YEARBOOK OF PHYSICAL ANTHROPOLOGY 33:195–214 (1990)

Study of Nucleic Acids Isolated From Ancient Remains

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KEY WORDS Ancient DNA, molecular evolution, DNA damage, DNA polymorphism, archaeology

ABSTRACT The examination of preserved, organic archaeological remains with the techniques of molecular genetics is providing direct access to the genetic components of these tissues. Although the nucleic acids are often chemically modified and substantially sheared, it has been possible to retrieve ancient DNA sequences. The polymerase chain-reaction method, which has been modified to overcome the inhibitory properties of lesion-containing DNA, is capable of reconstructing ancient DNA sequences from template molecules that are shorter than the amplified products. This has made it possible to analyze both ancient or extinct mitochondrial and genomic DNA sequences. These studies have resolved ambiguities in the phylogenies of extinct animals and have contributed to knowledge about the patterns of human population migration. The potential of ancient nucleic acid analysis to make contributions in paleopathology, molecular evolution, and population genetics depends on improved methodologies which, in turn, requires a more comprehensive understanding of postmortem chemical processes.

Organic archaeological specimens are routinely analyzed with techniques that are designed to probe molecular structures. The application of the techniques of molecular genetics focuses specifically on the nucleic acids preserved in ancient tissues (Table 1). It is the information encoded by these chemical components that may make it possible to study aspects of inheritance, paleopathology, and molecular evolution at the nucleotide level. Decoding the nucleotide sequence information in preserved materials presents some unique challenges, since the nucleic acids are often neither pure nor intact.

When soft tissues are available for study, they are usually preserved by mummification. Mummification is the desiccation or functional inactivation of tissues at a rate greater than the decomposition of the tissues. The action of bacteria, fungi, and autolytic enzymes requires an ambient, osmotically balanced aqueous environment. Dehydration arrests, to some extent, the degradation of the gross morphology of the tissue by inhibiting the degradation of its chemical constituents. The rate of this process can depend on climate (frozen or desiccated remains; Hansen et al., 1985; Allison, 1985), pH and anoxia (bog environment; Royal and Clark, 1960), or intra-corporeal osmolarity (artificial mummification with natron; Lucas, 1932).

CELL BIOLOGY

The subcellular structures which contain nucleic acids are poorly preserved in ancient remains. Although the identification of cellular and subcellular architecture has been technically challenging and riddled with artifactual contamination,

TABLE 1. List of cloned, amplified, or isolated ancient or extinct DNA sequences

Organism	Sequence	Age (Yr B.P.)	Reference
Human	Mitochondrial Region V	500, 800	Salvo et al. (1989)
		300–750	Hagelberg et al. (1989)
		7,000	Pääbo et al. (1988)
	Alu repeated sequence	500, 800	Rogan and Salvo (1990)
		5,000	Pääbo (1986)
	18S rDNA	500–2,000	Rogan and Salvo, in prep.
	28S rDNA	500–2,000	Rogan and Salvo, in prep.
	Mito. NADH dehydrogenase	300–750	Hagelberg et al. (1989)
	Mito. D-loop	4,000	Pääbo (1989a)
	Mito. cytochrome B2	7,000	Pääbo et al. (1988)
	Mito. positions: 13282–13286	7,000	Pääbo et al. (1988)
Woolly mammoth		40,000	Higuchi and Wilson (1984)
Quagga	Mito. cytochrome oxidase Mito. NADH dehydrogenase	140	Higuchi et al. (1987)
Marsupial wolf	Mito. 12S rDNA Mito. cytochrome B	120	Thomas et al. (1989)
Ground sloth (Mylodon)	Mito.	18,000	Pääbo (1989a)
Z. mays	Mito. cyto. C oxidase H2a repeated sequence	1,500	Rollo et al. (1988)
	Ribosomal RNA	1,000	Venanzi and Rollo (1990)
Cress	Ribosomal RNA	3,300	Venanzi and Rollo (1990)
E. virginensis		1,200	Rogers and Bendich (1985)
E. minutiflora		500	Rogers and Bendich (1985)
L. shockleyi		1,200	Rogers and Bendich (1985)
J. osteosperma		3,500–>45,000	Rogers and Bendich (1985)
O. ramosissima		11,000	Rogers and Bendich (1985)
Symphoricatpos		>45,000	Rogers and Bendich (1985)
Magnolia	Chloroplast rbcL gene	>17–20 myr	Golenberg et al. (1990)

in at least one case (Ramses II), different cell types could be distinguished (Cockburn and Cockburn, 1980). Histological identification of normal and diseased tissue has usually been made on the basis of connective tissue patterns. Although nuclei are rarely found, they have been observed in both Old and New World mummified remains. Hemotoxylin staining has identified nuclear detail in tissues obtained from at least one Egyptian mummy of 23 examined (Pääbo, 1985a), and analysis of Peruvian mummified tissues by Feulgen staining revealed that approximately one in 3,000 muscle cells contained intact nuclei (Allison, personal communication). The loss of compartmental boundaries is generally consonant with other autolytic processes which are initiated with tissue necrosis (see below).

NUCLEIC ACID ISOLATION AND RECOVERY

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) can be isolated from ancient remains using methodologies developed for contemporary, living tissue (Blin and Stafford, 1976). The tissue is mechanically disrupted, then treated extensively with emulsifying agents to dissolve lipid components, and with proteases to digest proteins. DNA has been recovered from ancient bone matrices by chelation of calcium with ethylenediamine tetraacetate (EDTA) at this stage (Hagelberg et al., 1989). After centrifugation, the supernatant fraction is extracted with phenol and chloroform to remove additional proteins and other hydrophobic components. A relatively purified preparation of nucleic acids can then be precipitated with alcohol. If sufficient quantities are obtained (~10 μg), the sample can also be size fractionated by gel electrophoresis.

Although nucleic acids are similarly prepared from both contemporary and ancient tissues, necrotic processes generally diminish DNA yields more than 100-fold. Postmortem decay of human bodies is an extremely complex and poorly understood process, beginning with autolysis and putrefaction then followed by slow aerobic and anaerobic microbial decomposition of organic material. Decomposition by microorganisms not only releases enzymes that digest polymeric organic com-

ponents (including nucleic acids) into small molecules, but also leaves contaminating nucleic acids derived from the invading genomes. In the analysis of frozen mammoth tissue, bacterial contamination was so extensive that no useful sequence information was obtained (Higuchi and Wilson, 1984; Johnson et al., 1985). In other cases, the burial context can contribute unrelated nucleic acids to archaeologically preserved isolates. For example, a major component of the DNA isolated from ancient human brains recovered from the Windover peat bogs in Florida (Royal and Clark, 1960; Doran et al., 1986) is, in fact, plant nucleic acids.

When decomposition is not immediately evident, the reduced yields are likely to be due to both endogenous biochemical activities and exogenous chemical reactions promoted by the environmental context. Release of autolytic enzymes by lysosomes has been shown to accompany cell death (Bär et al., 1988, Amenta and Baccino, 1989). Loss of enzyme regulation and lactic acidosis during autolysis increases hydrolase activity, a class of enzymes that includes endo- and exonucleases (Bradley, 1938). Endonucleases act on DNA by cleaving it internally into smaller fragments, whereas exonucleases sequentially remove nucleotides from the termini of these molecules (Lewin, 1987). The breakdown of the nuclear membrane presumably leaves most of the DNA and RNA susceptible to attack by endogenous nucleases. Proteolytic attack of other RNA-containing structures, such as ribosomes and splicesomes, can deprotect their nucleic acids, making them digestible by these same nucleases.

The rate of autolysis has been shown to be promoted by specific environmental conditions. This process is correlated with the moisture content of the necrotic tissue (Mackie, 1929), and may partially account for the remarkable preservation of long DNA molecules in some mummified remains excavated from arid environments (Rogan and Salvo, 1990; and Salvo, unpublished observations). In general, however, studies of preserved human remains from different archaeological sites around the world have determined that ancient DNA is markedly reduced in size (<500 bp), possibly, in part, as a consequence of nuclease action (Doran et al., 1986; Pääbo, 1985b). Although autolysis and microbial degradation cannot be clearly separated, both are maximal at temperatures between 34°C and 40°C. The excellent preservation of tissues recovered from permafrost may be due to the fact that both of these processes were inhibited at the time of death (Thuesen and Engberg, 1990).

The morphology of ancient human nucleic acids from both South America and Egypt reveals inter-strand cross-linking (Pääbo, 1989a; Salvo, unpublished observation), a characteristic not present in contemporary preparations. Although the chemistry of these structures is unknown, the inability of intercalating agents such as ethidium bromide to bind efficiently to ancient DNA (Pääbo, 1989a; Rogan and Salvo, 1990) is consistent with the possibility that the helix is severely deformed along much of its length. This may be a consequence of accumulated DNA damage during storage (see below).

THE CHEMISTRY OF ANCIENT DNA

Nucleic acid yields and sizes may also be affected by chemical reactions that occur independently of environmental context. Oxidation of macromolecular components appears to be the major form of modification. The analysis of protein components in ancient Egyptian tissues demonstrated that methionine and cysteine residues were often oxidized (Pääbo, unpublished results). Base compositional analysis of DNA from this sample was consistent with the loss of pyrimidine components and the addition of several unidentified compounds (Pääbo, 1985a). This result is typical of contemporary DNA which has been oxidatively damaged by radiation (Scholer et al., 1960).

Strand scission can also arise as a consequence of oxidative damage to nucleic acids. Using an enzymatic assay that detects the release of nucleotides from modified DNA, Pääbo found that ancient Egyptian DNA is extensively oxidatively damaged (Pääbo, 1989a). This is probably a major form of DNA damage, since oxygen radicals are both abundant and diffusible. Radicals are not only generated

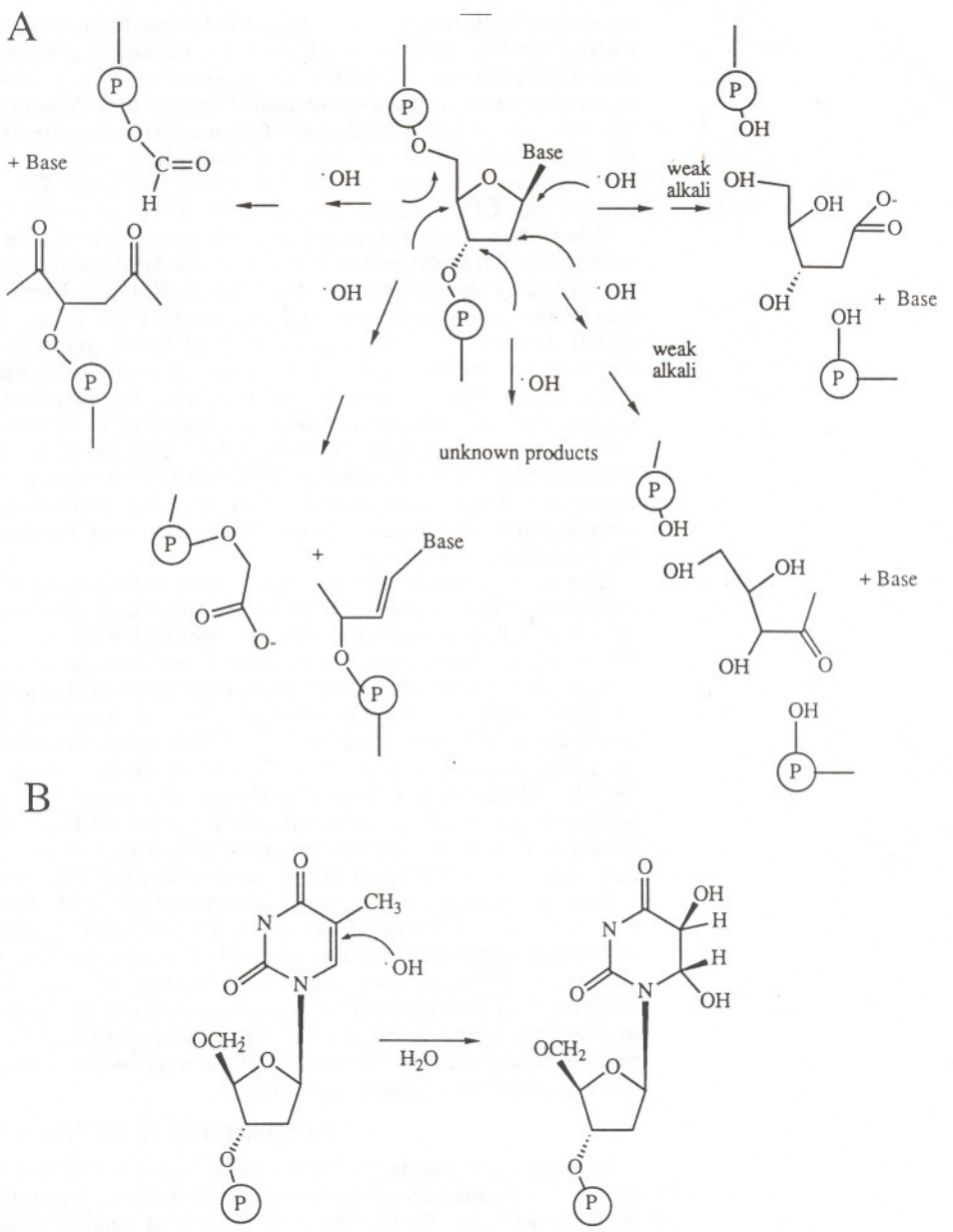

Fig. 1. Major products of hydroxyl radical attack on DNA in the presence of oxygen. **A:** Reaction with the 1, 2, 4, or 5 carbon in the deoxyribose ring. Note that strand scission usually occurs and that the base is often released. Adapted from Hutchinson (1985). **B:** Oxidation of thymine to 4,5-dihydroxythymine.

intracellularly in the mitochondria and chloroplasts, but are ubiquitous in the environment as well. They can be generated by metal catalysts present in the soil or by microorganisms which generate diffusible radicals as a normal function of their metabolism (Gibson, 1984).

Single-strand breaks in the DNA helix with blocked 3′ termini are among the most abundant products of free radical attack (Fig. 1A) (Hutchinson, 1985). They

result from an irreversible, diffusion-controlled reaction of hydroxyl radical with the deoxyribose ring, followed by hydrolysis and subsequent rearrangements. The products of this reaction are extremely labile, since mild incubation at 37°C can lead to a rapid increase in the proportion of broken DNA (Lafleur et al., 1979).

Only a small fraction of ends at the breaks have a 5′-OH (Bernhard, 1981), and few of the ends have a 3′-OH capable of acting as a primer for DNA polymerase (Landbeck and Hagen, 1973). Most 5′ ends are either phosphate groups, or very labile sugar fragments that hydrolyze under mild treatments (Henner et al., 1982). The major proportion of 3′ ends are stable sugar fragments or 3′ termini blocked by nucleotide fragments (Henner et al., 1983; Demple et al., 1986).

The nucleotide bases are also a major target for hydroxyl radicals. Although both purines and pyrimidines are susceptible, studies of chemical yields suggest that pyrimidine adducts are more common (see Fig. 1B). The principal adducts formed between the OH radical and pyrimidines come from addition to the 5,6-double bond. Oxidation of thymine favors the formation of thymine glycol, while cytosine is modified primarily to 5-hydroxycytosine and isodialuric acid (Teoule and Cadet, 1978). All of these reactions generate non-saturated ring systems that may sterically deform the helix and that somewhat destabilize the stacking interactions between successive bases. Three-dimensional model building suggests that these modifications do not interfere with base-pairing or completely disrupt stacking (P. Rogan, unpublished results).

Damage can occur throughout ancient DNA molecules, and artifactual sequence information is a real danger. Some of the damage can be "repaired" or by-passed by enzymes and methodology (see below), while multiple analyses of independent samples from the same individual (Pääbo et al., 1990a) can help verify specific sequence variants. Comparison of ancient DNA sequences obtained from genes that are known to be highly conserved throughout evolution can serve as an internal check on the integrity of other more interesting polymorphic regions.

SEQUENCE ANALYSIS OF ANCIENT NUCLEIC ACIDS

Although ancient nucleic acid may be heavily damaged and strand lengths might be reduced, the nucleotide sequence information does not appear to have been substantially corrupted. In his original study of ancient Egyptian human DNA, Pääbo determined that the sequence of a cloned 300 bp human AluI repeat element was representative of the entire family (Pääbo, 1985b). Similar results have been obtained for other genomic as well as mitochondrial DNA sequences (Doran et al., 1986; Pääbo et al., 1988; Rogan and Salvo, 1990). However, sequence analysis of DNA obtained from a museum sample of quagga tissue did produce some artifactual results (Higuchi et al., 1987) (see section headed "Fidelity of the PCR Technique"). The caveat in making conclusions regarding the sequence integrity of ancient nucleic acids is that the techniques employed in selecting templates for sequencing (i.e., cloning or polymerase chain reaction [PCR] amplification, see below) might only work with relatively intact templates. We can conclude that there must be stretches of uncorrupted nucleic acid sequences present in the ancient specimens, but from these results, we cannot determine what fraction of the total they represent.

Pääbo encountered numerous technical problems in cloning ancient nucleic acids. Damaged and sheared DNA is difficult to work with for several reasons. First, some enzymes employed in molecular biology are inactive on damaged DNA templates because modified nucleic acids are either not recognized by or inhibit DNA polymerases, ligases, and restriction endonucleases (Doran et al., 1986; Pääbo, 1986). Another problem arises when DNA ligation products containing damaged nucleotides are introduced into a bacterial host. These lesions probably reduce transformation efficiency by both inhibiting plasmid replication and by activating the host's own enzymatic repair machinery generating abasic sites or strand breaks in this region (Demple et al., 1986). This can result in the mutagenesis, and/or linearization with subsequent loss of the cloning vector. Finally, the aver-

Fig. 2. DNA has an anti-parallel complementary structure. Four nucleotides, adenine, cytosine, guanine, and thymine, abbreviated A, C, G, and T, are the basic building blocks and form specific pairings (A with T, and G with C), which hold the two DNA strands together via hydrogen bonds indicated in the diagram as dots. Information stored along one strand is present in a reverse-order complementary image on the other.

age fragment length of many preparations of ancient DNA is short, hence the information yield is severely limited using conventional cloning techniques. Since small human DNA fragments (<1,000 bp) are less likely to contain interesting DNA sequences than large ones, a direct cloning approach would require screening an enormous library of clones (tens of millions) in order to isolate a particular sequence. This is not practical with current technology.

THE POLYMERASE CHAIN REACTION IN ANCIENT DNA ANALYSIS

The polymerase chain reaction (PCR) has had a major impact on many facets of molecular biology (Saiki et al., 1988), but ancient DNA analysis, in particular, has been revolutionized. In order to analyze DNA sequences, a small amount of purified DNA must be prepared. With samples from living specimens, this is usually a straightforward procedure which involves grinding frozen tissue into a powder, removal of lipids and proteins by extraction with organic solvents, and precipitation of the DNA with ethanol. However, the yield of DNA from ancient sources is ~100-fold lower due to damage and modifications which occur soon after death. Polymerase chain reaction facilitates million-fold (or greater) amplification of the minute quantities of intact DNA sequences, making the use of subsequent molecular biological techniques possible.

The complementary structure of the DNA double helix is exploited during the amplification reaction. Information stored in the sequence of nucleotides along one strand of the double helix is present in a reversed complementary image on the opposing strand (Fig. 2). Short pieces of single-stranded DNA (oligonucleotide primers) can be synthesized chemically with defined sequences that match the outer edges of a genetic region of interest (e.g., an area known to contain polymorphisms). A thermostable DNA polymerase can be used to make faithful copies of the DNA sequence which lies between the flanking primers. The target DNA is denatured (typically 94°C for 2 min in buffer solution) and the large excess of primer molecules anneals to the complementary sequences at a lower temperature (55°C). The DNA polymerase can then copy the DNA sequences (at 72°C) adjacent to one end of each primer for up to several thousand nucleotides. The exact length of the amplified region is defined by the distance between the primers which are designed to bind to opposing strands of the double helix and direct synthesis toward the other primer (Fig. 3). The process is repeated (denaturation, annealing, extension) for 20–40 cycles, which theoretically doubles the amount of DNA between the primer binding sites of each cycle. In only 20 cycles, this process can amplify the target region over 1 million times.

DNA primers can be synthesized (commercially, e.g., Operon Technologies, San Pablo, CA) to match any known DNA sequence, and the automated PCR apparatus, enzymes and reagent kits are available from several sources (e.g., Perkin Elmer Cetus, Norwalk, CT). The actual amplification reaction is very fast (typically only a few minutes per cycle), so under ideal conditions a purified sample of ancient DNA can be amplified in several hours and ready for further analysis.

Amplification of ancient DNA sequences via PCR does not routinely generate the expected product(s), since it is often inhibited by some component in the template preparation (Pääbo et al., 1988; Rogan and Salvo, 1990). Pääbo et al. (1988)

have reported an extreme inverse dependence of the amplification efficiency on the length of the domain to be amplified. These authors propose that this general characteristic can be used to establish the authenticity of ancient DNA isolated from different environments. Although human DNA isolated from the Windover site is of high molecular weight due to the anaerobic preservation (Dickel et al., in press 1990), the amplification of mitochondrial sequences is still inhibited. The bone matrix may afford some protection from damage, since lengthy (600 bp) mitochondrial amplification products have been reported (Hagelberg et al., 1989). Since mitochondrial DNA is maintained in an environment that generates free-radicals during normal metabolism (Lehninger, 1982) and postmortem (Mann et al., 1978), it might be expected to exhibit more damage by hydroxyl radical attack than chromosomal sequences. Further analysis is needed to address this issue.

Successful PCR amplification has been achieved by modifying the standard PCR protocol (Saiki et al., 1988). Pääbo et al. (1988) were able to overcome inhibition of *Thermophilus aquaticus* (Taq) DNA polymerase by including bovine serum albumin (BSA) and increasing the enzyme concentration in the reaction mixture. Hagelberg et al. (1989) found that elevated enzyme levels were not required, though BSA was still included. The total DNA concentration was also elevated in order to increase the amount of intact ancient DNA template (Pääbo et al., 1988). Successful amplification with this protocol might be explained as the dilution of a competitive inhibitor or preferential binding of a soluble inhibitory factor (by BSA) that co-purifies with DNA.

The nature of PCR inhibition may, in fact, vary among archaeological samples and the procedures for purifying their nucleic acids. By amplifying mixtures of ancient and contemporary DNA preparations, Rogan and Salvo (1990) demonstrated that the inhibitor in their ancient DNA preparations was not an extraneous soluble factor, but was integral to the DNA template. The inhibitory lesions could be circumvented by displacement and resynthesis of the damaged DNA strand with either Klenow DNA polymerase or reverse transcriptase (Kornberg, 1980; Feinberg and Vogelstein, 1983). These enzymes, in contrast with Taq and several other DNA polymerases, can bypass oxidative damage in DNA (Clark and Beardsley, 1987). The profile of inhibition of several different DNA polymerases is consistent with the accumulation of oxidized nucleic acids in the DNA templates rather than contamination by a soluble factor. By incorporating a repair synthesis step prior to PCR amplification, Rogan and Salvo (1990) were able to generate *Alu* repeat products for specimens from 70% of the individual tombs surveyed. This approach also appears to partially overcome the size limitation observed in some PCR amplifications, since products as long as 250 bp were routinely obtained. Nevertheless, it should be remembered that products as long as 5,000 bp can be amplified from contemporary DNA.

THE FIDELITY OF THE PCR TECHNIQUE

The propagation of errors during the replication of specific nucleotide sequences has warranted concern about the accuracy of the polymerase chain reaction technique. When Taq DNA polymerase is used to copy contemporary DNA templates, incorrect nucleotides are introduced at a rate of less than one per 9,000 bp (Tindall and Kunkel, 1988). The lesions present in ancient DNA, in some cases, can contribute to substantially lower fidelity of PCR amplification. For example, the sequence of a mitochondrial DNA segment isolated from the 4,000 year-old mummified liver of an Egyptian priest showed two nucleotide changes in 45 bp (Pääbo, 1989a). When this amplification product was cloned and sequenced, 17 of the 25 pyrimidines were mutated in the derived clones (Pääbo et al., 1990a). The observation that 15 of the 17 base changes are pyrimidine substitutions is consistent with the preference for oxidative damage to occur at these sites. The presence of previously unknown sequence variants revealed by direct PCR amplification of ancient mitochondrial DNA should therefore be interpreted cautiously.

Pääbo et al. (1990a) offer a mechanism for the direct amplification of ancient

DNA. They suggest that amplification of ancient DNA proceeds in two stages: a slow "lag" phase followed by an exponential amplification reaction. Like other DNA polymerases (Friedberg, 1985), Taq DNA polymerase arrests (and may be error-prone) at the sites of DNA lesions. This generates incomplete extension products, which when melted and reannealed to other ancient templates during the reaction cycle can serve as primers for additional rounds of DNA synthesis. The process is repeated until another lesion is encountered by the polymerase. An amplification product can be synthesized only if this product is extended as far as the sequence complementary to the opposing oligonucleotide primer. The recombinant nature of amplification products suggests that the derived sequence may be chimera of multiple templates. Any non-uniformity among the templates will be homogenized in the final products. The mutagenic nature of lesions in ancient DNA can therefore account for the observed heterogeneity in the sequences of direct amplification products (Pääbo et al., 1988; Pääbo et al., 1990a).

The retention of contemporary mitochondrial polymorphisms in ancient DNA sequence suggests that direct PCR can demonstrate some fidelity in replication. DNA studies of prehistoric (7,000 [B.P.]) Amerindian DNA revealed conservation of both a 9 bp duplication and a restriction site polymorphism, both of which are present in the majority of contemporary descendants (Pääbo et al., 1988). In other studies, the nicotinamide adenine dinucleotide reduced form (NADH) dehydrogenase domain of mitochondrial DNA was faithfully amplified from medieval and 17th century human remains (Hagelberg et al., 1989), except for a single transitional variant (Vigilant et al., 1988).

A wide range of DNA modifications are known to inhibit DNA polymerases both in vitro and in vivo (Friedberg, 1985), but the extent of inhibition is both lesion- and polymerase-dependent. Copies of ancient DNA templates can be obtained by utilizing a replication system that recognizes altered nucleotides and faithfully replicates through them (Pääbo, 1986; Rogan and Salvo, 1990). In one study, we found that Klenow fragment of DNA polymerase I was able to utilize ancient DNA as a template for the incorporation of radionucleotides. Pretreating the ancient templates with Klenow fragment was found to improve the success of subsequent PCR amplifications. This result is consistent with Klenow enzyme proceeding past lesions that normally block 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. Reverse transcriptase, which is also capable of bypassing oxidized nucleotides, appears to be even more tolerant than Klenow DNA polymerase for pre-amplification of ancient DNA [Rogan and Salvo, in preparation]. A highly conserved, human genomic template (28S rDNA) was amplified to assay the fidelity of this procedure. PCR products were cloned and the resultant nucleotide sequences were compared with the sequence of a contemporary amplification product. The sequences of ancient ribosomal DNA from four unrelated individuals contained a single nucleotide discrepancy in 1,000 bp [Rogan and Salvo, in preparation]. Since replication past lesions in vitro is thought to be inefficient, only a small proportion of the least damaged ancient templates are presumably "repaired." The relatively strong inhibition of Taq DNA polymerase on damaged templates compared with its high processivity on intact templates probably accounts for the reliability of the modified PCR technique.

THE SENSITIVITY OF PCR AS A CAUTIONARY NOTE

Since PCR can synthesize many copies of a DNA fragment from minute quantities of template DNA, rigorous care must be exercised to avoid generating false-positive reaction products. Although there are many potential sources of contamination in both the field and the laboratory, common sense practices can minimize these effects (Kwok and Higuchi, 1989). In the laboratory, control PCR reactions can be carried out to assess introduced contamination at each stage of sample preparation.

While handling of preserved specimens is unavoidable during excavation, con-

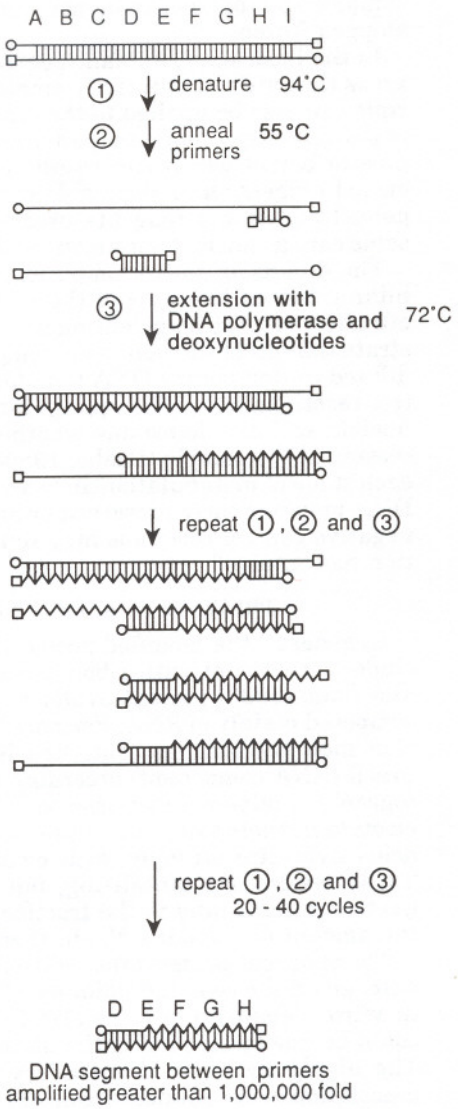

Fig. 3. The polymerase chain reaction can amplify specific domains of preselected DNA sequences from a small number of template DNA molecules (Saiki et al., 1985). This process is carried out iteratively by thermally melting the ancient DNA duplexes (step 1) and annealing the resulting single-stranded molecules to excess oligonucleotide primers (step 2) which initiate opposing DNA synthesis reactions in the presence of a thermostable DNA polymerase (step 3). The primers are synthesized chemically (in another process) to match known contemporary DNA sequences; thus, they define the length of the amplified interval. The major amplification product is a double-stranded DNA fragment which can then be gel purified, sequenced, cloned, or restriction-mapped. Newly synthesized DNA is indicated by wavy lines. The circles and squares at the end of the various DNA strands indicate chemically distinguishable ends. Letters above the DNA molecules indicate relative position, and could stand for genes or known polymorphisms.

tamination by the archaeologist can be limited if disposable gloves are worn. In this regard, samples found at disturbed excavation sites warrant caution because unknown human DNA contamination may have occurred. In order to prevent decomposition or microbial growth during transit to more humid climes, organic

samples can be sealed in non-porous containers with desiccant or, if possible, shipped frozen.

In the laboratory, human specimens can be processed in dedicated containment areas that are not ordinarily employed in the analysis of human DNA. This principle can also be applied to the study of other ancient organisms as well. The use of positive-displacement pipettes reduces the likelihood of aerosol formation in the pipette barrel which can result in cross-contamination between samples. Autoclaved reagents and disposable plasticware are employed wherever possible. Oligonucleotides and reagents used for amplification should be prepared with the same care as ancient specimens and in an area isolated from the PCR workstation.

The design of control amplification reactions can be a critical factor in determining the authenticity of the PCR product. When no product is observed (as is often the case with ancient nucleic acid), the positive control reaction can demonstrate that all of the non-DNA components of the reaction are functional. A highly diluted contemporary DNA template is often used for this purpose. Negative control reactions where the appropriate buffer solution is substituted for the ancient nucleic acid can determine whether contamination has occurred during the processing of the sample (Pääbo, 1989b). Several null reaction tubes can be set up at each stage of manipulation and carried through the remaining steps. Since ancient DNA may be poorly preserved or absent from preserved tissues, running multiple negative control reactions may limit the possibility that false-positive amplification has occurred.

PRESENCE OF RIBONUCLEIC ACID IN ANCIENT PREPARATIONS

In general, the modified protocols for isolating ancient nucleic acids do not include a treatment with ribonuclease to remove ribonucleic acid (RNA). Venanzi and Rollo (1990) present evidence that the nucleic acids in ancient samples are composed mainly of RNA. Formate hydrolysates of ancient maize, cress, and Egyptian mummy samples contained little thymine (DNA component) compared to uracil (RNA component) according to reverse-phase high-pressure liquid chromatography analysis. Ribonuclease (RNase) treatment degrades most of the polynucleotide in their samples. In the case of maize seeds, radiolabeled ancient nucleic acids hybridize strongly with cloned genes encoding abundant cellular RNAs. These results are tantalizing, but cannot yet be regarded as conclusive, since questions pertaining to the fraction of unpolymerized uracil in ancient seeds and the amount of ancient DNA in these samples remain to be answered.

The apparent preservation of RNA in ancient preparations is somewhat remarkable, given the seeming ubiquity of ribonucleases that contribute to RNA lability in vitro. Venanzi and Rollo (1990) suggest that the longevity of ribosomal RNA could be due to its substantial abundance relative to other cellular nucleic acids. The highly constrained secondary structures, modified nucleotides, and strong association with abundant cellular proteins might protect ribosomal RNA from cellular ribonucleases postmortem. However, there has not been an unequivocal demonstration that the amplified sequences carry remnants of post-transcriptional processing (i.e., intron excision or polyadenylation). Until then, the existence of ancient RNA should be considered tentative.

CURRENT AND PROSPECTIVE ISSUES IN MOLECULAR ARCHAEOLOGY

The use of ancient DNA to study genetic variation

Although there are many types of mutations that occur in eukaryotic genes, the majority of those in coding regions are nucleotide substitutions (Fitch, 1967). Genetic variability is expressed by the fixation of allelic mutations among members of a population. Although some nucleotide sequence differences are fixed by natural selection, most are silent and reflect natural polymorphism among the population (Kimura and Ohta, 1971).

Ancient DNA sequence information, in theory, is capable of charting the distribution of ancestral mutation events in time and space. Since most nucleotide

sequences have been obtained from preserved remains <10,000 years old, this limits the types of human polymorphism that can be studied. The fixation of new mutations by selection is not a frequent event in human evolution. This is because the mutation rate in eukaryotes is dependent on both generation time and the repair efficiency, rather than chronological time (Magni, 1969; Muller, 1959). The relatively small number of human generations that can be sampled during this interval and the extremely low mutation rate in humans (probably 0.1–1% per million years) makes it unlikely that nucleotide sequences of prehistoric remains, in practice, will reveal many novel ancestral mutation events. It is possible, however, that previously unknown polymorphisms may be discovered which occurred in extinct human lineages.

Populational polymorphism

The identification of contemporary polymorphic mitochondrial alleles has facilitated the analysis of human geographic origins and dispersal (Cann et al., 1984). Blood-typing determinants have been intensively studied in ancient remains because of their ubiquity in mammals (Boyd and Boyd, 1937; Harrison et al., 1969; Allison et al., 1976). In addition to their limited polymorphism, degradation and contamination of these antigen epitopes may have generated anomalous results (Flaherty and Haigh, 1986). The extremely polymorphic products of the human major histocompatibility complex antigens can also be used to chart ancient populations (Stastny, 1974; Hosseini and Allison, 1976), and a wide spectrum of serological reagents is available. The results obtained with this method are more consistent than those obtained with the ABO blood group tests. Caution in interpreting immunological experiments is warranted since the specificity of sera for potentially modified ancient antigens has not yet been evaluated.

The patterns and timing of migration can be reconstructed from highly polymorphic DNA sequences of contemporary populations (Cann et al., 1987). Phylogenetic inferences drawn from such sequences assume that different groups have similar mutation rates. This assumption is necessary because some of the mutations that occurred at rapidly evolving sites have been obscured by reversion of multiple nucleotide substitutions. In order to deduce a phylogeny, estimates must be made of the frequency of these invisible events. Such estimates are dependent on the accuracy of the mutation rate in each respective population. In theory, ancient nucleic acid sequences can be directly compared with those of descendant populations. While 2,000–10,000 years may not be a sufficient period to reveal invisible nucleotide substitutions, the fact that these sequences are internal nodes in the cladogram may improve the accuracy of a specific tree topology. In other words, having an ancestral sequence with associated geographical and chronological information may be able to resolve ambiguous bifurcations where descendant clades cannot be distinguished on the basis of mutation distance alone.

The hominid settlement of the Americas by Siberian people subsequent to the late Pleistocene is consistent with a variety of biological and linguistic analyses (Laughlin, 1963; Swadesh, 1962; Spuhler, 1972). The issue of how and when the migrations occurred is a focal point of studies on American origins. Cladograms of indigenous North Americans determined from genetic, linguistic, and dental evidence generally have a tripartite structure with Eskimo/Aleut, Na-Dene or Athapaskan, and Macro-Indian divisions (Turner, 1985; Williams et al., 1985; Greenberg et al., 1986). The relationships among these groups and their respective genetic distances appear to depend on the type of data analyzed, the method of analysis, and the number of informative characters.

The various alternative hypotheses are circumscribed by the following parameters: 1) geographic isolation mechanisms, i.e., the disappearance of the Bering land bridge, or the recession of the Cordilleran and Laurentide ice sheets (Brues, 1977); 2) the amount of distribution of genetic, biological, and linguistic variability in the ancestral and descendant populations (Spuhler, 1972); and 3) the degree to which genetic, archeological, and cultural markers can differentiate between dis-

crete population groups (Weiss and Maruyama, 1976; Dumond, 1979; Townsend and Brown, 1979). For example, constraints on generation time and heterogeneity probably exclude the possibility that this hemisphere was populated by the dispersal of descendants of a single, homogeneous, small founder population. However, entry of a single pre-differentiated ancestral Aleut-Eskimo population (Laughlin and Wolf, 1979) has been suggested as one of several plausible hypotheses which account for the observed diversity.

A heterogenous founding population could have generated satellite groups by several mechanisms. In one scenario, colonization by the fittest individuals among small groups migrating inland might have been selected by a harsh environment where food sources were less predictable. An alternative, but not exclusive, model suggests that different groups may have become geographically isolated by the opening and closing of an ice-free corridor out of Beringia along the eastern side of the Rocky Mountains (Szathmary, 1984). In this class of hypotheses, phylogenetic relationships might be expected to correlate with geographic distances. In fact, Szathmary and Ossenberg (1978) have suggested that Na-Dene and Eskimo groups are more closely related to each other than either is to the Macro-Indians. However, bootstrap analysis of classical genetic data show that the Na-Dene group can form a significant cluster with Macro-Indian populations (Cavalli-Sforza et al., 1988).

The suggestion that multiple independent groups migrated to North America implies that some populations resided here longer than others. Consequently, the older occupants might be expected to exhibit greater intra-populational diversity than the recent arrivals. Advocates of a tripartite migration argue that ancestors of the Macro-Indians were followed by the Na-Dene and then the Eskimo/Aleut peoples. While comparative linguistic and some genetic studies are consistent with this prediction (Greenberg et al., 1986; Williams et al., 1985), the dendrogram derived from dental characters suggests that the Eskimo/Aleut, Athapaskan, and Macro-Indians share a common ancestor (Greenberg et al., 1986), with the greatest variation occurring in the Eskimo/Aleut population.

These models describing the settlement of North America probably oversimplify the actual patterns of hominid migration, settlement, and extinction. Genetic, linguistic, and morphological data taken from current descendants has to be corrected for admixture, variable rates of change, population size (and historical bottlenecks), and the numbers of characters analyzed. Imprecision in estimating these parameters is translated into large standard errors on phylogenetic distances (Weiss and Maruyama, 1976). This makes cladistic bifurcations less meaningful, and makes it difficult to discriminate between alternative hypotheses of North American migration.

The ideal set of data would consist of multiple samplings of large numbers of discrete, compatible traits, each having specific linkage to a distinct identifiable Asian ancestral group. Mitochondrial DNA sequences may fit this profile quite well. There are estimated to be more than 3,000 polymorphic nucleotide sites in this genome. To date, only a small proportion of these markers have been examined for their linkage to specific populations (Caan et al., 1987), although several have been shown to be correlated with Asian ancestry. For example, ~20% of contemporary people of Asian ancestry carry a distinct 9 bp sequence in region V of the mitochondrial DNA (Wrischnik et al., 1987), while most of the world's population contains an exact duplication of this sequence. Although this genetic marker could have dispersed across the Asian continent subsequent to the migration of people to North America, the presence of this marker in DNA isolated from ancient or contemporary Indians would create a tentative link to this Asian subgroup. Mitochondrial DNA sequences from a prehistoric North American Indian from the 8,000 year-old Windover site in central Florida (Doran et al., 1986; Pääbo et al., 1988) and several pre-Columbian Chilean mummies 500–800 years B.P. [Rogan and Salvo, in preparation] have been found to retain the common duplication. Analysis of a greater number of samples will be required to pursue this potential

DNA link to Asia. However, the Windover specimen did include several informative sequence variants including an A to G transition in region V of the mitochondrial genome. The A to G transition is known to exist in a small group of Japanese lineages (Horai and Matsunaga, 1986) and this suggests a connection to the Windover individual. The excavation and sequence analysis of other specimens from the Windover site should indicate whether this purine transition reflects the true genotype. In contemporary populations of South American, Central American, and Northwestern Indians, the single 9 bp sequence element has been identified as predicted (Schurr et al., 1990; Wallace et al., 1985; Valencia and Ward, 1990; Pääbo et al., 1990b), but more extensive mitochondrial sequence information and other variants are needed to help define the number and extent of early migrations into the New World.

Multi-allelic loci

Chromosomal recombination generates genomic polymorphism at high frequencies (relative to nucleotide substitution) in eukaryotes. Conservation at highly polymorphic loci has been exploited to identify related members of a population, since it is unlikely that two randomly selected individuals share common alleles (Klein, 1986; Jeffreys et al., 1985a). These methods, and in particular, DNA fingerprinting analysis, can detect relationships between non-nuclear family members (Jeffreys et al., 1985b). Contemporary DNA-typing techniques are being adapted to study nucleic acids from individuals entombed in the same cemetery [Rogan, Allison and Salvo, in preparation]. This might reveal whether kin relationships were maintained with ancient burial practices, and may generate information about the heterozygosity of local populations. There is ample paleopathological evidence in ancient South American mummies for phenotypic defects usually associated with the expression of recessive genetic alleles (Allison et al., 1982). The extent of polymorphism at multi-allelic loci could potentially measure the level of inbreeding or homozygosity in these groups.

Informative non-polymorphic markers

DNA primers designed to amplify specific Y chromosomal sequences may potentially assist in the determination of sex in poorly preserved remains. Direct sequencing of PCR products derived from homogenous, multi-copy conserved DNA sequences (e.g., 28S rDNA) provide an internal control for the extent of damage, postmortem modification, or artifactual events arising during amplification.

The analysis of extinct animal remains

The impact of DNA sequences amplified from preserved, extinct museum specimens on evolutionary biology will be substantial. For extant organisms whose taxonomic assignments are controversial, dendrograms based on DNA sequences are already resolving indeterminate phylogenetic relationships.

The evoluation of the horse family from *Eohippus* to its contemporary descendant, *Equus,* is based on abundant paleontological evidence (Simpson, 1953). The classification of the quagga, a recent descendant of the equine radiation, has been the subject of some controversy. Whereas Bennett (1980) grouped the quagga in the same class as the domestic horse, Eisenmann (1979) suggested that it was more closely related to the plains zebra. Both quagga and zebra have also been proposed to be members of the same species (Rau, 1974). The phylogenetic tree derived by comparing a 229 bp ancient mitochondrial quagga sequence with those of contemporary perissodactyls places quagga and plains zebra on a common bifurcating branch, (Fig. 4) (Higuchi et al., 1984, 1987). A survey of polymorphic sites in the complete mitochondrial genomes of the modern species resulted in a tree having the same topology (George and Ryder, 1986). The two nucleotide discordancies between quagga and zebra sequences were interpreted as postmortem changes (Higuchi et al., 1987), since neither is a silent mutation and both of the supposed amino acid replacements are at conserved positions in higher vertebrates. The

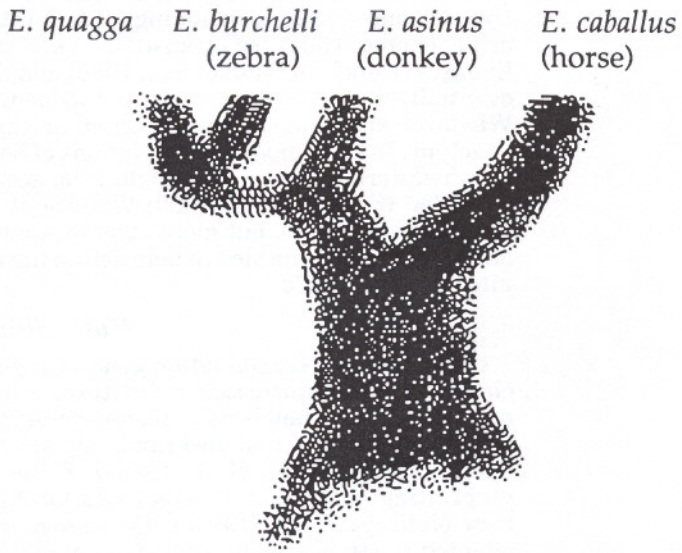

Fig. 4. A recent phylogeny of the Equidae based on data taken from Higuchi et al. (1987) (DNA sequence analysis of a region of the quagga and zebra mitochondrial genome) and Wilson et al. (1974) (comparisons of serum albumen immunological cross-reactivity in donkey, zebra, horse). Distance relationships are approximate.

absence of true sequence variation means that, in this case, the ancient sequence is not informative with respect to the mitochondrial mutation rate in this genus.

Molecular and morphological evidence is not necessarily in agreement regarding the origins of the extinct marsupial wolf (*Thylacinus cynocephalus*). The morphological characters appear to be inconsistent, since tooth and pelvic structures are similar to South American borhyaenids, while hindlimb features are shared with Australian dasyurids (Archer, 1982). Both immunological cross-reactivity of albumens (Lowenstein et al., 1981) and DNA sequence comparisons of thylacine mitochondrial 12S rDNA with modern species (Thomas et al., 1989) agree with the branching suggested by the thylacine fossil record (which began 12–15 million years ago (myr) in Australia (Archer, 1982). These taxa are thought to have diverged ~10–20 myr ago based on a mitochondrial DNA phylogeny or ~6–10 myr ago by antigenic criteria. Both estimates post-date the separation of Australia from Antarctica and South America, which suggests that the South American marsupial characteristics in the wolf are not ancestral, and may be an example of convergent evolution.

The analysis of extinct plant remains

The chemical and physical properties of nucleic acids extracted from ancient plant remains appear to be comparable to animal nucleic acids. In general, however, plant tissues appear to yield nucleic acids more often than animal remains do. Perhaps this is because the cell wall, a structure unique to plants, affords protection against some external agents of decomposition. The frequent fossilization of plant structures supports this notion.

Rogers and Bendich (1985) extracted frozen tissue homogenates with cetyltriammonium bromide to purify nucleic acids away from enzyme-inhibiting polysaccharides. Processing of whole seeds and grains gave DNA yields intermediate between storage organs and embryos. Although this protocol is optimized for isolation of high molecular weight DNA (Murray and Thompson, 1980), 18S and 25S ribosomal RNA bands were prominent in several of their preparations. DNA deg-

radation was apparent in all of the mummified seeds and embryos, but preparations of *Escholtzia minutiflora, Lycium shockleyi, Opuntia ramosissima,* and *Juniperus osteosperma* contained molecules longer than 10 kb. The Juniper samples were digestible with a restriction endonuclease, and if these preparations are not extensively damaged, it may be possible to clone restriction fragments directly for subsequent sequence analysis.

The DNA sequence analysis of polymorphic genetic loci and genes encoding desired traits of agriculturally significant ancient plant and animal species may help to trace the process of artificial selection. Sequence patterns may be able to distinguish the temporal and geographic emergence of annual from perennial varieties and domesticates from annuals (McCorriston and Hole, in press). The domestication process is thought to be correlated with the polyploidization of a wide variety of staple crops (Stebbins, 1950). This has resulted in greater genomic stability, but also has limited the overall rate of mutation in these organisms (Kahler et al., 1984). While polyploidization might tend to homogenize polymorphic chromosomal loci, it should not directly effect variability in organelle genomes.

Ancient specimens of maize are the predecessors of contemporary corn varieties, and probably represent an intermediate stage in the domestication process. Well-preserved specimens, often found in association with pre-Columbian burials, contain quantities of both genomic and mitochondrial DNA (Rollo et al., 1988). The mitochondrial amplification product obtained in this study conserved both length and restriction sites. The sequences of polymorphic genomic and mitochondrial domains are eagerly awaited, since the mutations could be corroborated with genealogies derived from morphological characters. Independently determined mutation rates could then be used to deduce the date when domesticated varieties were first cultivated.

As the cultivation of wild cereals in the Near East resulted in their domestication, new processing techniques were discovered which improved the nutritional value of these foods (Braidwood, 1953). The sprouting and fermentation of grains as well as the baking of bread were extensively recorded in ancient Egypt (Winlock, 1955). Recently, a pre-dynastic brewery site was excavated at Hierakonpolis and found to contain vats with residue thought to be derived from fermentation of beer (Geller, 1989). Contemporary cultivated strains of the yeast, *Saccharomyces cerevisiae,* which has a vital role in both baking and brewing, are genetically and biochemically quite distinct from wild-type varieties. By amplification of ribosomal DNA sequences, it may be possible to determine whether the fermentation agent at the Hierakonpolis site was *S. cerevisiae* [Rogan, Geller and Salvo, in preparation]. If this organism is identified, chromosomal loci thought to differ in wild-type and commercial yeast strains will be surveyed in the ancient specimen.

HORIZONS IN MOLECULAR ARCHAEOLOGY

Molecular paleopathology

> Paleopathology is the science of the diseases which can be demonstrated in human and animal remains of ancient times (Ruffer, 1913).

The study of human material from ancient times utilizes modern laboratory diagnostic procedures that Ruffer did not dream of. The application of radiographic, histological, and microbiological methods to mummified remains has generated insights into developmental abnormalities and infectious disease. Molecular biological approaches should complement these techniques, since they could reveal the nature of genetic anomalies or conclusively establish the causative agents of infection.

Discontinuous traits or non-metrical markers have been employed by physical anthropologists as a means of establishing blood relationships between deceased individuals. Using a mouse model, these anomalies have been shown to have a genetic basis (Berry, 1968). When skeletal variants (considered to be minor defects

TABLE 2. Technical requirements for the study of ancient nucleic acid sequences

Bypass non-mutagenic lesions present in DNA template
Discriminate between indigenous DNA or RNA sequences and contaminants derived from other organisms
Allow analysis of extremely limited quantities of nucleic acid
Be informative, i.e., generate sequence information from polymorphic domains (including single copy sequences) with high fidelity

in ossification) and other congenital malformations such as spina bifida and hip dysplasia have been diagnosed in groups of excavated individuals, the comparison of multiple traits have produced a statistically based measure of genetic divergence among groups (Sneath and Sokal, 1973). Until the development of procedures to isolate ancient DNA, it was not possible to determine the validity of genetic distances calculated from non-metric morphological data. The direct analysis of polymorphic genetic loci in mummified remains from several Chilean cemeteries will provide an independent reevaluation of the regional population diversity (Allison et al., 1982).

The demonstration that two ancient individuals were genetically related by analysis of their respective DNA sequences or restriction patterns has not yet been accomplished. Suitable test cases have been identified due to the high mortality associated with childbirth in ancient Andean cultures. Often the child was interred with its mother with the umbilical cord intact. The mother usually shows signs of a recent delivery, since swollen breasts and abdomen as well as the placenta may still be evident (Allison et al., 1982). We are currently analyzing DNA from such a mother/daughter pair provided by Dr. Arthur Aufderheide (Rogan and Salvo, in progress). At this point, several distinct non-polymorphic chromosomal sequences have been amplified in both specimens.

The consequences as well as the etiology of parasitic infections in ancient man are frequently detectable by standard laboratory procedures. The most common cause of death outside of urban localities appears to have been acute and chronic respiratory disease (Mims, 1987). The morphologies and histochemistry of pneumococci and mycobacteria are distinctive in mummified tissue, particularly if found in conjunction with characteristic pathology (Cockburn and Cockburn, 1980; Buikstra, 1981; Allison, 1985). In cases where diagnosis might be uncertain, PCR amplification of genomic or episomal DNA derived from the pathogen may be possible.

There may be an additional dividend from the analysis of ancient prokaryotic or fungal DNA. The determination of such sequences might reveal bona fide nucleotide differences (relative to the contemporary descendant) that correspond to mutation events that have been fixed by natural selection. The actual frequency of nucleotide substitutions over several thousand years could resolve a dispute over whether prokaryotic mutation rates depend more on chronological times (Drake, 1966) or the number of elapsed generations (Kubitschek, 1970).

Technological improvements

Only a few years ago, the retrieval of nucleic acid sequences from mummified tissues seemed out of reach. While the development of PCR methodology has made repetitive ancient DNA sequences accessible, amplification of polymorphic, low-abundance chromosomal loci are currently still a challenge. The presence of a 382 bp sequence similar to the first intron of the human major histocompatibility locus (HLA)-DQ α1 gene might indicate that unique sequences exist in mummy DNA preparations (Del Pozzo and Guardiola, 1989). As our understanding of the chemistry of ancient DNA increases, more sophisticated molecular approaches will be developed and make the sequences of many single-copy genes available. In this regard, we are continuing to survey DNA polymerases which are more refractory to template damage, while retaining nominal fidelity. An alternative approach of restoring ancient DNA relies on a class of DNA repair enzymes that recognize and

excise a broad spectrum of oxidative lesions (Demple et al., 1986). It may be possible to lightly treat ancient DNA with these agents prior to amplification. This will require the development of quantitative assays to measure the extent of oxidative damage in ancient samples (Pääbo et al., 1990a), since excessive levels of glycosylase treatment could potentially strip too many bases from DNA templates.

When the amplification of single-copy sequences becomes routine, it should become possible to clone libraries from ancient genomes. We envision a PCR-based approach that employs oligonucleotide primers derived from two different interspersed repetitive sequence families. Although this protocol will not generate a comprehensive genomic library, it will facilitate the sequence analysis of many polymorphic sites in ancient DNA, and also make ancient DNA analysis accessible to many more investigators.

The difficulties in amplifying ancient DNA have necessitated the design of experiments requiring a high degree of specificity in the desired PCR product. The inability to clearly identify an organic archaeological specimen would normally preclude the specific amplification of purified nucleic acids. The wealth of phylogenetic information based on ribosomal DNA sequences can provide a framework for the identification of DNA from unknown organisms. The development of universal oligonucleotide primers that can generate several species-specific PCR products (Rogan et al., 1990) from mixtures of divergent organisms might make it possible to identify individual components in complex archaeological specimens.

The limits of ancient DNA analysis appear to depend more on the integrity and preservation state of the sample, rather than its age. A variety of ancient DNAs have been analyzed from the remains of plants and animals determined to be several thousand years in age (Table 1), yet the most remarkable find to date is the analysis of chloroplast DNA from magnolia leaves estimated to be between 17–20 million years old (Golenberg et al., 1990). The leaves were found in sediments that provided a cool, hydrated anoxic environment which minimized oxidative damage. Sequencing of PCR-amplified regions of the rbcL gene (which encodes the large subunit of ribulose 1,5-bis-phosphate carboxylase) allowed a direct comparison to extant species. The time frontier may be pushed back even further if it becomes possible to amplify DNA sequences from insects preserved in amber. Chromatin-like structures have already been identified in thin sections of a 40 million year-old mosquito (Poinar and Hess, 1982). One cannot help but wonder what the limits of this molecular sleuthing will be and whether DNA sequences exist in some cool, anoxic, dinosaur graveyard.

Investigators now have molecular measures to help track the temporal and geographical influences on life. Molecular analyses of select fossil remains promise to give investigators a bright new perspective from which to approach both evolution and population genetics. Technical achievements have fostered these developments, but collaborations between a myriad of scientists in divergent fields will be required to define the limits of such pursuits.

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