace. Amino acid change associated with the major polymorphic Hinc II site of oriental and caucasian mitochondrial DNAs. *Am. J. Hum. Genet.* 35:167–176, 1983.
5. Bouchard, C., F. T. Dionne, J.-A. Simoneau, and M. R. Boulay. Genetics of aerobic and anaerobic performances. *Exerc. Sport Sci. Rev.* 20:27–58, 1992.
6. Bouchard, C., A. S. Leon, D. C. Rao, J. S. Skinner, J. H. Wilmore, and J. Gagnon. The HERITAGE Family Study: aims, design, and measurement protocol. *Med. Sci. Sports Exerc.* 27:721–729, 1995.
7. Clayton, D. A. Replication of animal mitochondrial DNA. *Cell* 28:693–705, 1982.
8. Cohen, J. *Statistical Power Analysis for the Behavioral Sciences*. Hillsdale, NJ: Lawrence Erlbaum Associates, 1988, pp. 215–271.
9. Dionne, F. T., L. Turcotte, M. C. Thibault, M. R. Boulay, J. S. Skinner, and C. Bouchard. Mitochondrial DNA sequence polymorphism, $\dot{V}O_{2max}$ and response to endurance training. *Med. Sci. Sports Exerc.* 23:177–185, 1991.
10. Foran, D. R., J. E. Hixon, and W. M. Brown. Comparisons of ape and human sequences that regulate mitochondrial DNA transcription and D-loop DNA synthesis. *Nucl. Acids Res.* 16:5841–5861, 1988.
11. Gollnick, P. D. and B. Saltin. Significance of skeletal muscle oxidative enzyme enhancement with endurance training. *Clin. Physiol.* 2:1–12, 1982.
12. Greenberg, B. D., J. E. Newbold, and A. Sugino. Intraspecific nucleotide sequence variability surrounding the origin of replication in human mitochondrial DNA. *Gene* 21:33–49, 1983.
13. Holloszy, J. O. and E. F. Coyle. Adaptations of skeletal muscle to endurance training and their metabolic consequences. *J. Appl. Physiol.* 56:831–838, 1984.
14. Johnson, M. H., D. C. Wallace, S. D. Ferris, M. C. Rattazzi, and L. L. Cavalli-Sforza. Radiation of human mitochondrial DNA types analyzed by restriction endonuclease cleavage patterns. *J. Mol. Evol.* 19:255–271, 1983.
15. Lesage, R., J.-A. Simoneau, J. Jobin, J. Leblanc, and C. Bouchard. Familial resemblance in maximal heart rate, blood lactate and aerobic power. *Hum. Hered.* 35:182–189, 1985.
16. Lortie, G., J.-A. Simoneau, P. Hamel, M. R. Boulay, F. Landry, and C. Bouchard. Responses of maximal aerobic power and capacity to aerobic training. *Int. J. Sports Med.* 5:232–236, 1984.
17. Ogawa, T., R. J. Spina, W. H. Martin III, et al. Effects of aging, sex, and physical training on cardiovascular responses to exercise. *Circulation* 86:494–503, 1992.
18. Prud'Homme, D., C. Bouchard, C. Leblanc, F. Landry, and E. Fontaine. Sensitivity of maximal aerobic power to training is genotype-dependent. *Med. Sci. Sports Exerc.* 16:489–493, 1984.
19. Strachan, T. and A. P. Read. *Human Molecular Genetics*. New York: Wiley-Liss, 1996, pp. 83–145.
20. Stromme, J. B., F. Inger, and H. D. Meen. Assessment of maximal aerobic power of specifically trained athletes. *J. Appl. Physiol.* 42:833–837, 1977.
21. Vilkki, J., M.-L. Savontaus, and E. K. Nikoskelainen. Human mitochondrial DNA types in Finland. *Hum. Genet.* 80:317–321, 1988.
22. Wallace, D. C., M. T. Lott, M. D. Brown, K. Huoponen, and A. Torroni. Report of the committee on human mitochondrial DNA. In: *Human Gene Mapping 1995: A Compendium*, A. J. Cuticchia (Ed.). Baltimore, MD: Johns Hopkins University Press, 1995, pp. 910–954.
23. Wilmore, J. H. and D. L. Costill. *Physiology of Sport and Exercise*. Champaign, IL: Human Kinetics, 1994, pp. 36–37.