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# DNA Damage Promotes Jumping between Templates during Enzymatic Amplification*

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**Pairs of templates and primers were designed so that only recombination events would lead to amplification via the polymerase chain reaction. This approach reveals that lesions such as breaks, apurinic sites, and UV damage in a DNA template can cause the extending primer to jump to another template during the polymerase chain reaction. By comparing sequences of amplification products that were determined directly or via bacterial cloning, it was shown that when the thermostable *Thermus aquaticus* DNA polymerase encounters the end of a template molecule, it sometimes inserts an adenosine residue; the prematurely terminated product then jumps to another template and polymerization continues, creating an *in vitro* recombination product. Consequently, amplification products from damaged templates such as archaeological DNA are made up of a high proportion of chimeric molecules. The illegitimate adenosine and thymidine residues in these molecules are detected when cloned molecules are sequenced, but are generally averaged out when the amplification product is sequenced directly. However, if site-specific lesions exist in template DNA or if the amplification is initiated from very few copies, direct sequencing also may yield incorrect sequences. The phenomenon of the "jumping polymerase chain reaction" can be exploited to assess the frequency and location of lesions in nucleic acids.**

The polymerase chain reaction (1) is becoming widely used in molecular biology because it can detect and amplify a few or even single copies of a DNA segment. Polymerase chain reaction is of particular value for the study of DNA in single cells (2), forensic samples (3), archaeological remains (4), and museum specimens (5). However, in the latter cases, the vast majority of DNA molecules present have been shown to be damaged (6). It may be speculated that these damaged molecules can contribute in various ways to the population of molecules that make up the final amplification product. Similarly, when amplifications are initiated from single template molecules, damage present in the template molecule could influence the results.

Such considerations prompted us to investigate the effects of various types of damage in the template DNA on the polymerase chain reaction as performed with the thermostable *Thermus aquaticus* (*Taq*) DNA polymerase, particularly with respect to insertions of incorrect bases and the generation of recombination products *in vitro*. This investigation also led us to design a polymerase chain reaction method that may prove valuable for detecting and measuring DNA damage.

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## EXPERIMENTAL PROCEDURES

Enzymatic amplifications were performed in 25-μl reaction mixtures containing 67 mM Tris/Cl, pH 8.8, 2 mM $MgCl_2$, 250 μM concentration each of dATP, dCTP, TTP, and dGTP and 1.25 units of *T. aquaticus* DNA polymerase (Perkin-Elmer-Cetus). In the experiments where ancient DNA was used, 2 μg/ml bovine serum albumin (Sigma, fraction V) was added to the reactions. Agarose gel electrophoresis and direct sequencing were performed as described by Pääbo *et al.* (4). Unless otherwise stated, 2 ng of each template construct was present in 25-μl reactions. Primers used were: the M13 "universal" primer (7), D3E and D18X (4), L14841 (8), H14876 (5′-CGCTGCAGAATAGGCCTGTTAGGATTTG-3′), L16175 (5′-GCA-AGCTTAGTACATAAAAACCCAATCCA-3′), DI5 (5′-AAGATC-TTTGAGAGATGTGA-3′), and SP1 (5′-TACCCGGGGCTGA-GCCATCACTCAA-3′). Forty cycles of polymerase chain reaction were performed as follows: denaturation at 92 °C for 40 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min. Sonication was done in a model H-IL sonicator (Ultrasonics) for 5 × 30 s on ice, which produced fragments ranging in size from 100–1500 nucleotides. Depurination was performed by incubating the DNA in 0.1 N HCl for 5 min at room temperature. Tris Cl, pH 8.8, was then added to a final concentration of 0.1 M. UV irradiation was performed by illumination of the DNA sample on a UV table (UVP) for 1 min. The depurinated and UV-irradiated templates showed no evidence of degradation when analyzed by agarose gel electrophoresis.

## RESULTS

*Jumping Induced by Breaks in Template*—In order to assess the tendency for forming recombinant amplification products, polymerase chain reaction was carried out from a set of two template molecules (Fig. 1). Template I was a cDNA containing the complete protein-coding sequence of cow lysozyme type 2b (clone λcBL42, Ref. 9). Template II was a genomic clone of a cow lysozyme type 3 gene (clone pLI, Ref. 10) which contains exons 2, 3, and 4 as well as introns 2 and 3. Within exon 2, template II differs from template I only by having cytidine residues at positions 290 and 293 (Fig. 2). These two template molecules were mixed with one primer specific to exon 1 (*primer A* in Fig. 1), which occurs in template I but not in template II, and another primer (*primer B* in Fig. 1) which is specific for intron 2 and occurs in template II but not in template I. Thus, no template molecule containing both of the primer sites was added to the reactions, and exon 2 is the only region located 3′ to the primers where sequence similarity between the two template molecules exists. After 40 cycles of polymerase chain reaction, no specific product could be detected when the amplification reaction was analyzed by ethidium staining of an agarose gel (Fig. 1, *bottom left, lane 4*).

When template I was cut with *Pst*I and/or template II with *Sau*3AI, an amplification product of the expected size for an *in vitro* recombination product between the two templates



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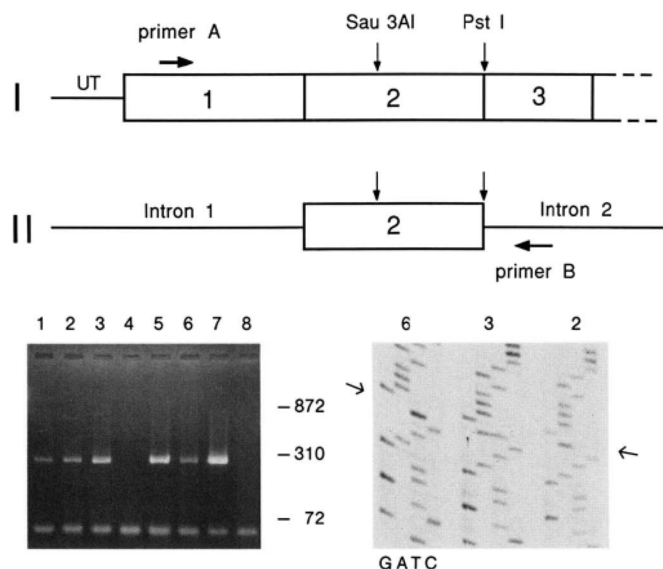


FIG. 1. **Jumping polymerase chain reaction illustrated with two lysozyme clones.** *Above*, parts of the two lysozyme clones as well as primers and restriction sites used are schematically illustrated. *Primer A*, specific for exon 1, is DI5, and *primer B*, specific for intron 2, is SP1 (see "Experimental Procedures" for details). *Numbers* indicate protein-coding exons, and *UT* denotes the 5′-untranslated region of the cDNA. *Below at the left*, agarose gel electrophoresis of amplification reactions are shown. The templates used were: *1*, template I *Pst*I-cut, template II unrestricted; *2*, template I unrestricted, template II *Sau*3AI-cut; *3*, template I *Pst*I-cut, template II *Sau*3AI-cut; *4*, both templates unrestricted; *5*, templates sonicated; *6*, templates UV-irradiated; *7*, templates depurinated; *8*, no template. The lower molecular weight bands are dimers of primers. The migration positions of molecular size markers are indicated in numbers of base pairs. *Below at the right*, part of the direct sequencing reaction around the *Sau*3AI site is shown for the UV-irradiated template (*lanes* marked *6*), templates I and II cut with *Pst*I and *Sau*3AI, respectively (*lanes* marked *3*) and uncut and *Sau*3AI-cut, respectively (*lanes* marked *2*). The *arrows* point to position 249.

was generated (Fig. 1). Upon direct sequencing (11), this product was shown to have the expected composite sequence where axons 1 and 2 were joined. In addition, at positions 290 and 293 where the two template sequences differ, direct sequencing of the amplification product yielded an equal mixture of the two sequences when both templates had been cut. When only one of the templates were cut, the sequence of that template was present at these sites. These results show that *in vitro* recombination events occur readily when breaks exist in the template molecules and that this phenomenon is dependent on sequence similarity between the two extended primers, since restriction of templates I and II with *Sau*3AI and *Pst*I, respectively, did not yield any relevant amplification product.

*Insertion of Adenosine Residues*—Direct sequencing of the amplification products showed that when template II had been cut with *Sau*3AI and template I with *Pst*I, an adenosine residue was present in about equal frequency with the expected thymidine residue at position 249 (Fig. 1, *lower right, lanes 3*). This represents the position at which template II had been cut with *Sau*3AI. When only template II was cut, only an adenosine residue was detected. This demonstrates that *in vitro* recombination between template molecules during polymerase chain reaction can cause unambiguous and incorrect sequences to be obtained. In experiments where the *Pst*I site was cut either alone or in conjunction with the *Sau*3AI, no aberrant bases were detected by direct sequencing.

The polymerase chain reaction product generated from the *Pst*I- and *Sau*3AI-cut templates was cut with *Pst*I and *Bgl*II and cloned in the vector M13mp19 in order to determine the sequence at the *Sau*3AI site. Nine clones were sequenced for the 234 bp[1] from the DI5 primer site to the *Pst*I restriction site (Fig. 2). Eight of these clones had cytosine residues at positions 290 and 293, which indicates that they originate from template II. Seven out of these eight clones carry a thymidine residue at position 249 instead of the expected adenosine. This confirms the results of the direct sequencing and shows that *Taq* polymerase can insert adenosines when it reaches the end of the template DNA strand. In addition to these substitutions, the polymerase chain reaction products display four substitutions where 2 cytosine, 1 adenosine, and 1 thymidine residues are gained in the 2106 bp sequenced. This reflects the normal error frequency that we and others (12, 13) observe when polymerase chain reaction products are cloned.

*DNA Damage Induces Jumping*—In order to determine whether random damage to the template DNA could induce jumping polymerase chain reaction, aliquots of the two unrestricted template DNA preparations were subjected to sonication, depurination by acid, and UV irradiation, respectively. In all cases, the resulting templates were able to generate a recombinant polymerase chain reaction product (Fig. 1, *bottom left*) that upon direct sequencing proved to contain the expected sequence.[2]

The products were cloned, and a total of 27 clones were sequenced (Fig. 2). The majority of these clones carry C at positions 290 and 293. This is expected, since the probability that a particular sequence would be originating from template II should increase with its proximity to primer B if the position within exon 2 at which the jumping between templates occurs is random. In one case (clone jU26), the jumping seems to have occurred between positions 290 and 293. Also, the number of substitutions seems to be elevated in intron 2. However, no net increase of adenosine or thymidine residues can be seen.

*Jumping PCR Promoted by Ancient DNA*—To elucidate whether the jumping phenomenon occurs in amplifications from ancient DNA, we amplified a region of the mitochondrial control region from the DNA extracted from the 4000-year-old mummified liver of an Egyptian priest. This DNA segment had previously been amplified and directly sequenced from this individual and shown to differ at two positions from a published human mitochondrial sequence (6). When the amplification product was cloned and four clones were sequenced (Fig. 3), one proved to be identical with the directly determined sequence. The other three clones displayed the same two differences from the reference sequence but in addition carried substitutions where cytidine residues had been replaced by thymidine residues in six cases and a guanosine residue had been replaced by an adenosine residue in one case. This distribution of changes is consistent with the nucleotide composition of this DNA segment as well as with the observation that in this as well as other archaeological DNA samples pyrimidines are predominantly modified or missing (6). At these sites, adenosines may have been inserted on the opposite strand by two different mechanisms: (*a*) during polymerization without jumping and (*b*) by eliciting jumping. In both cases, only the changes occurring opposite cytidine residues would lead to incorrect nucleotides being incorporated. Thus, all of the substitutions detected would be

[1] The abbreviation used is: bp, base pair(s).

[2] Also, high template DNA concentrations (in the order of 1 $\mu$M) may cause high frequencies of jumping in a system similar to the one used here (M. Dutreix, unpublished observation).

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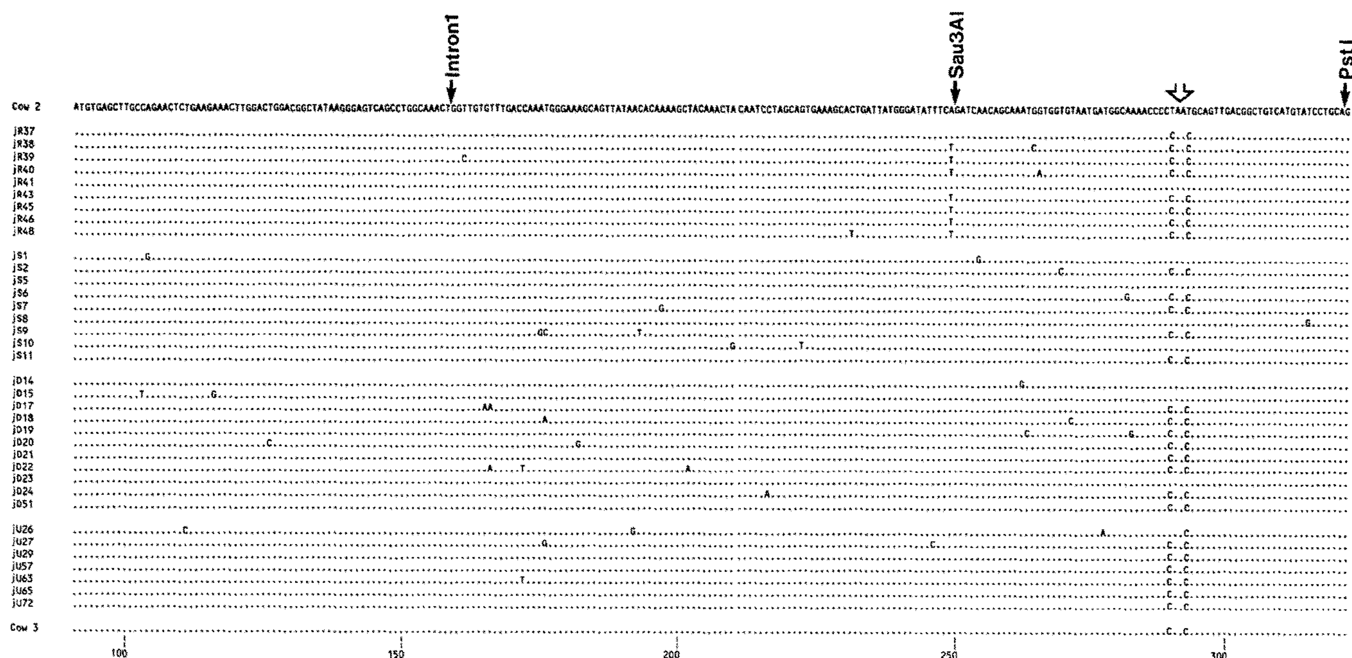


FIG. 2. **DNA sequences of cloned amplification products resulting from the jumping polymerase chain reaction.** The templates were the cow lysozyme 2 cDNA and lysozyme 3 genomic clones in Fig. 1 which had been restricted (*jR*), sonicated (*jS*), depurinated (*jD*), and UV-irradiated (*jU*), respectively. *Arrows* point to the positions at which indicated restriction enzymes cleave the DNA strand shown and where intron 1 exists in the genomic clone. The *open arrow* points to positions 290 and 293.

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Reference  ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAA
Direct Seq .............T.........................C.....
Clone 1    .............T.........................C.....
Clone 2    ....T.......TT....T...T................C.....
Clone 3    ...A.........T.........................C.....
Clone 4    .............T.T..T....................C.....
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FIG. 3. **DNA sequences of a 45-bp segment of the mitochondrial control region from a 4000-year-old Egyptian mummy.** The published human sequence (above; Ref. 22) is compared to the directly sequenced amplification product (identical with results in Ref. 6) and four clones isolated from the product of the amplification reaction.

transitions at positions occupied by guanosine and cytidine residues on the strand whose sequence appears in Fig. 3.

The fact that some clones contain numerous adenosine/thymidine substitutions in conjunction with the fact that "jumping" occurring at modified thymidines is expected to predominate indicates that the frequency of modified bases and strand breaks in the ancient DNA is extremely high, causing several "jumping events" or misincorporations to occur per molecule amplified. Since pyrimidines are predominantly damaged in ancient DNA (6) and since adenosines inserted at sites opposite modified thymidine residues will not cause substitutions, a quantitative estimate of the frequency of damage can be made for cytosine residues only. For these residues, the sequences in Fig. 3 indicate that a minimum of 1 residue in 11 (7 cytosine residues out of 76 sequenced) causes the misincorporation of an adenosine and thus is damaged or absent from the template DNA.

*An Assay for DNA Damage*—To demonstrate directly that jumping polymerase chain reaction occurs when amplification is performed from ancient DNA, a copy of the mitochondrial sequence cloned in M13 was used together with approximately 1 $\mu$g of the 4000-year-old DNA in a "jumping polymerase chain reaction assay" (Fig. 4). The primers for amplification were the M13 universal primer (*A*) and a primer (*B*) located in the mitochondrial control region outside the region cloned. Thus, any generation of a product by these primers must be due to an intermolecular amplification initiated by recombination between the mitochondrial segment present in the clone and corresponding sequences prepared from the ancient Egyptian individual. As a control, two additional primers (*C* and *D*) specific for a segment of the mitochondrial cytochrome *b* gene were added to the reaction. They are expected to give rise to a conventional, intramolecular amplification product of 97 bp and serve as an internal control for the amounts of noncloned template DNA added as well as for the overall efficiency of the reaction. Similar reactions were set up with total DNA prepared from contemporary autopsy material. The intramolecular amplification proved to be dramatically more efficient than the intermolecular amplification. In order to achieve comparable intensities of the two products, it was necessary to perform 15 cycles of polymerase chain reaction with primers intended only for the jumping polymerase chain reaction and then add the primers for the intramolecular amplification prior to performing 25 additional cycles. As can be seen in Fig. 4, the relative amount of the intermolecular polymerase chain reaction is appreciably greater in amplifications performed from the ancient DNA extract than from the contemporary DNA preparation. This demonstrates the existence of many more lesions inducing the partially extended amplification products to jump from one template molecule to another in the ancient DNA than in the modern DNA.

## DISCUSSION

Others have shown that DNA fragments can be fused for construction purposes (14) by the approach that we designate as the jumping polymerase chain reaction, that "shuffle clones" are sometimes detected when amplification products are cloned (15), and that *Taq* polymerase shares with other prokaryotic and eukaryotic DNA polymerases the propensity for inserting adenosines (16) when no template base is present. Furthermore, the lack of any 3′-5′ exonuclease activity in *Taq* polymerase (17) ensures that the additional base is not

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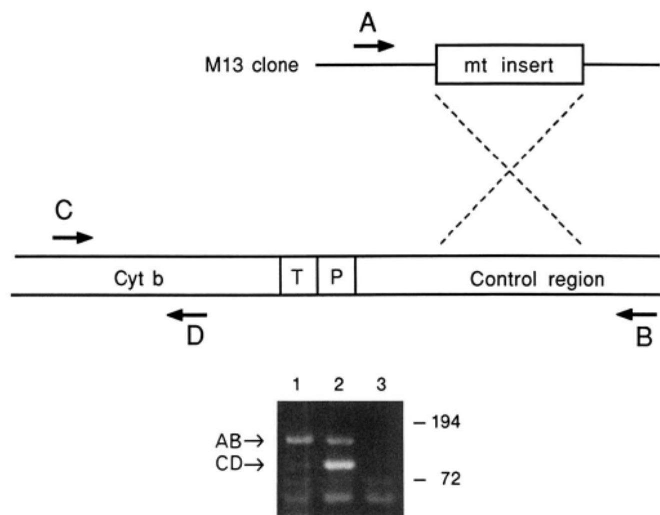


FIG. 4. **Demonstration of damage in ancient samples of DNA by the jumping polymerase chain reaction.** *Above*, a schematic illustration showing the cloned mitochondrial sequence; the universal M13 primer (*primer A*) and the mitochondrial *primer B* (L16175) used for the intermolecular amplification; *primers C* (L14841) and *D* (H14876) used as an internal control; the mitochondrial DNA analyzed for lesions by jumping polymerase chain reaction where the control region and the genes for $tRNA^{Thr}$, $tRNA^{Pro}$, and cytochrome *b* are indicated. *Below*, agarose gel electrophoresis of amplification reactions from the 4000-year-old liver DNA (*1*), total liver DNA from contemporary autopsy material (*2*), and no uncloned template DNA (*3*). *AB* denotes the 160-bp intermolecular amplification products, and *CD* the 97-bp intramolecular product. The identities of the former products were confirmed by direct sequencing. The migration positions of molecular size markers are indicated in numbers of base pairs.

removed after its incorporation into the nascent strand. However, our observation that no addition of adenosines occurs at the *Pst*I site indicates that this phenomenon is dependent not only on properties of *Taq* polymerase but also on the sequence context and underscores the need to sequence DNA constructs generated by polymerase chain reaction-induced joining of molecules.

From the experiments described above, it is clear that if amplifications are initiated from single or very few template molecules, any damage inducing the jumping polymerase chain reaction may be reflected in a large fraction or even all of the molecules making up the final amplification product. Furthermore, if site-specific breaks (*e.g.* induced by the HO endonuclease in the yeast *MAT* locus; Ref. 18) or nicks (*e.g.* induced by topoisomerase I; Ref. 19) occur in the DNA prepared from an organism, almost all molecules in the amplification product may be of incorrect sequences. However, if the damage in the template DNA is randomly distributed but so extensive that few or no intact molecules containing both primer sites exist, the jumping polymerase chain reaction will allow amplifications of longer DNA segments than are actually present in the sample. This is because the primers during the first cycles of amplification are extended on different templates until one or both of them reach the reciprocal priming site. After this initial lag phase, the length of which is proportional to the amount of damage present, a molecule containing both primer sites has been created and an exponential amplification will ensue (20). However, a substantial part of the product molecules will be rearranged and contain inserted adenosines. In fact, even the majority of the molecules may carry such abnormalities (Fig. 3), but when the product is directly sequenced, only a consensus sequence reflecting the unperturbed template sequence will be scored (21).

A serious concern is that if the amplification product is not sequenced but rather typed for particular alleles with allele-specific oligonucleotides, *in vitro* recombination phenomena may go undetected. In fact, new specificities may be created by the jumping polymerase chain reaction combining DNA segments from different alleles or loci. In particular, this may be the case when amplifications are performed from nuclear genes in heterozygous individuals, from genes belonging to multigene families such as the major histocompatibility complex, or from extracts that contain DNA from more than one individual or species.

The experimental design illustrated in Fig. 4 can be used to assess the amount of damage present in a particular DNA segment. The lesions can in many cases be determined to the exact base at which they occur by the cloning and sequencing of the product; it is to be expected that adenosine or thymidine residues should be present at sites of damage (*cf.* Fig. 3). However, these applications of the jumping polymerase chain reaction require further investigation into the molecular nature of the DNA modifications that cause *Taq* polymerase to stall or fall off its template. Also, finding conditions that promote stalling or falling off the template may improve the sensitivity of this assay system.

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