

Effect of highly fragmented DNA on PCR

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ABSTRACT

We characterized the behavior of polymerase chain reactions (PCR) using degraded DNA as a template. We first demonstrated that fragments larger than the initial template fragments can be amplified if overlapping fragments are allowed to anneal and extend prior to routine PCR. Amplification products increase when degraded genomic DNA is pretreated by polymerization in the absence of specific primers. Secondly, we measured nucleotide uptake as a function of template DNA degradation. dNTP incorporation initially increases with increasing DNA fragmentation and then declines when the DNA becomes highly degraded. We demonstrated that dNTP uptake continues for >10 polymerization cycles and is affected by the quality and quantity of template DNA and by the amount of substrate dNTP. These results suggest that although reconstruction of degraded DNA may allow amplification of large fragments, reconstructive polymerization and amplification polymerization may compete. This was confirmed in PCR where the addition of degraded DNA reduced the resultant product. Because terminal deoxynucleotidyl transferase activity of Taq polymerase may inhibit 3' annealing and restrict the length of template reconstruction, we suggest modified PCR techniques which separate reconstructive and amplification polymerization reactions.

INTRODUCTION

The primary concern of the forensic scientist using the polymerase chain reaction (PCR) on DNA extracted from forensic, ancient or preserved samples is to verify the authenticity of the DNA and the information it contains. Additional concerns include the likelihood that tissue samples, of different ages and conditions of preservation, will give positive results and whether the information obtained is complete.

DNA derived from aged or degraded tissue is often highly fragmented due to autolysis, bacterial degradation and background spontaneous depurination (1–3). This fragmentation severely reduces the efficiency of the PCR, although different reasons for this reduction are given (4,5). Indeed, both the expectations that aged DNA will be degraded and that degraded DNA will be recalcitrant to PCR analysis are so well accepted that the behavior of the PCR itself has been used as a criterion for authenticity of the template DNA (5–9). In particular, it is

expected that: (i) PCR of ancient or degraded DNA should only amplify small fragments because the template DNA itself is comprised only of small fragments; (ii) the amount of amplified product should be small compared with similar reactions with modern DNA. The implication is that if large products (>400 bp) are generated then the template DNA is at least partially contaminated.

In this paper, we will demonstrate that: (i) DNA fragments that are larger than the initial template DNA may result from authentic amplification; (ii) DNA polymerization on self-primed DNA fragments can increase the likelihood of successful amplification ostensibly by reconstruction of the template; (iii) that such background template reconstruction can deplete available dNTPs and act as a competitor for the reaction substrate itself. In the light of our results, we offer considerations both for amplification conditions and for critical review of PCR when working with highly fragmented DNA. These considerations may be useful in the development of protocols for reliable retrieval of high molecular weight genetic markers in forensic testing of low molecular weight DNA.

MATERIALS AND METHODS

Amplification of fragments larger than template DNA

We amplified fragments of the chloroplast gene *rbcl* using previously extracted genomic DNA from wild barley, *Hordeum spontaneum*. The paired primers used were Ltrbcl1 (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3') and Ltrbcl724R (5'-CTT CGC ATG TAC CTG CAG TAG C-3') and Ltrbcl 209 (5'-GGA CCG ATG GAC TTA CCA GCC TTG ATC G-3') and Ltrbcl 1366R (5'-CCT TCC ATA CCT CAC AAG CAG CAG C-3') (10). The targeted fragments were thus 751 (Fragment A) and 1184 (Fragment B) bp in length. (Refer to Fig. 1 for relative sizes and overlap of the fragments.) The 25 µl reactions consisted of 10.375 µl H₂O, 5 µl 5× buffer (2.5 mg/ml BSA, 5 mM MgCl₂, 250 mM Tris-HCl, pH 8.5), 2.5 µl 2.0 mM each dNTP (Promega), 2.5 µl each 5 µM forward and reverse primers, 1.0 µl 25 mM MgCl₂, 1.0 µl template DNA (~50 ng), 0.125 µl Taq polymerase (5 U/µl) (Promega). The reactions were loaded in glass capillary tubes and run on an Idaho Technologies Air Thermal Cycler, with a 3 min 94°C presoak then 30 cycles of 94°C for 8 s, 55°C for 8 s and 72°C for 35 s. The capillary tubes were inserted into the machine only after the chamber temperature was >80°C, to avoid false priming and extension. The products were separated by electrophoresis on a 1× TAE gel of 0.7% low melt agarose (Fisher) and 0.65% Synergel

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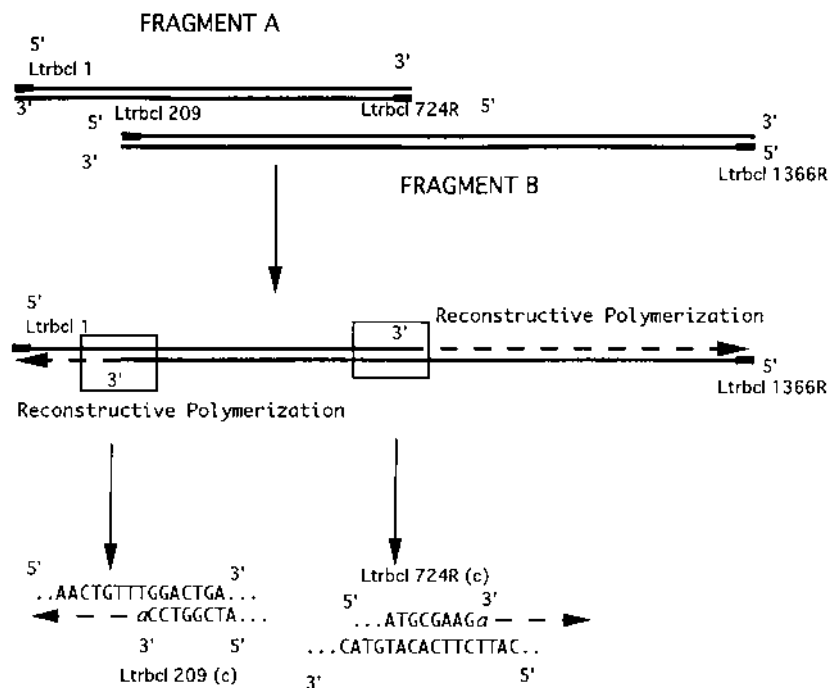


Figure 1. Diagram of relative sizes and regions of overlap of Fragments A and B from the first experiment. The diagram illustrates the hypothesized overlap and 3' annealing of the complementary strands of the two fragments after denaturation and re-annealing. Subsequent reconstructive polymerization is symbolized by the dashed line. The regions of 3' annealing are magnified at the bottom of the diagram. An adenosine is added at the 3'-ends, as would be expected for terminal deoxynucleotidyl transferase activity, and is symbolized by a lower case italicized *a*. In the case of the gene used, this additional base is complemented by a thymine on the opposite strand. It is hypothesized that in sequences where such added adenines are not complemented, 3' extension may be limited or prevented entirely unless the unannealed bases are removed by 3'→5' exonuclease activity.

(Diversified Biotech). The gel was stained with ethidium bromide and the bands excised from the gel with clean, new razor blades. The DNA was extracted into 50 μ l 1 \times TE buffer using the Wizard PCR Resin kit following the manufacturer's protocol (Promega).

The gel-purified PCR Fragments A and B were used as template DNA in subsequent PCR experiments. We ran a polymerase extension pretreatment of the template DNA by setting up 25 μ l reactions similar to those above except that no primers were added and 4 μ l of the gel-purified PCR products were added (either 4 μ l each fragment in separate reactions or 2 μ l each fragment together). The reaction mixtures were exposed to UV light for 45 min in a Stratalinker (Stratagene) prior to the addition of DNA and enzyme, to control against contaminating double-stranded DNA. A negative control reaction without DNA was included. The reactions were run with a 3 min 94°C presoak, followed by 20 cycles of 94°C for 8 s, 55°C for 8 s and 72°C for 20 s. The pretreatment reaction products were diluted 1:9 for use in further amplifications.

Eight test amplifications were set up using primers Ltrbcl1 and Ltrbcl1366R, which are expected to produce a 1393 bp fragment. The 25 μ l reactions were made up as above including the UV light treatment, but with different combinations of pretreated and untreated Fragments A and B as template. The gel-purified PCR products were diluted 1:49. We set up reactions using as template Fragment A untreated, Fragment A pretreated, Fragment B untreated, Fragment B pretreated, Fragments A and B together untreated, Fragments A and B pretreated, TE without DNA (negative control) and *H.spontaneum* DNA (positive control). The reactions were loaded in glass capillary tubes and run on an Idaho Technologies Air Thermal Cycler with a 3 min 94°C

presoak, then 30 cycles of 94°C for 8 s, 55°C for 8 s and 72°C for 35 s. The products were separated by electrophoresis on a 1 \times TAE gel of 0.7% low EEO agarose (Fisher) and 0.65% Synergel.

Reconstructive polymerization on genomic DNA and PCR amplification

To test whether polymerase extension pretreatment is effective on degraded genomic DNA, we artificially degraded high molecular weight DNA by digestion with 0.2 U DNase I (Sigma) incubated at 37°C in the presence of Mg²⁺ ions for 1 min. The reaction was stopped by the addition of 5 μ l 0.5 M EDTA and the DNase I was denatured by a 10 min incubation at 94°C. The DNA was extracted by a standard phenol/chloroform procedure (11). A 50 ng aliquot of the degraded calf thymus DNA was then pretreated by a Taq polymerase cycled extension. The reaction conditions included 0.2 mM dNTP solution, 2 mM MgCl₂ and the manufacturer's (Gibco) supplied reaction buffer. The pretreatment reactions, including a negative control, were loaded in 25 μ l capillary tubes and run on an Idaho Technologies Air Thermal Cycler set for a 2 min denaturation at 94°C followed by 20 cycles of 94°C for 4 s, 50°C for 4 s and 72°C for 40 s. A negative control without DNA was run alongside. Test PCRs were set up using the mitochondrial cytochrome B primers L14724 (5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3') and H15149 (5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3') (12). The reaction cocktails consisted of 6.25 μ l H₂O, 2.5 μ l 10 \times buffer (Gibco BRL), 2.5 μ l 2.0 mM dNTP, 2.5 μ l 5 mg/ml BSA, 2.5 μ l each of 5 μ M primers, 1 μ l 50 mM MgCl₂ and 0.25 μ l Taq polymerase (5 U/ μ l) (Gibco). Five

microliter treatment aliquots were added to each reaction. The treatments were water (negative control), 10 ng uncut calf thymus DNA (positive control), 10 ng digested calf thymus DNA, 10 ng polymerase pretreated digested calf thymus DNA and a carry-through negative control from the pretreatment reaction. Five microliters of the products were separated by electrophoresis on a 1×TAE gel of 0.7% low EEO agarose and 0.65% Synergel and then later stained with ethidium bromide for viewing.

Limitation of dNTP incorporation in template DNA: number of PCR cycles

Replicate 25 µl reactions were set up containing 2.5 µl (500 pmol) of a dNTP solution containing 200 µM each of dGTP, dATP, TTP and dCTP, in addition to 62.5 pmol of [³²P]dCTP. Accordingly, the labeled dCTP was not a limiting reagent. Sonicated herring sperm DNA was the only template DNA source. Two separate experiments were run with 10 and 100 ng of the DNA used as template respectively. The reaction solutions were made in batch and then aliquoted into separate tubes to increase uniformity among samples. The reactions were incubated at 94°C for 2 min before adding the Taq polymerase and run in a Coy Tempcycler II for 20 cycles of 94°C for 40 s, 50°C for 40 s and 72°C for 90 s. Replicate reactions were removed from the thermal cycler after 5, 10, 15 and 20 cycles. The reactions were stopped by adding 1 µl 0.5 M EDTA to the reactions after removal from the thermal cycler. Reaction solutions were then passed through a 1 ml Sephadex G-50 column equilibrated with TE and the final eluants were adjusted to an equal final volume of 50 µl. Two 10 µl aliquots from each reaction were measured on a Packard scintillation counter.

Limitation of dNTP incorporation in template DNA: dNTP concentration

Replicate 25 µl reactions were set up varying the amount of dNTPs available for the reaction. Three separate concentrations of dNTP were made at 500 µM and 5 µM and 50 nM for each of dGTP, dATP, TTP and dCTP; 2.5 µl of these solutions were used in the 25 µl reactions, resulting in final concentrations of 50 µM and 500 nM and 5 nM respectively. In each reaction 62.5 pmol labeled dCTP were added (2.5 µM final concentration). Thus, if the labeled dCTP was incorporated without discrimination, one of 20 cytosines incorporated in the 50 µM dNTP reactions would be labeled, four of five cytosines incorporated would be labeled in the 500 nM dNTP reactions and 499 of 500 cytosines incorporated would be labeled in the 5 nM dNTP reactions. Ten nanograms of sonicated herring sperm DNA were used as template in each reaction. The remainder of the reaction conditions, including the thermal cycling settings, were identical to the previous experiment. Replicate reactions were removed from the thermal cycler after 5, 10, 15 and 20 cycles. The reactions were stopped by adding 1 µl 0.5 M EDTA to the reactions after removal from the thermal cycler. Reaction solutions were then passed through a 1 ml Sephadex G-50 column equilibrated with TE and the final eluants were adjusted to an equal final volume of 50 µl. Two 10 µl aliquots from each reaction were measured on a Packard scintillation counter.

Limitation of dNTP incorporation in template DNA: degree of template DNA degradation

To test the hypothesis that amount of incorporation of dNTPs in non-specific template DNA reconstruction will be a function of the degree of template degradation, we set up replicate template reconstruction reactions (PCR without primers) using progressively degraded calf thymus DNA samples. Calf thymus DNA was degraded by digesting 4 µg DNA in a 100 µl volume containing 0, 0.2, 1 or 2 U DNase I. The reaction conditions (1×) included 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ and 50 µg/ml BSA. The reactions were incubated at 37°C for 1 min and then stopped by the addition of 5 µl 0.5 M EDTA. The DNase I was denatured by a 10 min incubation at 94°C and extracted once with phenol/chloroform. Replicate 12.5 µl dNTP incorporation reactions were prepared with 4.825 µl H₂O, 1.25 µl (2 mM each of dATP, dGTP, TTP and dCTP) dNTP solution, 1.25 µl 10× reaction buffer (Promega), 1.0 µl 25 mM MgCl₂, 2.5 µl (62.5 pmol) [α -³²P]dCTP (800 Ci/mmol, 10 mCi/ml), 0.125 µl Taq polymerase (5 U/µl) and 0.5 µl template. The template was varied for five treatment conditions: no DNA (negative control), 10 ng undigested DNA, 10 ng DNA digested with 0.2 U DNase I, 10 ng DNA digested with 1 U DNase I and 10 ng DNA digested with 2 U DNase I. Two replicate reactions were prepared for each treatment. The reactions were prepared in batch and aliquoted to separate tubes to ensure uniformity. The reactions were run on a Perkin Elmer GeneAmp PCR System 2400 with an initial 2 min denaturation at 94°C followed by 10 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 90 s. Upon completion of the 10 cycles, we placed the tubes immediately on ice and added 1 µl 0.5 M EDTA to stop the reaction. To measure the relative incorporation of labeled dCTP, 2 µl were sampled from each tube and mixed with 2 µl 0.5 M EDTA and 46 µl herring sperm DNA (100 ng/µl). Ten microliters were taken from each dilution and spotted on a glass filter. The filter was washed three times on ice for 5 min in 10% (w/v) TCA, 1% (w/v) sodium pyrophosphate. The filters were then washed for 5 min in 95% ethanol and air dried. The filters were then measured on a Packard scintillation counter. Two measurements were taken from each reaction tube.

Competitive effects of reconstructive polymerization on PCR efficiency

To assess whether polymerase-mediated template reconstruction *per se* interferes with specific PCR amplification by competition for dNTP and enzyme, we effectively separated the two reactions in a single tube by using calf thymus DNA as the intended reconstruction template DNA and barley DNA as the intended template for amplification of the chloroplast gene *rbcL*. The calf thymus DNA does not have a homologous gene to *rbcL* and therefore will not compete for *rbcL* primers. As treatments, we varied the template DNA in six PCR reactions as follows: no DNA (negative control), 10 ng high molecular weight calf thymus DNA, 10 ng calf thymus DNA digested with 0.2 U DNase I as above, 50 ng barley DNA, 50 ng barley DNA plus 10 ng undigested calf thymus DNA and 50 ng barley DNA plus 10 ng digested calf thymus DNA. The 25 µl reactions consisted of 8.825 µl H₂O, 2.5 µl (2 mM each of dATP, dGTP, TTP and dCTP) dNTP solution, 2.5 µl 10× reaction buffer (Promega), 2.0 µl 25 mM MgCl₂, 2.5 µl 5 mg/ml BSA, 2.5 µl primer LtrbcL1 (5 µM), 2.5 µl primer LtrbcL1201R (5 µM) (5'-CCT AAA GTT CCT CCA CCG AAC TG-3') (10), 0.125 µl Taq polymerase

(5 U/μl) and template DNA. The reactions were loaded in 25 μl capillary tubes and run on an Idaho Technologies Air Thermal Cycler set for a 2 min denaturation at 94°C followed by 30 cycles of 94°C for 4 s, 50°C for 4 s and 72°C for 40 s. Five microliters of the reactions were then characterized by separation on a 0.7% agarose, 0.65% Synergel TBE gel and stained with ethidium bromide.

RESULTS

Amplification of fragments larger than template DNA

Initial PCR amplifications from the *H.spontaneum* DNA give strong single bands of 751 bp (Fragment A) and 1184 bp (Fragment B) as expected. The bands were excised from the gel and the DNA extracted using Wizard PCR preps (Fig. 2, lanes 9 and 10). Three pretreatment reactions were set up using Fragments A and B in separate reactions and both together in a third reaction without primers. No visible bands were apparent when aliquots from these reactions were separated by electrophoresis (data not shown). Aliquots from these reactions along with comparable untreated template DNAs were then run in PCRs with primers Ltrbc11 and Ltrbc1366R, which generate a 1393 bp fragment. Reactions using Fragment A with or without pretreatment produced no visible products (Fig. 2, lanes 1 and 2). Fragment B alone with or without pretreatment (lanes 3 and 4) and Fragments A and B together without pretreatment (lane 5) gave products with a faster mobility than the double-stranded 1184 bp fragment (B) (lane 10). We interpret these products as being single-stranded DNA produced by extension of primer Ltrbc1366R using Fragment B as a template. Pretreated Fragments A and B together produced a strong band (lane 6) of equal mobility as the positive control (lane 8). The negative control (lane 7) did not produce any visible bands. The lack of the 1393 bp band in any other of the pretreated samples (Fragments A and B alone) indicates that neither the extra cycles themselves nor any systematic contamination contributes to amplifying the PCR product. Because only the template DNAs vary in either their composition or treatment and because only the pretreated combined Fragments A and B acted as sufficient template, it is apparent that pretreatment of the two fragments allows for the construction of a single larger piece by complementation and extension.

Reconstructive polymerization on genomic DNA and PCR amplification

Based on the previous results that indicated that DNA polymerization primed by overlapping fragments can reconstruct template DNA, we tested whether such reconstruction can occur using degraded genomic DNA. Calf thymus DNA was digested with DNase I to simulate degradation of DNA. The single-stranded nicks and digestion produced by DNase I in the presence of Mg²⁺ would be comparable with single-stranded breaks occurring following depurination. Using the approach used in the first experiment, we pretreated an aliquot of the digested DNA by using the DNA in 20 cycles of Taq polymerase-mediated polymerization in the absence of specific primers. This pretreated DNA then served as a template in a specific PCR. In comparison, uncut calf thymus DNA and digested but untreated calf thymus DNA were also used as target templates. The reaction using the uncut DNA as a template produced a high concentration of the 459 bp fragment (Fig. 3, lane 2). The digested DNA acted as a

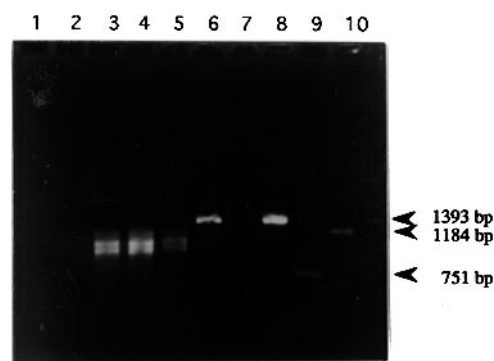


Figure 2. TAE agarose gel of PCR products and controls. Gel-purified Fragments A (*rbcL* 1–724R) and B (*rbcL* 209–1366) were pretreated (individually and mixed together) by performing 20 cycles of polymerization in the absence of primers. Pretreated and untreated fragments were then used as template in reactions containing primers Ltrbc11 and Ltrbc1366R. An aliquot from each of the primer-containing reactions was analyzed on a 0.7% agarose, 0.65% Synergel TAE agarose gel: lane 1, Fragment A untreated; lane 2, Fragment A pretreated; lane 3, Fragment B untreated; lane 4, Fragment B pretreated; lane 5, Fragments A and B untreated; lane 6, Fragments A and B pretreated. Lane 7 contains the ‘no template DNA’ negative control. Lane 8 contains *H.spontaneum* genomic DNA amplified with primers Ltrbc11 and Ltrbc1366R, resulting in a 1393 bp fragment. Lanes 9 and 10 contain the gel-purified Fragments A (751 bp) and B (1184 bp) respectively. Lanes 8–10 serve as size standards as the sequences of these fragments are known.

weak target template (lane 3). The expected product was produced in much lower concentrations, consistent with the expectation that some residual high molecular weight DNA is present after digestion with DNase I. In contrast, however, the same degraded template DNA that was pretreated by reconstructive polymerization produced a much higher concentration of specific PCR products (lane 4). Negative controls that were run in lanes 1 and 5 showed no products. Thus, the reconstructive polymerization found with specific DNA fragments in the first experiment apparently also occurs with random genomic fragments and can improve the success of PCR using degraded template DNA.

Limitation of dNTP incorporation in template DNA: number of PCR cycles

Genomic extension or reconstructive polymerization is not expected to follow the exponential product increase as found in PCR as products will not generate copies of themselves. Therefore, reconstructive polymerization may occur only during the first few cycles of a PCR. Alternatively, complete reconstruction may require many cycles to be complete and thus may compete with the intended amplification reaction for enzymes and dNTPs. Additionally, if dNTP incorporation is a function of the degree of fragmentation and amount of DNA available, differing amounts of DNA may have differing dNTP incorporation curves.

To monitor the time component of uptake, replicate reactions containing 50 μM of each dNTP and using sonicated herring sperm DNA as template were sampled after 5, 10, 15 and 20 cycles. Polymerization was measured as a function of uptake of labeled dCTP by scintillation counting. The results are summarized in Table 1 and plotted in Figure 4 as pmol incorporation. The scintillation counts were adjusted by the

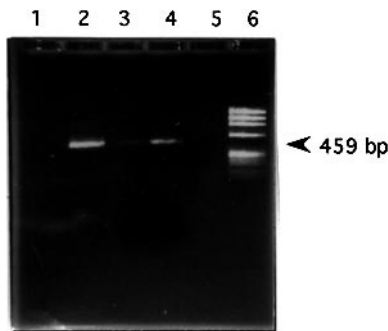


Figure 3. TBE agarose gel of PCR products and controls when highly polymerized, degraded or reconstructed genomic DNA serves as template. Calf thymus DNA was degraded by digestion with DNase I and then pretreated by performing 20 cycles of polymerization in the absence of primers. Undigested, digested and pretreated digested calf thymus DNA were templates in PCR containing mitochondrial cytochrome B primers L14724 and H15149, which are expected to define a 459 bp fragment. An aliquot from each PCR was analyzed on a 0.7% agarose, 0.65% Synergel TBE gel: lane 1, 'no template DNA' negative control; lane 2, undigested calf thymus DNA template; lane 3, digested calf thymus DNA template; lane 4, pretreated digested calf thymus DNA template; lane 5, 'pretreated no template DNA' negative control; lane 6, *Hae*III-digested ϕ X174 size standard.

half-life factor of the label to facilitate comparison. Both the amount of DNA in the reaction and the number of cycles had significant effects on incorporation of labeled dCTP at the $P < 0.001$ level. The interaction between cycles and initial amounts of DNA was not significant, indicating that both initial amounts of DNA responded similarly. Surprisingly, the lower amount of template DNA generally gave higher incorporation readings, although the reasons for this are not clear. It should be noted, though, that comparisons of the 95% confidence limits for the two DNA concentrations at each cycle overlap, which would imply lack of significant differences. In both experiments, an increase in incorporated labeled dCTP continued through cycle 15. Also, in all reactions there was a decrease in incorporated label in cycle 20.

Table 1. ANOVA table of incorporation of labeled dCTP as a function of polymerization cycles and amount of template DNA

Source of variation	df	MS	F_s
Template DNA	1	81024.9	52.11***
Cycles	3	34816.4	22.39***
Template DNA \times cycles	3	2496.5	1.61 (NS)
Error	8	1554.8	

$F_{0.001}[1,8] = 24.5$, $F_{0.001}[3,8] = 15.8$, $F_{0.05}[3,8] = 4.07$.
Scintillation counts were square root transformed.

Limitation of dNTP incorporation in template DNA: dNTP concentration

To test for differential dNTP incorporation into fragmented double-stranded DNA in a PCR, we varied the concentration of dNTPs while keeping the amount of double-stranded template constant at 10 ng. There were significant differences between tubes within treatments, indicating substantial inter-well variation in the temperature cyler (Table 2). This factor, though, contributed only 3% to the total variance. In this experiment, the interaction term, the

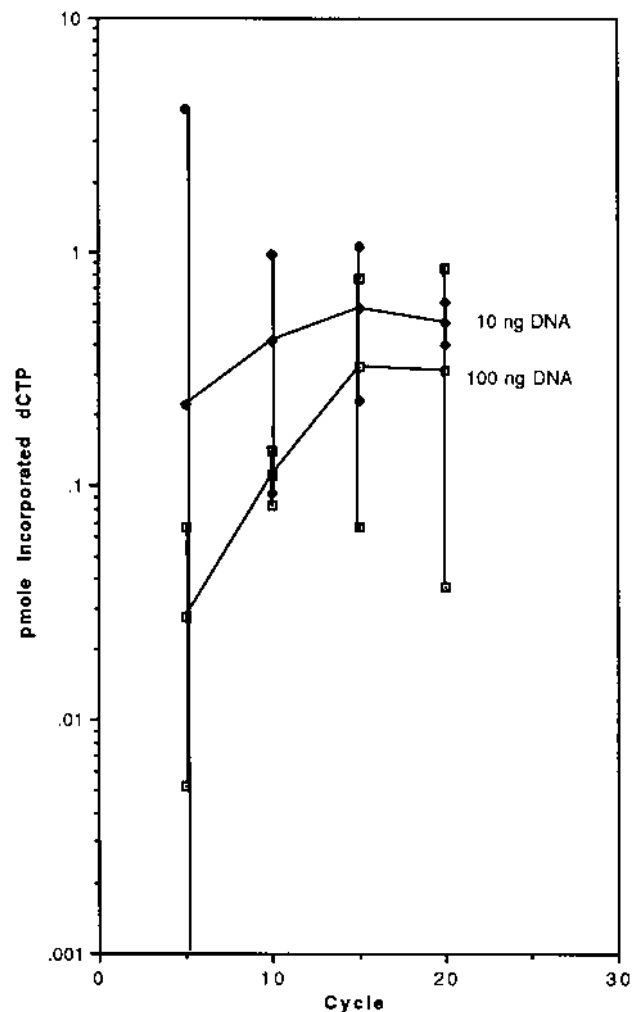


Figure 4. Plot of dNTP incorporation measured in pmoles as a function of number of polymerization cycles. Two initial concentrations of sonicated herring sperm DNA (100 ng and 10 ng) were used. Data are converted from back-transformed means and 95% confidence limits (vertical bars) of square root transformed counts (d.p.m.).

effect of cycles \times amount of dNTPs, was significant at the $P < 0.01$ level and is indicative of a differential incorporation change with cycles at the different dNTP levels. This effect can be seen clearly when incorporation is plotted against cycles for the different dNTP treatments (Fig. 5) and is most likely due to exhaustion of the dNTPs when in lower concentrations.

Under ideal conditions, the amount of amplified product A in a PCR will be expected to accumulate according to

$$\log(A) = \log(1 + E) \times n + \log(A_0),$$

where E is the efficiency of the reaction, n is the number of cycles and A_0 is the initial amount of DNA. In actuality, accumulation does not increase indefinitely with cycles, but approaches a plateau which will be seen as a change in E . Assuming that the amounts of initial DNA in the reactions, A_0 , are identical, differences in log c.p.m., $\log(A)$, will be directly proportional to the differences in $\log(1 + E)$,

$$\log(A_1/A_2) = n \times \log(1 + E_1/1 + E_2)$$

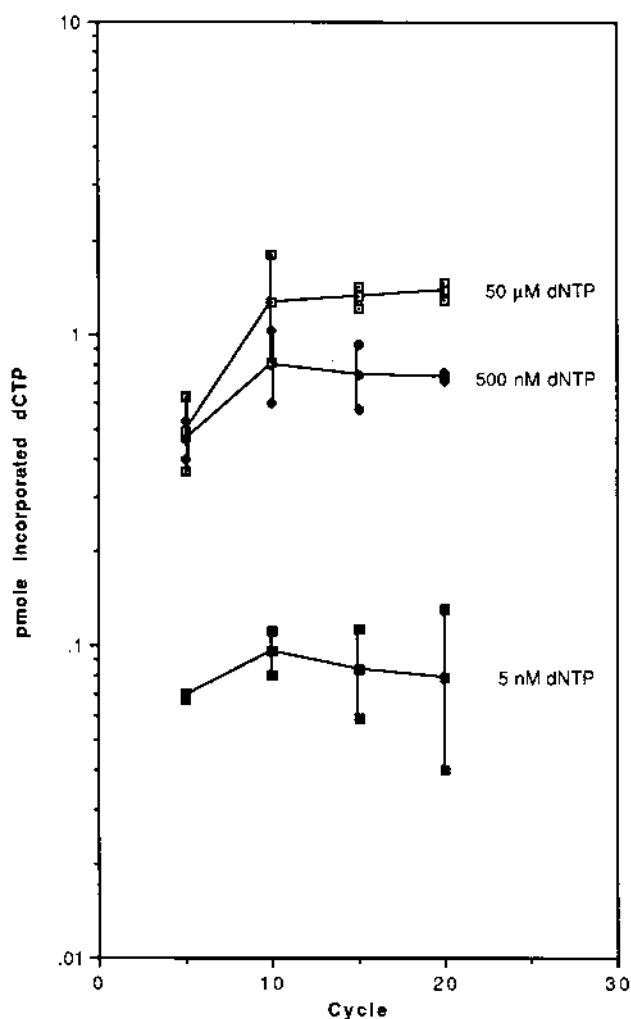


Figure 5. Plot of dNTP incorporation measured in pmoles for varying concentrations of dNTP as a function of number of polymerization cycles. Three initial concentrations of dNTP (50 μ M, 500 nM and 5 nM) were used. Data are converted from back-transformed means and 95% confidence limits (vertical bars) of square root transformed counts (d.p.m.).

and this difference in efficiency can be directly related to exhaustion of dNTP in our experiment. Therefore, unplanned comparison of transformed counts using the T method (13) will indicate significant changes in efficiency, E . It should be noted that equivalent efficiencies will occur either when the amplification is occurring at the same rate or if there is no further amplification because the substrates are exhausted. All incorporation values in the 5 nM dNTP experiment do not differ significantly, indicating that the dNTP substrate was exhausted within the first five cycles. Incorporation in the 50 μ M and 500 nM dNTP experiments were not significantly different after five cycles, but did differ significantly from all incorporation in the 5 nM dNTP experiment. Subsequent counts at 10, 15 and 20 cycles in the 500 nM dNTP experiment did not differ, indicating an exhaustion of dNTP before the tenth cycle. The 50 μ M dNTP experiment indicated a significant increase in incorporation through the tenth cycle, but no further significant increases, indicating that the substrate dNTP was exhausted before the fifteenth cycle.

It should be further noted that available Mg^{2+} may vary both among treatments and within treatments over time as dNTPs may

bind $MgCl_2$. This effective difference in $MgCl_2$ may affect Taq DNA polymerase activity. Given, however, the non-significant difference in dNTP incorporation between the reactions with 50 μ M and 500 nM initial dNTP concentrations, it is not apparent that the $MgCl_2$ concentration is influencing polymerization in these ranges. Also, while the low incorporation that occurred in reactions with 5 nM initial dNTP concentration or in the later cycles of the 500 nM dNTP reactions could be due to the higher available $MgCl_2$ concentrations or an interaction with available dNTP, our interpretation that the dNTP concentration is the predominant affect is supported in the following experiments, where $MgCl_2$ concentration is held constant among the treatments.

Table 2. ANOVA table of incorporation of labeled dCTP as a function of polymerization cycles and concentration of substrate dNTP

Source of variation	df	MS	F_s
Cycles	3	53036.2	16.4***
Concentration of dNTP	2	843889.4	261.4***
Cycles \times concentration	6	16194.6	5.0**
Tubes within groups	12	3228.3	33.3***
Error	24	97.0	

$F_{0.001}[12,24] = 4.39$, $F_{0.01}[6,12] = 4.82$, $F_{0.001}[2,12] = 13.0$, $F_{0.001}[3,12] = 10.8$. Scintillation counts were square root transformed.

Limitation of dNTP incorporation in template DNA: degree of template DNA degradation

Based on our demonstration of reconstructive polymerization, it is expected that dNTP incorporation will initially increase as overlapping DNA fragments become more degraded and then decrease as the remaining DNA fragments become too small to re-anneal or overlapping fragments no longer exist. To test this hypothesis, we artificially degraded calf thymus DNA by digesting with 0, 0.2, 1 and 2 U DNase I in the presence of Mg^{2+} cation for 1 min at 37°C. When Mg^{2+} is present, DNase I produces single-stranded rather than double-stranded nicks in the DNA (14). We confirmed a monotonic decrease in average DNA fragment size with increased DNase I by inspection of the DNA after electrophoresis on an agarose gel (data not shown). The four treatments of DNA served as templates in template reconstruction reactions, with the amount of reconstruction being measured by uptake of labeled dCTP. The treatment effects are significant at $P < 0.01$ (Table 3). Further *a posteriori* testing using LSD multiple range tests indicate that the incorporation of labeled dCTP in the non-degraded DNA did not differ significantly from that of the negative control. All the reactions with degraded DNA had significantly greater incorporation than the negative control. Additionally, the reactions with DNA having intermediate levels of degradation, i.e. templates treated with 0.2 and 1 U DNase I, had significantly greater incorporation than did the reaction with the non-degraded DNA. However, incorporation decreased in the reaction with the most digested template DNA and was not significantly different from incorporation within the undigested DNA. The means of label incorporation and the homogeneous subsets are displayed in Figure 6. The results demonstrate that template extension as indicated by dCTP incorporation first increases with greater template fragmentation and then decreases as the template DNA becomes highly degraded.

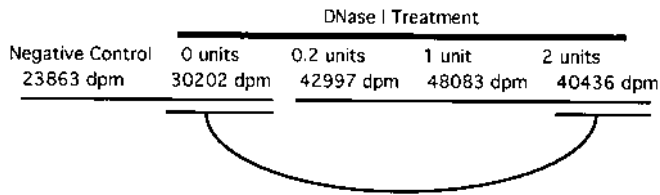


Figure 6. Diagram of homogeneous subsets of mean incorporation (scintillation counts) by DNase I treatment of template DNA. Mean d.p.m. values are listed under each treatment condition. The negative control contained no DNA, while the remaining reactions contained 10 ng calf thymus DNA that had previously been digested with 0, 0.2, 1 or 2 U DNase I. The lines under the mean d.p.m. values indicate homogeneous sets.

Table 3. ANOVA table of incorporation of labeled dCTP as a function of degree of template DNA degradation

Source of variation	df	MS	F_s
DNase treatments	4	2879.94	7.88**
Replicates within treatments	5	307.84	0.78 (NS)
Error	10	394.36	

$F_{0.05}[5,10] = 3.33$, $F_{0.01}[4,15] = 4.89$.

Scintillation counts were square root transformed.

Competition of template reconstruction and primer specific amplification

The previous results indicate that PCRs which use degraded DNA as a template may have reduced efficiency not only because a suitable target template DNA may be limiting but also because reconstructive polymerization may be competing for dNTP and enzyme. To separate the two effects (limited template and reaction competition), we set up reactions in which the PCR primers were specific to one DNA source while the reconstructive polymerization would be predominately centered on a second DNA source. As demonstrated above, degraded calf thymus DNA will incorporate dNTP in the presence of Taq polymerase without requiring specific primers for extension as a result of self-priming from overlapping fragments. Additionally, by using chloroplast gene *rbcl* primers, we would not expect calf thymus DNA to serve as a template for exponential PCR amplification or to compete with barley DNA when present for *rbcl* primer annealing. Therefore, when both are present, we would expect the degraded calf thymus DNA to preferentially drive the template reconstruction reaction and the barley DNA to drive *rbcl* amplification. The amplification reactions which contained only undigested or DNase I-digested calf thymus DNA (Fig. 7, lanes 3 and 4) did not result in a specific PCR product but appeared to be identical to the negative control (lane 2), having well defined primer-dimer bands. In contrast, the positive control with barley DNA alone (lane 5) resulted in a strong, well-defined 1.2 kb amplified product. Similarly, when high molecular weight calf thymus DNA is added to the barley reaction, a strong 1.2 kb band is present with only a slight reduction in intensity or quantity (lane 6). In contrast, when degraded calf thymus DNA is added to the barley reaction (lane 7), there is a distinct reduction in the quantity of the amplified product. These results clearly demonstrate that the degraded calf thymus DNA is reducing the

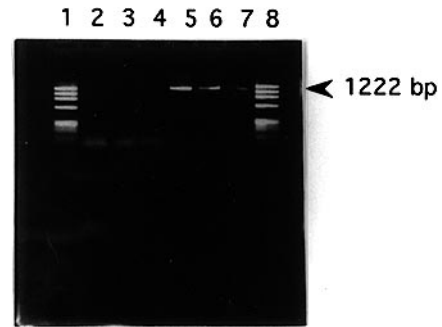


Figure 7. TBE agarose gel of chloroplast-derived PCR products and controls when highly polymerized or degraded calf thymus DNA is added to the reaction. Reactions were set up using the chloroplast primers *LtrbcL* 1 and *LtrbcL* 1201R, which define a 1222 bp fragment. An aliquot from each PCR was analyzed on a 0.7% agarose, 0.65% Synergel TBE gel: lane 1, *HaeIII*-digested Φ X174 size standard; lane 2, 'no template DNA' negative control; lane 3, 'undigested calf thymus DNA template' negative control; lane 4, 'digested calf thymus DNA template' negative control; lane 5, *H.spontaneum* genomic DNA template; lane 6, *H.spontaneum* genomic DNA and undigested calf thymus DNA template; lane 7, *H.spontaneum* genomic DNA and digested calf thymus DNA template; lane 8, *HaeIII*-digested Φ X174 size standard.

efficiency of the target PCR. As the high molecular weight calf thymus DNA does not reduce the efficiency of the barley amplification and calf thymus DNA does not appear to anneal to the chloroplast primers, it is likely that the uptake of dNTPs during template reconstruction is competing with the target barley PCR for limiting substrates.

DISCUSSION

The results lead to three important conclusions concerning PCRs in which highly fragmented DNA serves as template. First, amplified regions are not necessarily limited in size to the maximum lengths of template molecules introduced in the reaction. Template reconstruction can occur in which overlapping genomic fragments anneal and are extended by the DNA polymerase (Fig. 1). These reconstructed fragments may then complete a region to which both amplification primers can anneal and thus legitimate amplification of fragments larger than the original template molecules can occur. Second, it must be recognized that such reconstruction of fragments will occur not only in the region targeted for amplification, but also throughout the genome, wherever suitably overlapping fragments exist. The suitability of overlapping fragments is determined by the annealing conditions of the reaction. It is clear however, that the degree of initial fragmentation of the template DNA will grossly affect the amount of reconstruction polymerization which occurs. Incorporation of dNTP increases with greater fragmentation of the template up to the point at which either the fragment sizes are so small as to remain melted under experimental conditions or large regions of the genome are missing so that overlap and extension are restricted. Third, incorporation of dNTP may be extensive and can compete with an intended amplification, thereby reducing the efficiency of the PCR.

The general observation that successful amplification using degraded DNA is difficult to achieve and severely restricted in target size at best may appear to be in conflict with the results presented here. For successful amplification to occur, the target region must be complete between the two primers and sufficient

enzyme and substrate dNTP must be available. The results demonstrate that genomic reconstruction can take up considerable amounts of dNTP in the absence of primers. A corollary to this hypothesis is that within a given cycle the available enzyme is encumbered by the reconstructive polymerization. In opposition to these reconstruction polymerization reactions, it must be recognized that in regular PCR, primer extension will be driven by the high molar concentrations of primers. However, because complete target template may not be immediately available, incomplete, linear amplification of variable lengths will be produced by primer extension rather than exponential amplification of complete target regions. Thus, neither the genomic reconstruction nor the exponential amplification will proceed efficiently. Previous observations that increased amounts of Taq polymerase (5) or removal of low molecular weight DNA (4) increases amplification success with degraded samples may then be explained by our model in terms of the negative effects of competition of the two polymerase activities. Our results, which demonstrate that successful amplification of fragments larger than the template can be achieved if polymerization without primers is run before primer-driven amplification, supports these conclusions. It should be noted that nested PCR (PCR in which products from a primary reaction and internal primers are used in a secondary PCR) will produce the same effects. Thus, we suggest that increased success of amplification of degraded DNA may be achieved if the competitive reactions of genome reconstruction and exponential target amplification are separated.

These initial considerations of genomic reconstruction only partially explain why degraded DNA results in amplification of relatively small fragments of DNA. If genomic reconstruction can reproduce large molecules which may act as template, why are larger amplifications so difficult to obtain? Fragment extension necessary for genomic reconstruction is dependent on 3' annealing of overlapping fragments. Terminated fragments will often have a non-template-driven addition of a base, usually an adenosine, by terminal deoxynucleotidyl transferase activity of the DNA polymerase. The additional 3' base may not complement the base opposite it upon annealing, resulting in an unannealed 3'-end. This unbonded end will restrict or block polymerization. Thus, longer and more complete reconstruction, which is expected to occur after several rounds of denaturation, annealing and extension, will be restricted. [It should be noted that both of the bases immediately 3' of the fragments in our first experiment were adenosines in the original sequence, so that a spuriously added A may have annealed to an available T in the template (Fig. 1).] Barnes and colleagues (15,16) have suggested similar models for the limitation of long PCR. If the limitation on genome/template reconstruction is the same, then the use of thermally stable DNA polymerases with some 3'→5' exonuclease activity may allow increased success of longer reconstruction and subsequent PCR amplification. This would be achieved by the exonuclease removal of the 3' base when 5'→3' polymerization activity is held up because of the unannealed 3' nucleotide.

Clearly, the goals of the forensic scientist or evolutionary biologist are not limited to the size of possible amplification products, but also to the informational reliability of the products. One source of concern when working with degraded DNA is that the information may include polymerization-induced errors, i.e. incorporation of incorrect nucleotides by the polymerases. This source of error also exists when working with highly polymerized DNA, but may be

exacerbated by damaged template bases. However, as these errors should be randomly distributed, direct sequencing of PCR products greatly reduces this concern. A second source of concern may arise if products are chimeric, resulting from the amplification of a recombined template from two alleles. Previously, this has been suggested to be the result of 'jumping PCR', where incompletely extended primer extensions denature from their template strands and then re-anneal with a different template for completion of the extension (4,17-19). It is apparent that genomic reconstruction may also be a source for such artificial recombination. However, tests of the use of PCR in forensic analyses have largely proved these concerns to be exaggerated, with even degraded samples giving repeatable and reliable results (20-23). It remains to be tested whether amplification of largely reconstructed molecules will also produce reliable results.

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