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Editors

Ancient DNA

Recovery and Analysis of Genetic Material from Paleontological, Archaeological, Museum, Medical, and Forensic Specimens

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Dried Samples: Soft Tissues

12 High-Fidelity Amplification of Ribosomal Gene Sequences from South American Mummies

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1. Introduction

The condition of nucleic acids in mummified tissues depends on several variables, including the presence or absence of specific embalming treatments, the conditions of storage, and the natural chemical reactions that have occurred over time (Pääbo et al. 1989). These factors substantially reduce the yield of nucleic acids from ancient tissues; however, ancient DNA (aDNA) can be analyzed by amplifying specific sequences in vitro, with modifications of the polymerase chain reaction (PCR) (Pääbo et al. 1988; Rogan and Salvo 1991). Even when nucleic acids can be retrieved, manipulation of aDNA with molecular biological techniques is not always successful. Inhibitors of the PCR process that either copurify with or are components of the nucleic acid template have been shown to interfere with the processivity of the DNA polymerase used to amplify the template (Rogan and Salvo 1990).

Previous studies of nucleic acids derived from Atacamanian mummified tissues have shown that damaged nucleic acids, rather than soluble contaminants, prevent amplification (Rogan and Salvo 1990). Some of the chemical modification might be a consequence of oxidation of both deoxyribose and bases, although other reactions promoting decomposition may also have occurred. These processes affect both the yield and the integrity of the DNA sequences that are recovered. Nucleic acids from ancient specimens are often highly fragmented, and yields are typically reduced more than 1,000-fold relative to contemporary tissues (Rogan and Salvo 1991). Ancient nucleic acids are particularly susceptible to cleavage by endonucleases that excise oxidized pyrimidine moieties (Pääbo 1989). In some of the South American samples that we have analyzed, large genomic fragments greater than 10 kilobase pairs have been imaged by electron microscopy (Salvo unpubl. observations). However, interstrand crosslinking is also present and retards the migration of aDNA during agarose gel electrophoresis (Rogan and Salvo 1990; Salvo, unpubl. observations). We have previously shown that ancient nucleic acids can be enzymatically copied with certain DNA- or RNA-dependent DNA polymerases (AMV reverse transcriptase and Klenow DNA

polymerase) that bypass some of these damaged sites (Rogan and Salvo 1990). The replicates can then serve as bonafide templates for PCR amplification with *Taq* DNA polymerase.

Extensive DNA damage and limited yields of aDNA impose substantial constraints on successful polymerase chain amplification that are not encountered when working with contemporary specimens. Modification of these templates presumably can introduce mutations into the resulting sequence and limit the length of the longest sequences that can be successfully amplified (Pääbo et al. 1990). The PCR products may therefore not be of sufficient length or accuracy to confirm their ancient human origin.

PCR-based procedures capable of verifying the authenticity and homogeneity of ancient human templates can be applied to control against potential contamination from human or other eukaryotic, contemporary or ancient nucleic acids. Postmortem decay of human bodies, the burial context, or residual parasitic infections can lead to recovery of human nucleic acids contaminated with DNA derived from other genomes (Royal and Clark 1960; Higuchi and Wilson 1984). Human nucleic acids may be distinguished from orthologous sequences found in other organisms by amplification of loci, such as the ribosomal RNA gene loci, that are relatively monomorphic within each species but exhibit interspecies variability. On the other hand, ethnic and geographic variation in contemporary humans has been extensively catalogued by studying mitochondrial DNA (mtDNA) inheritance (Vigilant et al. 1991). It is conceivable that the mtDNA haplotypes of ancient-specimens might be sufficiently distinctive that contamination with contemporary human nucleic acids would be easily detected. Ultimately, the analysis of genomic polymorphisms, when accomplished, will uniquely identify each mummified individual.

Pretreatment of aDNA substrates with Klenow DNA polymerase reverse transcriptase (RT) greatly facilitates the subsequent retrieval of DNA sequences from these preparations (Rogan and Salvo 1990). Since there was the possibility that the pretreatment and amplification process could be mutagenic, a highly conserved sequence was studied in the experiments described below. The identification of other species that might have contributed to the pool of nucleic acids is clearly an important problem, since sequence errors introduced during the amplification of certain human sequences could, in theory, produce sequences resembling those of nonhuman species.

We wished to determine whether ancient nucleic acids might be inaccurately replicated, resulting in amplification products with sequences divergent from the original template. Redundant, multicopy sequences were selected to increase the probability of successful amplification and to simplify the detection of mutations which might have arisen during amplification. The 18S, 5.8S, and 28S ribosomal DNA (rDNA) genes together compose a single multicistronic sequence, with a similar number of copies of each present in the human genome (Fig. 1). There are at least 160 uniform copies of the rDNA multigene family (Schmickel 1973), organized in long tandem arrays at several sites in

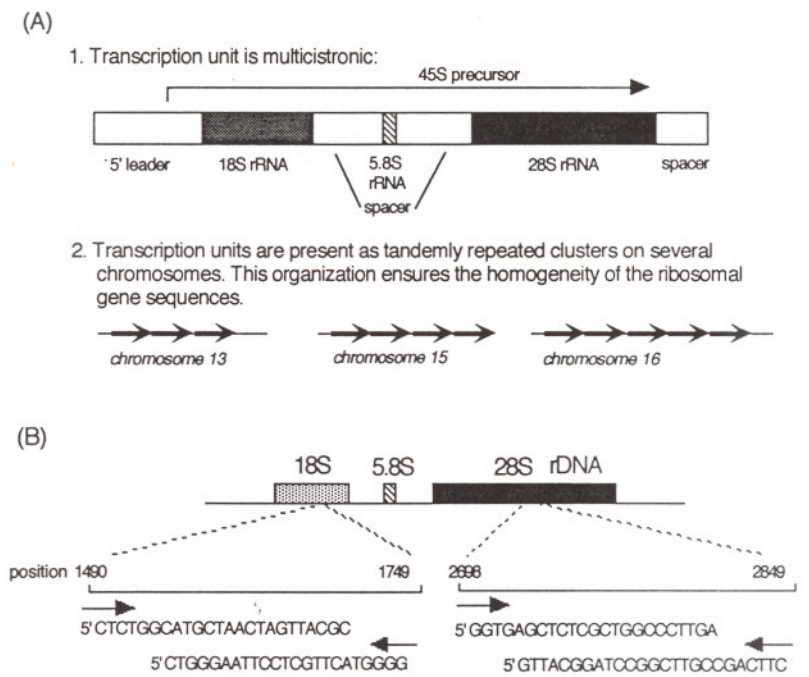

FIGURE 1. (*A*) Organization of large subunit rDNA genes in eukaryotes. The 18S and 28S rRNA genes are arranged on a multicistronic, tandemly repeated DNA segment in humans and other eukaryotes. Although several distinct clusters of rDNA repeats are found at different locations within the genome, the overall sequence relationships within the repeat unit are retained. The 18S and 28S rDNA sequences are conserved between species, although divergent sites may be used to establish taxonomic relationships (Rogan et al., 1993). (*B*) Locations of oligonucleotide PCR primers within the 18S and 28S rDNA genes. The PCR amplification products are defined by the corresponding coordinates in the 18S (GenBank locus HUMRGE) and 28S (GenBank locus HUMRGM) rDNA sequences.

the human genome (Henderson et al. 1972). These sequences are highly uniform, and the PCR products were predicted to have identical sequences unless the amplification reaction itself was mutagenic.

Any sequence heterogeneity found in the amplification products would be predicted to arise from chemical modification that has accumulated in the aDNA templates or from misincorporation during the PCR procedure. In order to ensure that human sequences were amplified, a 28S rDNA interval was selected which is known to exhibit a high degree of interspecies variability (Rogan et al., in press). The analysis of these loci therefore provides a means of assessing the overall integrity of the DNA, the accuracy of the amplification process, and potential contamination with other eukaryotic organisms.

2. Methods

2.1 DNA Isolation

Specimens were dissected with disposable scalpels and other autoclaved instruments. Only internal tissues were taken, to eliminate the possibility of contamination with contemporary DNA that could conceivably have arisen during excavation or subsequent handling of the mummy. Nucleic acids were purified from 2–11 g of skeletal muscle from eight different individuals based on a modification of the method described in Herrmann and Frischauf (1987). Samples were ground to a powder in liquid nitrogen with a Waring blender and digested overnight in a solution of proteinase K (100 μg/ml), sodium dodecyl sulfate (0.5%), 1 mM Tris-Cl (pH 8.0), and 100 mM EDTA at 37°C. The suspension was centifuged and the resulting supernatant was repeatedly extracted with a mixture of phenol and chloroform until the organic/aqueous interface was well defined. Nucleic acids were precipated from the aqueous fraction with ethanol or isopropanol, lyophilized, and dissolved in TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) at the approximate concentration of 0.2 ml per gram of tissue. A reddish-brown contaminant that coprecipitated in some of the samples was removed by Sephadex G-50 spin column chromatography.

2.2 Choice of Amplification Templates

The decision to select a reiterated, uniform sequence was in part based on previous results demonstrating the efficient amplification of such templates (Rogan and Salvo 1990). The 5′ and 3′ terminal coordinates of the 28S PCR product correspond to positions 2698 and 2849 of the published sequence (GenBank locus HUMRGM, accession number M11167; Gonzalez et al. 1985) while the 18S product spans nucleotides 1490 through 1749 (GenBank locus HUMRGE, accession number M10098; Torczynski et al. 1985). The predicted sizes of the 28S and 18S amplification products are 162 bp and 260 bp, respectively. The length of these amplified intervals does not exceed the limitations imposed by most aDNA templates. The left and right oligonucleotide primers derived from the 18S sequence contain natural *Sph*I and *Eco*RI restriction sites, which upon amplification were cleaved to generate cohesive termini suitable for subsequent cloning of the PCR-generated fragments.

One of the criteria used to select the rDNA primer sequences was their ability to amplify orthologous sequences from a wide variety of eukaryotic species. The sequences of rRNAs from different species can be aligned, and can be used to determine phylogenetic relationship between different organisms (Woese 1987). Differences between the aligned sequences form the basis for taxonomic classification schemes. The polymerase chain reaction has been used to amplify highly divergent sequence regions that are flanked by conserved domains corresponding to the oligonucleotide primers (Rogan et al., 1993).

The human 28S primer combination is capable of amplifying contemporary preparations of nucleic acids from a diverse set of plant, fungal, and animal species. The sequences of these amplification products have been used to generate a dendrogram illustrating the phylogenetic relationships between these organisms (Rogan et al. 1990).

2.3 Modified PCR Protocol

Experiments were carried out in a laboratory environment which, with the exception of control samples, was devoid of purified contemporary human genomic DNA. To prevent aerosol contamination between solutions, aDNA samples were manipulated with either a dedicated set of positive displacement pipettes or, when necessary, with conventional instruments equipped with cotton-plugged pipette tips.

In order to prepare intact genomic DNA templates for PCR amplification, replicates of aDNA were synthesized in an oligonucleotide-primed reverse transcriptase reaction. The ancient nucleic acid (5–10 µl) was denatured at 92°C for 5 min prior to annealing random hexanucleotide primers (1 mg), and addition of AMV reverse transcriptase (40 units). A 50 ml reaction containing 50 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 100 mM dNTPs, and 50 mg/ml BSA was incubated at 42°C for 2–3 hr. The reaction was stopped by adding 2 ml of 0.5 M EDTA. Oligonucleotide primers were then removed by Sephadex G-25 spin column chromatography, and the DNA was precipitated with ethanol, lyophilized, and resuspended in 25–40 µl of TE.

A 2–4 µl aliquot of pretreated aDNA was amplified by standard PCR for 40 cycles with *Taq* DNA polymerase (denaturation at 92°C, 1 min; annealing at 45°C, 1 min; extension at 72°C, 2 min; Saiki et al. 1985). Oligonucleotide primers were synthesized by Operon Technologies, Inc. (San Pablo, Cal.) and HPLC-purified prior to use. Primer sequences contained a GC-rich sequence at their 5′ terminus adjacent to a unique restriction site present in the rDNA sequence. The left and right 28S rDNA primers contained natural *Bam*HI and *Sac*I recognition sites, respectively, whereas *Eco*RI and *Sph*I sites were present in the 18S oligonucleotides. For each set of ancient specimens, both positive controls (contemporary DNA templates) and several negative controls (DNA template absent) were amplified in parallel.

One-fifth volume of each amplification reaction was analyzed by gel electrophoresis in a 4% gel consisting of a 3:1 mixture of Nusieve and molecular biology grade agarose. Gels were stained with ethidium bromide and the DNA amplification products were visualized by UV fluorescence.

2.4 DNA Cloning and Sequencing

For two different mummified individuals, 5 mg of the 18S amplification product was extracted with phenol/chloroform, reprecipitated, and doubly digested

with an excess of *Eco*RI and *Sph*I (100 units). Enzymes were inactivated by heat treatment. After adjusting the sample volume to 1 ml, oligonucleotides and short PCR products that could interfere with the subsequent cloning steps were removed by centrifugation through a Centricon-100 column (Amicon). The nucleic acids were then concentrated by ethanol precipitation and resuspended at 0.2–0.3 mg/ml. Forced ligation between a fivefold excess of the restriction-digested PCR products and similarly digested M13mp19 vector DNA was carried out according to standard protocols (Sambrook et al. 1990). Ligation products were transformed into *E. coli* strain JM103 and plated in YT soft agar containing IPTG and XGal (Messing1983). Colorless plaques containing recombinant phage were picked and single-stranded DNA was purified from independent cultures. The Sanger sequencing procedure was carried out with a modified T7 DNA polymerse (Tabor and Richardson 1989). Sequences were compared with the contemporary 18S consensus using the GCG sequence analysis package (Devereux et al. 1984).

3. Results

3.1 Efficiency of aDNA Amplification

Most of the specimens selected were successfully amplified regardless of age or geographic location of the burials. The study reported here included two individuals derived from the coastal Camerones site approximately 50 km south of Arica, Chile (CAM8-T8 and CAM8-T10), three mummies from the Morro site in the city of Arica (Mol-6 T7, Mol-T28 C9, and Mol-T22 C6), and two from San Miguel de Azapa approximately 15 km inland (AZ141-T249M, AZ141-T249I, and AZ28-T6B). Carbon-14 dating studies have demonstrated that all of these specimens are most likely pre-Columbian, with age estimates of approximately 550 years for the Camerones specimens, 1,200 for those from Santa Cruz de Azapa, and 2,000 years for those from the Morro cemeteries (M. Allison, pers. comm.). The climate and natural mechanism of preservation appear to be the only constants among the different specimens.

For each sample, both 18S and 28S rDNA amplification products of the correct size were synthesized. Figure 2 shows the 28S amplification products from seven different individuals separated by agarosed gel electrophoresis (4% Nusieve agarose). Generation of these products required a pretreatment with oligonucleotide-directed RT, consistent with our previous results (Rogan and Salvo 1990). A control amplification reaction with 50 ng of contemporary human placental DNA template shows the expected 28S rDNA product (Fig. 2, lane Jy). Multiple control reaction in which distilled water was substituted for the solution containing DNA at either the prerepair or the amplification step of the procedure did not generate 18S or 28S PCR products (for example, see Fig. 2, lane 0).

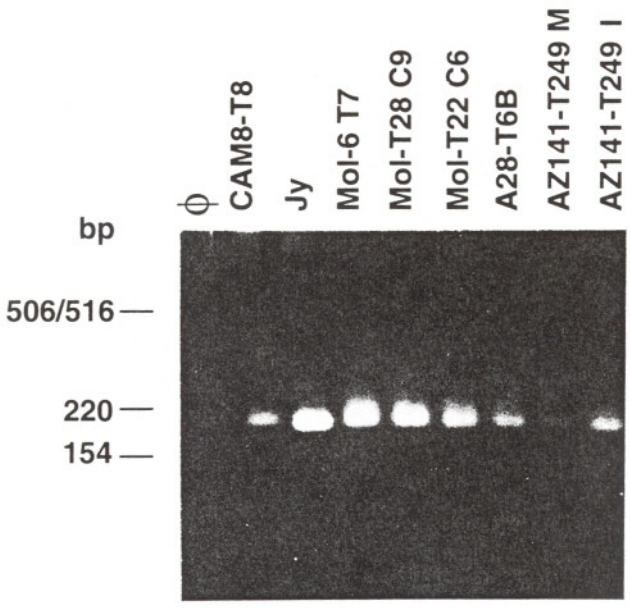

FIGURE 2. Amplification of a segment from human 28S rDNA. One specimen from the Camarones site (CAM8-T8), three from the Morro I cemetery (MoI-6 T7, MoI-T28 C9, and MoI-T22 C6), and three from San Miguel de Azapa (A28-T6B, AZ141-T249M, and AZ141-T249I) were amplified according to experimental conditions described in Rogan and Salvo (1990). The expected 162-bp product was generated by amplification of 50 ng of human placental genomic DNA (lane Jy). In the negative control reaction shown here (lane ϕ), 4 μl of double-distilled H_2O was substituted for template DNA at the AMV RT pretreatment step. The positions of size markers are shown in base pairs (bp).

3.2 Fidelity of aDNA Amplification

The accuracy of the amplified sequence is predicted to depend on both the integrity of the DNA template and the propagation of replication errors that could arise during the PCR process. This is because chemically modified sites can be misread by DNA polymerase during template replication (Clark and Beardsley 1987). It is conceivable that the PCR procedure could therefore incorporate mutations present on different replicated strands from one or more templates into the same amplification product.

To assess the fidelity of the PCR procedure, the sequences of individual, ancient 18S rDNA amplification products were compared with the contemporary rDNA sequence. The fragments were concentrated, restriction digested to generate cohesive ends and cloned into M13mp19 (Sambrook et al. 1990). Sequences of representative recombinant phage inserts were determined by Sanger dideoxy sequencing of seven different recombinant sequences

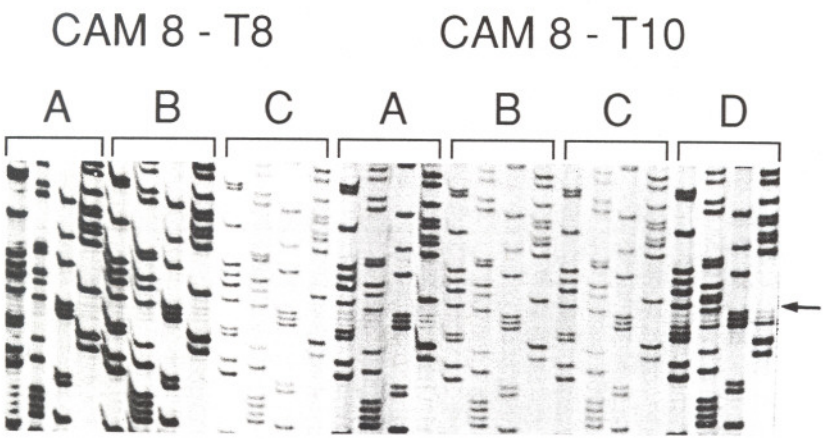

FIGURE 3. Sanger dideoxy sequencing the M13mp19 recombinants containing amplified 18S rDNA. 18S ribosomal RNA gene sequences were amplified from DNA isolated from two ancient specimens (CAM8-T8 and CAM8-T10), digested with *Eco*RI and *Sph*I, and cloned into M13mp19. Single-stranded DNA was prepared and sequences from seven clones were determined (three from CAM8-T8 denoted A, B, C, and four from CAM8-T10 denoted A, B, C, D). In each case, the order of sequencing reactions from left to right is TGCA. The arrow shows the position of the mutation in clone D from CAM8-T10. Sequencing reactions were carried out with Sequenase 2.0 (USB) and the M13 universal sequencing primer (GTTTTCCCAGTCACGAC) on 6% denaturing polyacrylamide gels. The immediate region around the discordancy is presented.

determined, four derived from amplification of CAM8-T10 and three from CAM8-T8 (Fig. 3).

The fidelity of an amplified sequence is predicted to be a function of the density and distribution of modified sites as well as the ability of RT and subsequently *Taq* DNA polymerase to bypass or correctly recognize modified positions. By analyzing the sequences of individual amplification products separately, it was possible to discern whether the amplification procedure was robust. Several different outcomes could be anticipated for this experiment. The presence of distinct mutations in each of the recombinant PCR products would suggest either that the amplification procedure is error-prone and/or that multiple, damaged templates were amplified. If most or all of the cloned sequences were similar to one another but differed from the reference sequence, it is likely that a limited number of damaged templates were amplified in a high fidelity PCR reaction. If the reference and cloned sequences were identical, either undamaged templates were present or RT presumably recognized the modified sites and incorporated the correct complementary nucleotide, effectively repairing the damage.

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position        1560
reference       GCGTTCAGCCACCCGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGA

ancient         ----------------------------------------*----------

                1610                                            1659
                TGTCCGGGGCTGCACGCGCGCTACACTGACTGGCTCAGCGTGTGCCTACC

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FIGURE 4. Sequences of cloned PCR amplification products derived from aDNA and the contemporary 18S rDNA reference sequence (Gonzalez et al. 1985). Comparison of seven independent recombinants with the reference sequence revealed one nucleotide discordancy in a single clone (a transition indicated with an asterisk at position 1599). All other sequences were identical (–) to the contemporary sequence. Although the complete insert was sequenced for each recombinant, only the interval between nucleotides 1560 and 1659 of the reference sequence is shown.

Except for a single-nucleotide discordancy in one of the clones (a transition at position 1599 of the 18S sequence), each of the sequences determined was identical to the contemporary reference sequence (Fig. 4). There is no evidence that these samples were contaminated by nucleic acids from any other eukaryotic species. Several interpretations of this result are possible. Either unmodified templates were present in these samples, or the pretreatment and amplification enzymes are capable of correctly reading damaged nucleotides and adding the appropriate base at those sites, or a full-length template was reconstructed from intact segments derived from different rDNA templates during the amplification reaction. The single-nucleotide mismatch that was detected could be consistent either with modification of the templates or with the introduction of mutations during the PCR procedure (see below).

4. Discussion

A multicopy homogeneous template derived from the 18S-28S rDNA transcription unit was successfully amplified with a two step replication reaction, utilizing AMV RT followed by thermal cycling with TAQ DNA polymerase. The sequences of seven independent recombinant 18S reaction products from two different individuals revealed one nucleotide discordancy in over 1,000 nucleotides. This high level of fidelity is comparable to that found in conventional PCR amplification and may be due to the multicopy nature of the template, pretreatment with AMV-RT, or a combination of both of these factors.

4.1 High Fidelity of Amplification

During the amplification of aDNA sequences in vitro, chemically modified nucleotides may be sometimes misread by DNA polymerase, resulting in

mutations in the amplified sequence (Pääbo et al. 1990). In the present study, the accuracy of a modified PCR protocol was assessed for two different highly conserved, multicopy genomic templates using ancient human DNA preparations from eight distinct individuals. Sequence analysis shows that both 18S and 28S rDNA products are generated in each of the DNA samples and that 18S sequences are accurately amplified. This suggests either the presence of intact genomic templates that were replicated with high fidelity or that the hexanucleotide-directed RT was capable of recognizing modified nucleotides and incorporating the appropriate complementary bases at these sites.

A single-nucleotide discordancy relative to the contemporary consensus was found in the sequence of one of the cloned PCR products. The error may have been introduced during the later stages of amplification rather than the prerepair step, since mutations arising during the RT reaction would be propagated throughout the remaining cycles, resulting in identical changes in multiple recombinant clones. This mutation represents approximately 0.1% of the total sequence determined. Since *Taq* DNA polymerase misincorporates approximately 1 in 104 nucleotides in vitro, the observed sequence error could have occurred during amplification (Tindall and Kunkel 1988). The difference between the observed and the experimentally determined enzymatic mutation rate is probably not statistically significant.

We cannot completely eliminate the possibility that the observed sequence change arose during replication of the aDNA template with RT. From these experiments, it is not possible to determine which strand was mutated, only that a A→G or a T→C transition occurred. RT may have misread and/or misincorporated at this site as thymidine is a predominant target for oxidative damage to DNA (Hutchinson 1985). Pyrimidine substitutions represent the majority of oxidative damage to DNA (Hutchinson 1985). They also represented the majority of mutations produced in the amplification of ancient Egyptian mtDNA sequences (Pääbo et al. 1990).

4.2 *High Efficiency of Amplification*

Our current and previous efforts demonstrate that human repetitive DNA sequences can be amplified in many of the pre-Columbian, mummified specimens that have been analyzed (Rogan and Salvo 1990). Successful amplification at these loci may be due, in part, to the multicopy stoichiometry of the template. Although the copy number of rDNA templates is at least 5,000-fold lower than that of the *Alu* elements previously studied, this sequence is more highly conserved. The similar copy number of rDNA and mtDNA in the cell may explain why ancient mtDNA sequences can be consistently retrieved (Pääbo et al. 1988; Rogan and Salvo 1990). The inability to amplify these sequences in some samples by conventional approaches suggests that most of the templates in these preparations may be chemically modified. In each repetitive

sequence family there may be a small number of nearly intact copies, which are preferentially amplified when treated with a DNA polymerase that bypasses the damaged sites.

These DNA lesions appear to partially inhibit the PCR amplification reaction by blocking extension by some DNA polymerases (Clark and Beardsley 1987). A full-length amplification template is thought to be generated by the repeated annealing and replication of overlapping, complementary partial extension products (Pääbo et al. 1990). The kinetics of the amplification reaction are predicted to be biphasic: exponential amplification would not be expected to occur until the full-length product was synthesized. The formation of a detectable PCR product would be predicted to require additional cycles of amplification.

DNA synthesis using oligonucleotide-directed RT prior to amplification may provide a machanism for bypassing some of the chemically modified sites present in aDNA. Annealing of hexanucleotides composed of random sequences permites AMV RT to intiate DNA synthesis at many different priming sites along the single-stranded aDNA templates. RT has a propensity to generate incomplete extension products from RNA or DNA templates both during retroviral replication and in vitro. Both Klenow DNA polymerase and RT recognize and replicate beyond sites of oxidative damage in vitro, although this reaction is inefficient (Clark and Beardsley 1987). This might result in longer partial extension products from aDNA templates than those generated by *Taq* DNA polymerase. The increased length of these intermediate species should facilitate the formation of complementary duplexes capable of serving as full-length templates. Consistent with this prediction, major extension products can be detected in the early stages of PCR amplification using ancient templates pretreated with RT. These intermediates appear to be converted into full-length products with retarded kinetics compared to contemporary DNA controls, and are not present in the control reactions.

Although these studies were designed to utilize the DNA-dependent DNA polymerase activity of RT, it is possible that preserved ribosomal RNA species may have been amplified. The protocol for isolating ancient nucleic acids does not degrade RNA by treatment with ribonuclease A. 18S and 28S rRNAs are among the most abundant nucleic acid species in living cells, and it is conceivable that sufficient template may have remained to generate DNA products from a random-primed reverse transcription reaction. Venanzi and Rollo (1990; cf. Rollo et al. Chapter 16) have presented evidence that ancient nucleic acids from plant specimens are composed mainly of RNA. The apparent preservation of RNA in ancient preparations is remarkable, given the seeming ubiquity of ribonucleases that contribute to RNA lability in vitro. The integrity of rRNA could have conceivably been protected from nuclease action either by endogenous or postmortem chemical modification or by interactions with preserved ribosomal proteins. Future studies will address this issue.

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