59. *DNA Amplification from Ancient Human Skeletal Remains and Their Sequence Analysis*

By Satoshi HORAI,[†),*)] Kenji HAYASAKA,[*)] Kumiko MURAYAMA,[*)] Noriyuki WATE,[**)] Hiroko KOIKE,[**)] and Nobuyuki NAKAI[***)]

(Communicated by Motoo KIMURA, M. J. A., Dec. 12, 1989)

Introduction. The development of polymerase chain reaction (PCR) makes it possible to amplify DNA of aimed region from a very small amount of it. By applicating this technique for archaeological or ancient samples, it was successful to amplify DNA and analyze a part of the nucleotide sequences only in case of using soft-tissues such as frozen, mummified or stuffed tissue, haired skin and muscular tissue.[1)-5)] Such samples generally have been preserved artificially or accidentally. Most of those preserved to this day as human remains are hard-tissues, bones. So if we can amplify DNA extracted from bones and analyze them, we are likely to get some quite important information to explain the process of human evolution, divergence of human races, restoration of human populations of the past, and their migration or dispersal.

We used the samples dated from the first term of the Showa Era (60 years B.P.) back to the far past, and at last we succeeded to amplify DNA extracted from a human skull whose age is estimated at about 6000 years B.P. and determine the nucleotide sequence. It is the noncoding region of mitochondrial DNA (mtDNA) that we amplified and sequenced. It is known as the region whose evolutionary rate is several times as high as other parts of mtDNA.[6)-8)] Furthermore, mtDNA is evolved several times as rapidly as nuclear DNA.[9)] So this is a very suitable region to study about individual variations. Concerning the region, we determined and compared the nucleotide sequences for over a hundred of individuals including contemporary Japanese, Asians, Caucasians and Africans.[6),10)]

Materials and method. *DNA extraction from ancient bones.* The surfaces of bone samples (0.5 to 1 g) were washed with 0.1 N-NaOH and then rinsed with ultra-pure double distilled water. The bone fragments were ground to about 1 mm^3 particles by the dental drill. The bone powders were placed into a visking tube and decalcified with several changes of 1 N-HCl solution, and dialyzed against TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After centrifugation of the dialyzates, the supernatants were concentrated by the macrosolute concentrator Minicon-B (Amicon) to the volume of 3 ml and the DNA was extracted three times with phenol : chloroform (1 : 1). The extracted DNA was further concentrated by centrifugation-driven dialysis using Centricon 30 (Amicon).

PCR amplification of mtDNA from ancient and contemporary DNAs. A fragment of mtDNA was amplified from 10 μl of the extracted DNA in 100 μl of reaction mixture containing 200 μM of each of four dNTPs, 0.2 μM of each

†) To whom all correspondence should be addressed.
*) Department of Human Genetics, National Institute of Genetics, 1,111 Yata, Mishima, Shizuoka 411, Japan.
**) College of Liberal Arts, Saitama University, 255 Shimo-okubo, Urawa, Saitama 338, Japan.
***) Faculty of Science, Nagoya University, Chigusa-ku, Nagoya, Aichi 464, Japan.

primer, 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.01% gelatin.[11] A 19-base and a 20-base oligonucleotide primers were synthesized by using the Applied Biosystem model 380B DNA synthesizer and had the following sequences: primer A= 16190–5′–CCCCATGCTTACAAGCAAG–3′–16208; primer B=16422–5′–ATTGATTTCACGGAGGATGG–3′–16403 (the notation of Anderson *et al.* (1981) for base numbers).[12] A 233-bp fragment of mtDNA in the major noncoding region is amplified by using these primers. The PCR were carried out for a total of 30 cycles with the use of a Thermal Cycler (Perkin Elmer Cetus). The cycle times were as follows: denaturation, 10 sec at 94°C; annealing, 10 sec at 45°C; primer extension, 15 sec at 72°C. The amplified fragments were separated by electrophoresis on 1.5% agarose gels and detected fluorographically after staining with ethidium bromide. For the second PCR, one-tenth of the first PCR products (10 μl) were used as templates and performed the PCR exactly the same manner as the first PCR. The direct sequencing from the agarose extracts of the second PCR products[13] was performed with primer B and the Sequenase sequencing Kit (US Biochemical Corp.).

Results and discussion. We have tried to amplify mtDNA from 22 samples of human bones derived from different eras. In the first PCR we could amplify DNA only in the positive control as shown in Fig. 1a. However, when we took

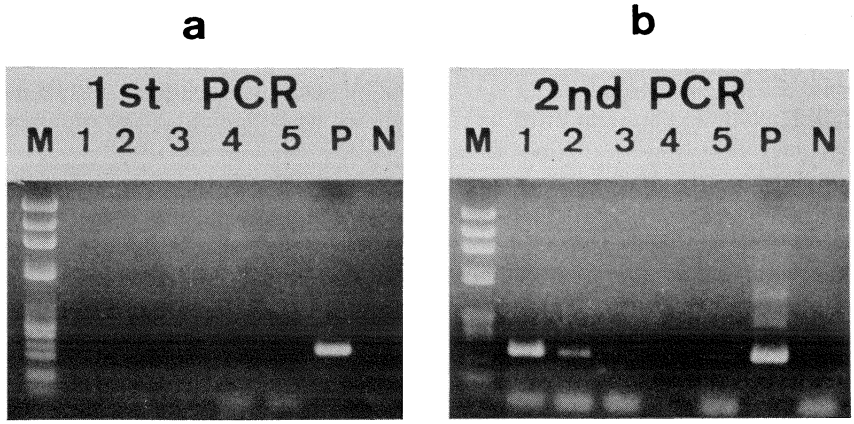

Fig. 1. Agarose gel electrophoresis of PCR amplified mtDNAs from ancient and contemporary DNA. Lane M contains the size marker ϕX174 digested with HaeIII. Lanes 1 to 5 contain samples extracted from bones. Lane 2 is the extraction of Urawa-1. Lane P contains a contemporary DNA. Lane N is a blank control with no DNA. a; the first PCR products and b; the second PCR products, respectively.

the portion of the first PCR products and performed the second 30-cycle PCR, we could amplify DNA in some samples of bone extracts. But no band can be seen in the negative control even in the second PCR (Fig. 1b). This negative control is very crucial to check the contamination of contemporary DNA during the experimental procedure as well as to evaluate the appropriate combination of primers. Because in some primer combinations positive signals were obtained in the negative control without template DNA after the second PCR. Although

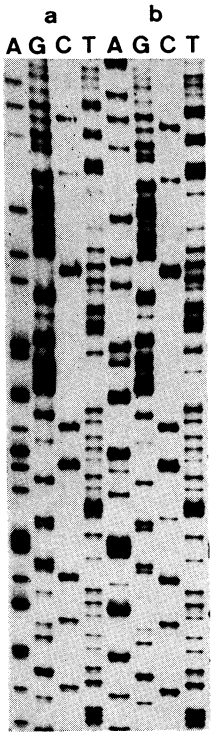

Fig. 2. Sequence of PCR amplified mtDNA. Autoradiogram of a 6% polyacrylamide gel containing the sequence of part of the noncoding region. a; amplified DNA from a contemporary Japanese. b; amplified DNA from Urawa-1.

```
          16190
            |                                                    50
Anderson  CCCCATGCTT ACAAGCAAGT ACAGCAATCA ACCCTCAACT ATCACACATC
Urawa-1   .......... .......... .......... ...T...... ..........

                                                               100
Anderson  AACTGCAACT CCAAAGCCAC CCCTCACCCA CTAGGATACC AACAAACCTA
Urawa-1   .......... .......... .......... .......... ..........

                                                               150
Anderson  CCCACCCTTA ACAGTACATA GTACATAAAG CCATTTACCG TACATAGCAC
Urawa-1   .T........ .......... .......... .......... ..........

                                                    190
Anderson  ATTACAGTCA AATCCCTTCT CGTCCCCATG GATGACCCCC
Urawa-1   .......... .......... ..C....... ..........
```

Fig. 3. Nucleotide sequences of the 190-bp region. The sequence reported by Anderson *et al.* (1981) is shown in the upper lines with the initial base number. Base substitution observed in Urawa-1 are shown by letters in lower lines. Dots indicate the identity of nucleotides between the reference sequence and the sequence from Urawa-1.

we could amplify DNA by the second PCR from the majority of samples, it was impossible to amplify DNA in some samples, which are probable due to the condition of preservation of bones. However, we succeeded to amplify mtDNA from the oldest one of them, a skull named Urawa-1, excavated from five meters deep ground in Urawa city, the central part of Japan, in 1988.

Using collagen which was simultaneously isolated from this skull, the absolute age of the Urawa-1 is estimated at 5790±120 years B.P. by the direct detection of ^{14}C using accelerator mass spectrometry.[14),15)] This age is equivalent to the early phase of Jomon-period in the Japanese prehistory. We determined the

nucleotide sequences from the second PCR product of Urawa-1 by the direct sequence method as shown in Fig. 2. We observed nucleotide substitutions at three positions when we compared a 190-bp region sequence determined for Urawa-1 with that originally reported by Anderson *et al.* (1981)[12] as shown in Fig. 3.

Recently, we have determined nucleotide sequences of the major noncoding region of mtDNA from 95 humans from three racial groups.[6] These sequences include a 482-bp long region encompassing most part of the D-loop forming region of mtDNA. Comparison of these sequences with six sequences previously determined[7),12] have revealed remarkable features of nucleotide substitutions and insertion/deletion events. The nucleotide diversity among the sequences is four-fold higher than the corresponding value estimated from restriction enzyme analysis of whole mtDNA genome. The PCR amplified region of mtDNA with the two primers in the present study was completely included in the 482-bp long region mentioned above.

The nucleotide sequence from Urawa-1 was compared with those from 101 contemporary humans and six additional sequences determined for individuals from Southeast Asia.[10] Table I summarizes number of nucleotide differences

Table I. Sequence differences between Urawa-1 and each of 107 contemporary humans in the 190-bp region (bp 16190–16379)

	Polymorphic position*			race	No. of nt differences			
					0	1	2	3–8
Urawa-1	16223	16291	16362	J	0	14	8	39
				M	2	1	0	13
				C	0	0	1	19
				N	0	0	0	10

J, M, C and N stand for Japanese, non-Japanese Mongoloids, Caucasoids and Negroids, respectively. * nocleotide positions which are different from those in the reference sequence reported by Anderson *et al.* (1981).

between the sequences from Urawa-1 and those from each of the 107 individuals in the 190-bp region. The three nucleotide positions which are different from the reference sequence of Anderson *et al.* (1981) are also presented in Table I. In comparing the sequence from Urawa-1 with those from 107 individuals, we found the complete identity in two Southeast Asians (a Malay and an Indonesian) out of 16 non-Japanese Asians. However, we never observed the identical sequence to Urawa-1 among the 61 contemporary Japanese. Namely, 14 Japanese differed at one position and 8 Japanese differed at two positions. Furthermore, 39 Japanese differed at three to eight positions even in this short region. These observations indicate that the ancestor of Japanese who presumably lived in the central part of Japan about 6000 years ago had the common origin with the some contemporary Southeast Asians. It is striking that the 61 contemporary Japanese do not show the complete identity of sequence, though about one-third of individuals exhibited only one or two nucleotide differences in the 190-bp region.

Restriction enzyme analysis revealed that the Japanese population could be separated into at least two distinct groups: a group with the smaller frequency (Group I) first diverged from the other group with the larger frequency (Group II).[16)–18] This is also confirmed by our recent sequence analysis of the major

noncoding region for Mongoloids including non-Japanese Asians.[6] In this connection the ancient Japanese (Urawa-1) belongs to one of the two groups (Group II) of the modern Japanese. Part of people who migrated from the continent during the period of two to three thousand years ago may be representative of the other group (Group I) of the modern Japanese, because the sequences from Group I individuals showed three to eight nucleotide differences in the 190-bp region in comparison with that from Urawa-1. Although our finding was obtained through determining nucleotide sequence from one ancient individual, this archaeological and molecular genetical study gives a new source of perspectives on the evolutionary history of human populations.

Acknowledgments. This work was supported by Grants-in-Aids for General Scientific Research, and Scientific Research on Priority Areas of Bioenergetics, Development of Evolutionary and Population to Genetics Incorporating Newer Molecular Findings, and From Asia to America: Prehistoric Mongoloid Dispersal to S.H. from the Ministry of Education, Science and Culture, Japan.

References

1) Higuchi, R., and Wilson, A. C. (1984): Fed. Proc., **43**, 1557.
2) Pääbo, S. *et al.* (1988): Nucleic Acids Res., **16**, 9775–9787.
3) Pääbo, S. (1989): Proc. Natl. Acad. Sci. U.S.A., **86**, 1939–1943.
4) Higuchi, R. *et al.* (1984): Nature, **312**, 282–284.
5) Pääbo, S. (1986): ibid., **314**, 644–645.
6) Horai, S., and Hayasaka, K. (1990): Am. J. Hum. Genet. (in press).
7) Greenberg, B. D. *et al.* (1983): Gene, **21**, 33–49.
8) Aquadro, C. F., and Greenberg, B. D. (1983): Genetics, **103**, 287–312.
9) Brown, W. M. *et al.* (1979): Proc. Natl. Acad. Sci. U.S.A., **76**, 1976–1971.
10) Horai, S. *et al.*: (unpublished).
11) Saiki, R. K. *et al.* (1988): Science, **239**, 487–791.
12) Anderson, S. *et al.* (1981): Nature, **296**, 457–465.
13) Gyllensten, U. B., and Erlich, H. A. (1988): Proc. Natl. Acad. Sci. U.S.A., **85**, 7652–7656.
14) Nakai, N. *et al.* (1984): Instr. and Meth., **233**, 171–174.
15) Nakamura, T. *et al.* (1985): Japan J. Appl. Phys., **24**, 1716–1723.
16) Horai, S., and Matsunaga, E. (1986): Hum. Genet., **72**, 105–117.
17) Horai, S. *et al.* (1986): Jpn. J. Genet., **61**, 271–275.
18) —— (1987): Human Genetics (eds. Vogel F., and Sperling, K.). Proceeding of the 7th International Congress, Springer-Verlag, Heidelberg, pp. 177–181.