

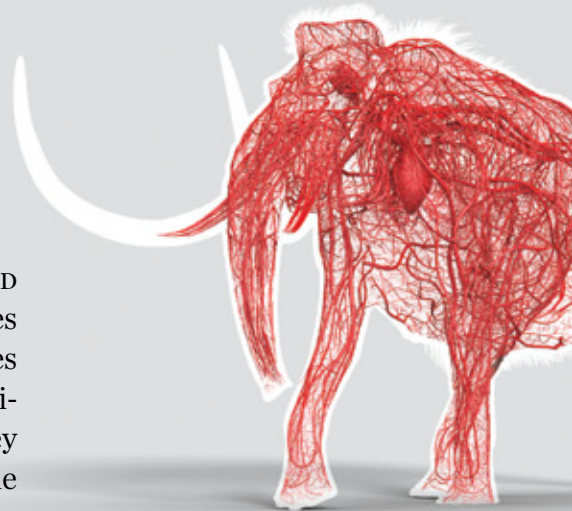
EVOLUTIONARY BIOLOGY

New Life for Ancient DNA

Biotechnology reveals how the woolly mammoth survived the cold and other mysteries of extinct creatures

By Kevin L. Campbell and Michael Hofreiter

FOR MORE THAN 150 YEARS SCIENTISTS HAVE PRIMARILY RELIED on fossilized bones and teeth to reconstruct creatures from deep time. Skeletons divulge the sizes and shapes of long-ago animals; muscle markings on bones indicate how brawny the creatures were and how they may have moved; tooth shape and wear attest to the kinds of food eaten. All in all, researchers have managed to extract extraordinary quantities of information from these hard parts. On rare occasions, they have chanced on exquisitely preserved mummies and frozen carcasses that have



IN BRIEF

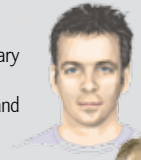
Scientists' understanding of extinct creatures has long relied almost entirely on fossils of their bones and teeth.

But recent advances in ancient DNA research are revolutionizing studies of ancient beasts.

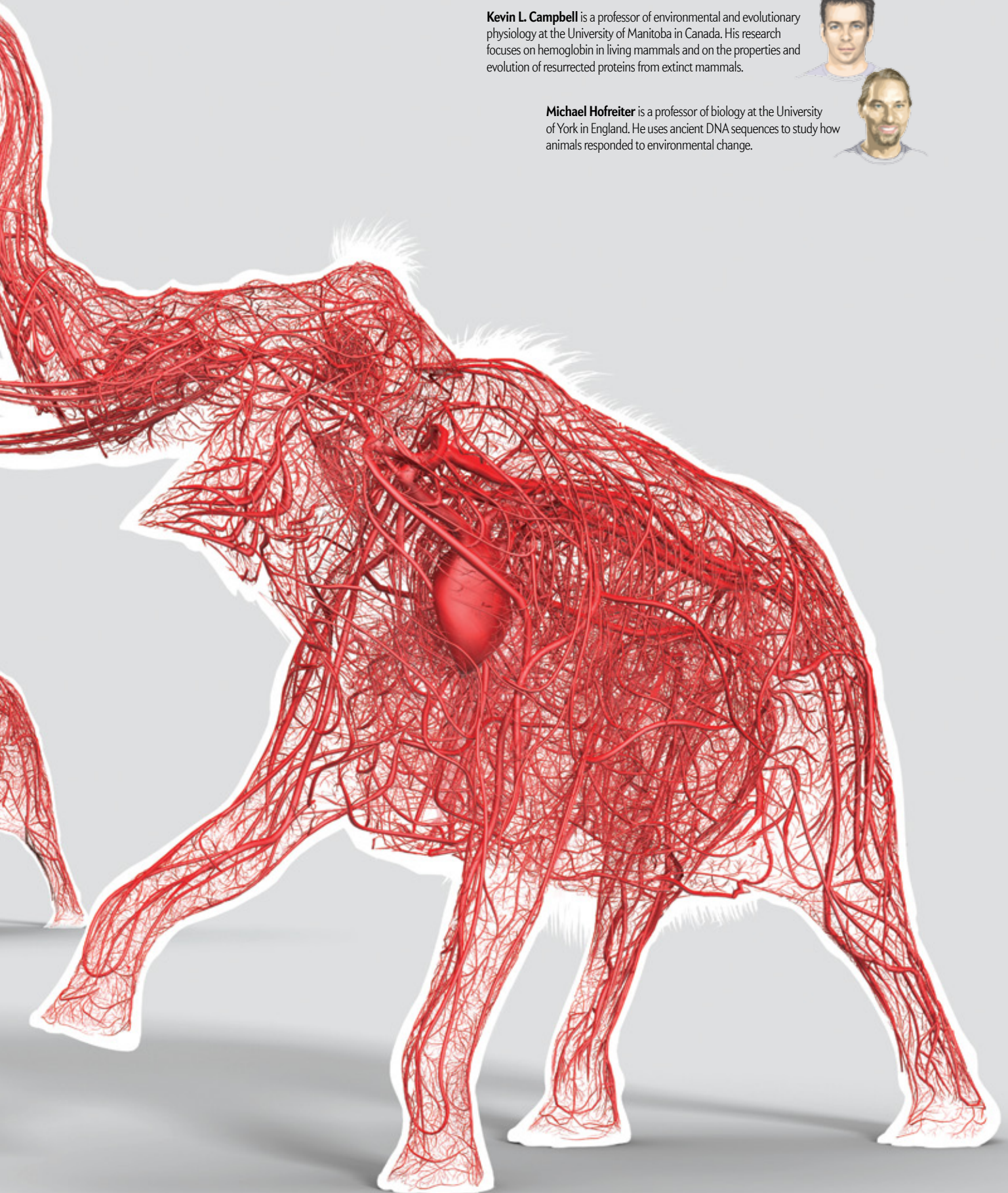
Researchers can now re-create the genes of these animals and study the proteins they encoded.

That scientists might one day be able to study such paleophysiology was unthinkable just a decade ago.

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Michael Hofreiter is a professor of biology at the University of York in England. He uses ancient DNA sequences to study how animals responded to environmental change.



allowed them to add more detail to their reconstructions, such as the length of the fur, the shape of the ears, the specific contents of an animal's last supper. Yet for all that scientists have been able to deduce about the physical characteristics of life-forms from past eras, we know very little about the physiological processes that sustained them.

That gap is closing, however. Recent advances in biotechnology now allow us to reassemble ancient genes from extinct animals and resurrect the proteins those genes encode—proteins that both form and drive the cellular machinery that underlies life-giving processes. The work heralds the dawn of a thrilling new scientific discipline: paleophysiology, the study of how the bodies of bygone organisms functioned in life. We are still in the earliest days of this research, but already we have gained stunning insights into how one iconic beast of prehistory—the woolly mammoth—adapted to the brutal conditions of its Ice Age world. Although the *Jurassic Park* dream of cloning prehistoric animals remains out of reach, our work has demonstrated the feasibility of observing key physiological processes that took place in creatures that have long since vanished from the face of the earth.

COLD CASE

FOR ONE OF US (Campbell), the inspiration for this venture began one evening in 2001 while watching a television show documenting the exhumation of woolly mammoth remains from Siberian permafrost. Given the highly publicized cloning of Dolly the sheep, announced in 1997, pundits on the show speculated—wrongly, it turns out—that DNA from this mammoth might soon permit scientists to bring these creatures back to life. Campbell's own vision was far more targeted than that colossally complicated enterprise and, ultimately, more feasible. He wanted to find out how these extinct cousins of today's Asian elephants managed to adapt to the cold climate in the high latitudes where they lived.

The fossil record shows that the ancestors of woolly mammoths originated in the subtropical plains of Africa and only moved into Siberia less than two million years ago, just as the

earth was entering one of the most profound cooling events in its history: the Pleistocene ice ages. As is true of African elephants, the main physiological challenge the mammoth ancestors would have faced in their homeland was avoidance of overheating. Once the lineage migrated north and the world cooled, however, conservation of body heat became paramount.

Because almost everything we know about the biology of extinct species has been inferred from detailed studies of their fossilized, frozen or mummified remains, discussions of mammoth cold adaptation have primarily been limited to physical attributes that are directly observable from recovered carcasses, such as the thick, woolly undercoat for which these mammoths are named. Physical features are only one part of the story, however—and probably a minor one at that. Indeed, a network of physiological processes was undoubtedly essential for their survival in the cold. Unfortunately, these processes leave no traces in the fossil record, so our only hope of studying them is to recover tattered bits of DNA from ancient remains, piece the genes together in their entirety, insert them into living cells and coax the cells to re-create the proteins that once controlled these processes. We can then observe precisely how the proteins of extinct animals functioned compared with those of their living relatives.

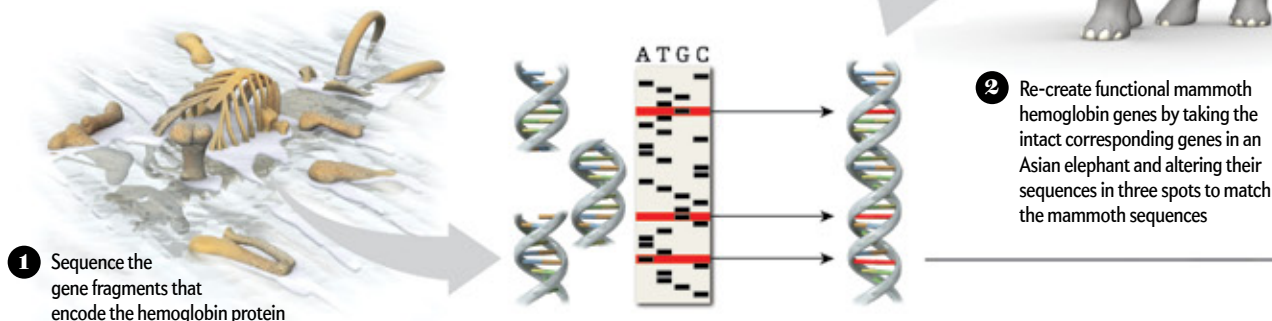
Thus, Campbell's idea of studying cold adaptation in mammoths using preserved DNA, though orders of magnitude simpler than actually raising the beasts from the dead, was still going to require a massive amount of fancy biotechnological footwork. As luck would have it, major advances in ancient DNA research were around the corner that would help pave the way to realizing his goal.

Even under the best circumstances, DNA in long-dead specimens, if it has been preserved at all, persists in exceedingly small amounts. It is also highly fragmented and riddled with chemical damage. The cells of living organisms contain two kinds of DNA: simple loops of DNA in the cell's energy-producing organelles, or mitochondria, and the much more complex DNA in the cell nucleus. Early studies of ancient DNA focused on the mitochondrial va-

HOW IT WORKS

Breathing Life into Mammoths

By reconstructing ancient genes, scientists can re-create the proteins they encoded and observe how they behave, thereby gaining insights into the physiology of extinct animals. For instance, resurrection of the red blood cell protein hemoglobin from a woolly mammoth (*below*) has shown that the temperature-sensitive protein evolved adaptations that enabled it to do its job of delivering oxygen to body tissues in the cold conditions these beasts faced.



riety because it is much more abundant than nuclear DNA: each cell has hundreds of mitochondria but only one nucleus. Yet mitochondrial DNA accounts for a minute fraction of all the genetic material in a cell; it encodes only a handful of proteins, all used only in mitochondria. The real action is in nuclear DNA. Scientists initially believed it was impossible to recover enough ancient nuclear DNA to study it. Yet in 1999 Alex Greenwood, now at the Leibniz Institute for Zoo and Wildlife Research in Berlin, and his colleagues reported that they had found evidence in permafrost remains showing that small fragments of nuclear DNA could survive for tens of thousands of years in amounts sufficient for analysis.

Although Greenwood's work demonstrated that it was possible to obtain short snippets of nuclear sequences (that is, pieces containing up to 70 nucleotides—the “letters” of the genetic code) from creatures as old as woolly mammoths, it remained largely impractical to sequence the hundreds to thousands of nucleotides that make up each complete gene. Furthermore, Greenwood's approach entailed the destruction of large amounts of hard-won ancient DNA. By borrowing a technique called multiplex PCR that molecular biologists use to generate multiple copies of DNA from extant organisms, though, one of us (Hofreiter) came up with a solution to these problems, thus clearing a key hurdle to studying the physiology of extinct organisms. In a first proof of principle, his research team assembled the first complete mitochondrial genome (a 16,500-nucleotide sequence) from an Ice Age species—the mammoth—publishing the findings in 2005.

BLONDES AND REDHEADS

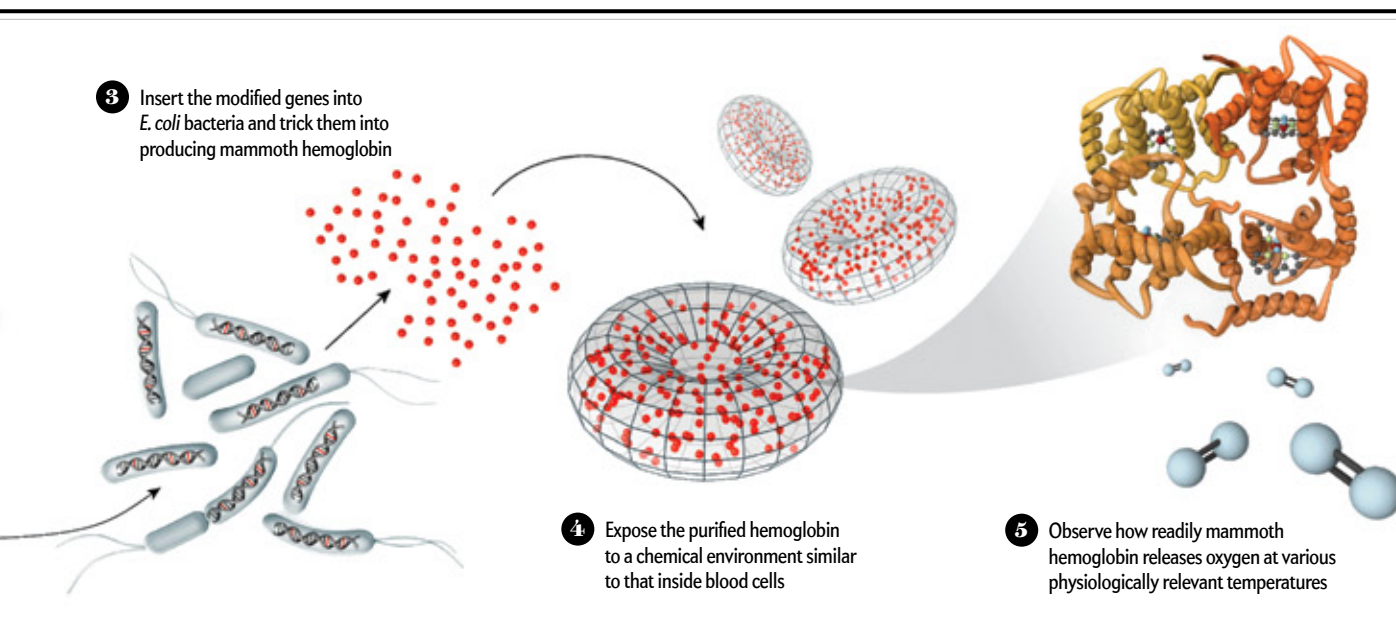
HAVING HONED its ancient DNA-sequencing technique, Hofreiter's team in Leipzig, Germany, then used it to reconstruct the first complete nuclear gene from an extinct species. Once again, the source of the DNA was a mammoth, specifically an exceptionally well-preserved 43,000-year-old thigh bone that Eske Willerslev of the University of Copenhagen found in northern Siberia. The team chose a gene called *melanocortin 1 receptor* (*MC1R*) that is known to help determine coloration in bird feathers and mam-

malian hair. *MC1R* was appealing because it is short and easy to insert into cells where its molecular activity could be measured, enabling investigators to link DNA sequences to observable traits.

Given that the hair recovered from permafrost-preserved mammoths tends to be either light or dark in color, Hofreiter and his collaborators postulated that differences in gene function—as opposed to chemical factors in the sediments to which the hairs had been exposed for tens of thousands of years—might have underlain these two distinct hair colors. Sequencing all 1,236 nucleotides making up the complete *MC1R* gene revealed two separate gene variants, or alleles. The first allele differed from the corresponding African elephant gene at a single nucleotide, whereas the second allele contained three additional mutations, all of which produced substitutions of amino acids (the building blocks of proteins) in the resulting protein.

Although Hofreiter and his collaborators were intrigued to find that two of these substitutions occurred at positions in the protein that have rarely changed over the course of evolution, the absence of comparable mutations in other mammals made it impossible to gauge whether these unusual replacements influenced mammoth coat coloration. Analysis of the gene's activity in cells, however, showed that one of the three mutations in the second allele produced a substitution that made a less active version of the pigmentation gene. To judge from the molecular activity of pigmentation genes of other mammals, this weaker variant probably helped to make the fur of some mammoths blond.

By remarkable coincidence, Hopi Hoekstra, then at the University of California, San Diego, and her colleagues simultaneously discovered that some populations of modern-day beach mice carry an *MC1R* gene variant that produces the same key amino acid exchange found in the second mammoth allele. More important, the mice carrying this variant had light-colored fur, which provides natural camouflage in the sandy environments they inhabit. For mammoths the benefit of being blond is much less clear because blond individuals would still have been highly conspicuous on the treeless landscape of primeval Siberia. It is con-





RARE CARCASSES such as this 42,000-year-old baby mammoth found in Russia contain a wealth of information, but only DNA can reveal the exact biological processes that sustained these animals during life.

ceivable, however, that a pale pelage helped these animals stay warm in this cold, windy environment, as has been shown for extant birds and mammals with light coloration. That may sound counterintuitive in that light-colored hair reflects a lot of solar radiation, but such hair also scatters some of the incoming radiation toward the skin, where it is absorbed as heat. In contrast, dark fur absorbs solar radiation at its outer surface, where wind rapidly dissipates the heat it provides.

Fresh off its success in reconstructing ancient nuclear genes from mammoths, the Hofreiter group turned its attention to Neandertals, relatives of *Homo sapiens* that lived in Eurasia and went extinct around 28,000 years ago. The team obtained a 128-nucleotide fragment of the *MC1R* gene that coded for an amino acid substitution not seen in humans today. As with the mammoth allele, functional analysis indicated that this single change makes the protein less active than the standard human version. Given that *MC1R* gene variants with similar reductions in function occur in modern-day humans of European descent who have red hair and fair skin, we speculated that some Neandertals might also have had red hair and fair skin (albeit because of a different mutation with similar effects on the protein's activity). At the high latitudes where Neandertals lived, the ultraviolet light needed to synthesize vitamin D is in short supply. Fair coloration may have helped Neandertals absorb enough ultraviolet light, which penetrates dark skin less readily.

These pioneering studies unambiguously demonstrated that the genetic reconstruction of observable traits had now become a practical reality. We were now ready to use this powerful new tool to follow the living processes of extinct species—true paleophysiology.

WHEN BLOOD RUNS COLD

ALL LARGE cold-adapted mammals around today—from reindeer to musk ox—possess a system of closely packed arteries and veins that run antiparallel to one another along the limbs and extremities. This arrangement, known as a rete mirabile, or “wonderful net,” forms a highly efficient countercurrent heat exchanger in which warm, oxygenated arterial blood exiting the body core transfers most of its heat to cold venous blood returning toward the heart. The resulting thermal gradient permits the temperature of extremities in contact with cold surfaces, such as the footpad, to be maintained just above freezing, drastically reducing overall heat loss. These heat savings mean fewer calories are required to keep warm, thereby providing a crucial advantage for Arctic species during winter, when calories are often hard to come by. Paradoxically, this anatomical adaptation deprives the extremities of the heat energy needed to ensure that hemoglobin functions properly. In vertebrate animals, the red blood cell protein hemoglobin collects oxygen from the lungs and then delivers it to tissues. Breaking the weak chemical bond between hemoglobin and oxygen requires energy, however, so hemoglobin's ability to deliver oxygen to tissues plummets with declining temperature.

To compensate for this shortcoming, the hemoglobins of cold-tolerant mammals require a supplementary heat source. Although the precise molecular mechanisms underlying this trait are not well understood, they generally appear to involve the binding of other molecules inside the blood cells to the hemoglobin. The formation of chemical bonds between these molecules and hemoglobin releases heat energy that can be donated to help discharge hemoglobin's oxygen to the tissues.

Campbell's team—which until then was working indepen-

GETTY IMAGES

dently from the Hofreiter group—hypothesized that mammoth hemoglobin, too, evolved changes that facilitated oxygen release in the cold. Sequencing of mammoth hemoglobin genes and comparison of those sequences with those of Asian elephant hemoglobin genes could presumably reveal if such changes occurred and what they were.

Early attempts in collaboration with Alan Cooper of the University of Adelaide in Australia to sequence the two mammoth genes that produce the different so-called globin chain proteins that form the backbone of hemoglobin met with major setbacks: most available mammoth samples were simply not of high enough quality to obtain workable segments of DNA. At this point, Campbell and Cooper's group joined forces with Hofreiter's group, and using the same DNA extract involved in the *MCIR* study, we soon obtained the complete coding sequence of the two mammoth hemoglobin genes and thus learned the amino acid sequences of the globin chains.

The initial DNA-sequencing results revealed that one of the mammoth globin chains differed from Asian elephants at three of 146 amino acid positions—a finding that quickly became a source of great excitement because we were convinced this trio of amino acid substitutions contained the clear genetic signature of physiological cold adaptation. Preliminary support for this hypothesis came in the form of a rare human hemoglobin variant, termed hemoglobin Rush, which carries one of the mutations found in the mammoth sequence. Although the Rush protein differs from the normal human blood protein at only a single amino acid position, the difference radically alters the biochemical properties of hemoglobin in a way that markedly reduces its temperature sensitivity and thus allows it to release its oxygen more readily in the cold, just as the hemoglobins of cold-adapted mammals do.

The next step toward establishing that the changes evident in the mammoth hemoglobin were adaptations to a cold climate was to resurrect the ancient hemoglobin and watch it in action. To make copies of the genes for mammoth hemoglobin components, we obtained intact hemoglobin genes from Asian elephant blood and altered them at the three mutation sites to match the mammoth sequences. We then inserted the resulting mammothlike genes into *Escherichia coli* bacteria, tricking them into assembling mammoth hemoglobin indistinguishable in form and function from that once circulating in the blood of the 43,000-year-old specimen that yielded up its DNA.

For the first time in history we were now in the enviable position of analyzing an important physiological process of an extinct species in precisely the same manner that we would use to study that process in a modern animal. We carefully measured the ability of both mammoth and elephant hemoglobins to bind and off-load oxygen at various physiologically relevant temperatures in solutions that mimicked the chemical environment found inside red blood cells. As predicted from the hemoglobin Rush studies, the mammoth protein did indeed relinquish oxygen much more readily than Asian elephant hemoglobin did at cold temperatures (both hemoglobins functioned the same at normal core body temperature of around 37 degrees Celsius). Intriguingly, the ability of the mammoth hemoglobin to bind to additional molecules and thus create the supplemental heat source needed to deliver its oxygen payload arose by completely different genetic changes than those found in the hemoglobins of modern Arctic mammals, as comparisons of the mammoth hemoglobin gene sequences

with sequences from their modern counterparts show. It bears mention that whereas the mammoth mutation is adaptive for cold tolerance, the human Rush variant is not, because it destabilizes the protein such that carriers are chronically anemic. The question of why this undesirable property arises in human, but not mammoth, hemoglobin still needs to be answered.

RAISING THE MAMMOTH?

OF COURSE, the hemoglobin adaptation is only one piece of the puzzle of how woolly mammoths adapted to life in the cold; many other biochemical adaptations of these animals, not to mention those of dozens of other extinct species, remain to be elucidated. Unfortunately, the spate of ancient genomes that scientists have sequenced in recent years are unlikely to be of much help in this regard because the so-called shotgun-sequencing technique used to obtain them yields a random assortment of sequences that, though good for big-picture assessments, are generally not accurate or complete enough to offer physiological insights unless the sequencing is repeated so many times as to be relatively cost-prohibitive.

A new approach called hybridization capture generates deeper coverage of target genes at a much lower cost and so may resolve that issue, allowing for large-scale studies comparing the important gene networks of, say, Siberian mammoths from relatively warm interglacial periods with those from the frigid glacial maximums, when the glaciers were at their thickest. Hybridization capture could also enable investigators to compare geographically disparate populations of the same species—Siberian and Spanish mammoths, for instance. Such studies would not only allow an assessment of genetic variability within the species but could provide insight into novel physiological adaptations in response to local geographic and climatic conditions. Exciting as these future prospects are (imagine watching 50,000 years of evolution unfold before your eyes), our ability to analyze paleophysiology is somewhat limited. Ideally, we would study extinct proteins *in vivo* because many properties of proteins become visible only in a living organism. Such studies are unlikely to occur anytime soon, however, because they would require re-creating an extinct species.

For now we will have to content ourselves with observing ancient proteins in test tubes and cell cultures. Already we are using the techniques to probe the physiology of other vanished creatures—among them, the mastodon and a more recently extinct Arctic marine mammal known as Steller's sea cow. The infinitely more complex possibility of cloning these animals will remain in the realm of fantasy for the foreseeable future. Meanwhile we will continue breathing life into these long-dead beasts one ancient protein at a time. ■

MORE TO EXPLORE

Multiplex Amplification of the Mammoth Mitochondrial Genome and the Evolution of Elephantidae. Johannes Krause et al. in *Nature*, Vol. 439, pages 724–727; February 9, 2006.

A Melanocortin 1 Receptor Allele Suggests Varying Pigmentation among Neanderthals. Carlos Lalueza-Fox et al. in *Science*, Vol. 318, pages 1453–1455; November 30, 2007.

Substitutions in Woolly Mammoth Hemoglobin Confer Biochemical Properties Adaptive for Cold Tolerance. Kevin L. Campbell et al. in *Nature Genetics*, Vol. 42, pages 536–540; June 2010.

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To view an interactive explanation of mammoth hemoglobin resurrection, visit ScientificAmerican.com/aug2012/mammoth