ramp. The hind feet of the mice were painted with children's poster paints of contrasting colors. The tracks left by the mice as they ran up the ramp were recorded on paper tape.
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Molecular Genetic Analyses of the Tyrolean Ice Man

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An approximately 5000-year-old mummified human body was recently found in the Tyrolean Alps. The DNA from tissue samples of this Late Neolithic individual, the so-called "Ice Man," has been extracted and analyzed. The number of DNA molecules surviving in the tissue was on the order of 10 genome equivalents per gram of tissue, which meant that only multi-copy sequences could be analyzed. The degradation of the DNA made the enzymatic amplification of mitochondrial DNA fragments of more than 100 to 200 base pairs difficult. One DNA sequence of a hypervariable segment of the mitochondrial control region was determined independently in two different laboratories from internal samples of the body. This sequence showed that the mitochondrial type of the Ice Man fits into the genetic variation of contemporary Europeans and that it was most closely related to mitochondrial types determined from central and northern European populations.

In September 1991, the frozen mummy of a man was found in the Tyrolean Alps. Radiocarbon dates of skin and bone samples indicated an age between 5100 and 5300 years (*1*). Because no comparable archaeological discovery exists, this find has attracted considerable scientific and public interest. It has also been the subject of various rumors and even allegations of fraud (*2*). Molecular genetic investigations of the Ice Man could address some of the questions surrounding the find. Comparisons of DNA sequences from the body with contemporary populations may reveal aspects of his ethnic affiliation. Molecular studies of other organisms such as viruses or bacteria associated with the body may furthermore illuminate the evolution of these organisms. As a first step toward such investigations, we have analyzed the state of preservation of the DNA in the Ice Man and determined the sequence of a hypervariable segment of the mitochondrial control region from numerous samples removed from the body.

Ancient DNA has been retrieved from a variety of plant, animal, and human remains (*3*, *4*) that go back a few tens of thousands of years as well as from some fossils that are millions of years old (*5*–*7*), although the latter results are partially controversial (*8*). In most cases, work on archaeological DNA has been limited to mitochondrial DNA because its high copy number increases the chance of survival of a few molecules in the face of molecular damage that accumulates post mortem. Because the body of the Ice Man has been frozen with the exception of a short period after its discovery, its DNA may be preserved better than that of other finds. This unusual condition might allow nuclear markers such as microsatellites to be studied in addition to mitochondrial DNA and thus open several additional avenues of study.

A total of eight samples of muscle, connective tissue, and bone were removed under sterile conditions from the left hip region of the body, which had been damaged during salvage of the mummy. Additionally, parts of one sample that has been radiocarbon dated (*1*) were analyzed. Extracts of DNA were made from 10 to 200 mg of each sample by a silica-based method that is highly efficient in the retrieval of ancient DNA (*9*). Enzymatic amplifications from the mitochondrial control region were attempted. Because this region encodes no structural gene products and evolves faster than other parts of the mitochondrial genome, it is particularly suited for the reconstruction of the history of human popula-

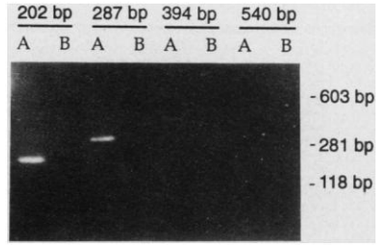

Fig. 1. Agarose gel electrophoresis of mitochondrial DNA amplification of different lengths from the Ice Man. For every primer pair, amplifications from (**A**) an extract of the Ice Man and (**B**) an extraction control are shown. The primer pairs used are as follows: L16055/H16218 (202 bp), L16055/H16303 (287 bp), L16055/H16410 (394 bp), and L15997/H16498 (540 bp), where L and H refer to the light and heavy strand, respectively, followed by the number to the nucleotide position (*14*) at the 3′ end of the primer. Migration positions of molecular size markers are given in numbers of base pairs.

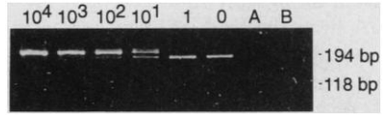

Fig. 2. Quantitation of mitochondrial DNA in an extract from the Ice Man. A dilution series of a competitor template, containing a 20-bp insertion in a mitochondrial fragment, was added to a constant amount of extract, and a PCR that used primers L16068/H16218 was performed as described in (*10*). The numbers above the lanes indicate the numbers of competition molecules added to the amplifications. (**A**) An extraction control and (**B**) a control where no template was added. Migration positions of molecular size markers are given in numbers of base pairs.

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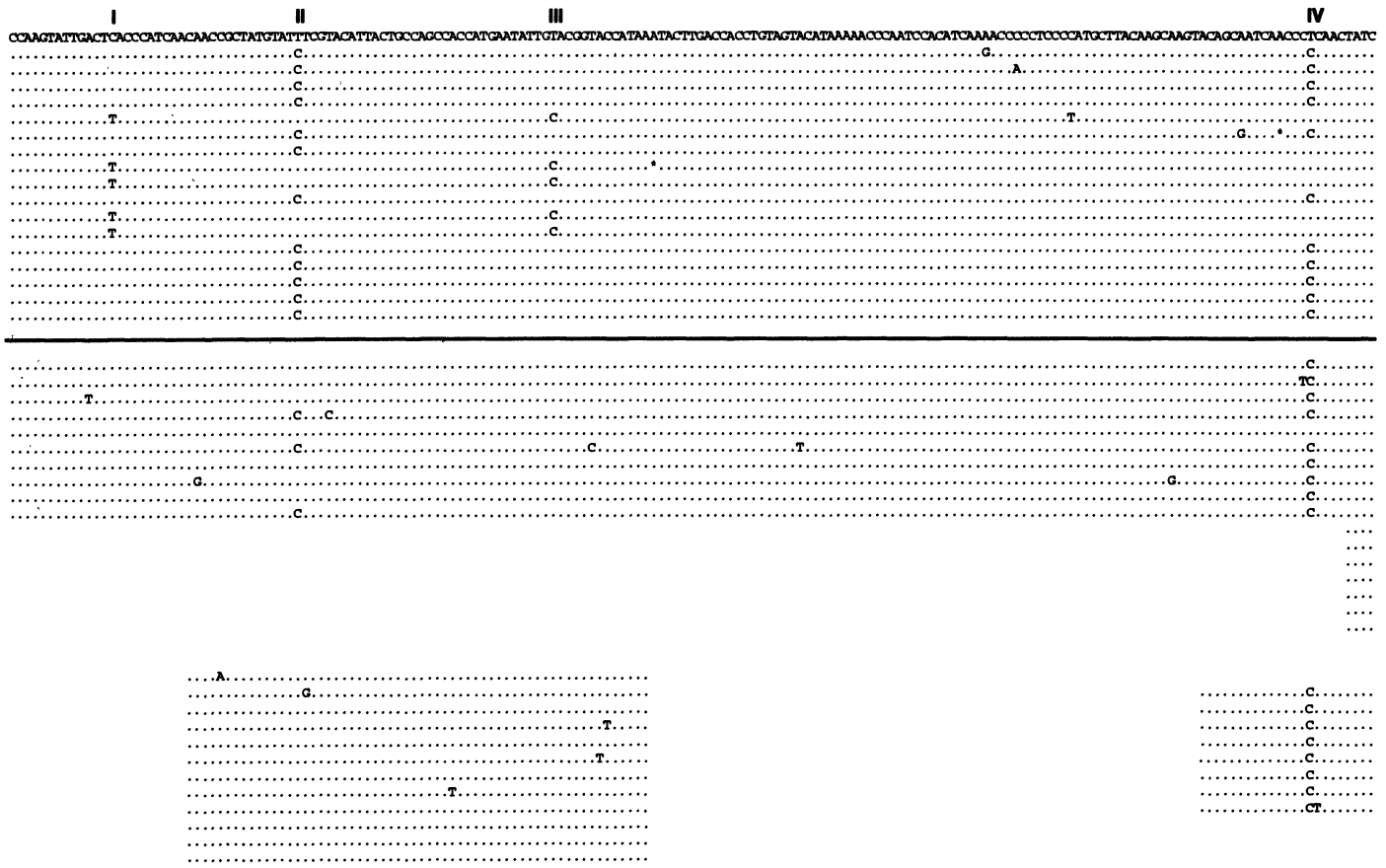


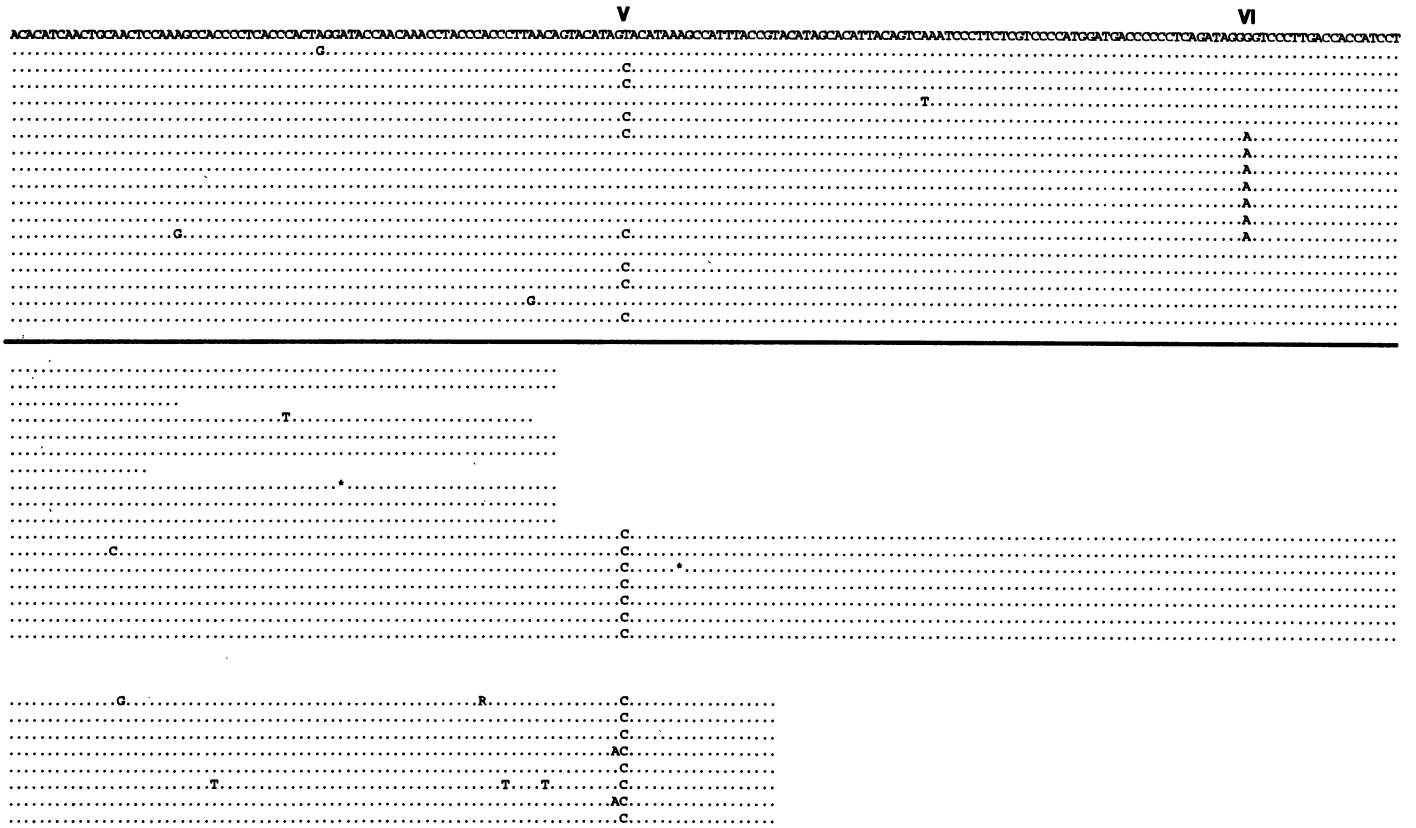
Fig. 3. DNA sequences from cloned PCR products of different lengths. Positions I to VI correspond to bases 16069, 16093, 16126, 16224, 16311, and 16390 (*14*), respectively. The lengths of the cloned amplification products were 394, 287, 221, 161, and 100, respectively. The sequence

tions. Primers spanning DNA segments of different lengths were used (*10*) (Fig. 1). A segment that is 202 base pairs (bp) long (including the primers) amplified efficiently, whereas a 287-bp fragment was less well amplified, a 394-bp fragment was barely detectable, and a 540-bp fragment was not amplified at all. Such an inverse relation between the efficiency of amplification and the length of the product amplified is typical of DNA retrieved from archaeological remains and results from damage and degradation of the DNA. However, mummified human remains generally allow amplification of no more than 200 bp (*11*). The DNA from the Ice Man may thus be more similar to exceptionally well preserved material found, for example, at some cave sites (*12*) or in Florida sinkholes (*13*).

Because the majority of the DNA extracted from archaeological remains may stem from contaminants such as microorganisms that could have infested the body, a quantitative polymerase chain reaction (PCR) assay was used to estimate the number of amplifiable human mitochondrial DNA molecules that survive in the body. In this assay, primers for a 189-bp fragment of the mitochondrial control region were used in amplifications to which a constant amount of the Ice Man extract was added as well as a dilution series of a competitor template. The competitor template consisted of a mitochondrial DNA fragment into which a 20-bp insert had been introduced. An aliquot representing one-sixtieth of an extract from 70 mg of tissue contained approximately 10 copies of endogenous template molecules (Fig. 2). On the assumption that there are approximately 500 mitochondrial DNA molecules per human cell, this aliquot indicates that on the order of 10 nuclear genome equivalents exist per gram of tissue. This distribution is more than six orders of magnitude less than would be expected from undegraded DNA. Two additional extracts, which were similarly analyzed, contained approximately 10 and 20 genomes per gram of tissue, respectively. Thus, the vast majority of the endogenous DNA of the Ice Man is degraded, and single-copy genes should not be amplifiable from the small samples now available. This conclusion was confirmed in experiments in which primers that can detect single copies of the human amelogenin gene, located on the X and Y chromosomes, failed to show any amplification.

When the mitochondrial amplification products (Fig. 1) were sequenced directly, several ambiguous positions were observed in the sequencing reactions. To elucidate the reason for this ambiguity, amplification products from five extracts were cloned and 57 clones were isolated and sequenced. In addition to substitutions present in single clones, which may be attributed to polymerase errors during PCR, there were 10 positions among 354 scored where more than one clone had a substitution when compared to a human sequence commonly used as a reference (*14*). Some of these variable positions were different among the different tissue samples. Furthermore, the sequences from the individual extracts were attributable to several different sequence types, as judged from the occurrence of the substitutions in contemporary sequences. Homoplasmy of mitochondrial DNA is the rule in humans (*15*), which indicates that the mummy has been contaminated by DNA from several individuals, presumably when handled during discovery and retrieval. The presence of many different sequences made it impossible to use these extracts to determine which, if any, of the sequences represents the in vivo Ice Man sequence.

To remove contaminating DNA, two of the samples were selected that were of sufficient size to allow the superficial parts to be removed. From predominantly internal portions of these samples new extrac-



between the primers is given. Nucleotides identical to the reference sequence (upper line) (*14*) are represented by dots, and deletions are represented by asterisks. The solid line separates the longest PCR product from the shorter ones.

tions were performed, and a 394-bp fragment was amplified and cloned. Representative results from one of the extracts are shown in Fig. 3. In addition to two deletions, six transitions, and two transversions that occurred in single clones, multiple substitutions occurred at six positions (positions I to VI) among the 17 clones sequenced. These substitutions are all transitions and are known to occur in contemporary individuals. There is a tendency for substitutions to be associated with each other in a fashion that correlates with the distance between the variable positions. For example, positions I and III are separated by 56 nucleotides. In all 12 clones that carry C at position I, position III carries a T, and in the five clones that have a T at position I, position III has a C. On the other hand, at positions IV and V, which are separated by 86 nucleotides, the nucleotides are not consistently associated with each other, and at positions even further apart no correlation is seen. Thus, it seems that variable positions that are located further apart in the amplification product are randomized with respect to phase. Because damage in archaeological DNA has been shown to cause jumping by partially extended PCR products between template molecules (*16*), these long amplification products can be assumed to be due to in vitro recombination involving a minimum of two different sequences.

In vitro recombination between templates should be reduced if shorter amplifications are performed. Furthermore, if DNA template A is more abundant in the tissue sample than template B and is simultaneously more degraded than template B, then template A should dominate quantitatively in shorter amplification products. Therefore, amplifications of 100, 161, 221, and 287 bp were performed from the same extracts and several clones sequenced in each case (Fig. 3). The 287-bp amplification encompassed positions I to IV. All clones carried a C and a T at positions I and III, respectively, whereas position II carried C in three and position IV carried T in one of the 10 clones. Position II was included in the 100-bp amplification in which a T was observed in 12 out of 12 clones. Eight out of eight clones had a C for position IV in the 161-bp amplification. Position V was included in the 161- and the 221-bp amplifications and showed C in all 16 clones sequenced. Finally, position VI had a G in all eight clones for the 221-bp amplification. Thus, the short amplification products showed no ambiguities at the six positions that in the longer amplifications varied among clones. Two differences from the reference sequence remain in the shorter clones: C at position IV and C at position V.

The strong consensus for one sequence in amplification products of 161 bp and shorter agrees well with the observation that amplifications of short length function much more efficiently than longer ones (Fig. 1). It indicates that the Ice Man's DNA may have been degraded to pieces with an average length of less than 150 bp such that when amplifications shorter than this were performed, consistent results were obtained. In longer amplifications, the in vitro recombination of partial extensions of these ancient molecules with contaminating and less abundant modern molecules of longer average length may have occurred (*16*). The substitutions observed at positions I, II, and III, which stem from the contaminating molecules, show that they were derived from a minimum of two individuals.

To provide independent corroboration of these results, one bone sample was analyzed in Oxford, England. There, two extractions were performed by different workers (*17*), and the mitochondrial control-region sequence between positions 16,056 and 16,402 (*14*) was obtained by amplification and the direct sequencing of two overlapping segments of 182 and 194 bp. The sequences from both extractions were un-

Table 1. Comparison of the Ice Man sequence with some contemporary populations. The numbers of individuals investigated in each population are given as well as the mean pair-wise sequence differences (mean) to the Ice Man sequence, the standard deviation of the mean, and the numbers of sequences identical to the Ice Man. The sources of the sequence data are as follows: sub-Saharan Africans (*21*), Siberians (*22*), Native Americans (*19, 23, 24*), the Mediterranean area (includes Sardinians, Italians, Egyptians, Saudi Arabians, and Turks) (*25–27*), and northern Europe (includes North Germans, Danes, Britons, and Icelanders) (*26, 28, 29*). To elucidate if the Ice Man sequence might be particularly well represented in the contemporary Alpine population, individuals from the Alpine region were sequenced (14 from Boscogurin; 26 from Oberwallis, Unterwallis, and Wallis; 15 Raetoromans; 6 from Tessin; and 5 and 6 each from German- and Italian-speaking areas close to the Alps, respectively). To investigate the relation to the current population of the Alpine valley where the mummy was found, sequences were determined from 16 unrelated individuals in Oetztal, whose ancestors had lived in the valley as far back as could be genealogically verified (in most cases three generations). The sequence used for the analyses was between positions 16,056 and 16,408 (*14*).

Population	Sample size	Mean	SD	Shared sequences
Sub-Saharan Africa	120	7.45	2.37	0
Siberia	143	6.87	1.42	0
Americas	419	6.64	1.66	0
Mediterranean	228	5.35	2.34	3
Northern Europe	255	3.73	1.95	9
Alpine region	72	3.38	1.85	1
Oetztal	16	3.38	1.67	0

ambiguous and identical to that reported above. Taken together, these results rule out laboratory-specific contamination of the samples. We thus identify the sequence described above, which carries two changes from a reference sequence (*14*), as the sequence that the Ice Man carried when alive.

The rate of evolution of this segment of the mitochondrial control region has been estimated to approximately 1% divergence between two lineages per 10,000 (*18*) to 30,000 years (*19*). One mitochondrial lineage may therefore have changed by 0.4 to 1 substitution in the region sequenced over 5000 years. Thus, it can be assumed that a Late Neolithic sequence would fall within the variation of contemporary sequences. The putative Ice Man sequence was compared to data sets of mitochondrial sequences from various regions (Table 1). The sequence of the mummy occurs most frequently among present-day Europeans north of the Alps, having been found in 7 of 155 individuals from northern Germany, Denmark, and Iceland and twice among 100 British Caucasoids. It has also been found once in Swiss populations and three times in the Mediterranean region but not among 120 sub-Saharan Africans, 143 Siberians, 419 Native Americans, and 16 individuals from the valley (Oetztal) where the body was found. When the mean pair-wise sequence difference between the Ice Man sequence and sequences from various regions of the world is determined, we find that it is the furthest from the sub-Saharan Africans (7.45) and the closest (3.38) to the individuals from the Alpine area. An average of 3.73 and 5.35 substitutions distinguish the sequence from the North European and Mediterranean sequences, respectively, whereas differences of 6.64 to 7.45 occur relative to the non-European populations. Thus, the sequence seems to fit within the European gene pool (*20*). Within Europe, it tends to be more closely related to Alpine and northern populations. A more precise determination will have to await both more sequence information from the Ice Man and a much more extensive survey of genetic diversity among European populations.

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