LETTER
Mutations Induced by Ancient DNA Extracts?

D. Serre, M. Hofreiter, and S. Pääbo

Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany

We have investigated whether some factor in ancient DNA extracts induces site-specific mutations in modern DNA. We find no evidence for higher mutation rates when extracts from three different Pleistocene mammals are added to modern DNA than when water or extraction blanks are added. We also fail to find evidence that any such factor affects ancient DNA sequences determined from the same extracts. This as well as the patterns of nucleotide substitutions seen in DNA sequences determined from hundreds of other specimens leads us to doubt that a previously unknown mutagenic factor can be a general feature of extracts from old tissues.

Pusch and Bachmann (2004) have made extracts from the remains of 14 animals and plants that vary in age from 60 years to 55 Myr. To these extracts they add contemporary human DNA and find that nine of the extracts inhibit the polymerase chain reaction–mediated amplification, particularly of long DNA fragments. That ancient DNA extracts can inhibit the PCR is well known (e.g., Höss and Pääbo [1993] and Hänni et al. [1995]) and that this may affect longer fragments more than shorter fragments is not surprising, given the low processivity of the Taqpolymerase (Merkens, Bryan, and Moses 1995).

What is unusual and potentially worrying about the results presented by Pusch and Bachmann (2004) is that when they add modern human DNA to the extracts, amplify a 148-bp segment of the mitochondrial control region, and sequence clones from the amplification products, they see substitutions that are so numerous that 77% of all clones differ from the DNA sequence putatively added to the extracts. The substitutions tend to fall at certain positions, many of which are known to vary among contemporary humans. As a consequence, in 11 of the 14 experiments, an incorrect consensus DNA sequence would be determined. They interpret this result as the effect of a mutagenic factor that must occur in at least 13 of the 14 extracts and conclude that DNA sequences determined from ancient extracts may be incorrect because of this putative mutagenic effect.

Because we and others have shown that ancient specimens as well as laboratory reagents are almost always contaminated by human DNA, often stemming from multiple individuals (Handt et al. 1996; Kolmann and Tuross 2000; Hofreiter et al. 2001*b*; Wandeler et al. 2003; Serre et al. 2004), we worry that contamination of the specimens and experiments may yield results that can be interpreted as "mutagenesis" because multiple DNA sequences would be retrieved after amplification and cloning. We, therefore, first tested whether a mutagenic effect as described by Pusch and Bachmann (2004) can be detected when we amplify ancient mitochondrial DNA sequences from animal remains. To do this, we extracted DNA from a brown bear bone dated to 14,000 years before present and a cave bear bone that is 28,000 years old, both found in the same cave in southwestern Germany. Because the former sample represents a species that still exists, but the latter sample is from a related but extinct species, we expect, in the absence of mutagenic effects, the endogenous mtDNA sequences of the Pleistocene brown bear to be similar or identical to mtDNA sequences of the modern brown bear and the endogenous DNA sequences of the cave bear to be potentially different but related to mtDNA sequences of the modern brown bear. By contrast, if a mutagenic factor in the extracts influences the results, we would expect both mtDNA sequences to differ from modern brown bear mtDNA sequences.

We performed two independent amplifications of a 44-bp segment of the mitochondrial control region from each bone and sequenced 19 clones from the brown bear and 13 clones from the cave bear (fig. 1). All brown bear clones are identical, whereas one cave bear clone carries a single substitution from the other clones. The brown bear sequence is identical to some contemporary brown bears, whereas the cave bear sequence differs at eight positions from the brown bear. At two of these eight positions, all other cave bears sequenced to date in our laboratory (Hofreiter et al. 2002, 2004) as well as other laboratories (Hänni et al. 1994; Loreille et al. 2001; Orlando et al. 2002) have been shown to differ from brown bears. Moreover, the cave bear DNA sequence differs at six to 10 positions from over 100 brown bear DNA sequences determined to date, whereas it differs at zero to two positions from 65 cave bear DNA sequences. Thus, there is no indication that a putative mutagenic effect in the extract has affected the brown bear sequence.

However, if such an effect exists in the cave bear extract, these results may be produced if the cave bear extract was contaminated by brown bear DNA and the mutagenic effect was so efficient and specific that it changed 103 out of 104 nucleotides at the positions indicated by arrows in the figure while not affecting any other positions. Moreover, such a mutagenic effect must have failed to affect all ancient brown bears for which DNA sequences have been determined (Leonard, Wayne, and Cooper 2000; Barnes et al. 2002) but have affected all cave bears studied to date because the cave bear sequence is identical or very similar to all such DNA sequences.

To test whether such a mutagenic effect exists in the cave bear extract, we repeated the experiment described by Pusch and Bachmann (2004). We, thus, added 50 ng of contemporary human DNA to the cave bear extract. In addition, we performed a number of controls not performed by Pusch and Bachmann (2004). First, we added 50 ng of chimpanzee DNA to an independent amplification from the extract to test if the putative mutagenic factor affects only human but not closely related DNA sequences. Second, we added 50 ng of modern human and chimpanzee DNA to

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E-mail: paabo@eva.mpg.de.

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                                          ↓ ↓        ↓ ↓↓ ↓↓                      ↓
Modern brown bear (reference)             TACTATTTTACCCCGTGTCCTATTTATTTCATATATACCATCTT
Ancient brown bear     1-1                ..........................................
Ancient brown bear     1-2                ..........................................
Ancient brown bear     1-3                ..........................................
Ancient brown bear     1-4                ..........................................
Ancient brown bear     1-5                ..........................................
Ancient brown bear     1-6                ..........................................
Ancient brown bear     1-7                ..........................................
Ancient brown bear     1-8                ..........................................
Ancient brown bear     1-9                ..........................................
Ancient brown bear     1-10               ..........................................
Ancient brown bear     2-1                ..........................................
Ancient brown bear     2-2                ..........................................
Ancient brown bear     2-3                ..........................................
Ancient brown bear     2-4                ..........................................
Ancient brown bear     2-5                ..........................................
Ancient brown bear     2-6                ..........................................
Ancient brown bear     2-7                ..........................................
Ancient brown bear     2-8                ..........................................
Ancient brown bear     2-9                ..........................................
Ancient cave bear      1-1                C.T........T.TA.AC.........................C
Ancient cave bear      1-2                C.T........T.TA.AC.........................C
Ancient cave bear      1-3                C.T........T.TA.AC.........................C
Ancient cave bear      1-4                C.T........T.TA.AC.........................C
Ancient cave bear      1-5                C.T........T.TA.AC.........................C
Ancient cave bear      1-6                C.T........T.TA.AC.........................C
Ancient cave bear      1-7                C.T........T.TA.AC.........................C
Ancient cave bear      1-8                C.T........T.TA.AC.........................C
Ancient cave bear      2-1                ..T........T.TA.AC.........................C
Ancient cave bear      2-2                C.T........T.TA.AC.........................C
Ancient cave bear      2-3                C.T........T.TA.AC.........................C
Ancient cave bear      2-4                C.T........T.TA.AC.........................C
Ancient cave bear      2-5                C.T........T.TA.AC.........................C
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FIG. 1.—Alignment of DNA sequences of cloned PCR products from a cave bear and a brown bear bone from Hohle Fels Cave, Ach Valley, Germany. For both individuals, two independent PCRs were done (indicated by the first numbers) and five to 10 clones (second numbers) were sequenced. As a reference, we used the closest matching modern brown bear DNA sequence from GenBank (which is identical to the ancient brown bear DNA sequence). Dots indicate sequence identity. Arrows mark the positions where the two consensus sequences differ, red arrows mark the two positions of fixed differences between brown and cave bears. (GenBank accession numbers AJ632271 for the cave bear and AJ632272 for the brown bear).

blank extractions performed in parallel with the extraction of the cave bear remain to see if a putative mutagenic effect results from the extraction procedure itself. Third, we added 50 ng of modern human and chimpanzee DNA to a PCR performed without any ancient extract to determine the cumulative rate of nucleotide misincorporations occurring during the PCR, cloning, and sequencing reactions. When human DNA is added to the experiments (fig. 2), the cave bear extracts yields one substitution among 16 clones analyzed, whereas when chimpanzee DNA is added, the same extract yields two substitutions among 11 clones (figure S1 in Supplementary Material online). This is not different (Fischer's exact test, $P > 0.2$) from either the blank extraction (one substitution in 15 clones and zero in 12 clones, respectively) or the amplification where just water was added (one in 14 clones and four in 14 clones, respectively). Thus, the numbers of substitutions do not differ significantly between the amplifications performed in the presence of ancient DNA extracts and those in which no ancient extract was added. We, furthermore, tested extracts from a cave bear from which endogenous bear sequences cannot be amplified and a Pleistocene ground sloth from South America using the same controls. In neither case did we observe any difference in substitution frequencies between extracts and controls (figure 2 and figure S1 in Supplementary Material online).

We find the difference in results between Pusch and Bachmann's (2004) experiments and our experiments puzzling, given the fact that they perform their extractions using the same methods as we do and that they find the putative mutagenic effects in all or almost all of their 14 extracts, whereas we see it in none of three extracts. To our mind, there are three possible and not mutually exclusive explanations to the results obtained. First, it is possible that there is something in how Pusch and Bachmann (2004) perform their experiments that causes the PCR to induce more mutations than commonly seen. We note, for example, that the carry-over of silica from the extraction will inhibit the PCR and affect ion concentrations in the PCR, something that may well affect the fidelity of the Taq-polymerase. We find this a plausible possibility because the silica extraction procedure does in our hands eliminate most inhibitory factors from environmental samples, whereas nine of 14 extracts performed by Pusch and Bachmann (2004) show PCR inhibition. Second, we note that contaminations of the extracts or any experimental reagents with DNA from humans will produce apparent substitutions that occur primarily at positions known to vary among

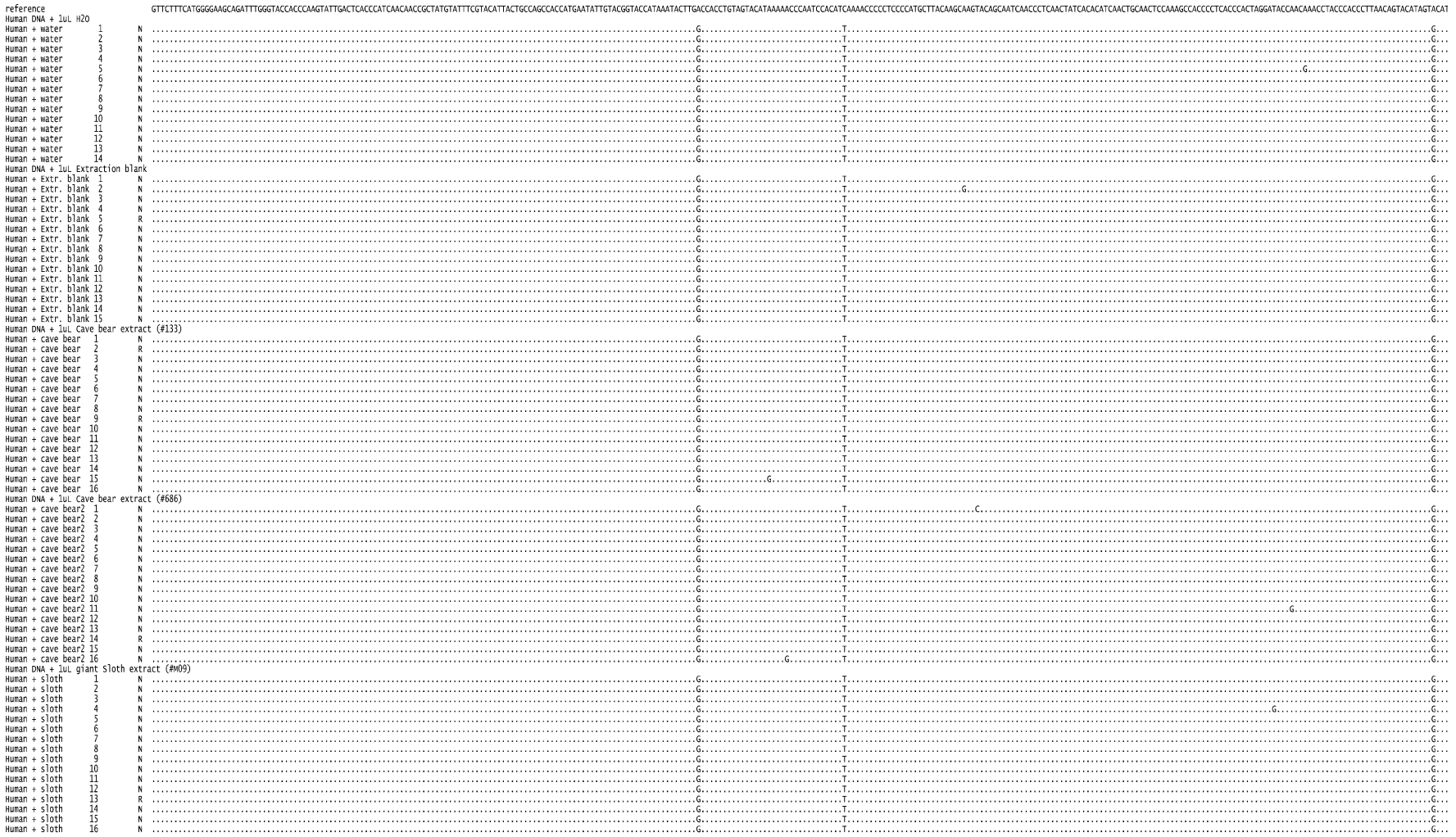

FIG. 2.—Clone sequences obtained from the spiking experiments using human DNA. Dots indicate identity to Anderson et al. (1981). In each experiment, 50 ng of modern human DNA was added to 1 ul of (from top to bottom) water, extraction blank, cave bear bone extract from Hohle Fels, cave bear bone extract from Switzerland, and giant ground sloth feces extract (see text for details).

humans, as is the case in Pusch and Bachmann's (2004) experiments. Third, it may be that there is some mutagenic effect of the type Pusch and Bachmann (2004) describe in many ancient extracts. However, this would mean that it was present in at least 13 of 14 of the extracts Pusch and Bachmann (2004) performed and in none of the three extracts we performed, which is unlikely (Fischer's exact test, $P < 0.006$). Furthermore, for it to have affected the determination of ancient DNA sequences, it would require not only that a site-specific mutagenic effect was present in many or most ancient extracts but also that those extracts were contaminated with DNA from the relevant contemporary animal (and of no other animal in all cases where general primers were used) so that this DNA could become "mutagenized." Alternatively, this would require that endogenous DNA sequences were "mutagenized" in extinct animals, but when ancient remains of extant animals such as the brown bear in figure 1 are analyzed, such a mutagenic effect is absent. We find this scenario unlikely because DNA sequences from over 200 extinct animals and from thousands of museum specimens have by now been determined without any reports of unexpected DNA sequences. For example, complete mitochondrial genome sequences have been determined from four moas (Cooper et al. 2001; Haddrath and Baker 2001) without the appearance of unexpected substitutions for example introducing stop codons in protein-coding regions. Furthermore, although molecular damage in ancient DNA molecules can yield incorrect sequences on rare occasions (Hofreiter et al. 2001*a*), it has been shown that no acceleration of the rate of evolution relative to related extant organisms can be detected in ancient DNA sequences determined from Neandertals, cave bears, and ground sloths, as would be expected if unrecognized mutagenic factors affected the results (Hofreiter et al. 2001*a*).

It is worthwhile to note that for Neandertal remains, contaminating modern human DNA that could potentially be "mutagenized" to look "Neandertal-like" is present in almost all specimens and extracts. However, we find it extremely unlikely that this would explain the results published, because such a putative mutagenic effect would then affect only Neandertal remains and not numerous human remains of similar age as well as even more numerous human contaminations of animal remains that have been analyzed without the detection of "Neandertal-like" mtDNA sequences. Thus, in a recent study (Serre et al. 2004) in which four Neandertal remains and five early modern humans were studied, all hominid remains as well as six cave bears analyzed in parallel contained human DNA. However, only the Neandertals contained a combination of two substitutions seen exclusively in Neandertals to date. Note that this does not exclude the fact that if enough clones amplified from modern humans are sequenced, eventually some clones that contain one or both of these substitutions will be found because of Taq polymerase errors.

In conclusion, we do not believe that the "mutagenic" effect that Pusch and Bachmann (2004) observe in their extracts is a general feature of extracts from ancient tissues. We look forward to a more careful biochemical characterization of the factor assumed by Pusch and Bachmann (2004) to cause mutations in their extracts. In the meantime, it is fortunate that it is easy to test for this putative effect by the addition and amplification of modern DNA to ancient extracts as Pusch and Bachmann (2004) and we have done. Given the ubiquity of human DNA as well as the occasional occurrence of mitochondrial heteroplasmy (Chinnery et al. 2000), we suggest that some DNA other than total human DNA should be used for such experiments. For example, a cloned mitochondrial control region from an ape or indeed any other DNA sequence not likely to contaminate experiments would be suitable for this purpose.

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