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Genetics

Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification

(oxidative damage/electron microscopy/*Alu* sequences/mitochondrial DNA/polymerase chain reaction)

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ABSTRACT **Several chemical and enzymatic properties were examined in the DNA extracted from dry remains of soft tissues that vary in age from 4 to 13,000 years and represent four species, including two extinct animals (the marsupial wolf and giant ground sloth). The DNA obtained was invariably of a low average molecular size and damaged by oxidative processes, which primarily manifest themselves as modifications of pyrimidines and sugar residues as well as baseless sites and intermolecular cross-links. This renders molecular cloning difficult. However, the polymerase chain reaction can be used to amplify and study short mitochondrial DNA sequences that are of anthropological and evolutionary significance. This opens up the prospect of performing diachronical studies of molecular evolutionary genetics.**

DNA from ancient organic remains has been extracted in a number of cases (e.g., see refs. 1–5). On two occasions, molecular cloning made it possible to obtain DNA sequences from such remains (6, 7). However, a serious concern pertinent to the study of ancient DNA is the occurrence of postmortem damage in DNA extracted from archaeological specimens, since such damage may make impossible the application of many molecular biological techniques and/or cause erroneous sequence information to be obtained (8, 9).

To make general statements about the state of preservation of DNA in ancient dry remains of soft tissues, I have extracted nucleic acids from 12 specimens representing a wide variety of geographical regions and different time periods. Here I report the nature of the chemical modifications present in these DNA samples and show that, whereas these modifications render molecular cloning techniques difficult and possibly error prone, the recently developed polymerase chain reaction (PCR) is likely to produce reliable sequence information from many ancient tissue remains. This has wide implications in that it opens up the possibility of using museum specimens as well as archaeological finds to address questions of historic, evolutionary, and taxonomic significance.

MATERIALS AND METHODS

Samples. Twelve specimens (A–L) were used for DNA extraction: A, a 4-year-old piece of dried pork from Løten, Norway; B, a piece of skin from a stuffed marsupial wolf (*Thylacinus cynocephalus*), kept since 1869 in the Zoological Museum of the University, Zürich; C, a 13,000-year-old skin from a ground sloth (*Mylodon*) originating from the Ultima Esperanza Cave, Chile, now in the British Museum (Natural History), London; D, skin from a natural mummy from Sayala, Egyptian Nubia, 6th–12th century AD (body K9/7); E, same as D (body K13/2); F, skin from a 5000-year-old natural mummy from Gebelein, Egypt (British Museum, no. 32754) (10); G, same as F (no. 32753); H, same as F (no. 32755); I, colon tissue from Egyptian mummy ROM I, *ca.* 1200 BC, in the Royal Ontario Museum, Toronto (11); J, large organ package, found to the left in the chest cavity of Egyptian mummy ÄS73b, 3rd century BC, belonging to the Institute for Anthropology and Human Genetics, University of Munich (12); K, a mummified human head from Peru, 4th–5th century AD (Las Trancas 83, new number 606), in the Institute for Anthropology and Human Genetics, University of Munich (13); L, liver tissue from canopic jar of Egyptian mummy 21470, 20th–19th century BC, in the Manchester Museum, Manchester, U.K. (14).

DNA Extraction and Miscellaneous Methods. DNA was extracted by a modification of the Blin and Stafford procedure (15) that has been described by Pääbo *et al.* (16). After ethanol precipitation, a brown contaminant, probably representing Maillard products of reducing sugars (17), was removed from the nucleic acids by centrifugation through a 10–40% sucrose gradient in 10 mM Tris·HCl, pH 8.0/1 M NaCl (18) at 30,000 rpm (100,000 × *g*) (15°C for 24 hr). The eluted fractions were ethanol precipitated and resuspended in 50 μl of Tris salt buffer. Fractions containing nucleic acids were pooled from the middle third of each gradient. The brown contaminants remained on top of the gradients. For the extracts used for enzymatic amplification, ethanol precipitation and sucrose gradients were replaced by centrifugation on Centricon 30 filters (Amicon) to minimize losses of DNA. In addition, control extractions were performed in an identical manner except that no tissue was added to the extraction mixture.

Agarose gels, ethidium bromide staining, nick-translation of the probe containing an *Alu* repeat (19), end-labeling (without prior phosphatase treatment), precipitation with trichloroacetic acid (TCA), hybridization, and autoradiography were by standard techniques (18). Hydrolysis of DNA and high-performance liquid chromatography (HPLC) of the bases were performed as described (20).

Quantitation of the ancient DNA was performed by an ethidium bromide dot assay (18) as well as estimations from ethidium bromide-stained gels. Determinations of concentrations by absorbance at 260 nm proved impossible to perform in most extracts because of an unknown component that exhibited peak absorption at ≈215 nm. All determinations of ancient DNA concentrations should be regarded as tentative since it is not known how the lesions present in the DNA affect its ability to intercalate fluorescent dyes.

Alkali sensitivity was assayed by adding 0.5 μg of salmon sperm DNA to the end-labeled sample followed by NaOH to a final concentration of 0.3 M. Incubation was at room

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Abbreviations: PCR, polymerase chain reaction; TCA, trichloroacetic acid; endo III and IV, endonucleases III and IV; AP, apurinic/apyrimidinic.

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temperature overnight. Endonuclease IV (endo IV) digestion was performed in 10 mM NaCl/50 mM Hepes, pH 7.8/5 mM dithiothreitol; endonuclease III (endo III) digestion was in 100 mM KCl/50 mM Hepes, pH 7.8/1 mM EDTA/1 mM dithiothreitol; and uracil-DNA glycosylase digestion was in 70 mM Hepes, pH 7.8/1 mM EDTA/1 mM dithiothreitol. All enzymatic digestions were for 1 hr at 37°C. In the case of uracil-DNA glycosylase, NaCl was then added to a final concentration of 100 mM and endo IV was added for an additional incubation of 1 hr. Before TCA precipitation, 1 μg of salmon sperm DNA was added to the digests. Control digestions were performed as described above except that no enzyme was added to the incubation mixtures. The purification of endo IV (21), endo III (22), and uracil-DNA glycosylase (23) has been described.

Electron microscopy was performed essentially as described (24).

Molecular Cloning and Enzymatic Amplification. Mung bean nuclease treatment was performed according to the manufacturer's directions. Cloning in pUC19 (25) was done by standard techniques; isolated supercoiled plasmid DNA was sequenced by the dideoxynucleotide chain-termination method (26).

Enzymatic amplification by the PCR (27) was performed as described (16) using heat-resistant *Thermus aquaticus (Taq)* DNA polymerase (28). The primers used to amplify and sequence human mitochondrial DNA were as follows: D3E, 5′-GCGAATTCCTAGTGGGTGAGGGGTGGC-3′ 16255; D18X, 5′-GCTCTAGACCATGCTTACAAGCAAGT-3′ 16209; cytb2, 5′-AAACTGCAGCCCCTCAGAATGATATT-TGTCCTCA-3′ 15149; M14725(H), 5′-CGAAGCTTGATAT-GAAAAACCATCGTTG-3′ 14724. Numbers at 3′ ends refer to Anderson *et al.* (29). Underlined sequences were added to the 5′ ends to create restriction sites. Primers A and B, specific for region V of the mitochondrial genome, have been described (30). Single-stranded template for sequencing was generated by the unbalanced primer method (31).

RESULTS

DNA Extraction. DNA was prepared from 0.1–0.5 g of the samples and 8% of each extract was analyzed by agarose gel electrophoresis. In all extracts discussed here and in >90% of all extracts prepared from well-preserved desiccated tissues, DNA could be visualized by ethidium bromide staining of agarose gels (Fig. 1). The DNA invariably proved to be degraded to an average molecular size of 100–200 base pairs (bp) with substantial amounts of DNA migrating in the range of 40–500 bp. The yield of DNA as determined by ethidium bromide fluorescence varied between 1 and 200 μg per g of dry tissue. The majority of the samples seemed to be double-stranded, since they stained green with acridine orange (ref. 2; data not shown). However, when the amount of human DNA in the extracts of ancient human tissue was determined by probing aliquots of the extracts, which had been immobilized on nitrocellulose filters, with a nick-translated probe containing a human *Alu* repeat, the amounts of extracted human DNA varied between 0 and 200 ng per g of tissue—i.e., 3 orders of magnitude lower (data not shown). Since one explanation for this discrepancy could be a substantial amount of damage occurring in the ancient DNA, the extracted DNA samples were analyzed for base damage.

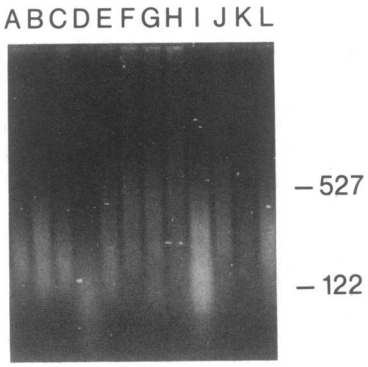

FIG. 1. Agarose gel electrophoresis of DNA extracted from the remains of 12 old dry tissues. The gel contained 2% agarose and DNA was visualized by ethidium bromide. Letters refer to the samples analyzed (see *Materials and Methods*). Migration positions of molecular size markers are indicated in bp.

DNA Damage. Approximately 400 ng of DNA from each of the extractions was hydrolyzed under acid conditions and the released nitrogenous bases were analyzed by reversed-phase HPLC. As shown in Fig. 2, a sample extracted from 4-year-old dried porcine muscle contained the four unmodified bases as well as two additional minor peaks. This chromatographic picture was indistinguishable from a DNA sample extracted from a human cell line. In contrast, the ancient DNA samples showed a drastically different pattern. Whereas adenine and guanine could still be resolved in relative amounts approximately similar to those present in the contemporary sample, cytosine and thymine were present in greatly reduced amounts, the latter at <5% of the expected amount. In addition, a number of new peaks appeared in the analyses of the ancient samples. Most of these peaks eluted early in the chromatogram and presumably represent ring-fragmented and/or ring-saturated pyrimidine derivatives, which are known to elute early on reversed-phase HPLC analysis (e.g., see ref. 32).

Since pyrimidines, in particular thymine, are known to be substantially more sensitive to oxidative damage than purines (33), it was speculated that a large proportion of the base modifications observed might be accounted for by oxidative damage. Therefore, aliquots of the extracted DNA samples were end-labeled and analyzed for lesions by treatment with

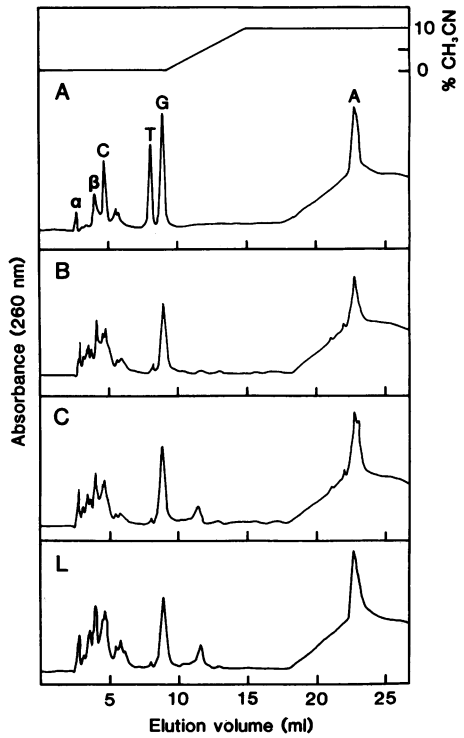

FIG. 2. HPLC analysis of hydrolyzed DNA. Samples analyzed were four-year-old dried pork (*A*), skin of marsupial wolf (*B*), skin of ground sloth (*C*), 4000-year-old mummified liver (*L*). α and β, injection artifacts.

alkali or various enzymes. TCA precipitations were then performed to determine the extent of sensitivity of the DNA samples to the various treatments. Representative results are given in Table 1. The 4-year-old pork sample was not sensitive to alkali to any greater extent than a contemporary salmon sperm DNA. However, the ancient DNAs were highly alkali sensitive. Since oligonucleotides >20 bases long can be precipitated by TCA (18), it was estimated that more than one alkali-sensitive site per 20 bp must exist in the ancient DNA.

Alkali-sensitive sites in DNA can be caused by apurinic/apyrimidinic (AP) sites as well as by modifications of sugar residues (34). To distinguish between these classes of lesion, the samples were further assayed by endo IV, an enzyme specific for AP sites (21). Approximately half of the labeled molecules were shown to be sensitive to endo IV and thus contain AP sites. The remainder of the alkali-sensitive sites are inferred to represent modified sugar residues. Furthermore, the DNA samples were analyzed by endo III. This enzyme specifically removes 5',6'-saturated pyrimidine products (35) from DNA creating AP sites, which are then susceptible to β-elimination promoted by endo III. All ancient DNA samples proved to be highly sensitive to the action of endo III, whereas the contemporary pork sample was essentially resistant. Therefore, at least 1 pyrimidine in 10 was modified in the ancient DNA. In contrast, the ancient DNA was to only a limited and variable extent sensitive to the action of uracil-DNA glycosylase, an enzyme that removes uracil from DNA, creating AP sites susceptible to endo IV. This indicates that deamination of cytosine has played only a minor role in the postmortem modification of these DNA samples. Taken together, these data show that a high amount of oxidative damage is present in old DNA.

To further analyze possible damage in the ancient DNA, one sample was subjected to electron microscopy. In Fig. 3, representative results are shown. It can be seen that the vast majority of the molecules are double stranded and relatively small. Based on an average length of 3.4 Å per base pair (36), the majority of the DNA molecules are ≈100 bp long, even if occasional molecules up to 1500 bp long can be found. Furthermore, DNA–DNA cross-links are present in rather high frequency as well as occasional complex structures, which apparently consist of large amounts of cross-linked and condensed DNA (data not shown).

Molecular Cloning. Molecular cloning was performed on DNA extracted from the ≈4000-year-old liver of Nekht-ankh, an Egyptian priest of the 12th Dynasty (sample L; ref. 14). In different experiments, 1–5 μg of ancient DNA was made blunt-ended and ligated to 25 ng of a plasmid vector (pUC19) that had been cut with *Sma* I and treated with phosphatase. Aliquots of the ligation mixture were transformed into highly competent JM101 bacteria. Approximately 500 clones per μg of insert DNA were obtained. The insert size among 24 clones picked at random varied between 40 and 270 bp, with a mean of 90 bp. When the clones were probed with a human *Alu* repeat, several positive clones were identified, four of which were sequenced. As illustrated in Fig. 4, the inserts of these clones were of an average length of 53 bp and displayed a high degree of similarity to an *Alu* consensus sequence. In fact, of the 11 single nucleotide positions at which these clones differ from the consensus sequence, 10 are known to occur frequently in *Alu* repeats (38). Furthermore, the insertion of the dinucleotide CT at position 64 of the consensus sequence is diagnostic of a class of *Alu* sequences (37). Thus, no evidence of major postmortem changes can be seen in the DNA sequences of these clones. However, clone 11:2 carries a T at position 30, which is not common among *Alu* repeats, and clone 12:2 is truncated from the 5' end at approximately position 25 and joined to a sequence that displays no similarity to an *Alu* sequence. Even if the latter situation is not unprecedented (39), these features could represent cloning artifacts.

Table 1. Analyses of lesions present in ancient DNA samples

Treatment	Percentage of TCA precipitable DNA		
	A	D	L
Alkali	74	5	11
Endonuclease IV	98	37	49
Endonuclease III	81	8	7
Uracil-DNA glycosylase + endonuclease IV	86	35	55

Aliquots of samples A (4-year-old pork), D (≈1000-year-old mummy), and L (≈4000-year-old liver) were end-labeled and subjected to indicated treatments. Then, TCA precipitations were performed. Numbers indicate the fractions of radioactivity that were precipitable when compared to mock-treated samples.

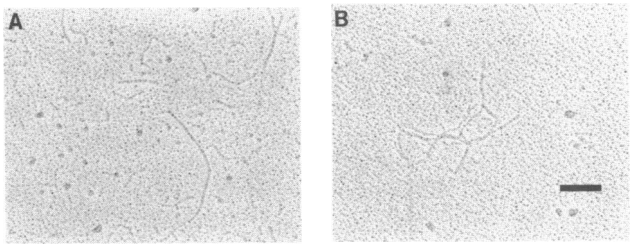

FIG. 3. Electron microscopy of DNA extracted from the 4000-year-old liver of Nekht-ankh. An aliquot of the sucrose-gradient purified DNA (sample L) was prepared by a modified magnesium method (24) and shadowed with carbon/platinum evaporated from an electron gun. (Bar = 105 nm.)

Enzymatic Amplification. Approximately 1 μg of DNA extracted from the same sample used for molecular cloning was subjected to enzymatic amplification by PCR. To overcome an inhibitory activity present in the ancient DNA extract, large amounts of the *Taq* polymerase had to be added to the amplification reaction mixtures (cf. ref. 16). Mitochondrial DNA sequences were chosen for amplification since mitochondrial DNA polymorphisms are highly suited for anthropological studies (40) and because mitochondrial genomes occur at a high copy number in most cells, which may facilitate their survival and detection. Three pairs of primers specific for various segments of the mitochondrial genome were used. These primers are expected to amplify DNA segments of 84, 121, and 471 bp.

Fig. 5 shows an electrophoretic analysis of the amplification products after 40 cycles of PCR. A single specific band of the expected size was resolved as well as a dimer of the primers used in the case of the 84- and 121-bp-long amplifications from the ancient extract. The high enzyme concentration present caused additional nonspecific amplification products to arise in some of the amplifications performed from control extracts, where no tissue had been added to the extraction buffer. These products do not contain the relevant DNA sequence and are not observed when small quantities of human DNA, containing adequate templates, are added to the extract (data not shown). Also shown in Fig. 5 are analogous amplifications from 1 ng of contemporary human DNA. It can be seen that the 84-bp and the 121-bp-long amplifications from ≈1 μg of the ancient extract yielded specific products in approximately the same amounts as did 1 ng of contemporary DNA. In the case of the 471-bp-long product, only the contemporary DNA yielded any product. In fact, the longest amplification product that could be generated from this extract was 140 bp long. Thus, the size reduction of the DNA (Fig. 1) and the oxidative damage present are setting limits to the size of the fragments that can be amplified.

Alu sequences:

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                  10        20        30        40        50        60        70        80
Consensus GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCGGATCAC--GAGGTCAGGAGATC
   11:2                              T.....................A...A.............
   12:2         .ATCA.TAAC.C..AAAAG..T.....................................
   12:4                              ..........................T......T.........
   12:5       .A...A..........A.........................T.....A..A.......CT....
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D loop:

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 Reference 16210-ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAA-16254
Nekht-ankh        ............T.........................C.....
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Region V:

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 Reference 8216-CTAGAATTAATTCCCCTAAAAATCTTTGAAATAGGGCCCGTATTTACCCTATAGCACCCCCTCTACCCCCTCTAGAGCCCA-8296
Nekht-ankh        ..................................A.........................................
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FIG. 4. DNA sequences obtained by molecular cloning and enzymatic amplification from the 4000-year-old liver of Nekht-ankh. (*Upper*) The four clones of *Alu* repeats are compared to relevant parts of an *Alu* consensus sequence (37). (*Lower*) Two amplified mitochondrial sequences from Nekht-ankh are shown and compared to the published human sequence (29). Dots indicate identical nucleotide positions and dashes represent gaps. The two direct repeats in region V are underlined.

The specific PCR products generated from the ancient DNA were isolated from an agarose gel and their sequences were determined by direct sequencing. Unambiguous and identical results were obtained in several amplifications from the same extracts as well as from three additional extracts prepared from different parts of the mummified liver. The sequences derived from the displacement (D) loop as well as region V of the mitochondrial genome appear in Fig. 4. The D-loop sequence differs at two positions from the published human mitochondrial sequence (29). At position 16223 it carries a T instead of a C, which is present in 18 of 22 human D-loop sequences determined in this laboratory (T. Kocher and L. Vigilant, personal communication). At position 16249, it carries a C instead of a T, which is a sequence variant that has not been seen in any other human sequence determined to date. The region V sequence differs at one position (position 8251) from the published sequence. This sequence represents a silent G to A transition, which has been described in several types of mitochondrial DNA from various geographical groups (16, 41–43).

DISCUSSION

It is evident from the data presented in Fig. 1 as well as from previous work (44) that DNA can be retrieved from a large proportion of ancient soft tissue remains that are desiccated and macroscopically well preserved. The DNA extracted from such remains invariably is of a small average molecular size (e.g., see refs. 2 and 3). However, within the time range investigated here, the extent of the reduction of size is not correlated to the age of the samples. For example, the DNA extracted from the 4-year-old dried pork and the ≈100-year-old *Thylacinus* sample are degraded to a comparable extent as the DNA from the 13,000-year-old *Mylodon* extract, whereas the predynastic 5000-year-old Egyptian mummy samples seem to be degraded to a lesser extent. Since nuclear DNA is degraded rapidly after death by endogenous hydrolytic processes (45), it seems likely that the rapidity with which the body has been desiccated immediately after death is a major factor that determines the extent of size reduction of the DNA.

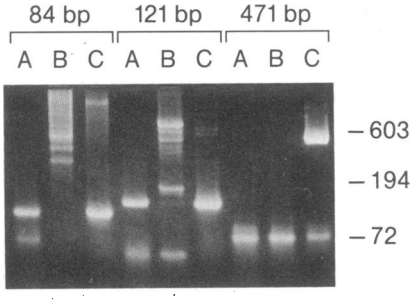

FIG. 5. Agarose gel electrophoresis of amplification products obtained from the 4000-year-old liver as well as contemporary human DNA. Amplifications were performed from sample L (lanes A), a control extract (lanes B), and 1 ng of contemporary human DNA (lanes C). Primers used were D3E and D18X (84 bp), primers A and B (121 bp), and primers cytb2 and M14725(H) (471 bp). The migration positions of molecular size markers are given in bp. Amplification products of 40–60 bp are dimers of primers.

After the initial desiccation of the tissue, the DNA can be expected to have been largely protected from the hydrolytic damage affecting DNA in hydrated tissues. Instead, however, the DNA from desiccated soft tissues is severely damaged by oxidation. A large proportion of pyrimidines, in particular thymine, was shown to be modified in ancient DNA when analyzed by reversed-phase HPLC. Furthermore, assays for various lesions and electron microscopy demonstrated that oxidation products of pyrimidines, AP sites, damaged sugar residues, as well as intermolecule cross-links were present at high frequency in the ancient DNA. This is reminiscent of the pattern of damage described in DNA irradiated by γ-radiation (46, 47). It is noteworthy that no correlation with age could be detected, neither in the extent of sensitivity to lesion-specific enzymes nor in the HPLC patterns of the bases. This may be due to the fact that the oxidative damage reaches a plateau in a comparatively short time span, after which it has only minimal additional effects. Alternatively, the conditions under which the individual specimens have been preserved may be of decisive importance.

The ancient DNA was ligated to a plasmid vector and transformed into the bacterial host. Cloning efficiencies invariably proved very low. This may be speculated to be due to the large excess of damaged DNA present to which a majority of the vector molecules may become linked. That the clones obtained contain human DNA was confirmed by identifying and sequencing four clones containing *Alu* repeats. The size of the inserts was small. This, together with the low cloning efficiency, made the prospect of isolating anthropologically informative genes from ancient samples bleak. In addition, the presence of a vast excess of heavily modified DNA raised the concern that individual clones might contain cloning artefacts induced by postmortem changes (cf. ref. 8 and clone 12:2). Therefore, use was made of the PCR, which is able to amplify a specific DNA segment starting from only a few or even a single copy of the relevant sequence (48) and preferentially replicates undamaged molecules (9). When 1 μg of extracted DNA was used to amplify a mitochondrial sequence of 121 bp, the intensity of the amplified band indicated that ≈200 intact copies of the relevant sequence were present. However, when longer

amplifications were attempted, the amounts of specific product generated rapidly became smaller and no amplification of ancient DNA sequences longer than 140 bp was successful.

The main problem encountered in using the PCR to amplify DNA sequences from ancient human tissue specimens is contamination. Small amounts of exogenous human DNA contaminating the reagents used for the extraction and PCR can present themselves as double sequences at polymorphic positions. These problems can, however, be overcome by rigorous precautions taken in the preparation and handling of all solutions as well as the use of disposable glassware throughout. In addition, multiple extracts must be performed, preferably from different tissues of the same individual to control for contamination of the specimen. By fulfilling these criteria, mitochondrial sequences can be obtained from a large proportion of the samples from which DNA can be extracted. This has so far been achieved from a number of ancient human individuals (ref. 16; data not shown) as well as remains of extinct (ref. 9; R. G. Higuchi and A. C. Wilson, personal communication) and extant (W. K. Thomas and S.P., unpublished data) animals in museum collections.

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