Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 264, No. 17, Issue of June 15, pp. 9709-9712, 1989

Ancient DNA and the Polymerase Chain Reaction

THE EMERGING FIELD OF MOLECULAR ARCHAEOLOGY*

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Molecular evolution is a historic process through which genes accumulate changes due to stochastic events as well as selective processes. Students of molecular evolution suffer from the frustration of trying to reconstruct this historic process from only a knowledge of the present-day structure of genes. Until recently, there has been no hope of escaping this "time trap." However, advances in molecular biological techniques have enabled us to retrieve and study ancient DNA molecules and thus to catch evolution red-handed. In consequence, we can now study the genealogical relationships of extinct species and vanished populations. In addition, it seems likely that we shall be able to monitor fast genetic processes such as recombinational events. Our review discusses older attempts to obtain molecular genetic data from archaeological remains as well as recent achievements and emerging vistas.

Studies of Ancient Proteins

The first indications that molecular genetic information might persist in ancient materials were early demonstrations that the peptide bond can last for up to 10^8 years in fossil shells and bones (1–3) and that subcellular detail implying the survival of ribosomes and chromatin is evident in insects from 40 million-year-old amber (4). Indeed, these findings inspired the hope that genetic information should be retrievable from the amino acid sequences in ancient remains, and substantial efforts over the past two decades went into such endeavors.

Unfortunately, the major proteins in bone (collagen) and shell (conchiolin) are likely to be genetically uninformative because collagen has a repetitious primary structure and is encoded by multiple genes (5), whereas conchiolin is a complex mixture of proteins whose genetic basis is unknown (6). Second, the proteins in ancient remains are structurally heterogeneous because of post-mortem modifications (7, 8).

Even in exceptionally well preserved remains, such as frozen muscle from an extinct Siberian mammoth, extensive modifications were evident from elemental analysis, electron microscopy, and amino acid analysis of the 40,000-year-old tissue (9, 10). In the case of albumin, one of the most stable globular proteins known in animal tissues (8), only about 2% of the mammoth molecules could dissolve in water, and 80% of the latter were modified in charge, size, or antigenicity (9).

* Work done in the laboratory of A. C. W. received support from the National Science Foundation and the National Institutes of Health.

‡ Supported by a long-term fellowship (ALTF 76-1986) from the European Molecular Biology Organization.

Immunological Distances—The first comparisons of the primary structure of a gene product of an extinct species to that of living species were achieved indirectly by using polyclonal antisera raised against a homogenate of mammoth muscle. Such an antiserum was tested for its ability to form precipitates and complement-fixing lattices with a panel of native albumins from several living species (9, 10). The reactions were strong with Indian and African elephant albumins, weak with sea cow albumin, and weaker still with other mammalian albumins. Since the cross-reaction specificity of the anti-mammoth serum matched exactly those of antisera to the pure albumins of elephants, the native albumins of these three species (mammoth, Indian elephant, and African elephant) are nearly identical in primary sequence (*cf.* Ref. 11).

Radioimmunoassay, which does not require lattice formation and thus does not demand that a cross-reactive antigen bear more than one antigenic site, confirmed the close relationship of elephant and mammoth albumins (12); it also placed the extinct Tasmanian wolf within the genealogical tree for extant carnivorous marsupials (12) and Steller's sea cow within the tree for extant sea cows (13) on the basis of tests with antisera to their albumins. However, this method, like other univalent methods, can be less reliable as a predictor of sequence divergence than is microcomplement fixation (14).

Immunological methods are especially likely to give misleading results when employing antisera that are raised and later also tested against mixtures of poorly defined antigens. For this reason, we consider most antigenic studies reported on ancient materials to be of questionable genetic value.

Gene Frequencies—At the population level, anthropologists and paleozoologists are often interested in determining the frequencies of alleles at polymorphic loci in ancient populations. Determinants of the ABO system have been of most interest (15, 16) because they are present on nearly all cells in mammalian tissues. However, blood group serology performed on ancient tissues has many pitfalls because of the possibility of differential degradation of polysaccharide antigens as well as contamination of the old tissues by plant and microbial antigens which may cross-react with the antibody or bind to blood group determinants (*cf.* Ref. 17). In well preserved human remains, serological typing of proteins encoded in the major histocompatibility complex (HLA antigens) may be more informative than ABO. HLA has the additional advantage of fewer risks of anomalous cross-reactions caused by proteins from other organisms (*e.g.* Ref. 18). Nevertheless, the inherent difficulty of interpreting the reaction of serological reagents with antigens that are modified by unknown processes remains.

DNA in Old Tissue Remains

Following the realization that DNA may survive in ancient tissues (19–21), DNA has been extracted from a wide variety of such remains (whether dry, frozen, or preserved wet in peat), ranging in age up to 45,000 years (Table I). Although DNA can be extracted from most soft tissue remains that are well preserved morphologically, post-mortem modifications made it hard to clone such DNA in bacteria.

Higuchi *et al.* (22) succeeded in cloning mitochondrial DNA sequences from the extinct quagga, a member of the horse genus (*Equus*). This represented the first retrieval of phylogenetically informative DNA sequences from a museum specimen and allowed the quagga to be placed into a phylogeny of

TABLE I

DNA from eight kinds of ancient remains

Ancient DNA has usually been detected by its staining with ethidium bromide in electrophoretic gels and its activity as a template that can direct the incorporation of radioactive nucleotides into DNA in the presence of DNA polymerase and random primers. Hybridization with DNA from a modern species is used to determine whether the DNA detected originates from the species under study or from a contaminating source. The specific hybridization test has not been applied to the oldest plant remains or the insects in amber.

Sample	Maximum age	Study
	years	
Dry remains		
Museum skins	140	Refs. 22, 43[a,b]
Natural animal mummies	10,000	[b]
Human mummies	5,000	Refs. 24, 25, 27
Plants	45,000	Refs. 41, 44, 45
Insects in amber	26 million	Ref. 21[c]
Frozen remains		
Mammoth muscle	40,000	Refs. 21, 46[c]
Wet remains		
Pickled museum specimens	100	[d]
Human brain	8,000	Refs. 26, 32

[a] R. Higuchi and B. Bowman, unpublished data.

[b] W. K. Thomas, R. H. Thomas, and S. Pääbo, unpublished observations.

[c] R. Higuchi and A. Wilson, unpublished data.

[d] P. Basasibwaki, T. Kocher, A. Meyer, and A. Wilson, unpublished data.

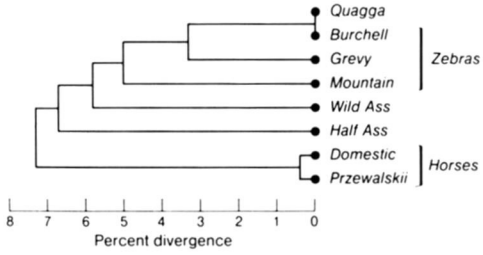

FIG. 1. **Phylogenetic tree relating mitochondrial DNA from the extinct quagga to mtDNAs from other members of the horse genus.** Percent divergence refers to the estimated number of base substitutions/hundred base pairs compared for any pair of the eight species. These estimates come from restriction mapping of the whole mitochondrial genome (42), sequencing of two cloned segments (23), and, in the quagga case, sequencing of two enzymatically amplified segments (30).

the horse and its relatives, the zebras and asses (Fig. 1) (23). The results argue for a close relationship between the quagga and Burchell's zebra and against the view that the quagga is related more closely to the domestic horse. The cloning of repetitive DNA sequences from an ancient Egyptian mummy (24) showed that far older DNA may be preserved in a clonable form and that nuclear DNA as well as mitochondrial DNA may persist for millenia.

Little progress followed these initial successes because, like all macromolecules extracted from old tissue remains, ancient DNA is heavily modified. This manifests itself by a reduction in size down to an average of only a few hundred base pairs (*e.g.* Refs. 25 and 26) and by an abundance of lesions, such as baseless sites, oxidized pyrimidines, and cross-links, many of which can be attributed to oxidative processes (27). These modifications are so extensive that less than 1% of the DNA molecules extracted from museum specimens or archaeological finds can be expected to be undamaged.

In attempts to clone ancient DNA in living bacteria, one is confronted with this vast excess of damaged DNA in two ways. First, extremely low cloning efficiencies are obtained since the majority of the vector molecules becomes ligated to

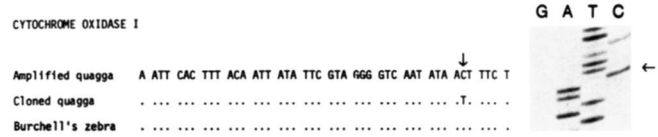

FIG. 2. **Mitochondrial DNA sequences obtained in two ways from skin of a 140-year-old museum specimen of the extinct quagga.** *Left panel* (base sequences), the *upper* result comes from directly sequencing the product obtained when PCR was applied to a DNA preparation from quagga skin. The *middle* result comes from sequencing a cloned fragment of unamplified quagga DNA (22). The *lower* result was obtained in the same way as the middle one except that the DNA was cloned from highly purified mitochondrial DNA of a Burchell's zebra (23). *Dots* indicate identity with the amplified quagga sequence. The *arrow* denotes a position at which a cloning artifact (a T residue) occurs in the cloned quagga sequence. *Right panel* (photograph), part of a sequencing gel highlighting with an *arrow* the position in the amplified sequence at which a C (rather than a T) residue occurs in the quagga. Modified from Ref. 30.

damaged molecules whose modifications preclude replication in bacteria. Second, some damaged molecules become repaired in bacteria, often by processes that are error-prone and thus likely to distort the information extracted from cloned sequences. Recent advances stemming from Kary Mullis's invention of the polymerase chain reaction (28) have eliminated some of the problems caused by both the low cloning efficiencies and cloning artifacts arising from modifications in ancient DNA.

The Polymerase Chain Reaction in Molecular Archaeology

The polymerase chain reaction (PCR)[1] can amplify preselected segments of DNA up to quantities which permit direct sequencing, starting from extremely small amounts of DNA or even single molecules (for a review, see Ref. 29). The amplification is done with two synthetic oligodeoxynucleotide primers, each about 25 bases long, a thermostable DNA polymerase, and the four deoxyribonucleotide triphosphates. The first primer matches part of the Watson strand at one end of the segment, while the second primer matches the Crick strand at the other end. Since the 3′ ends of the two primers point toward each other, repeated cycles of heating and cooling lead to a chain reaction, *i.e.* an exponential synthesis of many copies of the specific segment bounded by the two primers.

PCR is an ideal tool to amplify a small number of intact ancient DNA molecules present in a vast excess of damaged molecules. In addition to its ability to detect extremely small quantities of DNA, PCR has the advantage of being an *in vitro* system, which has no capacity for repair or misrepair. For example, in bacterial clones of quagga mitochondrial DNA, two replacement substitutions were found when the quagga sequences were compared to other vertebrate sequences (22, 23). These positions in the quagga were later shown not to differ from the general vertebrate sequence when they were directly sequenced from amplification products ((30) Fig. 2). The replacements observed in the cloned sequences were thus due to cloning artifacts, possibly resulting from misrepair of damaged molecules.

In contrast, during enzymatic amplification, most damaged molecules will either not be replicated at all, *e.g.* due to inter- or intramolecular cross-links, or will be at a replicative disadvantage because many lesions, such as baseless sites, slow down the DNA polymerase. Intact molecules will thus amplify preferentially. Some damaged molecules will of course have only minor lesions, such as deaminated bases, which can

[1] The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s).

generate replication errors without retarding replication. These errors as well as those introduced by the polymerase at undamaged sites will be present in the final population of molecules produced by PCR. However, since each error is specific to one molecule in the starting population (and its descendants), its contribution to the result, *viz.* the sequence determined on the whole descendant population of amplified molecules, is likely to be negligible. Such errors would be encountered only if one were to clone individual molecules from the final population before carrying out the sequencing reactions (30, 31). An additional advantage of PCR is that its speed allows for easy reproduction of results.

Ancient DNA Sequences Revealed via PCR

Using PCR and direct sequencing, it has been possible to obtain mitochondrial sequences from a 7000-year-old brain excavated in Florida (32). Preserved brains exist in association with human skeletal remains at several sites in Florida (26) and owe their excellent state of preservation to anaerobic and neutral conditions in the waters of Florida peat bogs. Extracts from the archaic brain contained DNA that allowed amplification of 140-bp-long mtDNA fragments. Longer amplifications were unsuccessful. This is in sharp contrast to contemporary DNA, where fragments of up to 1 kilobase and longer amplify efficiently. It seems that the damage present in old DNA causes a strongly inverse correlation between amplification efficiency and size of the amplification product, which sets a limit to the length of amplifications that can be performed.

Three informative mitochondrial polymorphisms were studied in the Florida brain. One is a direct repeat of 9 bp which occurs in only one copy in many Asians as well as some Native Americans (32, 33). Furthermore, a *Hinc*II site at position 13259, shown to be absent in many Amerindians (34), and a *Hae*III site at position 8250 were amplified and sequenced. From the sequences obtained, the archaic Floridian could be compared to the mtDNAs of more than 100 present-day Native Americans. This ancient sequence does not match any of the three mitochondrial lineages found to date in America. Further work is in progress to characterize present-day Amerindian populations as well as further archaic Florida brains, and these studies can be expected to yield a fuller picture of the population structure and genealogical history of Amerindians.

The same procedures revealed DNA sequences from ancient humans that are preserved in the form of mummies. For example, a 4000-year-old Egyptian priest was shown to carry an unusual D-loop sequence (27). It is not yet known if this was a common genotype in ancient Egypt and whether it is represented today in Egypt or elsewhere.

Another area where paleomolecular biology is producing information is zoology. In addition to the quagga discussed above, the 40,000-year-old Siberian mammoth (also referred to above) has yielded PCR products contaminated with human sequences, presumably originating from handling of the mammoth after its discovery. Fortunately, the mammoth sequences were separable from the human sequences and, as expected, proved to be closely related to those of elephants.[2]

Since mtDNA is present in many copies/nucleated cell, it can be assumed that this high copy number facilitates its survival and retrieval. In fact, attempts to amplify specific nuclear single-copy genes from ancient remains (*e.g.* part of the β-globin gene from human mummies) have been unsuccessful, presumably due to their lower abundance in DNA extracts. Furthermore, the fast evolution and maternal mode of inheritance of mtDNA make it ideal for studying ancestor-descendant relationships (35, 36).

[2] R. Higuchi and A. Wilson, manuscript in preparation.

Authenticity of Amplified Sequences

Contamination by Modern DNA—The main concern pertinent to the amplification of ancient DNA sequences is contamination of the DNA extracts or reagents by contemporary DNA. When extinct species are studied, such contamination can often be detected by mere inspection of the sequences when a phylogenetic analysis fails to place the species under study close to its biological relatives. For example, the authenticity of the DNA sequences obtained by bacterial cloning from the quagga was demonstrated by the fact that they proved the quagga to be a close relative of extant zebras but not identical to any of those tested (22, 23). Similarly, mtDNA sequences from the Siberian mammoth show the mammoth to be closely related to elephants but not identical to either elephant species.

Although contaminating sequences of human origin are easily detected in ancient remains of nonhuman species, contamination by nonhuman sequences may be harder to detect. Therefore, the phylogenetic criterion of authenticity discussed above cannot be solely relied upon to distinguish contaminating DNA.

Accordingly, the following additional criteria must be set up and adhered to in order to detect any source of contaminating DNA. We have found it imperative always to do control extracts (i) in parallel with the extracts of the old specimens in order to detect contamination in solutions and reagents (32, 37). Furthermore, several independent extracts (ii) from every individual should be prepared, and the sequences obtained should be unambiguous and identical.

The strong inverse correlation between amplification efficiency and size of the amplification product (iii), which is observed for ancient but not modern DNA, can to some extent serve as a further criterion of authenticity. In our experience, archaeological remains generally do not yield any products above 150 bp in size, whereas better preserved specimens,

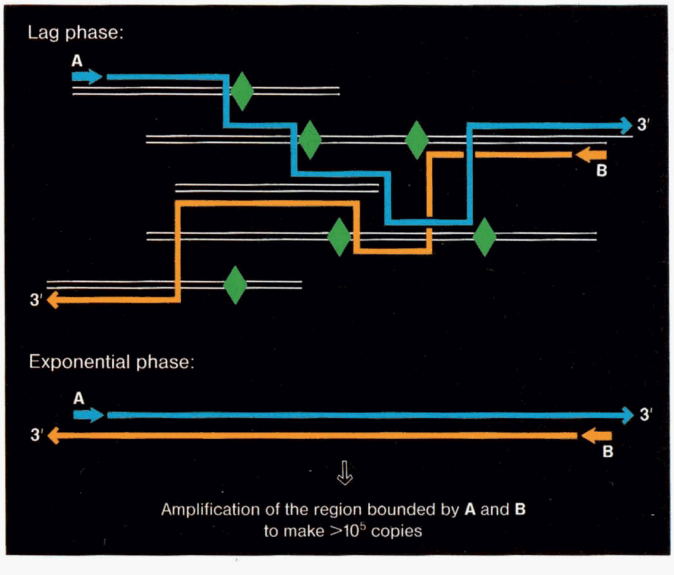

FIG. 3. **Concept of "jumping PCR."** In this hypothetical example, two primers (*A* and *B*) use undamaged parts of five damaged templates to amplify a mosaic product. In an amplification reaction where each template DNA is so damaged that no molecules allow the DNA polymerase to proceed directly from one primer site (*A*, *blue*) to the other (*B*, *yellow*), the primers will be extended during the first PCR cycle up to points where lesions (*green*) or ends of fragments cause the polymerase to stop. During subsequent cycles, these extended primers can anneal to other template molecules and be further extended. After a sufficient number of cycles the two primers have grown so long that their 3′ ends overlap and a full-length double-stranded molecule is formed. This molecule can now serve as a template for a conventional chain reaction.

such as museum skins, may allow for the amplification of up to 500-bp pieces. Sequences longer than this have invariably proved to originate from contamination of the specimens by modern DNA.

When the three above criteria (i, ii, and iii) are fulfilled, a given sequence is considered likely to be of ancient origin.

Errors because of Post-mortem Changes—As noted above, specific errors stemming from post-mortem changes are not expected to predominate in an amplified population of molecules. If they were to predominate and thus cause incorrect sequences to be determined, the pattern of substitution would be affected in a predictable way. The substitutions would be distributed at random with respect to position within codons. In fact, however, the ratio of silent to replacement changes in DNA from the extinct quagga as compared to modern DNA from the extant mountain zebra is 10 to 0. If the differences had occurred at random, a ratio of 2 to 8 would have been expected.

Mosaic Sequences via Jumping PCR—An additional property of PCR may also affect the authenticity of the amplified sequences. If no template molecules spanning the entire segment defined by the primers exist in the old extract, the amplification may in fact start from shorter fragments of the DNA that are complementary to one or the other of the primers (Fig. 3). These fragments serve as templates in the first extension, and the partially extended primers can in the next cycle be further extended upon hybridization to other fragments of the region that is to be amplified. Only when enough extension steps have been performed so that the two extended primer products overlap with their 3′ ends will a conventional exponential amplification reaction ensue. These initial steps may allow the amplification of regions that are longer than the longest intact molecule in an extract.

The building of a mosaic sequence via PCR poses no problem in the analysis of mammalian mtDNA sequences for which homoplasmy is the rule (35). However, in the case of chromosomal genes from ancient remains of heterozygous individuals, "jumping PCR" may generate erroneous sequences that result from recombination during amplification.

Vistas for Molecular Archaeology

The recently achieved ability to study DNA from museum specimens and archaeological finds via PCR (27, 30, 32, 38) opens up the possibility of studying molecular evolution by actually going back in time and directly approaching DNA sequences that are ancestral to their present-day counterparts. This allows us to address many questions involving the identity and relationship of extinct species to other extinct and extant species. In these cases, even rare examples of single preserved specimens are of great value. Second, the evolution of populations can be studied and will most often involve the comparison of ancestral and descendant populations, attributing changes in gene frequencies to factors such as variations in population size, migration, selection, and genetic drift.

In addition to organismal and population questions, promising avenues of research on molecular evolution may now be approachable. We refer especially to the study of those molecular genetic processes that may occur rapidly enough to be studied on a time scale of 10^4 years, in contrast to the slow accumulation of base substitutions, which in nuclear and organelle genomes is generally less than 1% of sequence divergence/10^5 years. Faster processes include recombination in minisatellite sequences which cause apparent mutation frequencies on the order of 10^{-2}/locus/generation (39, 40). Other cases where rapid evolution may allow molecular processes to be observed directly can be provided by human parasites (especially viruses) and the evolution of domestic plants (41). A particularly important goal for the future is to refine the polymerase chain reaction so that it becomes possible to study ancient nuclear single copy genes as well as DNA sequences from bones, teeth, shells, and amber.

Acknowledgments—We thank numerous colleagues for discussion and sharing unpublished data with us, in particular C. E. Beck, D. M. Irwin, T. D. Kocher, C. Orrego, E. M. Prager, W. K. Thomas, and F. X. Villablanca, as well as E. Myers for expert graphical work.

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