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Genetics

PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates

WAYNE M. BARNES

Department of Biochemistry and Molecular Biophysics 8231, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110

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ABSTRACT **A target length limitation to PCR amplification of DNA has been identified and addressed. Concomitantly, the base-pair fidelity, the ability to use PCR products as primers, and the maximum yield of target fragment were increased. These improvements were achieved by the combination of a high level of an exonuclease-free, N-terminal deletion mutant of *Taq* DNA polymerase, Klentaq1, with a very low level of a thermostable DNA polymerase exhibiting a 3′-exonuclease activity (*Pfu*, Vent, or Deep Vent). At least 35 kb can be amplified to high yields from 1 ng of λ DNA template.**

Amplification of DNA spans by the polymerase chain reaction (PCR) has become an important and widespread tool of genetic analysis since the introduction of thermostable Taq (*Thermus aquaticus*) DNA polymerase for its catalysis (1). Two limitations to the method are the fidelity of the final product and the size of the product span that can be amplified. The fidelity problem has been partially addressed by the replacement of *Taq* DNA polymerase by *Pfu* (*Pyrococcus furiosus*) DNA polymerase (2), which exhibits an integral 3′-(editing)-exonuclease that apparently reduces the mutations per base pair per cycle from about 10^{-4} to about 10^{-5}. However, I have found that this enzyme is unable to amplify certain DNA sequences in the size range of 1.5–2 kb that Klentaq1 (N-terminal deletion mutant of *Taq* DNA polymerase analogous to the Klenow fragment of *Escherichia coli* DNA polymerase I; unpublished work) or AmpliTaq (full-length *Taq* DNA polymerase; ref. 3) can amplify handily, and *Pfu* is no more able (i.e., is not able) to amplify DNA product spans in excess of 5–7 kb than is any form of *Taq* DNA polymerase. For full-length *Taq* DNA Polymerase and its N-terminally truncated variants Klentaq1, Klentaq5 (4), and Stoffel fragment (3), PCR amplification rapidly becomes inefficient or nonexistent as the length of the target span exceeds 5–6 kb. This is true even if 30 min (10 times longer than seemingly necessary) is used during the extension step of each cycle (data not shown). Although there are several reports of inefficient but detectable amplification at 9- to 10-kb target length (5–9) and one at 15 kb (10), most general applications are limited to 5 kb. Apparently something has been blocking extension to longer lengths.

MATERIALS AND METHODS

DNA Polymerases. Vent and Deep Vent DNA polymerases were supplied by New England Biolabs. *Pfu* DNA polymerase and its exonuclease-negative (exo$^-$) mutant were supplied by Stratagene at 2.5 units/μl. Klentaq1 is an N-terminal deletion variant of *Taq* DNA polymerase (unpublished work). The deletion endpoint is between that of Klentaq5 (4) and Stoffel fragment (3). Purified Klentaq1 was as supplied by Ab Peptides (St. Louis) at 25–35 units/μl (a protein concentration of about 0.7 μg/μl). One unit of DNA polymerase activity incorporates 10 nmol of nucleotide in 30 min at 72°C with activated (partially degraded) calf thymus DNA as template. Since activated calf thymus DNA is a somewhat undefined substrate and is structurally different from PCR substrate, this assay was routinely eschewed in favor of a PCR-based assay to set the above stock concentration of Klentaq1: the concentration of Klentaq1 stock was adjusted so that 0.25 μl effectively (but 0.12 μl less effectively) catalyzed the amplification of a 2-kb target span from 10 ng of plasmid substrate with cycling conditions including 7 min of annealing/extension at 65°C. The mixture of 15/16 μl Klentaq1 and 1/16 μl of *Pfu* DNA polymerases is designated KlentaqLA-16.

Agarose Gel Electrophoresis. This employed 0.7–1% agarose in 1× GGB (TEA) buffer (40 mM Tris acetate, pH 8.3/20 mM sodium acetate/0.2 mM EDTA) at 2–3 V/cm, with 3% Ficoll instead of glycerol in the loading dye. For Fig. 3*C*, 1% agarose pulsed-field clamped homogeneous electric-field (CHEF) electrophoresis (11) with a switching time of 4 sec was used. Standard DNA fragment sizes in every figure are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb. Figs. 3*C* and 4 also have a full-length λplac5 standard band, 48,645 bp.

All agarose gels were run or stained in ethidium bromide at 0.5 μg/ml and photographed (35-mm ASA 400 film) or videographed (Alpha Innotech or Stratagene Eagle Eye) under UV illumination. In printing the gel photographs, the left halves of Fig. 3 *A* and *B* were exposed 50% less.

DNA Primers. Primers, most supplied by Integrated DNA Technologies (Coralville, IA), were as follows (sequences 5′ to 3′): BtV5 no. 1703 (43-mer), CCG-AGA-TCT-CCA-TGG-ATC-CAA-AGA-ATC-AAG-ATA-AGC-ATC-AAA-G; lacZ536 (36-mer), CTA-TGA-CCA-TGA-TTA-CGG-ATT-CAC-TGG-CCG-TCG-TTT; lacZ333 (33-mer near 3′ end of *lacZ*), ACC-AGC-CAT-CGC-CAT-CTG-CTG-CAC-GCG-GAA-GAA; lacZ′533 (33-mer), CGA-CGG-CCA-GTG-AAT-CCG-TAA-TCA-TGG-TCA-TAG; λL36, GGG-CGG-CGA-CCT-CGC-GGG-TTT-TCG-CTA-TTT-ATG-AAA; λR36, AGG-TCG-CCG-CCC-CGT-AAC-CTG-TCG-GAT-CAC-CGG-AAA; MBL no. 8757 (left side of EMBL phage inserts, 27-mer ending at 27,940), GCT-TAT-CTG-CTT-CTC-ATA-GAG-TCT-TGC; MBL-1.7 (27-mer), TTT-TGC-TGG-GTC-AGG-TTG-TTC-TTT-AGG; MBL002 (33-mer), GCA-AGA-CTC-TAT-GAG-AAG-CAG-ATA-AGC-GAT-AAG; MBL101 (top strand 33-mer ending at 27,840), ATC-ATT-ATT-TGA-TTT-CAA-TTT-TGT-CCC-ACT-CCC; MBR (27-mer), ATA-ACG-ATC-ATA-TAC-ATG-GTT-CTC-TCC; MBR001 (33-mer ending at 34,576), GGA-GAG-AAC-CAT-GTA-TAT-GAT-CGT-TAT-CTG-GGT; MBR202 (bottom-strand 33-mer ending at position 34,793), GCG-CAC-AAA-ACC-ATA-GAT-TGC-TCT-TCT-GTA-AGG; MSA19 [28-mer (only 22 bp of homology) at 3′ end of *lacZ*′], GGA-AGC-TTA-TTT-TTG-ACA-CCA-GAC-CAA-C; MSA1933 (33-mer at 3′ end of *lacZ*′), CCC-GGT-TAT-TAT-TAT-TTT-TGA-CAC-CAG-ACC-AAC.

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Abbreviation: exo$^-$, exonuclease-negative.

λ DNA Templates. λvacAI, a gift from S. Phadnis (Washington University), is a λEMBL4-vectored clone of the cytotoxin gene region of *Helicobacter pylori* DNA. This DNA was extracted and stored frozen. The other phage template DNAs λplac5 (12) and λK138 (13) were added as intact phage particles that had been purified by CsCl equilibrium centrifugation, dialyzed, and diluted in 1× PC2 buffer.

Long and Accurate PCR. PC2 reaction buffer (4) consisted of 20 mM Tris·HCl (pH 8.55 at 25°C), bovine serum albumin at 150 μg/ml, 16 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, and 250 μM each dNTP. For success above 28 kb (at 35 kb), 1.5 μl of 2 M Tris base was added to each 100 μl, corresponding to pH 9.1 measured for the Tris·HCl component only at 20 mM in water at 25°C. Each 100 μl of reaction volume contained 20 pmol of each primer and 0.1–10 ng of phage DNA template. It was found that 0.8 and 1.2 μl of KlentaqLA-16 were appropriate for under 20 kb and over 20 kb, respectively. Reaction volumes per tube were 33–50 μl, under 40 μl of mineral oil in thin-walled (PGC or Stratagene) plastic test tubes.

PCRs utilizing the primers at the ends of λ required a preincubation of 5 min at 68–72°C to disrupt the phage particles and to allow fill-in of the λ sticky ends to complete the primer homology. Optimal cycling conditions were in a multiple-block instrument (RoboCycler, Stratagene) programmed per cycle to 30 sec at 99°C, 30 sec at 67°C, and 11–24 min at 68°C, depending on target length over the range shown in Table 1. The second-best cycler was the Omnigene (Hyb-Aid), programmed under tube control per cycle to 2 sec at 95°C, then 68°C for similar annealing/extension times. Unless otherwise stated, all of the experiments reported here used 24 cycles.

For reported results of comparison of conditions such as cycling temperatures and times, thermal cycler machines, thick- and thin-walled tubes, etc., reactions were made up as 100 μl complete and then split into identical aliquots of 33 μl before PCR cycling.

Megaprimer. This consisted of gel-purified 384-bp PCR product DNA homologous to the region between the *Bam*HI site and *Eco*RI site of the gene coding for the CryV insecticidal crystal protein of *Bacillus thuringiensis* (15) and was primer-modified to remove these restriction sites. The PCRs in Fig. 1 each employed megaprimer (300 ng), primer BtV5, 20 ng of genomic DNA from *B. thuringiensis* strain NRD12 (16), and enzyme as indicated in the legend. Cycling conditions were 30 sec at 95°C, and 7 min at 60°C for 20 cycles.

Table 1. Primer and template combinations.

Product size, kb	Left primer	Right primer	Template DNA
5.8	MBL101	MSA1933	λK138
6.657	MBL	MBR	λplac5
8.386	MBL-1.7	MBR	λplac5
8.7	MBR001	λR36	λK138
12.1	lacZ333	MBR202	λK138
12.5	MBL 27-mer or MBL101 33-mer	MBR 27-mer or MBR202 33-mer	λvacAI
13.971	MBR001 33-mer	λR36	λplac5
15.560	MSA19 28-mer or MSA1933 33-mer	MBR202	λplac5
18.0	MBL101	MBR202	λK138
19.8	L36	MBL002	λK138
20.707	MBL101	λR36 36-mer	λplac5
19.584	λL36	lacZ333	λplac5
22.0	λL36	lacZ′533	λK138
24.6	λL36	MSA1933	λK138
22.495	λL36	lacZ536	λplac5
26.194	lacZ′533	λR36	λplac5
28.083	L36	MBL002	λplac5
34.968	L36	MBR202	λplac5

Product sizes given to 0.001 kb are as predicted from the sequence and structure of λ and λplac5 as documented in GenBank accession no. J02459 and ref. 14. Product sizes given to 0.1 kb were determined by comparison with these products and with the λ *Hin*dIII size standards labeled λH3.

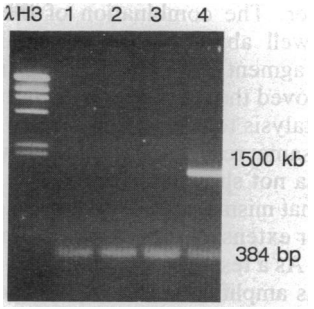

FIG. 1. PCR amplification attempted with a 384-bp megaprimer (double-stranded PCR product) paired with a 43-mer oligonucleotide primer BtV5. Per 100 μl of reaction volume, the following enzymes (see *Materials and Methods* for unit concentrations) were used to catalyze amplifications: lane 1, 1 μl of *Pfu* DNA polymerase; lane 2, 1/16 μl of *Pfu*; lane 3, 1 μl of Klentaq1; lane 4, both enzymes together (1 μl of Klentaq1 plus 1/16 μl of *Pfu*). The 384-bp band near the bottom of the gel is the megaprimer, which was originally amplified by Klentaq1. λH3, λ DNA digested with *Hin*dIII. The only successful amplification resulted from the combination of the two enzymes (lane 4). Vent DNA polymerase could substitute for *Pfu* with the same result (data not shown).

Test of exo⁻ *Pfu*. Each 100 μl of reaction mixture (incubated as 33 μl under 40 μl of oil) contained 2 ng of λplac5 DNA as purified phage particles, 20 pmol each of primers MBL-1.7 and MBR, reaction buffer PC2, and 1 μl of Klentaq1 (0.7 μg), except for reaction mixture 6, which contained 1 μl of *Pfu* DNA polymerase (2.5 units) alone. Other details are in the legend to Fig. 4. Thermal conditions were 24 cycles of 2 sec at 94°C and 11 min at 70°C.

RESULTS

The discovery reported here was made during attempts to utilize in PCR a primer with a mismatched A–A base pair at its 3′ end. In fact the primer was itself a PCR product "megaprimer" of 384 bp, and the mismatched A had been added by Klentaq1 using nontemplated terminal deoxynucleotidyltransferase activity common to DNA polymerases (17). Neither Klentaq1 (Fig. 1, lane 3) nor *Pfu* DNA polymerase (lanes 1 and 2 and other levels of enzyme not shown) could catalyze amplification of the 1500-bp target that lay between the PCR-product megaprimer and a 42-mer oligo-

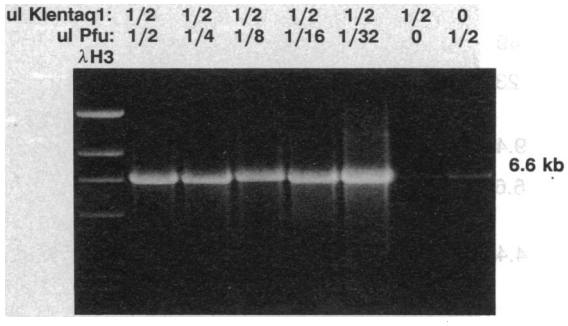

FIG. 2. Only a combination of Klentaq1 and *Pfu* can catalyze efficient amplification of 6.6 kb. Per 100 μl, the indicated level of each enzyme was used to catalyze PCRs templated with 19 ng of λplac5 DNA and primers MBL and MBR. Each amplification consisted of 20 cycles of 2 min at 94°C, 2 min at 60°C, and 10 min at 72°C.

nucleotide primer. The combination of the two enzymes, however, was well able to catalyze amplification of the desired target fragment (Fig. 1, lane 4). Evidently, the *Pfu* polymerase removed the presumed 3′ A–A mismatch, allowing Klentaq1 catalysis to proceed efficiently for each step of the PCR. The same result was obtained with Vent DNA polymerase (data not shown).

I speculated that mismatched 3′ ends are a general cause of inefficient primer extension during PCR of targets larger than a few kilobases. As a test system I employed a 6.6-kb λ DNA target which was amplified detectably but poorly by AmpliTaq, Klentaq1, or *Pfu* DNA polymerase in a variety of standard conditions. Per 100-μl reaction volume, 1/2 μl of Klentaq1 was combined with various amounts of *Pfu* polymerase, from 0.005 to 0.5 μl. Since the *Pfu* stock was at least 10 times less concentrated than the Klentaq1 stock, the actual ratios tested were 1:20 to 1:2000 in DNA polymerase units. Representative results of these tests are shown in Fig. 2. A high yield of target band was observed for all tested combinations of the two enzymes, yet several levels of each enzyme on its own failed to catalyze more than faintly detectable amplification. The lowest level of *Pfu* tested, 0.005 μl, exhibited only a slight beneficial effect. The apparent broad optimum ratio of Klentaq1 to *Pfu1* was 16 or 64 by volume, which is about 160 or 640 on the basis of DNA polymerase incorporation units. When tested at 6–8 kb (data not shown), other combinations of 3′-exo$^-$ and 3′-exo$^+$ thermostable DNA polymerases also showed the effect, including Amplitaq/*Pfu*, Klentaq1/Vent, Klentaq5 (DeltaTaq, United States Biochemical)/*Pfu*, Stoffel fragment/*Pfu*, Klentaq1/Deep Vent (my second choice), and *Pfu* exo$^-$/*Pfu* exo$^+$. Although comparatively few trials and optimizations were carried out, no other combination tried was as effective as Klentaq1/*Pfu*. It remains likely that other combinations can be found with equal or even

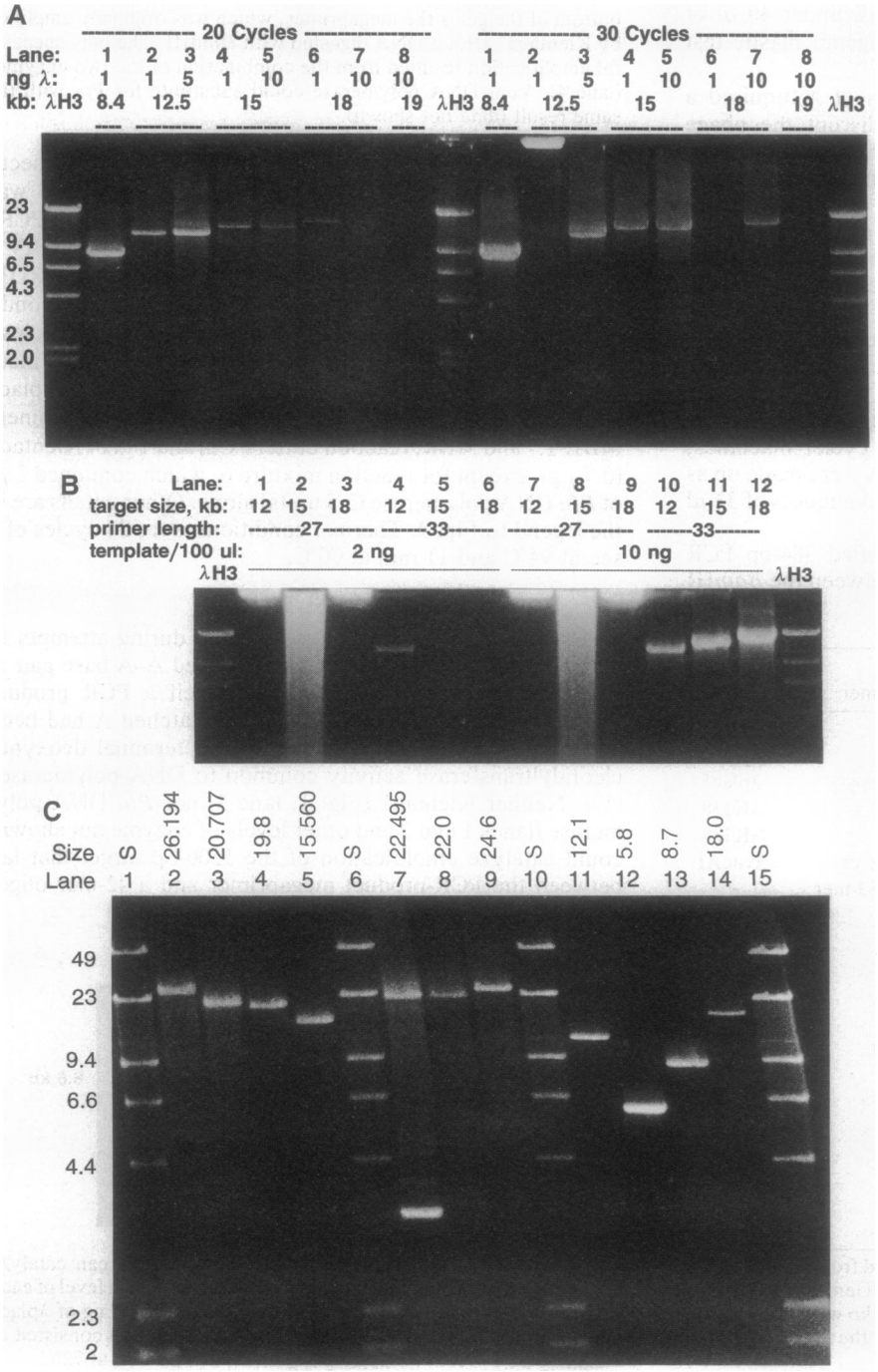

FIG. 3. (*A*) Amplification of 8.4–18 kb. Target product size (kb) is indicated above each lane. Level of template per 100 μl is indicated as ng λ of DNA. Twenty or 30 cycles of PCR were each 2 sec at 94°C and 11 min at 70°C. These early amplifications were non-optimal in several respects compared with the current optimal procedure (see *Materials and Methods*): thick-walled tubes were used instead of thin, catalysis was by KlentaqLA-64 (1 μl) instead of KlentaqLA-16, the 27-mer primers were used (see Table 1) instead of longer primers, the extension/annealing temperature was 70°C instead of 68°C, and the Omnigene thermal cycler was used. (*B*) The 33-mers are better than the 27-mers. Per 100 μl of reaction volume, 2 ng (lanes 1–6) or 10 ng (lanes 7–12) of λ transducing-phage template was amplified with 27-mer primers (lanes 1–3 and 7–9) or 33-mer primers (lanes 4–6 and 10–12). Besides being longer, the 33-mer λ primer sequences were situated 100 bp to the left of primer MBL and 200 bp to the right of primer MBR on the λ genome. KlentaqLA-16 in the amounts of 1.2, 1.4, and 1.6 μl was used to catalyze the amplifications of 12.5, 15, and 18 kb, respectively. Aliquots (15 μl, equivalent to 0.3 or 1.5 ng of λ template) were analyzed by 0.8% agarose electrophoresis. (*C*) CHEF pulse-field analysis (ref. 11; 4-sec switching time) of large PCR products amplified by KlentaqLA-16 (1.2 μl) under conditions which were suboptimal with respect to pH (unmodified PC2 buffer was used) and thermal cycler (Omnigene). Starting template (see Table 1) was at 0.1 ng/μl and the time at 68°C in each cycle was 21 min for products over 20 kb, 13 min for lanes 4 and 5, and 11 min for lanes 11–14. The volumes of PCR product loaded were adjusted to result in approximately equal intensity; in μl: 12, 12, 4, and 2; 10, 10, and 10; 2, 2, 4, and 1. The size-standard lanes (S) show full-length λplac5 DNA (48,645 bp) mixed with a *Hin*dIII digest of λ DNA. As for Table 1, the sizes given to 0.001 kb were predicted from the primer positions on the sequence of λplac5 DNA, and sizes given to 0.1 kb were determined from this gel.

superior performance or that different enzyme combinations will be appropriate for different amplifications.

A Very Short Heat Step Is Necessary. I next attempted to amplify DNA of 8.4–18 kb from λ transducing-phage template. An early cycling protocol employed a denaturation step of 1 or 2 min at 95°C or 98°C, but no useful product in excess of 8.4 kb was obtained until the parameters of this heat step were reduced to 2 or 20 sec at 93°C or 94°C. In an experiment with the denaturation step at 94°C for 20, 60, or 180 sec, the 8.4-kb product exhibited decreasing yield with increased length of this heat step (data not shown). Apparently, a component of the reaction is at its margin of thermostability (see speculation below). Fig. 3*A* shows that with the short, 2-sec denaturation step, target fragment was obtained for some reactions at all sizes in the range 8.4–18 kb, with very high product yields up to 15 kb when 30 PCR cycles were employed. Fig. 3*A* also shows some failed reactions which I cannot explain. The failure mode that gives rise to massive ethidium staining in the sample well (30-cycle lane 2) was particularly common, especially at high enzyme levels.

Longer Primers. A change in primer length from 27 to 33 nt greatly reduced the frequency of failed reactions. Fig. 3*B* demonstrates improved reliability for amplification of 12.5, 15, and 18 kb with the 33-mer primers, under conditions of otherwise optimally high enzyme levels in which the 27-mer primers failed to give rise to desirable target product. This result does not represent an extensive survey of primer length, and it has not yet been repeated with the improvements below. Therefore the optimum primer length for long PCR remains to be determined. Some of the amplifications analyzed in Fig. 3*C* utilized 36-mer primers from the very ends of λ. A 2- to 5-min preincubation at 68–72°C was necessary to release the template DNA from the phage particles and to fill in the sticky ends of λ to complete the template homology with primers λL36 and λR36.

Rapid Cycling. A change to thin-walled tubes, which have lower heat capacity and conduct heat more efficiently, further improved the reactions. Fig. 3*C* shows a CHEF pulsed-field agarose gel analysis of successful amplifications of DNA spans 6–26 kb in size. The target of 28 kb was not amplifiable in the Omnigene thermal cycler (data not shown) but did appear (Fig. 4, lane 2) when the RoboCycler was used.

Several models of thermal cycler have been used, and although not all have been optimized, some are clearly preferable to others for long PCR. As may be concluded from the advantage of thin-walled tubes noted above, success seems to be positively correlated with a high speed of temperature change made possible by the design of the thermal cycler. The RoboCycler achieves rapid temperature change by moving tubes from block to block, and observations with a thermistor temperature probe indicate that it raises the reactions to 93–95°C for only 5 sec under the denaturation conditions employed (30 sec in the 99°C block), before rapidly (within 30 sec) returning the reaction to 68°C. Targets over 20 kb do not appear reliably unless the extension time exceeds 20 min and the extension temperature is limited to 68°C; 69°C is too high for reliable long PCR with this system.

Higher pH. The current record of 35 kb (Fig. 4, lane 3) was only amplifiable when the pH was increased (at the suggestion of S. Cheng, personal communication). A preliminary scan of higher pH was carried out (data not shown), and this resulted in the appearance of the 35-kb band at pH 8.8–9.2, with the optimum at 9.1. Further improvement to a high yield of the 35-kb product was achieved by lengthening the extension time to 24 min. Other than the higher pH, the long PCR procedure has not yet realized any potential benefits from changes in buffer conditions from those optimized for 8.4 kb.

Identity of Long PCR Products. Fig. 3 shows that the mobilities of the successful large DNA products agree with those predicted in Table 1 from the known map positions of the primers used.

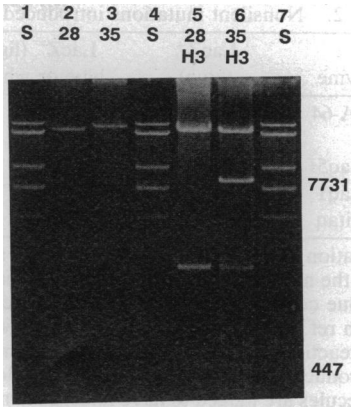

FIG. 4. Twenty-eight-kilobase and 35-kb products without (lanes 2 and 3) and with (lanes 5 and 6) digestion by *Hin*dIII. Before *Hin*dIII digestion, the 28-kb product was amplified with 21 min of extension time per cycle, and the 35-kb product was cycled with 24-min extension times, both in the RoboCycler at optimum pH. Lanes S (1, 4, and 7) contained undigested and *Hin*dIII-digested λplac5 DNA.

*Hin*dIII restriction enzyme digestion of the unpurified 28- and 35-kb products (Fig. 4, lanes 6 and 7) resulted in the expected left arm of λ (23 kb) and 2.3-kb band from both, and the predictable bands terminated by the right PCR primer: 447 bp (barely visible) from the 28-kb product and 7331 bp from the 35-kb product.

Exonuclease Mutant. To test the theory that it is the 3′-exonuclease activity of *Pfu* DNA polymerase which is conferring the advantage of long-distance PCR, I tested the available 3′-exo⁻ mutant of *Pfu* polymerase (9). The 3′-exo⁻ mutant failed to promote efficient amplification of a long DNA target (Fig. 5), supporting the hypothesis that it is the 3′-exonuclease activity which is important and necessary for the efficiency of PCR amplification in this size range.

Fidelity Test. Since the biological purpose of 3′-exonuclease is to edit base-pair mismatches for high replication fidelity, the fidelity of the PCR product was tested in an assay involving the amplification and molecular cloning of an entire *lacZ* (β-galactosidase) gene flanked by two selectable markers (4). Heretofore the highest reported fidelity of PCR amplification is that catalyzed by *Pfu* DNA polymerase (2). Table 2 shows that the fidelity of the product amplified by the 640:1 mixture of Klentaq1 and *Pfu* polymerase at least matches that obtained for *Pfu* polymerase alone, when each is used for 16 cycles of PCR. The designation of the enzyme mixture as Klentaq-LA (KlenTaq long and accurate) reflects this high fidelity.

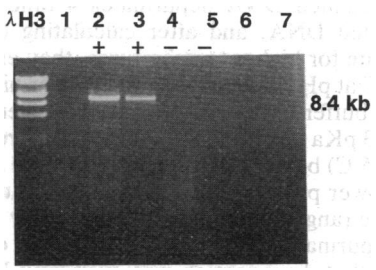

FIG. 5. Pfu exo⁻ mutant test. PCR amplification of 8.4 kb by 30 units (0.7 μg) of Klentaq1 alone (lanes 1 and 7) and in combination with a very small admixture (1/16 μl or 1/64 μl, equivalent to 1/6 or 1/25 unit) of archaebacterial *Pfu* wild-type exo⁺ DNA polymerase (+; lanes 2 and 3) or a mutant thereof lacking the 3′-exonuclease activity (−; lanes 4 and 5). Lane 6 resulted when 1 μl (2.5 units) of wild-type *Pfu* DNA polymerase (exo⁺) was employed alone.

Table 2. Nonsilent mutations introduced into the *lacZ* gene by 16 cycles of PCR

Enzyme	LacZ$^+$ (blue)	LacZ$^-$ (light blue or white)	% mutant	Effective cycle no.*	Errors per 10^5 bp†	Fold improvement over *Taq*
KTLA-64	571	34	5.6	12	1.05	12.7
Pfu	528	37	6.5	8	1.9	6.9
Klentaq5‡	442	85	16.1	8	5.1	2.6
Klentaq1	3225	985	26.4	8	9.0	1.5
Amplitaq	525	301	36.4	8	13.4	1.0

*Equation 1 of ref. 4 was rearranged to be as follows to solve for errors per base pair: $X = -[\ln(2F^{1/m-1} - 1)]/1000$, where X is the number of errors per base pair incorporated, 1000 is the effective target size in the *lacZ* gene (4), F is the fraction of blue colonies, and m is the effective cycle number.

†As in ref. 4, the effective cycle number was estimated at less than the machine cycles to reflect the actual efficiency of the reaction, yet higher than the minimum calculated from the fold amplification. Strand loss due to incomplete synthesis of product strands is a probable cause of lower-than-ideal amplification efficiency. Therefore, successful (not lost) product molecules are judged to have undergone more than the calculated minimum number of replications. KTLA-64 (Klentaq1/*Pfu* ratio of 64:1 by volume) was assigned a higher effective cycle number because its reactions started with 10 times less DNA (1.5 ng vs. 15 ng of plasmid pWB305 for the other four) to result in comparable levels of product.

‡The N-terminal deletion mutant of *Taq* DNA polymerase described in ref. 4.

DISCUSSION

The previous length limitation for PCR amplification is postulated to have been caused by low efficiency of extension at the sites of incorporation of mismatched base pairs. Although it would have seemed that the cure for these mismatches would be to use enzymes with 3′-(editing)-exonucleases, I believe that when *Pfu* and Vent DNA polymerase are used to catalyze amplifications on their own, their failure is due to degradation of the PCR primers by their 3′-exonucleases, especially during the required long synthesis times and at optimally high DNA polymerase levels. Evidently, very low levels of 3′-exonuclease are sufficient and optimal for removal of the mismatches to allow the Klentaq1 amplification to proceed. The optimally low level of 3′-exonuclease can be set effectively, conveniently, and flexibly by mixing and dilution rather than by mutation.

It is apparently critical that the length and temperature of the heat denaturation step of the PCR be kept to a minimum. This indicates that some component of the reaction is heat labile. The heat-labile component either is one of the two enzymes or is the DNA template. Klentaq1 is fully heat stable at 98°C and partially stable at 99°C when tested in 20 cycles of PCR employing 2-min denaturation steps (unpublished work), so Klentaq1 is not the heat-labile component. Although the heat stability of the exonuclease activity of *Pfu* polymerase has not been tested distinctly from its DNA polymerase activity (2), I speculate that the DNA template is the most likely heat-labile component for the following reasons. *Taq* DNA polymerase stops at sites of depurination (11). *In vivo* in *E. coli*, unexcised apurinic sites are thought to be lethal, indicating that DNA polymerase cannot cross them (18). Lindahl and Nyberg (19, 20) measured the rate of depurination of DNA at 70°C and pH 7.4. After considering that single-stranded DNA depurinates 4 times faster than double-stranded DNA, and after calculating the expected increase in rate for higher temperatures, they estimated that 1 min at 100°C at pH 7.0 would cause one apurinic site per 100 kb. Tris, the buffer used in PCRs, has a temperature coefficient of −0.03 pKa unit per degree (21), so that reactions with pH 8.55 (at 25°C) buffer may be expected to be at pH 6.45 at 95°C. This lower pH would cause even faster depurination, perhaps in the range of one every 20–30 kb per minute. This estimated depurination rate is within an order of magnitude of indicating that depurination may well limit long-distance PCR. The improvement obtained by increasing the pH slightly may correspond to a decrease in template depurination. If so, further improvements may result if depurination can be reduced, or if a majority DNA polymerase component can be found which is able to bypass depurination sites.

The short denaturation time found to be optimal (estimated 5 sec in the reaction itself at 95°C) is surprisingly effective for the amplification of 35 kb, whereas it might have been expected that longer PCR targets would need longer denaturation time to become completely denatured.

These amplifications were successful with several different target sequences, with several primer combinations, and with product sizes up to nearly twice the maximum size of inserts cloned into λ. Whole viruses and plasmids up to 35 kb in length should now be amplifiable with this system. Should this method prove applicable to DNA of higher complexity than λ, it could prove a boon to genomic mapping and sequencing applications, since *in vitro* amplification is convenient and avoids the DNA-rearrangement and genetoxicity pitfalls of *in vivo* cloning.

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