MOLECULAR GENETICS OF PRE-COLUMBIAN SOUTH AMERICAN MUMMIES

Peter K. Rogan* and Joseph J. Salvo†

*National Cancer Institute-Frederick Cancer Research Facility
Frederick, Maryland 21701
†GE Corporate Research & Development, Biological Sciences
Schenectady, NY 12301

ABSTRACT We have isolated human deoxyribonucleic acid (DNA) from the mummified remains of pre-Columbian South American Indians. Ten different individuals from two coastal Chilean cultures separated geographically and temporally (Azapa: 800 years before present [B.P.], Camarones: 500 years B.P.) were selected for our preliminary screen. High molecular weight DNA (>12000 base pairs) was recovered from nine of the specimens. Southern blot hybridization indicated that some of these samples contained human DNA of both genomic and mitochondrial origin. Further analysis of the ancient nucleic acid was hampered by low DNA yields and lesions that appear to inhibit its efficient replication *in vitro*. These difficulties were overcome in part by first repairing the DNA, and then amplifying specific target sequences with a polymerase chain-reaction protocol.

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INTRODUCTION

Although man has continuously occupied the northern coast of Chile for more than 8,000 years, the population density has been sharply limited by the lack of arable land and dependable water supplies. Due to the large number of small, isolated populations scattered along the northwest coast of South America prior to the 1500's, the early history of colonization and cultural interactions is rich but fragmentary, and often controversial. With the advent of molecular biological techniques, it has become possible to directly analyze the genetic material of mummified pre-Columbian Indians which suggests that studies of their genetic relatedness (between individuals or among neighboring communities) may now be feasible. In addition, the analysis of preserved animal, plant, and microbial life associated with the mummies may give us a better perspective on the presence and evolution of pathogens that coexist with inbred populations, and perhaps even aid in tracing the early development of agriculture. Paradoxically, the climatic conditions that limited the number of early settlers has also protected the materials required for modern molecular genetic analyses.

Our preliminary study of pre-Columbian Indians has focused on individuals excavated near Arica, Chile. The early mummification practices and the extremely arid climate has resulted in a legacy of well-preserved tissues in ancient coastal cemeteries. The oldest Chilean mummies (6,000 years B.P.) were treated with preservatives and eviscerated, whereas the more recent specimens (<3,000 B.P.) were buried intact and adorned only with pigments and ritual garments (Bird, 1943). Initially, we decided to concentrate our attention on specimens <1,000 years old, which are more readily available.

Nucleic acids were extracted from individuals found in two ancient cemeteries separated by about 100 km (Allison, 1985). The mummies found in the coastal Camarones Valley have been radiocarbon-dated to about 500 years B.P. (Allison, M., private communication), and were likely to have been Incan subjects. The other specimens (~800 B.P.) were excavated from San Miguel de Azapa, located north and 20 km inland from the Camarones site. Both groups presented us with an opportunity to develop and test molecular techniques that could be applied to future studies of ancient human genetics and pathology.

METHODS

DNA Isolation.

Nucleic acids were isolated from 2-11 gm of calf muscle from ten different mummies according to the method of Herrmann et al. (1987) with minor modifications. Samples were ground to a powder in liquid nitrogen,

digested in a solution of proteinase K and SDS, and extracted with phenol/chloroform until the organic/aqueous interface was well defined. The aqueous layer was mixed with sodium acetate (pH 6.5) and either ethanol or isopropyl alcohol to precipitate the nucleic acids. Although a reddish-brown contaminant was found to co-precipitate in some of the samples, it could be removed by Sephadex G-50 chromatography. However, this last step proved to be unnecessary for the successful execution of the experiments described below.

DNA Manipulations.

Random-hexanucleotide-primed synthesis of radiolabelled DNA with the Klenow fragment of DNA polymerase I and [α-^{32}P] dCTP (3,000 Ci/mMol) was carried out according to the method of Feinberg and Vogelstein (1983), except that the incubation was carried out overnight. Synthetic oligonucleotides were end-labelled with T4 polynucleotide kinase and [γ-^{32}P] ATP (6,000 Ci/mMol) as described by Maniatis et al. (1982).

DNA hybridization analysis on nylon support membranes (Hybond, Amersham) was performed according to standard procedures (Southern, 1975). Incubations with ^{32}P-labelled oligonucleotide probes were carried out in a cocktail containing 6× SSPE (1× SSPE: 0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 1× Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrollidine, 0.1% bovine serum albumin), 1% SDS, and 100 μg/ml carrier DNA at 37°C overnight. Blots probed with oligonucleotides were washed at low stringency (final: 2× SSC [0.3 M NaCl, 0.03 M sodium citrate] 37°C). Hybridizations with random-hexanucleotide-labelled, linear DNA probes were done in 5× SSC, 1× Denhardt's solution, 1% SDS, and 100 μg/ml carrier DNA at 60°C overnight.

Polymerase Chain-Reaction.

The standard polymerase chain-reaction (PCR) protocol (Saiki et al., 1985) was modified by pretreating ancient nucleic acids prior to the amplification step. Ancient DNA samples were first annealed to random-hexanucleotide primers, and replicated with Klenow fragment overnight at 25°C. The DNA was purified by phenol extraction and isopropanol precipitation after which aliquots of each sample were amplified using a standard PCR protocol for 40 cycles with Taq DNA polymerase, (annealing: 45°C, 3´; denaturation: 92°C, 1´; extension: 72°C, 4´). Amplification primers were synthesized by Operon Technologies, Inc. (San Pablo, CA) and purified by high performance liquid chromatography (HPLC) prior to use. For each set of ancient specimens, both positive (contemporary DNA templates) and negative controls (no DNA template) were amplified in parallel. This ensured that the conditions allowed faithful amplification of contemporary DNA, and guarded against low level contamination of reaction solutions or labware with extraneous DNA.

RESULTS

Detecting Nucleic Acids in Ancient Human Extracts.

Attempts to detect the ancient nucleic acids in agarose gels with ethidium bromide stain were disappointing. Based on the intensity of staining (relative to control DNA samples of known concentration) yields were $<1\ \mu g$ DNA per gram of ancient tissue, i.e., ~1,000-fold less than that obtained from extractions of contemporary tissues. However, the DNA was easily detected with a sensitive replication assay. Radioactive nucleotides were readily incorporated into ancient DNA with Klenow fragment and random-hexanucleotide primers. Although this reaction normally produces DNA fragments in excess of 750 base pairs in length with contemporary DNA templates (Feinberg and Vogelstein, 1983), CAM9-T29 and CAM8-T8 extension products averaged <200 bp when assayed on alkaline agarose gels (Figure 1). This was the first indication that some component or property of the ancient DNA preparations appeared to limit the processivity of DNA polymerases.

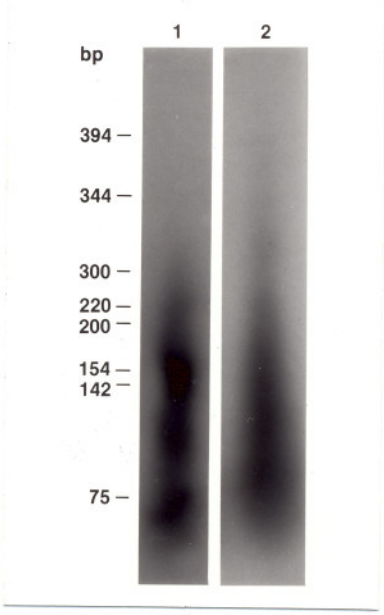

FIGURE 1. Preliminary detection of ancient DNA. Purified ancient DNA's were radiolabelled (with Klenow fragment, [α-^{32}P] dCTP and random-hexanucleotide primers) and separated on a 3% alkaline agarose gel. Unincorporated label was removed by gel chromatography with G-25 resin prior to gel electrophoresis. Specimens are designated first by location (CAM = Camarones, AZ = San Miguel de Azapa), then by cemetery number, and last by tomb number: lane 1 CAM8-T8, lane 2 CAM9-T29.

Identification of Human DNA Sequences by Reverse Genomic Blots.

Sequences of two different human interspersed repetitive DNA families are represented in the ancient samples. The ^{32}P-labelled CAM8-T8 and CAM9-T29 DNA's (see above) were used to probe a Southern blot with serial dilutions of: 1) a digest containing five different Alu elements, each on a distinct DNA fragment (Calabretta et al., 1982), and 2) a purified 1.6 kilobasepair (kbp) fragment carrying the 3´ end of an L1 repeat (Rogan et al., 1987). Figure 2A indicates that ancient DNA probes were able to detect as little as 0.05 μg (0.26 pmol) of Alu and 0.1 μg (0.1 pmol) of L1 repeat DNA, respectively. These probes are thus much less sensitive than contemporary genomic DNA, labelled to the same specific activity (results not shown). Presumably, the short fragments generated from the ancient templates contain fewer full-length repeat elements per labelled strand, accounting in part for the reduced level of hybridization.

Southern Blotting of Ancient DNA with Genomic and Mitochondrial Probes.

To determine the size of the ancient human DNA molecules extracted from the tissue samples, we separated ~25 ng of nucleic acid from each individual on 1% agarose gels. Southern blot analysis (Figure 2B) indicated that all of the samples, except AZ140-T69, hybridized to an Alu consensus probe. In general, all of the Azapa samples exhibited a lower signal than the Camarones specimens. Although this would normally indicate that less DNA was present in these lanes, we cannot rule out the possibility that the hybridization of probe and genomic sequences is inhibited by lesions in the ancient Azapa DNA. In fact, the Camarones DNA may be damaged as well, since the signal intensity of 25 ng of contemporary genomic DNA was substantially stronger (Figure 2B, lane 1). The major hybridization signals in eight of the ten specimens indicate that most of the DNA molecules appear to be larger than 12.1 kbp. One sample, AZ140-T69, did contain shorter fragments, though most of the DNA is >9.1 kb long.

Mitochondrial sequences could be detected in the Camarones samples, but signals were much weaker than those seen using the Alu probe (results not shown). The Azapa specimens did not hybridize to human mitochondrial DNA.

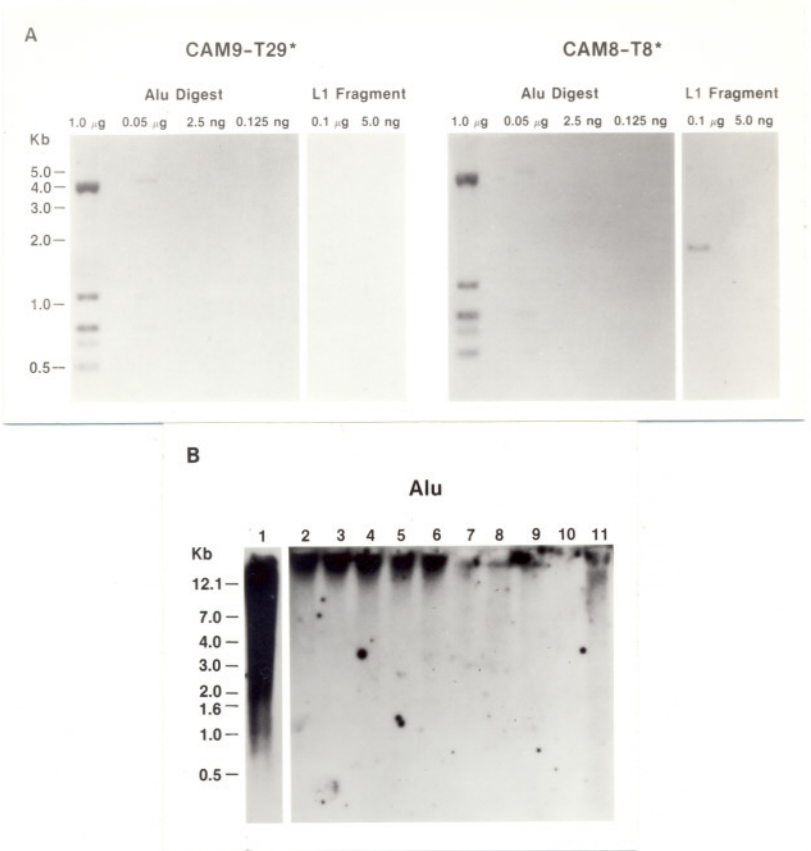

FIGURE 2. (A) Reverse genomic Southern hybridizations. The radiolabelled extension products characterized above (Figure 1) were hybridized to members of both Alu and L1 repetitive sequence families. A dilution series of either a purified DNA fragment derived from the 3´ terminus of the L1 family, or a restriction digest of plasmid DNA containing four Alu repeats, was separated on an agarose gel and transferred to a nylon filter. Each of the four Alu sequences is present on a distinct restriction fragment. Fragments in the digestion composed solely of plasmid sequences failed to hybridize to the probe. (B) Southern hybridizations with an Alu repetitive sequence probe. Undigested ancient DNA (<25 ng) was separated on a 1% agarose gel, transferred to a nylon membrane and hybridized to a purified ^{32}P-labelled human DNA probe containing five Alu repetitive elements. The specimens represented are: lanes 2-CAM9-T29, 3-CAM9-T23, 4-CAM8-T8, 5-CAM8-T10, 6-CAM9-T39, 7-AZ140-T51, 8-AZ140-T63, 9-AZ140-T71, 10-AZ140-T69, 11-AZ140-T114. A filter with BamHI-digested, contemporary human DNA (lane 1) was included as a positive control. No signal was seen in lanes containing λ DNA (not shown).

Amplification of Specific Human Genomic DNA Sequences.

We devised a strategy to partly overcome the low yield of DNA isolated from ancient tissues that hampers the application of most routine molecular biological techniques. Previously, the polymerase chain-reaction has been applied specifically for this purpose (Higuchi et al., 1988; Paabo et al., 1988). However, oligonucleotide primers from the conserved segments of an Alu sequence (Slagel et al., 1987) or from region V of the human mitochondrial genome (Wrischnik et al., 1987) failed to produce the expected amplification products from ancient DNA templates (Figure 3), although contemporary DNA was amplified as predicted (not shown). Amplification was observed with mixtures of ancient and contemporary DNA templates (Figure 3, lanes 3 and 6).

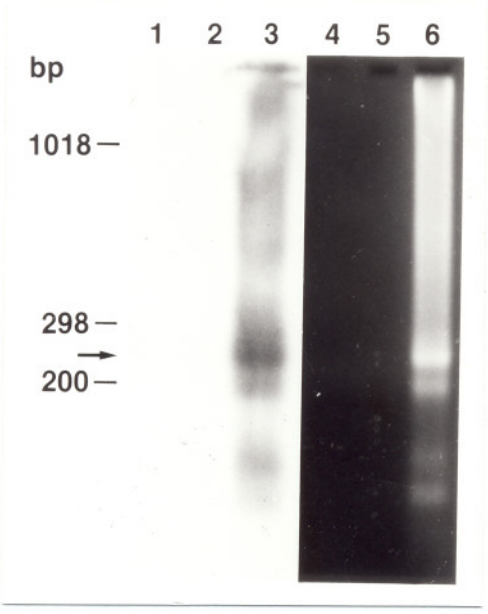

FIGURE 3. Inhibition of the polymerase chain-reaction using ancient DNA templates. PCR amplification reactions were carried out with ancient DNA alone, or with a mixture of ancient and contemporary DNA. The PCR primers used correspond to regions of an Alu consensus sequence (Slagel et al., 1982). PCR reaction products were separated on a 4% agarose gel, stained with ethidium bromide (lanes 1-3), and hybridized with an Alu probe specific for the amplified sequences (lanes 4-6). DNA samples in the various PCR reactions were as follows: lanes 1 and 4, CAM8-T8; lanes 2 and 5, CAM9-T29; lanes 3 and 6, a mixture of CAM8-T8, CAM9-T29 and JY contemporary DNA. The arrow marks the position of the 240 bp Alu-dimer amplification product.

To bypass some of the DNA modifications that are presumed to exist in ancient DNA, we pretreated the DNA samples prior to PCR amplification. The ability of Klenow fragment to generate radiolabelled extension products from ancient DNA's suggested that this enzyme was able to recognize and use the ancient DNA as a template for polymerization. Thus, a random-hexanucleotide-primed synthesis step was introduced prior to the reiterative cycles of DNA replication with Taq DNA polymerase. Figure 4A indicates that PCR amplification with Alu primers and pretreated ancient DNA yielded amplification products of the expected size (240 bp) for two of the Azapa specimens. In addition, discrete 125 and 105 bp species were also produced. Both of the short fragments (which correspond to the amplification of an Alu monomer unit) and the 240 bp band hybridize to a 21 mer probe derived from an Alu consensus sequence within the amplified domain (Figure 4A). These results suggest that ancient repetitive Alu sequences can be specifically amplified by this modified PCR protocol.

Mitochondrial DNA Amplifications.

Although mitochondrial DNA appeared to be present in all of the Camarones specimens, it was present at relatively low levels as indicated by the intensity of the Southern hybridization signal. We attempted to amplify a 118 bp sequence from region V that is known to carry a 9 bp deletion polymorphism in some contemporary Asian and African human populations (Wrischnik et al., 1987; Vigilant and Wilson, 1989). Using the modified PCR protocol, both the CAM8-T8 and CAM9-T29 isolates generated the intact 118 bp fragments (Figure 4B). Ancient DNA preparations from five other individuals also produce the non-deleted form of this sequence (manuscript in preparation).

DISCUSSION

The isolation and analysis of ancient DNA has become accessible via modifications to standard molecular biological techniques. Previous investigators have isolated DNA from museum specimens of extinct animals (Higuchi et al., 1984) as well as Egyptian mummies (Paabo, 1985). In each case, nucleic acids appeared to have been sheared and chemically damaged. Although multiple chemical modifications are present in the ancient DNA, oxidative damage appears to be the predominant lesion (Paabo, 1989).

A wide range of DNA modifications are known to inhibit DNA polymerases both *in vivo* and *in vitro* (Friedberg, 1985), but the extent of inhibition is both lesion- and polymerase-dependent. This suggested to us an avenue that might bypass some of the lesions present in the ancient Chilean DNA samples described here. By using a polymerase that is capable of recognizing and replicating through oxidatively-damaged DNA templates, we hoped to generate replicas of ancient templates that could then be amplified with standard PCR technology and Taq thermostabile DNA polymerase. Initially, we found that the Klenow fragment of DNA polymerase I was able to utilize the ancient DNA as a template for the incorporation of radionucleotides. It soon became apparent that pretreating the ancient templates with Klenow fragment improved the success of subsequent PCR amplifications. This result is consistent with Klenow enzyme proceeding past lesions that normally block the Taq DNA polymerase. Clark and Beardsley (1987) have demonstrated the tolerance of Klenow fragment to lesions using DNA templates oxidized *in vitro* with OsO_4. Their study suggests that other enzymes may prove superior to Klenow for this purpose, and we are currently screening all available polymerases and repair systems. Sequence analysis of the amplification products should help determine the fidelity of the PCR protocol when used in conjunction with any preamplification step(s). The observation that mixtures of ancient DNA and contemporary DNA can be amplified without pretreatment suggests that any polymerase inhibitors present in these ancient samples are an integral part of the DNA (lesions) or, are so tightly bound, that they do not act in *trans*.

Because of the small quantities of ancient DNA that can be isolated, our efforts to identify the specific types of lesions present have necessarily been indirect. Current efforts are focused on a careful microanalysis of these lesions with the hope that a better understanding of ancient DNA damage may lead to a superior PCR approach. The large size (>12 kbp) of these ancient DNA molecules as determined by gel electrophoresis is puzzling based on the results of other studies of ancient DNA (Paabo, 1985; Doran et al., 1986). We are now examining the DNA by electron microscopy to search for interduplex crosslinks (Paabo, 1989) that could account for the slow electrophoretic mobility observed in this study.

We have successfully amplified both genomic (Alu elements) and mitochondrial sequences (region V) from individuals described in this report, as well as from other specimens from the Azapa and Camarones cemeteries (manuscript in preparation). Seven of the ten individuals tested have yielded PCR-amplified products of the predicted size for both genomic and mitochondrial sequences. This suggests that large-scale genetic analyses of ancient cultures may now be feasible. The ability of this modified PCR protocol to amplify mitochondrial sequences in samples

with a very weak Southern hybridization signal indicates that it may be a suitable method for routinely manipulating low levels of damaged, ancient DNA. In addition, the kinship analysis of ancient humans based on mitochondrial sequences can be immediately integrated into the wealth of genealogical data already collected with respect to contemporary human populations (Cann et al., 1987).

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