Repair of degraded duplex DNA from prehistoric samples using *Escherichia coli* DNA polymerase I and T4 DNA ligase

Carsten M. Pusch*, Ian Giddings and Michael Scholz[1]

Molecular Genetics Laboratory, University Eye Hospital, University of Tübingen, D-72076 Tübingen, Auf der Morgenstelle 15, Germany and [1]Institute of Prehistoric Research, Department of Archeobiology, University of Tübingen, D-72072 Tübingen, Eugenstrasse 40, Germany

Received October 13, 1997; Revised and Accepted December 5, 1997

ABSTRACT

The most notable feature of DNA extracted from prehistoric material is that it is of poor quality. Amplification of PCR products from such DNA is consequently an exception. Here we present a simple method for the repair of degraded duplex DNA using the enzymes *Escherichia coli* DNA polymerase I and T4 DNA ligase. Adjacent sequences separated by nicks do not split up into intact strands during the denaturation step of PCR. Thus the target DNA is refractory to amplification. The proposed repair of nicked, fragmented ancient DNA results in an increase of amplification efficiency, such that the correct base order of the respective nuclear DNA segment can be obtained.

While scientific approaches to investigating prehistoric material such as bones, teeth or mummified tissue are feasible, they are highly complex. Three factors commonly prevent PCR-mediated amplification of ancient DNA (aDNA); the presence of contamination, a low copy number for the desired DNA segment or that the isolated DNA is heavily degraded (i.e. present in small fragments).

A number of the contaminants found in aDNA extracts have been shown to have a proven record as PCR inhibitors (e.g. humic acid, proteins and sugar remnants) (1–5). Even highly purified extracts (e.g. cleaned by silica, proteolytic enzymes, phenol extractions and/or repeated ethanol precipitations) still result in a very low rate of nuclear-specific amplification products. Therefore, we focused on the state of preservation of the chemically altered DNA.

A straightforward and reliable model for explaining these alterations, posits damaged DNA consisting of nicked double strands due to the influence of DNase activity, mechanical and/or physical actions (e.g. by hydrolysis, oxidation or enzymatic destruction) (3). The typically small fragmented duplex aDNA does not separate into two intact strands during the initial denaturation of PCR, but rather into a number of smaller fragments. This greatly minimises the chance of two complementary-oriented PCR primers amplifying a common DNA segment.

In order to assess this 'damage' hypothesis, we performed repair reactions (RRs) with the DNA polymerase I enzyme from *Escherichia coli* (Stratagene) and T4 DNA ligase (Boehringer Mannheim). This allows an increase in the number of informative PCR-based amplifications of single-copy DNA segments of the human nuclear genome.

Escherichia coli DNA polymerase I translates the nicks in the DNA, and the remaining gaps are closed by the subsequent use of T4 DNA ligase (Boehringer Mannheim).

This strategy was successfully applied to aDNA samples where an amplification product could reasonably be expected, but from which no PCR products were obtained. It was also used when highly purified isolates remained non-informative throughout 6–10 independent PCR assays, or when the total yield of isolated DNA was too low (<50 ng).

The individuals from whom samples were taken were buried at the Alamannic burial site at Neresheim (FRG) between ~450 and 700 AD (6). For bone sampling and DNA extraction, the Mix and Clean (MCM) guidelines were followed (7). All possible precautions to exclude contamination with modern DNA were observed (3,7). For each sample, contamination monitoring was performed by the use of two to three extraction blank controls and one water control. In addition PCR amplifications were carried out upon all samples, including negative controls, using mitochondrial specific primers. Due to the high copy number of mitochondrial genome, this is currently the most sensitive contamination monitoring protocol available.

A typical repair reaction contained 2.5 U *E.coli* DNA polymerase I, 5 µl 10× nick translation buffer, 50–1000 ng aDNA isolate, 0.4 mM each dNTP and H_2O_{dd} to a total volume of 50 µl. The reaction was carried out for 60–90 min at 37°C, and terminated with a 20 min incubation at 70°C. Subsequently, 18 µl of the polymerase-treated aDNA (50–300 ng) were mixed with 2 µl of 10× ligase buffer and 0.5 U ligase enzyme. A 1 h ligation reaction was then performed using a temperature-cycle ligation (TCL) protocol on a trio-thermocycler (Biometra) (8). The ligation reaction cycled between 10 and 30°C, holding at each temperature for 10 s. Following enzymatic treatment, the aDNA isolates were purified three times with glass wool/Sephadex

*To whom correspondence should be addressed. Tel: +49 7071 29 87618; Fax: +49 7071 29 5725; Email: carsten.pusch@uni-tuebingen.de

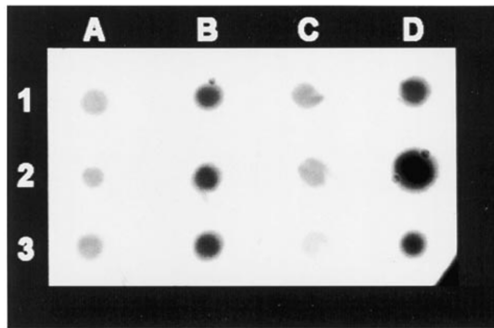

Figure 1. Autoradiographic image of differently prepared and spotted DNAs using 'hot' pseudo-nick translation (without DNase I). Three independent experiments were performed for each sample (1–3). (**A**) aDNA treated by polymerase I (*E.coli*) and ligase as described (weak signal). (**B**) aDNA raw extracts dotted onto nylon membrane without further modifications (strong signal). (**C**) Recent DNA nick translated in native condition (weak signal). (**D**) Recent DNA sample depurinated by HCl treatment (strong signal).

G-50 columns, then subjected to a phenol/chloroform/isoamylalcohol (25:24:1) extraction and finally precipitated with 2.5 vol $EtOH_{abs}$ and 1/10 vol 3 M NaOAc, pH 5.3. The pellet was washed twice with 300 µl 70% EtOH, air dried and subsequently dissolved in 10–15 µl of TE buffer, pH 8.0 prior to amplification by PCR.

To test RR, radioactively labelled dNTPs were used in place of unmodified dNTPs (Fig. 1). MCM-prepared aDNA raw isolates (200–800 ng) were incubated with 0.5 pmol each [α-^{32}P]dATP (ICN), dTTP, dCTP, dGTP and the aforementioned components, at 37°C for 5 min, prior to purification through glass wool/Sephadex G-50 columns.

Approximately 1/1000 vol (~200–800 pg) of the sample was dotted on a nylon membrane (Genescreen) and exposed to X-ray film (Fuji) (1.5 h at –70°C) (Fig. 1A). As reference samples, we used untreated aDNA (Fig. 1B), as well as recent DNA (Fig. 1C) extracted from peripheral blood as described by Miller *et al.* (9). The strongest autoradiographic signal was obtained with (untreated) aDNA raw extract (Fig. 1B), whereas identical autoradiographic intensities were obtained from both spotted recent (Fig. 1C) and enzymatically treated DNA samples (Fig. 1A). This appears to confirm the hypothesis that the nicks and breaks present in the aDNA could be filled-in by the incorporation of radioactive dNTPs, but modern DNA (both treated and untreated) having few gaps, exhibits little incorporation.

In order to model the proposed repair of degraded duplex DNA, artificial (raw) aDNA was produced (Fig. 1D). Pelleted and dried modern genomic individual DNA (10 pg) were dissolved in 20 µl of prewarmed (40°C) 0.25 M HCl. Following depurination, the sample was recovered by ethanol precipitation. The pellet was dissolved in 10 µl TE, pH 8.0 for subsequent fill-in using radioactive dNTPs. The intensity of the autoradiographic signal (Fig. 1D) was found to correlate with that of the non-modified aDNA sample (Fig. 1B).

Escherichia coli DNA polymerase I and T4 DNA ligase are enzymes which significantly modify aDNA. In order to further test the integrity of the method and exclude sequence rearrangements, a single-copy locus of the nuclear genome was amplified, cloned and sequenced. Primer sequences (5′-AGGATCTTCATCTCTCCCAAACC-3′ and 5′-TGAGCTTCCTACTGCCCAAGCTG-3′) were chosen to amplify a 107 bp segment of the human

```
                       .    :    .    :    .    :    .    :    .    :    .    :    .    :    .    :    .    :    .    :    .      107
             M69197    AGGATCTTCATCTCTCCCAAACCTGAGAACCAACACCTTCTCAGCAGTACTTTTTATCGCCTGGTCTACTTCCTTTTTGCTAGTCAGCTTGGGCAGTAGGAAGCTCA  37227

             Clone 1   ...........................................................................................................  107
Indiviual 1
             Clone 2   ..........................................CG...............................................................  107

             Clone 3   ...........................................................................................................  107
Indiviual 2
             Clone 4   ...........................................................................................................  107

             Clone 5   ...........................................................................................................  107
Indiviual 3
             Clone 6   ...........................................................................................................  107

             Clone 7   ...........................................................................................................  107
Indiviual 4
             Clone 8   ...........................................................................................................  107

             Clone 9   ..............................................................................T............................  107
Indiviual 5
             Clone 10  ...........................................................................................................  107

             Clone 11  .......................................................-...................................................  106
Indiviual 6
             Clone 12  ...........................................................................................................  107
```

Figure 2. DNA sequence alignment between a segment of the haptoglobin related gene (EMBL accession no. M69197, position 37121–37227) and cloned aDNA fragments from Neresheim individuals. The amplified aDNA fragments are ordered in pairs e.g. clones 1 and 2 are derived from individual 1. Dots indicate identity of ancient nucleic acids with the reference sequence M69197. The 12 sequences shown are representative of over 40 sequences derived from at least 20 individuals. Of the sequences obtained, 75% are correct, underlining the efficiency of the RR technique. By analysing at least three clones per individual, the absolute correct sequence is extrapolated.

haptoglobin related gene (EMBL accession no. M69197; position 37121–37227). Using the non-modified aDNA raw extracts of 10 different Neresheim individuals (each tested in three independent PCRs) we did not succeed in amplifying this particular portion of the ancient nuclear genome. Six of the non-informative DNA extracts were modified by RR and new PCRs performed on the aforementioned locus. Under the assumption of having amplified a pool of slightly different sequences, we isolated the PAGE-purified PCR products (10) and cloned them into pBluescript.

Twelve recombinants (two clones per individual) randomly chosen for subsequent sequencing on an automated 373A sequencer (Perkin-Elmer/Cetus) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit are described. Figure 2 shows an alignment of the aDNA sequences as compared with the corresponding DNA segment of modern template. The lack of variability over time in this gene, means that identity between aDNA and modern DNA is expected. Nine of the cloned segments were identical to the modern sequence, but three contained alterations in the base order. A single base pair deletion (position 37177) and two minor basepair exchanges (positions 37200 and 37165) were observed in clones 11, 9 and 2 respectively. No nucleotide insertions were observed. The result overall favours correct reproduction of the original sequence. In order to reliably construct a reproducible base order of the desired aDNA segment, however, it is vital to investigate several PCR products for each individual.

The use of RR on degraded aDNA is a powerful tool for obtaining amplifiable single-copy sequences. With badly degraded aDNA no major rearrangement of the base order of nucleic acids was observed. It is not known if the changes seen in the base order (Fig. 2) are due to errors in the activity of *Pfu* polymerase enzyme (Stratagene) or to cloning/sequencing artifacts (11). Since this particular thermostable polymerase is known for its proofreading activity, the latter may be favoured in this respect.

In summary, this straightforward technique modifies prehistoric DNA by a simple repair approach, producing significantly higher quality DNA, and guaranteeing efficient DNA amplification in otherwise non-informative PCRs.

ACKNOWLEDGEMENTS

We are grateful to Dr A.Czarnetzki (Osteological Collection, UoT) for generously donating ancient bone samples and also, to S.Hengst for technical assistance rendered. M.S. holds a fellowship from the VCS (NM).

REFERENCES

1 Hagelberg,E. and Clegg,J.B. (1991) *Proc. R. Soc. Lond. (Biol.)*, **244**, 45–50.
2 Pusch,C.M. and Scholz,M. (1997) *Technical Tips Online* (http://www.elsevier.com/locate/tto) T01217.
3 Hänni,C., Brousseau,T., Laudet,V. and Stehelin,D. (1995) *Nucleic Acids Res.,* **23**, 881–882.
4 Pääbo,S., Higuchi,R.G. and Wilson,A.C. (1989) *J. Biol. Chem.*, **264**, 9709–9712.
5 Pääbo,S. (1989) *Proc. Natl. Acad. Sci. USA*, **8**, 1939–1943.
6 Knaut,M. (1993) Forschungsberichte zur Vor-und Frühgeschichte in Baden-Württemberg, **48**, 29, 188–191.
7 Scholz,M. and Pusch,C.M. (1997) *Technical Tips Online* (http://www.elsevier.com/locate/tto) T01045.
8 Pusch,C., Schmitt,H. and Blin,N. (1997) *Technical Tips Online* (http://www.elsevier.com/locate/tto) T40071.
9 Miller,S.A., Dykes,D.D. and Polesky,H.F. (1988) *Nucleic Acids Res.*, **16**, 101–102.
10 Pusch,C.M. (1997) *Electrophoresis*, **18**, 1103–1104.
11 Krings,M., Stone,A., Schmitz,R.W., Krainitzki,H., Stoneking,M. and Pääbo,S. (1997) *Cell*, **90**, 19–30.

RELATED PAPERS RECENTLY PUBLISHED IN *NUCLEIC ACIDS RESEARCH*

Taberlet,P. *et al*. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res*. **24**, 3189–3194.

Hoss,M. e*t al*. (1996) DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Res*. **24**, 1304–1307.

Hanni,C. *et al*. (1995) Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucleic Acids Res*. **23**, 881–882.