J. Biochem. Mol. Biol. Vol. 30. No. 1. pp. 33~36 January 31. 1997

Multiple Age-Associated Mitochondrial DNA Deletions in Mouse Brain

Jinsun Kim, Minjung Kim, In Sook Kwon[1] and Eunsook Song*

Department of Biology, Sookmyung Women's University, Seoul 140-742
[1]National Institute of Agricultural Science and Technology, Division of Molecular Genetics, Suwon 441-707, Korea
(Received October 29, 1996)

Abstract : Age-dependent deletion of mitochondrial DNA (mtDNA) was detected in mouse brain using PCR method. The size of the deleted fragment was 0.5 kb, 0.9 kb, 1.7 kb and 4.3 kb in the region between cytochrome b gene and ATPase 6 gene. The deleted fragment was increased gradually from 3-month to 22-month. Direct repeat sequence flanking the deletion in 0.5 kb PCR product was TAAT.
Key words : aging, brain, mitochondrial DNA, multiple deletion.

Mitochondria are the cell organelles which supply most of the energy to the cell through oxidative phosphorylation. A mitochondrion has covalently closed circular DNA on which genes for 2 rRNAs, 22 tRNAs and 13 polypeptides for oxidative phosphorylation are located (Harman, 1972; Bibb *et al.*, 1981).

Aging is a biological process which drives cells into less efficient and less energetic states. As energy is thought to play a causative role in aging, mitochondria as energy producer can initiate the aging process (Torii *et al.*, 1992). Recently quite a few instances have been found in which mutation of mitochondrial DNA (mtDNA) is closely related with aging and age-associated diseases (Wallace *et al.*, 1988; Kadenbach, 1990; Corral-Debranski *et al.*, 1992; Ozawa, 1995). The effect of mutation can be diluted out by cell division, yet cells should suffer from it during its lifetime, once mutation occurs (Corral-Debranski, 1992). It is known that mutation rate is 16 times higher in mtDNA, because of less efficient repair system, than in nuclear DNA. Hence somatic mutation will affect genes on mtDNA more than on nuclear chromosome. As there are more than a thousand mitochondria in a cell, any mutation in mitochondria would not be revealed until significant portion is affected (Arnheim and Cortopass, 1992).

Consequently postmitotic tissue is more severely affected than mitotic tissue as cellular division can not efficiently dilute the somatic mutation which has been accumulated (Ikebe, 1990; Mecocci *et al.*, 1993).

In this report, we investigated mtDNA deletion in brain as this tissue is known as typical postmitotic tissue in relation to aging.

Materials and Methods

Sample

ICR mouse was originally purchased from KIST and fed ad libitum under natural condition in the animal room for its life time. It was sacrificed by spinal dislocation. Brain and liver were used immediately.

Preparation of mtDNA

Brain and liver were ground into powder in liquid nitrogen with mortar and pestle and mitochondria were prepared using the method by Zimmerman *et al.* (1988). 1 ml lysis buffer (120 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 1.2% SDS) was added to 10 mg washed mitochondria. Homogenized mixture was incubated at 37°C for 2 h. Then equal volume of phenol was added and DNA was extracted 3 times. After isopropanol precipitation, DNA was dried, solubilized in distilled water and stored at −20°C until use.

PCR analysis for deletion

PCR incubation mixture was purchased from Promega (Madison, USA) and mtDNA was added as template. Reaction was started by 10 min incubation at 95°C, denatured at 94°C for 1 min, annealed at 54°C for 1 min and synthesized at 72°C for 1 min for 35 cycles to detect deletion. Last extension was carried out

*To whom correspondence should be addressed.
Tel : 82-331-710-9417, Fax : 82-331-718-2337.
E-mail : eunsong@egret.sookmyung.ac.kr

at 72°C for 10 min. Ten microliter of PCR mixture was loaded on 1% agarose gel. Sense primer was L8231 and antisense primer was H14140. Fig. 1 shows the primer sequence we used. The location of these primers is indicated in Fig. 2.

DNA sequencing analysis

PCR products were purified by preparative agarose gel electrophoresis and treated with *E. coli* polymerase I and T_4 DNA kinase. The blunted and phosphorylated PCR products were cloned into the *Sma*I digested pBluescript (Stratagene, La Jolla, Calif.). Sequencing reactions were performed using alkaline denatured double-stranded DNA as a template, according to the manufacturer's instructions provided with the Sequenase Version 2.0 kit (Pharmacia Biotech, Uppsala, Sweden) with the pUC/M 13 universal or reverse primers.

Results and Discussion

Under the conditions we have employed (1 min for extension time), PCR products less than 5 kb were produced (Fig. 3B). If incubated for longer time (in this case, 4 min and 20 sec) at 54°C, normal 5.9 kb was obtained (Fig. 3A), which was expected from the primer sites (Fig. 2). As the age increased from 3 months to 22 months, increasing numbers of deletion bands were found in brain (only 1 PCR band at 3 month of age, while 3 more bands emerged at older age). In addition, the band intensity was gradually increased when the amount of PCR products was normalized with that of D-loop fragment amplified under same conditions.The multiple deletions are often observed in human (Cortopassi and Arnheim, 1990) and mouse (Chung *et al.*, 1994). Presently it cannot be determined whether they arise independently in different cells or in different mitochondria of a cell. Similar deletions appeared in liver mitochondria but only 2 products (0.5 kb and 0.9 kb) were obtained under the same conditions as brain. The deletion was detected in 7-month old mouse but not in 3-month old mouse (data not shown).

Though mutation seems to occur ubiquitously in mitochondria of most tissues, the onset time revealed on agarose gel may primarily depend on the critical amount of accumulated mutation. If the rate of somatic mutation is same in any instance in a cell, then the determining factor for mutation can be cell division rate. Since any type of tissue in an organism has its own cell division time and rate, the proportion of mutation in mtDNA will be higher in those tissues where cell division seldom occurs. Consequently, more mutation events will be found in long living cells (postmitotic tissue) than actively dividing, short living cells (mitotic tissue). The effect of mutation is more drastic in brain and muscle because defective mitochondria cannot be diluted out by continuous cell proliferation (Linnane *et*

	Primer site	Sequence
L8231	L8231-L8250 (ATPase 6)	5′-TAAGTATAGCCATTCCACTA-3′
H14140	H14140-H14120 (cyt b)	5′-ATTAGGTGTTTCTGTAGTTG-3′

Fig. 1. Primer sequence for detection of deleted mtDNA for PCR reaction.

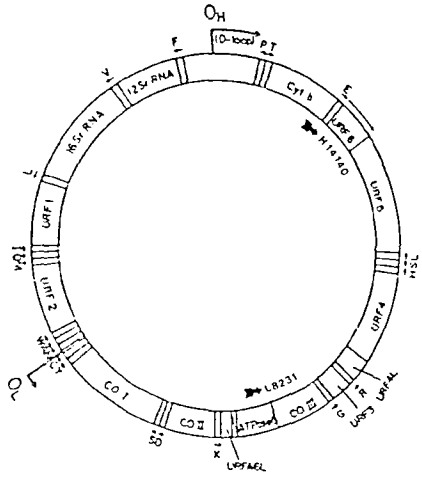

Fig. 2. DNA map of mouse mitochondria. Arrows are shown for primers to detect deletions.

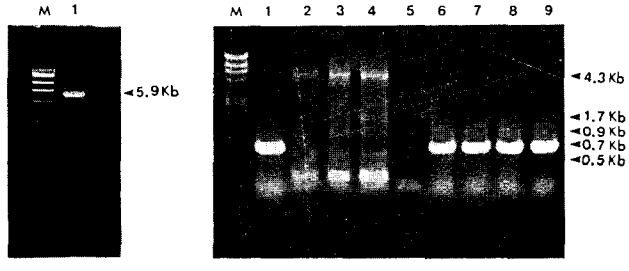

Fig. 3. Deletion product of mtDNA of mouse brain by PCR. (A) Normal PCR product. The reaction condition for PCR was same as that of deletion except synthesis time, which was 4 min and 20 sec. (B) Deletion was amplified by PCR. Lane M for lamda DNA digested by *Hin*dIII. Lanes 1, 2, 3, and 4 represent deletion PCR products from 3-, 7-, 12-, and 22-month old mouse brain, respectively. Lane 1 contains additional D-loop fragment amplified separately using D-loop specific primers and electrophoresed with deletion product from 3-month mouse brain. Lane 5 is negative control in which template mtDNA is not included. Lanes 6, 7, 8, and 9 are D-loop fragment produced by PCR to normalize the deletion products from 3-, 7-, 12- and 22-month old mouse brain, respectively.

ATTAGGTGTTTCTGTAGTTGATTTATAGTGAAATCATATTACTAGACCTG
ATGTTAGAAGGAGGGCTGAAAAGGCTCCAGTTAATGGTCATGGACTTGGA
TTAACTATGTGATATGCATGAGTTTGGTGGGTCATTATGTATTATCATGT
AGATATAGGCTTACTAGGAGGGTGAATACGTAGGCTTGAATTAATGCTAC
TGCAAATTCTAGAATTGTGAGTAGAAGTAGAATAATAAATGTAATGGTA
GCTGTTGGTGGGCTAATATTTATTAATACTAGAGTAGCTCCTCCGATTAG
GTGTATTAATAAGTGTCCTGCAGTAATGTTAGCTGTAAGCCGGTCTGCTA
ATGCCATTGGTTGAATAAATAGGCTAATTGTTTCAATAATAATAAGTATT
GGAATTAGTGAAATTGGAGTTCCTTGTGGAAGGAAGTGGGCAAGTGAGCT
TTTTAGTTTGTGTCGGAAGCCTGTAATTACGGCTCCAGCTCATAGTGGAT
GGCTATACTTA

Fig. 4. The DNA seqence of 0.5 kb deletion product. The 0.5 kb deletion product was amplified from 15 month-old mouse brain. electrophoresed and eluted for sequencing. The arrow is indicated where 5.4 kb deletion occurred.

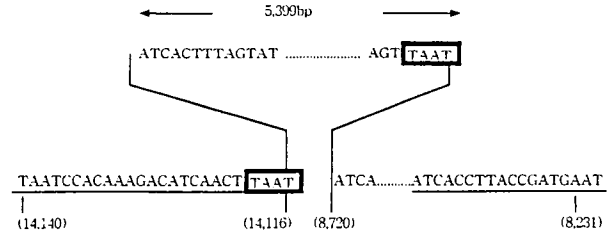

Fig. 5. The flanking sequence of 0.5 kb deletion product.

al.. 1992). Present results showing more deletions in brain mitochondria are consistent with this idea. Likewise this aspect may emerge as age-difference as shown in Fig. 3. We confirmed similar tendency in skeletal muscle which is also known as postmitotic tissue (unpublished observation). If mutation of mtDNA accumulates above threshold level. then the cell will not survive because of inefficient mitochondrial function. After a while. the proportion of affected cells in a tissue will be increased. which leads to tissue malfunctioning. This event may be called age-associated disease and aging (Ames and Gold. 1991). Ultimately death will result. Alternatively. shorter DNA fragments can be synthesized more efficiently than normal DNA under the same PCR reaction conditions.

Two short deletion products (0.5 kb and 0.9 kb) obtained from liver can be obtained.

Direct repeat sequence in deletion region

In most cases. direct repeat sequences ranging from 4 to 13 bases are associated with deletion in mitochondria. Common deletion occurs near between D-loop region and CO III gene. where subunit genes for oxidative phosphorylation are located (Bibb *et al.*, 1981). Most frequent deletion in human mitochondria in this region produces 4.977 bp deletion. which may be similar type to one of our deletions (5.4 kb deletion) obtained in this study with mouse tissue. The flanking sequence is TAAT direct repeat (Fig. 4 and 5), which has not been reported in mammalian tissues. near cyt b and COIII gene (Yuzaki *et al.*, 1989; Mita *et al.*, 1990). We have not yet determined the base sequences of the deletions other than 0.5 kb. Similar mechanisms such as slip-mispairing is assumed to be responsible for these deletions. As there are discrete deletion bands, recognition of specific site (or sequence) by enzymes for recombination may be a necessary step. Whether the enzymes or the activities of them are varied among various tissues are not known. But non-identical deletion mechanism may exist in different tissue type. as up to 25 deletion bands were found in aged mouse muscle. the flanking sequence of which did not contain direct repeat in most cases (Chung *et al.*, 1994). Alternatively. the event such as slip-mispairing between direct repeat sequences inside mitochondria or between neighboring mtDNA does occur more often in certain tissues. Further studies should be done to elucidate the responsible enzymes or factors.

Acknowledgement

This research was supported in part by Sookmyung Women's University Research Fund for 1996.

References

Ames, B. N. and Gold, L. S. (1991) *Mut. Res.* **250**, 3.
Arnheim, N. and Cortopassi, G. (1992) *Mut. Res.* **275**, 157.
Bibb, M. J., van Etten, R. A., Wright, C. T., Walberg, M. W. and Clayton, D. A. (1981) *Cell* **26**, 167.
Chung, S. S., Weindruch, R., Schwarze, S. R., McKenzie, D. I. and Aiken, J. M. (1994) *Aging Clin. Exp. Res.* **6**, 193.
Corral-Debranski, M. (1992) *Nature Genetics* **2**, 324.
Corral-Debranski, M., Shoffner, J. M., Lott, M. T. and Wallace D. C. (1992) *Mut. Res.* **275**, 169.
Cortopassi, G. A. and Arnheim, N. (1990) *Nucleic Acids Res.* **18**, 6927.
Harman, D. (1972) *J. Am. Geriatr. Soc.* **20**, 145.
Ikebe, S. (1990) *Biochem. Biophys. Res. Commun* **170**, 1044.
Kadenbach, B. (1990) *Naturwissenschaften* **77**, 221.
Linnane, A. W., Zhang, C., Baumer, A. and Nagley, P. (1992) *Mut. Res.* **275**, 195.
Mecocci, P., Macgarvey, U., Kaufman, A. E., Koontz, D., Shoffner, J. M., Wallace, D. C. and Beal, M. F. (1993) *Ann. Neurol.* **34**, 609.
Mita, S., Rizzuto, R., Moraes, C. T., Shanske, S., Arnaudo, E., Fabrizi, G. M., Koga, Y., DiMauro, S. and Schon, E. A. (1990) *Nucleic Acids Res.* **18**, 561.
Ozawa, T. (1995) *Exp. Gerontol.* **30**, 269.

Torii, K., Sugiyama, S., Takagi, K., Satake, T. and Ozawa, T. (1992) *Am. J. Respir. Cell. Mol. Biol.* **6**, 88.

Wallace, D. C., Zhang, X., Lott, M. T., Shoffner, J. M., Hodge, J. A., Kelley, R. I., Epstein, C. M. and Hopkins, L. C. (1988) *Cell* **55**, 601.

Yuzaki, M., Ohkoshi, N., Kanazawa, I., Kagawa, Y. and Ohta, S. (1989) *Biochem. Biophys. Res. Commun.* **164**, 1352.

Zimmerman, E. G., Akins, D. R., Planz, J. V. and Schurr, M. J. (1988) *Gene Anal. Tech.* **5**, 162.