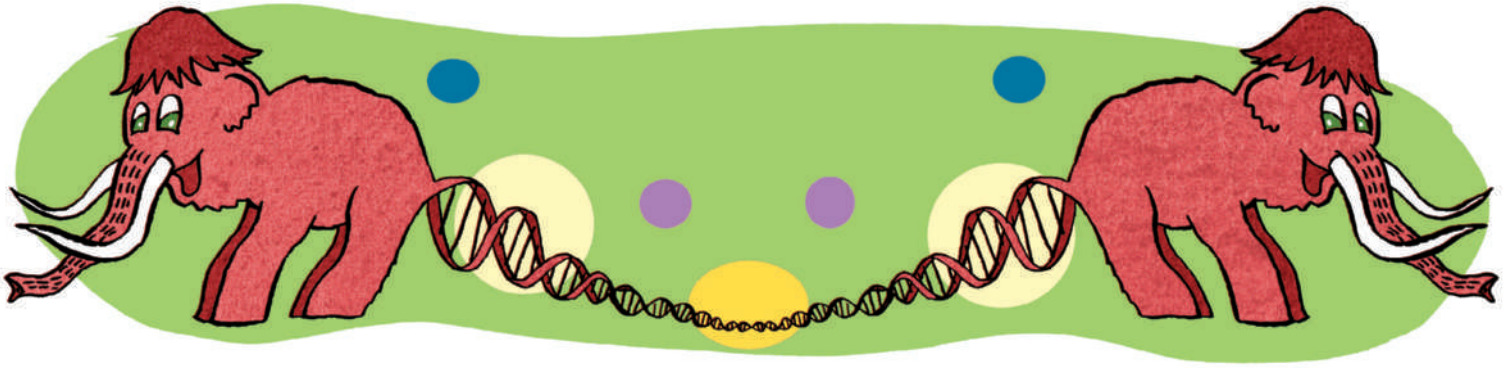
The order of the reference list and citations have been corrected in this PDF.



# LET'S MAKE A MAMMOTH

Evolution assumes that extinction is forever. Maybe not. **Henry Nicholls** asks what it would take to bring the woolly mammoth back from the dead.

In 1990 the late Michael Crichton gave the idea of reviving extinct species a slickly plausible and enormously entertaining workout in his novel *Jurassic Park*. At that time the longest genome that had ever been sequenced was that of a virus. The best part of 20 years on, hundreds of animal genome sequences have been published. This week, for the first time, the genome of something undoubtedly charismatic and definitively extinct joins the list: the mammoth (*Mammuthus primigenius*)[1]. If you want to bring a species back to life, the mammoth would be almost as dramatic as a dinosaur. And unlike *Tyrannosaurus rex*, the mammoth has close living relatives to lend a hand.

It is a fair bet that a complete genome and closely related species would make it easier to pull a Crichton on a mammoth than on a dinosaur. But easier is far from easy. To put flesh on the bones of the draft sequence — to go from a genome to a living, breathing beast — would require you to master, at the very least, the following steps: defining exactly the sequence or sequences you want for your creatures; synthesizing a full set of chromosomes from these sequences; engulfing them in a nuclear envelope; transferring that nucleus into an egg that would support it; and getting that egg into a womb that would carry it to term. None of those steps is currently possible. From getting a definitive sequence to harvesting eggs from an elephant there are all-but-insurmountable obstacles at every stage, and no evidence that anyone is going to work very hard to solve them. But do any of them actually make the dream impossible?

**"Genome synthesis is further developed today than genome sequencing was when Crichton wrote *Jurassic Park*."**

Darwin200

## The sequence

The first stop in this mammoth challenge is to obtain a sequence good enough for us to contemplate using it as the basis for a living being. The sequencing of long-dead DNA such as that of mammoths uses fragments at various levels of degradation. To detect and correct the base-pair changes that can occur after death and to avoid the inevitable errors involved in assembling millions of these tiny fragments into a coherent sequence, it is necessary to sequence much more than a single genome's worth of DNA. "If we want fewer than 1 error in 10,000 base pairs — a reasonable quality genome — we would need to sample in the order of 12-fold coverage," says Svante Pääbo, director of the genetics department at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, who has worked on Neanderthal genomes[2]. The genome published today has roughly 0.7-fold coverage. 'Reasonable quality' for science does not mean the sort of genome you would want to live with: in a human genome that error rate would mean 300,000 mutations.

Coverage can be improved as long as there's the money to do it, but old samples offer particular challenges: a lot of contamination by bacterial, fungal and other species' DNA. Thirty-five-fold coverage, which Pääbo says is as good as it gets, would be "extremely costly and extremely time-consuming", according to Eske Willerslev, head of the Ancient DNA and Evolution Group at the University of Copenhagen. Ever-cheaper sequencing, however, and the possibility of better preserved and prepared samples, mean that those expenses of cost and time will shrink. Willerslev sees nothing to stop researchers from producing a mammoth genome as good as any genome today at some point in the future. Whether such a genome would be good enough for a living being remains a somewhat open question — but with time and effort, it's plausible that a sufficiently error-free genome can be arrived at.

A sequence on its own, though, is not enough: researchers will need to work out exactly how it divides up into chromosomes. The obvious solution would be to tot up the number of chromosomes in an intact mammoth cell and sift through the genomic data looking for their beginnings and endings. But even the very best mammoth material falls short of this kind of preservation (see 'You need to do more than thaw'). "We have no idea — yet — how many chromosomes mammoths had," says Hendrik Poinar, a geneticist at McMaster University in Ontario, Canada. Kerstin Lindblad-Toh, co-director of the genome sequencing and analysis programme at the Broad Institute in Cambridge, Massachusetts, says that the institute will release a sequence of the African elephant (*Loxodonta africana*) to seven-fold coverage some time in 2009. When they do, the mammoth geneticists

ILLUSTRATIONS BY: S. KAMBAYASHI

will be all over it. But it will take an immense amount of work to identify and locate all the chromosome changes, gene deletions, duplications and rearrangements that mammoths will have acquired since they diverged from their African ancestors 7.6 million years ago. A sequence for the Indian elephant (*Elephas maximus indicus*), which is more closely related to the mammoth, would be of further help.

One chromosome offers particular problems. In mammals the Y chromosome is typically very small and hard to sort out, in part because it is remarkably repetitive. Researchers have sidestepped the issue in the elephant genome by sequencing a female. "The X chromosome is hard enough to assemble and the Y chromosome is the hardest chromosome out there," says Lindblad-Toh. The dinosaurs in *Jurassic Park* were designed to all be female, to avoid unwanted breeding; cloned mammoths might all be females, too, at least for the first generation, just because it would be easier.

There are other repetitive regions that will be hard to sequence with confidence, most notably the centromeres, which help chromosomes to get where they are meant to go in cell division. It is almost impossible to work out the centromeres' exact sequence, says Bill Earnshaw of the Wellcome Trust Centre for Cell Biology at the University of Edinburgh, UK. "You just get hopelessly lost," he says. "It's like being in a forest where all the trees look identical."

But this need not be a sticking point, as artifice can make do for accuracy. Just this year Earnshaw and his colleagues created a human artificial chromosome that contained a synthetic but fully functional centromere[3]. In principle, that could work for a synthetic mammoth chromosome, says Earnshaw. Replacements for the shorter repetitive sequences at the end of chromosomes, called telomeres, are also doable. And although the sequences will need specific sites at which chromosome replication can start, too, Earnshaw says that "any long enough strand of DNA will have sequences that can function as origins of replication".

Finally, there is the question of genetic variation. Most published mammalian genomes — the mammoth draft included — provide only a single version of all the genes and other sequences in the genome. But mammals have two versions of each gene — one from their mother and one from their father. Building a mammoth with chromosome pairs in which the two chromosomes were identical would be a recipe for trouble, as the effects of any bad gene would be felt to their fullest. Identifying different versions of genes would add yet more to the sequencers' to-do list, but it would be crucial to success.

## You need to do more than thaw

Making a nucleus will be difficult; why bother if some are already lying around? Some argue that finding frozen mammoth cells that have well-preserved nuclei is the obvious starting point for making a mammoth.

In Japan, scientists[1,2] have recently succeeded in cloning mice from cells that had been frozen for 16 years. This led them to venture that "nuclear-transfer techniques could be used to 'resurrect' animals ... from tissues frozen for prolonged periods without any cryopreservation". They mentioned mammoths specifically in this context. But those who work with ancient DNA on a daily basis are unconvinced. Sixteen years in a –20 °C laboratory freezer is a poor substitute for ten millennia or more lying in permafrost — and who knows how long unfrozen before that?

Eske Willerslev, head of the Ancient DNA and Evolution Group at the University of Copenhagen, points out that it takes time, and repeated cycles of tissue-damaging freezing and thawing, before a dead animal gets incorporated into the permafrost. That gives plenty of opportunity for DNA to degrade. What's more, Willerslev says, many microbes are active even at subzero temperatures, causing the samples to degrade further. All this, he thinks, explains why he has never seen or heard of a mammoth cell with a nucleus that's even intact, let alone transferable. Hendrik Poinar of McMaster University in Ontario, Canada, puts the problem with frozen-tissue approaches more succinctly: "I am currently unaware of –20 ° freezers that date to 20,000 years." **H.N.**

### DNA synthesis

With the genome sequenced in painstaking detail, glitches corrected, chromosomes identified, key repeat sequences written in appropriately and genetic variation introduced, it's time to turn towards the DNA synthesis itself. The largest totally synthetic genome produced so far is that of the bacterium *Mycoplasma genitalium*[4]. This contained 582,970 base pairs; the mammoth weighs in at 4.7 billion, half as many again as are found in the human genome. Assuming mammoths turn out to have the same number of chromosomes as elephants, the task would be broken down into making 56 separate chromosomes, each an average of some 160 million base pairs long.

Short strings of bases that are made in the test tube can be assembled into respectable double-stranded stretches of DNA about 8,000 base pairs long without too much error, says Ralph Baric, a microbiologist at the Carolina Vaccine Institute in Chapel Hill, North Carolina; a range of companies will synthesise such sequences for less than a dollar a base pair, and the reagents cost much less. But as neighbouring stretches are joined together *in vitro*, the DNA molecules become increasingly unstable. The team that put together the *M. genitalium* genome at the J. Craig Venter Institute in Rockville, Maryland, dealt with this by inserting the unstable chunks of DNA into 'bacterial artificial chromosomes'. The various components could then be stitched together in the relatively welcoming environment of *Escherichia coli*. For the last steps, the researchers took the largest pieces assembled in *E. coli* and inserted them into yeast artificial chromosomes, which are larger. These were then recombined in living yeast cells to produce constructs that had the entire genome in them.

This approach is impressive in

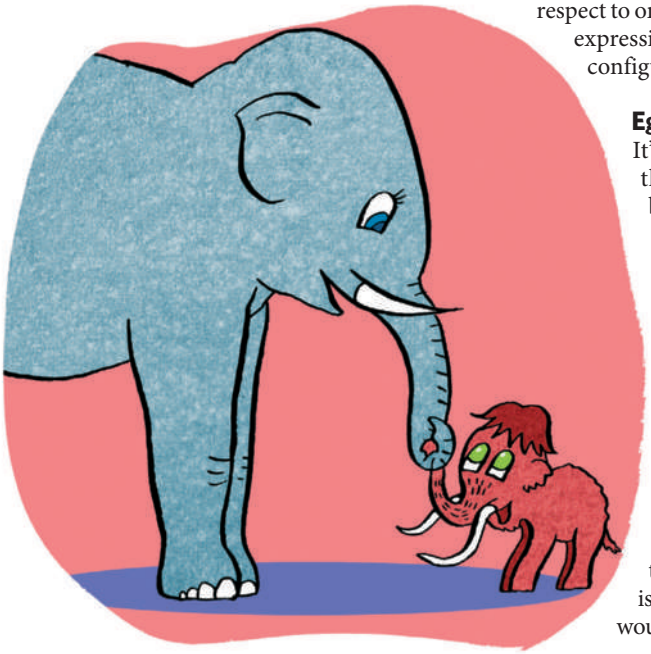
terms of speed and cost, says Drew Endy of the Department of Bioengineering at Stanford University in California. But it's unlikely to be scaled up to accommodate mammoth-sized chromosomes in any straightforward way. After all, he says, at just over 12 million base pairs, the entire yeast genome is much smaller than a medium-sized elephant chromosome: "I would wonder if yeast could handle so much exogenous DNA." This concern is echoed by Baric: "The bigger it is, the quicker there are going to be nicks or breaks, allowing for degradation or deletions in essential genes."

Even if it became possible to synthesize a stable mammoth chromosome in sufficient quantities, this would then have to be repeated for all the other chromosomes. "I don't think we're going to be seeing any mammoths any time soon," concludes Baric. But it will not be long before synthetic biologists develop a certain confidence in synthesizing microbial genomes from scratch, he says. The technologies that are used to do so will, he predicts, give a good indication of whether it might one day be possible to reconstruct the genome of a large mammal such as a mammoth. It is worth remembering that genome synthesis is further developed today, in terms of the maximum lengths achieved, than genome sequencing was when Crichton wrote *Jurassic Park*. And look how sequencing has progressed since then.

## Wrapping it up

Once the chromosomes have been synthesized, they need to be put into a nucleus. Cells wrap chromosomes back up into nuclei whenever they divide, so this part of the process has been fairly well studied. In the 1980s, researchers discovered that naked DNA added to extracts from the eggs of frogs quickly becomes wrapped up in proteins that condense it into chromatin; then membrane fragments bind and fuse to form a functional nuclear envelope around the chromatin. "Such artificial nuclei are capable of DNA replication and some DNA transcription," says Douglass Forbes, professor of cell and developmental biology at the University of California, San Diego. Forbes thinks that anyone trying to make a mammoth nucleus in the foreseeable future would be best advised to stick with frog extracts, rather than use fragments of the nucleus from some more similar creature such as an elephant. "Mammalian eggs, which are fertilized internally, might have much less ability to assemble nuclei," she says. When later transferred into the cytoplasm of an elephant cell, elephant nuclear proteins might complement or replace the frog-specific counterparts used to make the mammoth pseudo-nucleus, she says, giving it a more properly mammalian look and feel.

It would also be a challenge to ensure that the nuclear membrane engulfs all the mammoth chromosomes. "You will somehow need to keep them together when you inject them," says Eric Schirmer at the Wellcome Trust Centre. If you don't, miniature nuclei may form that contain a random rabble of chromosomes. The way these chromosomes sit with respect to one another might also affect gene expression; how to engineer the correct configuration, nobody knows.



## Egg collection

It's almost time to contemplate the vagaries of nuclear transfer, but not before you have sourced your elephant eggs, and these are likely to be in pretty short supply. Female elephants ovulate on a 16-week cycle, although they regularly skip five or so years owing to gestation and lactation. Stopping these natural breaks in cycling would be both cruel and unproductive; cow elephants that don't gestate have a strong tendency to develop massive ovarian tumours. When they do ovulate, only one oocyte is released; a litter of little elephants would be a death sentence, and even twins are remarkably rare.

On the positive side, though, elephants' infrequent ovulations are preceded by an early warning; uniquely among mammals that have been studied to date, elephant ovulation involves not one but two surges in luteinizing hormone, separated by 18–20 days. "The first hormone peak dissolves the vaginal mucous and forwards just one follicle for development," says Thomas Hildebrandt of the Institute for Zoo and Wildlife Research in Berlin, Germany. "The second peak stimulates ovulation."

In other creatures it would be quite straightforward to get the follicle in which an egg is developing out of the ovary after this surge of harbinger hormones; you use ultrasound to guide a harvesting implement up the reproductive tract, or perform a laparoscopy, during which the abdominal cavity is inflated to make room for the job to be done surgically.

Unfortunately, quirks of elephant biology rule out both these approaches. Whereas the entrance of the vagina is pretty simple to locate in most mammals, elephants have more than a metre of urogenital canal between the outside world and the hymen. This canal is as far as a bull-elephant's penis gets; the hymen remains intact even after copulation, which may be an evolutionary hangover from the elephant's aquatic past. Hildebrandt and his colleagues have developed a way to navigate an instrument up the canal, through the tiny aperture in the hymen that lets sperm in, along the vagina and into the uterus; they use it to perform artificial insemination with sex-selected sperm. But Hildebrandt says that even with such instruments threaded into the womb it would be almost impossible to locate a single mature follicle without some extra guidance, and the ovaries are too deep inside the abdominal cavity for the precise position of the follicle to be

## Let's fake a mammoth

The complete genome sequence of the African elephant (*Loxodonta africana*), expected soon from the Broad Institute in Cambridge, Massachusetts, should be a great help in identifying regions of the mammoth genome that vary from those of the elephant and starting the search for the key genetic differences that are unique to mammoths. But it might also provide a more direct and plausible way to make what one might call pseudo-mammoths.

With some idea of the genes involved in hair formation, coat colour and tusk development, for example, it might be possible to use genetic engineering and selective breeding to produce an elephant that looks remarkably mammoth-like, says Svante Pääbo of the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany. It wouldn't be a mammoth in a way that satisfied a purist, or an ecologist or a dreamer set on restoring something magnificent and lost to the world. But it could perhaps be good enough for, say, amusement-park work; and it would avoid some of the trickiest synthetic biology. "That's the furthest I could imagine I would see in my lifetime," says 53-year-old Pääbo. **H.N.**

revealed by ultrasound. Laparoscopic surgery is also out of the question, as elephants have no pleural space between their body wall and lungs. "Inflating the abdominal cavity during laparoscopy would compress the lungs and kill the animal," says Hildebrandt.

There may, however, be an ingenious way out of this bind. Cryobiologists have in the past transplanted tissue from the ovaries of one animal into the ovaries of another. The procedure has worked even if the tissue had been frozen and thawed out between leaving the donor and being grafted into the recipient. Not only has this work led to the treatment of infertility after chemotherapy, it has also raised the possibility that ovarian tissue from endangered species could be transplanted into laboratory animals, making them a source of eggs-on-demand. For Hildebrandt, the only realistic way of getting a steady source of elephant eggs would be to collect tissue from the ovary of a recently deceased elephant and graft slivers of it into a laboratory mouse or rat with a suppressed immune system that won't reject them.

This has in fact already been done. In the 1990s, frozen samples of ovarian tissue from African elephants culled in South Africa's Kruger National Park were thawed and transplanted into mice, and afterwards researchers saw what seemed to be mature follicles develop[5]. "But we didn't have enough material to assess whether those eggs were competent," recalls John Critser, now professor of comparative medicine at the University of Missouri in Columbia, who led the study.

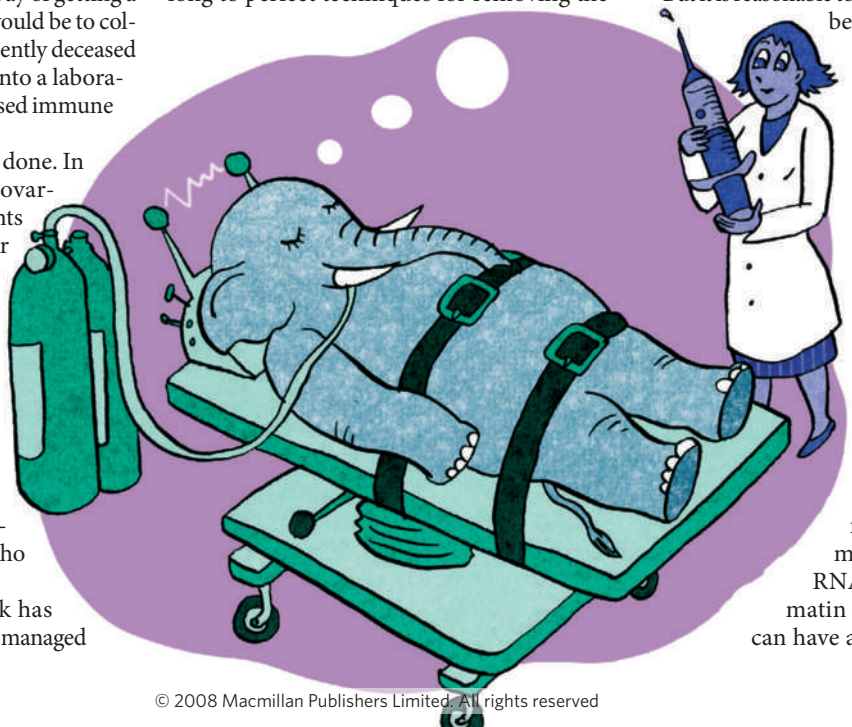
Although the elephant work has gone no further, researchers have managed to produce live and apparently healthy offspring from eggs that have come from tissue transplanted into another species. Such successes, though, have been with closely related species — rats acting as hosts for mouse tissue[6], for example. It's harder to see how elephant eggs would mature successfully in a mouse, not least because the mouse's oestrus cycle lasts just 4–6 days. It would probably be necessary to remove the mouse's pituitary gland and subject it to a hormonal cycle that approximates an elephant's with hormone treatments, says Critser. "It's not something that you could use easily right now for the production of oocytes. But that's not to say that it couldn't be developed."

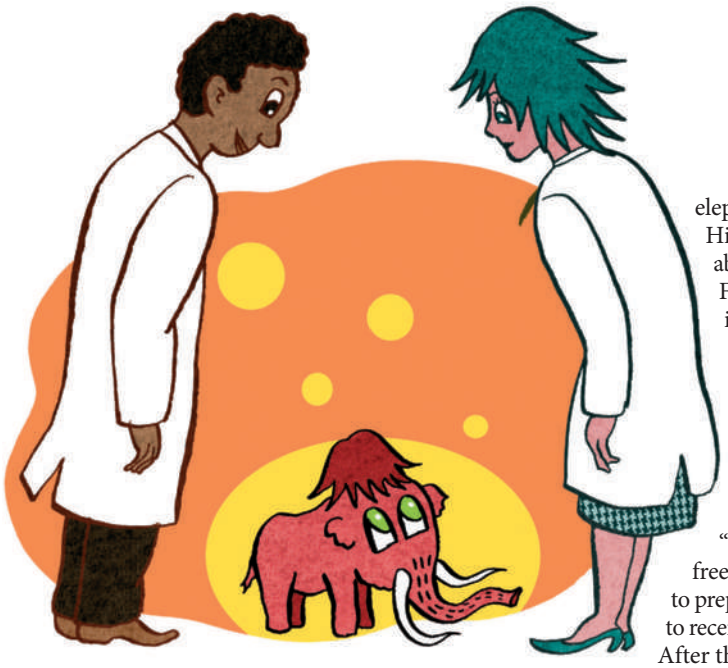
### Nuclear transfer

With a ready supply of eggs, it should not take long to perfect techniques for removing the native elephant nucleus to make space for the synthetic mammoth nucleus. But that might not be all the preparation needed for the nuclear transfer. The mitochondria — organelles that provide cells with energy through respiration — have their own genomes, specific to each species. Hybrids with a nucleus from one species and mitochondria from another can be viable — an embryo with sheep mitochondria but nuclear DNA from a mouflon developed into an animal, called Ombretta, in 2001 (ref. 7) — but there are risks of incompatibility. The close evolutionary relationship between elephants and mammoths reduces the chances of incompatibility, but Stefan Hiendleder, professor of animal science at the University of Adelaide in Australia, cautions that there would still be risks: human cells that have had their mitochondria replaced with those from other primates have failed to show proper respiration[8]. This means that researchers might need to rustle up some synthetic mammoth mitochondria and insert them into the elephant cytoplasm. With the mammoth mitochondrial genome already well sequenced[9], this should be relatively simple. And all the elephant mitochondria would have to be cleaned out, in case they hybridize with the newcomers, warns Hiendleder.

Nuclear transfer remains a fickle and inefficient way of producing new mammals even without such concerns. Only a few of the transfers result in embryos and not all of those embryos manage to establish a placenta. Of those that do, many abort spontaneously and the few successful live births frequently have developmental abnormalities.

But it is reasonable to hope that things may get better, says Xiuchun Tian of the Center for Regenerative Biology and Department of Animal Science at the University of Connecticut in Storrs. The most likely explanation for the inefficiency of nuclear transfer is that the genes are not being expressed in a manner appropriate for embryonic development. This, says Tian, is probably down to errant 'epigenetic' signals — inherited patterns of DNA methylation, histone modification, microRNA presence and chromatin structure, all of which can have a crucial effect on gene





expression[10]. The recent discovery that the nuclei of normal body cells can be induced into an embryonic-like state without the need for nuclear transfer will help researchers to understand the effect of these epigenetic signals and how to manipulate them, she says.

Better still, there may be an alternative way to dress up a synthetic nucleus in suitably epigenetic garb. Even if an early embryo is doomed not to go all the way to term, it can still be used as a source of stem cells. These could then be introduced into normal elephant embryos to create chimaeras — in which some cells are mammoth and some elephant. Such chimaeras may stand a better chance of developing to term, and although they wouldn't be mammoths, they would be a way by which mammoths might then be made.

**"The urogenital canal is as far as a bull elephant's penis gets."**

If enough chimaeras are created in this way, some should end up with mammoth cells in their ovaries or testes, giving you elephants that produce mammoth eggs or sperm. 'Germ-line chimaeras' of this type have already been created in several species. In 2004, Japanese scientists[11] made salmon with trout cells in the testes that produced sperm capable of fertilizing trout eggs and producing bona fide baby trout.

Gametes from germline chimaeras would be much more likely to be properly imprinted, says Tian — indeed, once you have a supply of mammoth eggs and mammoth sperm you might well consider the project close to bearing fruit.

## Embryo transfer

With a fertilized mammoth egg — either made through direct nuclear transfer or through the *in vitro* fertilization of mammoth eggs with mammoth sperm from chimaeras — the mammoth challenge comes to its final stage. Although the inaccessibility of elephant eggs means that nobody has ever performed elephant embryo transfer, Hildebrandt has thought about what it would take. First, he recommends inserting an arm up the urogenital canal to inject some sperm-free elephant ejaculate in the direction of the hymen on the day that hormones reveal the elephant is ovulating. "We think the sperm-free component is needed to prepare the female's uterus to receive an embryo," he says. After that, the transfer of the cloned mammoth embryo into the uterus, a total distance of some 2.5 metres, should be possible using the same apparatus used for artificial insemination, says Hildebrandt. "We are coming quite close to the oviduct, which would be the place to put a cloned embryo back into an elephant." An embryo at the four-cell stage would need to be transferred about two days about ovulation, he says.

At that point, the last concern becomes whether a mammoth fetus would be suited to the uterus of its surrogate mother. Evidence from preserved mammoths suggests that size, at least, should not be a problem. An Indian elephant calf can weigh around 120 kilograms at term and stands around 1 metre tall. Dima — a famous mammoth calf unearthed in northeastern Siberia in 1977 — is estimated to have been about the same mass and height when he died aged 7–8 months. It's a similar story for Lyuba, a calf discovered in Russia's Yamal Peninsula last year. Although Lyuba's mass when she died has not yet been definitively ascertained, initial reports suggest she was only 90 centimetres tall and 4 months old, says Daniel Fisher, professor of ecology and evolutionary biology at the University of Michigan in Ann Arbor and one of an international team poring over her spectacularly preserved remains. Most evidence indicates that newborn woolly mammoths were about the same size as newborn elephants, he says. "They could even have been smaller." Mercifully, we probably need not concern ourselves with how to incubate a preterm mammoth fetus.

## Birth and after

A single artificial mammoth would be a freak, not a species; once she was born others — including, ideally, males — would have to follow. Their genomes would have to be tweaked to ensure a certain diversity. A place for them to live would have to be found, as would an ecosystem into which they could integrate. It would be a huge undertaking — just as synthesizing mammoth chromosomes and reprogramming them into embryo-friendly nuclei would be. Perhaps the whole idea will remain too strange, too expensive, too impractical, even too unappealing for anyone to take seriously. But the fact that just 15 years ago cloning mammals was confidently ruled out by many as being impractical should give people pause before saying any such thing is impossible. On Darwin's 200th birthday in 2009, reoriginating extinct animal species will still be a fantasy. By 2059, who knows what may have returned, rebooted, to walk the Earth? ■

**Henry Nicholls is a science writer who lives in Greenwich, England. His most recent book is *Lonesome George*.**

1. Miller, W. *et al. Nature* **456,** 387–390 (2008).
2. Green, R. E. *et al. Cell* **134,** 416–426 (2008).
3. Nakano, M. *et al. Dev. Cell* **14,** 507–522 (2006).
4. Gibson, D. G. *et al. Science* **319,** 1215–1220 (2008).
5. Gunasena, K. T. *et al. Anim. Reprod. Sci.* **53,** 265–275 (1998).
6. Snow, M., Cox, S.-L., Jenkin, G., Trounson, A. & Shaw, J. *Science* **297,** 2227 (2002).
7. Loi, P. *et al. Nature Biotechnol.* **19,** 962–964 (2001).
8. Hiendleder, S., Zakhartchenko, V. & Wolf, E. *Reprod. Fert. Dev.* **17,** 69–83 (2005).
9. Gilbert, M. T. *et al. Science* **317,** 1927–1930 (2007).
10. Yang, X. *et al. Nature Genet.* **39,** 295–302 (2007).
11. Takeuchi, Y., Yoshizaki, G. & Takeuchi, T. *Nature* **430,** 629–630 (2004).
12. Wakayama, S. *et al. Proc. Natl Acad. Sci. USA* Advance online publication doi:10.1073/pnas.0806166105 (2008).

**See also pages 281, 295, 330 and 387, and online at www.nature.com/darwin.**