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# Effective amplification of long targets from cloned inserts and human genomic DNA

(cosolvents/genome mapping/PCR/thermostable 3′-to-5′-exonuclease)

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ABSTRACT **We have used the polymerase chain reaction (PCR) to amplify up to 22 kb of the β-globin gene cluster from human genomic DNA and up to 42 kb from phage λ DNA. We have also amplified 91 human genomic inserts of 9–23 kb directly from recombinant λ plaques. To do this, we increased pH, added glycerol and dimethyl sulfoxide, decreased denaturation times, increased extension times, and used a secondary thermostable DNA polymerase that possesses a 3′-to-5′-exonuclease, or "proofreading," activity. Our "long PCR" protocols maintain the specificity required for targets in genomic DNA by using lower levels of polymerase and temperature and salt conditions for specific primer annealing. The ability to amplify DNA sequences of 10–40 kb will bring the speed and simplicity of PCR to genomic mapping and sequencing and facilitate studies in molecular genetics.**

PCR (1, 2) and molecular cloning are powerful tools for the amplification of genetic sequences; yet PCR can be quicker, simpler, and less costly to perform. As a result, PCR has been widely applied in molecular biology, molecular evolution, genetics, and forensic biology (3). PCR has also had broad impact on genome mapping and sequencing projects (4, 5). PCR would have an even greater role, however, if sequences longer than 10 kb—sequences currently cloned with phage λ or cosmid vectors—could be amplified reliably.

Although recent reports (6–11) described amplifications of 5–15 kb, reported yields were low. Our goals were to amplify targets of at least 20 kb with high yields, even from single-copy genes within complex genomes, and to better understand the most critical parameters for longer amplifications.

We surveyed various thermostable DNA polymerases, reaction buffers and additives, and thermal cycling profiles, guided by the following likely requirements for a reliable "long PCR": (*i*) complete denaturation of target sequences, as longer targets may become increasingly difficult to denature; (*ii*) extension times sufficient for complete strand synthesis in each PCR cycle; (*iii*) protection of template DNA against damage [e.g., depurination (12)] during thermal cycling; and (*iv*) retention of specificity necessary for single-copy gene amplifications from genomic DNA.

One of us (W.M.B.) has also hypothesized that misincorporated nucleotides reduce the efficiency of amplifying long targets. A mismatched 3′-terminal base may cause prematurely terminated strand synthesis (13). Even the low levels of nucleotide misincorporation estimated for *Taq* DNA polymerase [≤1 in 10–50,000 bases (14)] will affect sequences longer than 10 kb. A small amount of thermostable 3′-to-5′-exonuclease activity removes such mismatched nucleotides and permits the predominant polymerase activity to complete strand synthesis (15). The use of such proofreading activity, combined with conditions identified in our survey, resulted in the highest yields of the longest products.

## MATERIALS AND METHODS

**Primer Design and Analysis.** Primer sequences are available from the authors upon request. Each primer was composed of 12 (G+C) and 8–11 (A+T) bases. The primers were chosen to share an optimum annealing temperature of ≈68°C, as defined by the "Tp" algorithm (16). The MELT program (J. Wetmur, Mt. Sinai School of Medicine, New York; also see ref. 17) was also used to calculate melting temperatures. OLIGO 4.0 software (National Biosciences, Plymouth, MN) was used to evaluate secondary priming sites and inter- and intraprimer complementation. Template sequences were from GenBank [accession nos. M17233 (phage λ) and J00179 (human β-globin gene cluster)].

**Target DNA and Nucleotides.** λ DNA (*cI587ind1sam7*; 1 ng/μl) and solutions containing all four dNTPs (pH 7.0 with NaOH) were from Perkin–Elmer. Total genomic DNA was from human placenta (Sigma) or the KAS011 β-lymphoblastoid cell line (gift of C. Aldrich and A. Begovich, Roche Molecular Systems). Primer stocks (10 μM) and λ DNA dilutions were made with 10 mM Tris·HCl, pH 8, at 25°C/0.1 mM EDTA.

A library of human genomic clones in λ FIX II was purchased from Stratagene and grown as recommended. Randomly selected plaques were stored in 30 μl of 25 mM Tris·HCl, pH 8.3/10 mM $MgCl_2$ at 4°C. Aliquots of 1 μl were used for PCR.

**Thermostable DNA Polymerases.** AmpliTaq DNA polymerase [recombinant (r) *Taq* from *Thermus aquaticus* (18)] and r*Tth* DNA polymerase [from *Thermus thermophilus* (19)] were purchased from Perkin–Elmer; Hot Tub was from Amersham; Vent [from *Thermococcus litoralis* (20)] and Deep Vent [from *Pyrococcus species* GB-D (21)] were from New England Biolabs; and *Pfu* [from *Pyrococcus furiosus* (22)] polymerase was from Stratagene. r*Tma* (from *Thermatoga maritima*)§ polymerase was a gift of D. Bost, S. Stoffel, and D. Gelfand (Roche Molecular Systems). Klentaq1 was as described (15).

**Additional Components.** Standard r*Taq* (10 mM Tris·HCl, pH 8.3/50 mM KCl) and r*Tth* [5% (vol/vol) glycerol/10 mM Tris·HCl, pH 8.3/100 mM KCl/0.75 mM EGTA/0.05% Tween 20] polymerase buffers for PCR were from Perkin–Elmer. The pH (at 25°C) of additional buffer stocks was measured at 1.0 M; Tricine was adjusted with KOH. Molecular biology grade dimethyl sulfoxide (DMSO) and glycerol

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Abbreviations: DMSO, dimethyl sulfoxide; FIGE, field-inversion gel electrophoresis; r, recombinant; U, polymerase unit(s).
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§Lawyer, F. C. & Gelfand, D. H., Abstracts of the 92nd General Meeting of the American Society for Microbiology, May 26–30, 1992, New Orleans, p. 200, no. H-104.

were from Sigma and J. T. Baker, respectively. Glycerol concentrations do not include contributions (≤1%) from enzyme storage buffers.

**PCR Methods.** Amplifications (50 or 100 μl) were performed in a Perkin–Elmer GeneAmp PCR System 9600, using MicroAmp tubes. All four dNTPs were at 0.2 mM, but other components were varied as discussed in the text. For a manual "hot-start," $Mg^{2+}$ [$MgCl_2$ or $Mg(OAc)_2$] was withheld until samples had been at 75–80°C for ≈90 sec and then added from a 25 mM stock. Cycles were as follows: denaturation at 94°C for 10 sec and annealing and extension at 68°C for a variable 5–22 min. For times longer than 12–14 min, the autoextension feature was used to add 15–20 sec per cycle, to a final 16–22 min. Depending upon the target copy number and length, 25–38 cycles were used. Most runs (total 6–10 hr) included an initial 10-sec hold at 94°C and a final 10-min hold at 72°C.

Human genomic inserts cloned in λ FIX II were amplified using 100-μl reaction mixtures in 25 mM Tricine, pH 8.7/85 mM KOAc/9.6% glycerol/all four dNTPs (each at 0.2 mM)/each primer at 0.4 μM/1.75 units (U) of r*Tth* polymerase/0.02 U of Vent polymerase/1.15 mM $Mg(OAc)_2$. The two-step profile described above was used, with an initial 12 min at 68°C, extended by 15 sec per cycle for 32 cycles.

Targets within the human β-globin gene cluster were amplified in 50-μl reaction mixtures with 37 ng of KAS011 DNA in 20 mM Tricine, pH 8.7/85 mM KOAc/8% glycerol/2% (vol/vol) DMSO/all four dNTPs (each at 0.2 mM)/each primer at 0.2 μM/0.9 U of r*Tth* polymerase/0.02 U of Vent polymerase/1.1 mM $Mg(OAc)_2$. The two-step profile described above was used with 12 min at 68°C for 12 cycles and then autoextending 15 sec per cycle for 24 cycles.

**Analysis of PCR Products.** Samples (4–10 μl from each PCR mixture) were routinely analyzed on standard horizontal gels of 0.6–0.8% SeaKem GTG agarose (FMC). For greater size resolution, field-inversion gel electrophoresis (FIGE, ref. 23) was performed using a Hoefer system. Sharper FIGE gel bands were obtained if PCR samples were supplemented with 1–3 mM $MgCl_2$ and incubated at 37°C for 10 min before being loaded. This effect has not been explained, but additional $MgCl_2$ appeared more critical than the temperature and time of incubation. Molecular size markers were λ/*Hin*dIII (New England Biolabs or GIBCO/BRL), λ/mono-cut mixture (New England Biolabs), and 1-kb ladder (GIBCO/BRL). PCR products from λ DNA were digested with *Bcl* I, *Bss*HII, and *Mlu* I (New England Biolabs) or *Bam*HI, *Eco*RI, and *Hin*dIII (GIBCO/BRL) and analyzed on standard gels. PCR products from phage plaques were digested with *Not* I (Stratagene) and analyzed by standard and FIGE gels.

## RESULTS

Long PCR protocols were first evaluated using target sequences from λ DNA. Targets of 1.5–42.2 kb were defined within the 48.5-kb genome of λ by pairing a fixed left-hand primer with right-hand primers chosen at 1- to 3-kb intervals. Fig. 1*A* shows PCR products of between 22.8 and 39 kb, resolved by FIGE. Even the 39-kb product is readily detected on a gel stained with ethidium bromide. In comparison with the molecular size marker bands, yields (per 50-μl reaction mixture) can be estimated at between 0.7 and 1 μg for the 22.8-kb product and between 0.2 and 0.3 μg for the 39-kb product. A 42.2-kb target has since been amplified, with lower yields (data not shown). Digestion with *Bss*HII and *Mlu* I confirmed products between 22.8 and 33.9 kb (Fig. 1*B*); *Eco*RI and *Hin*dIII were more informative for larger products (data not shown). All fragments observed were as expected for each target, and each PCR product was digested cleanly—evidence of the specificity of each reaction. As discussed below, these yields were obtained by increasing extension times, adding cosolvents, adding a polymerase with proofreading activity, and raising the pH of the reaction buffer. Specificity was maintained by optimizing enzyme and salt levels, carefully choosing primers, and using a hot-start method and relatively high annealing temperatures.

**Thermal Cycling Profile.** Two-temperature thermal cycling profiles were used. The short time at a moderate denaturation temperature (94°C) was chosen to minimize DNA damage such as depurination. The relatively high annealing temperature (68°C) minimized yields of products from secondary priming sites. Adding a 70–75°C step did not significantly improve yields, and even if the total time for annealing and extension remained constant, if the time at 68°C was less than or equal to 5–6 min, yields were reduced. More complex extension steps with temperature spikes to accommodate G+C- and A+T-rich stretches were not beneficial. A hot-start method (24) was used to minimize undesired primer interactions leading to short products that would amplify more efficiently than the desired long product.

**Extension Time.** Complete strand synthesis, a requirement for efficient PCR, is facilitated on long templates by sufficiently long extension times and addition of a proofreading activity. The r*Taq* and r*Tth* DNA polymerases proceed at as much as 60 nucleotides per sec in a standard PCR (R. Abramson, personal communication; ref. 25), suggesting that

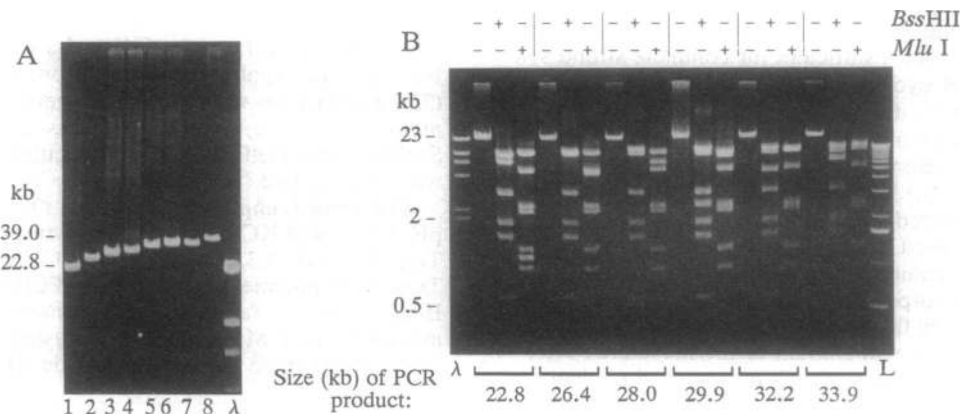


FIG. 1. Long PCR products (22.8–39.0 kb) from λ DNA. (*A*) FIGE gel. Reaction mixtures (50–μl) contained 20 mM Tricine (pH 8.7), 85 mM KOAc, 6.4 or 8% glycerol, 1, 3, 3.5, or 5% DMSO, 1.0–1.1 mM $Mg(OAc)_2$, 0.5 ng ($10^7$ copies) of λ DNA, 0.45 μM each primer, 2.5 U of r*Tth* polymerase, and 0.02 U of Vent polymerase (26–28 cycles). Lanes: 1, 22.8; 2, 26.4; 3, 28.0; 4, 29.9; 5, 32.2; 6, 33.9; 7, 35.0; 8, 39.0 kb. Aliquots of 3.5–6 μl were loaded, with 150 ng of λ/*Hin*dIII as molecular size standard (lane λ). (*B*) Digestions of the 22.8- to 33.9-kb products with *Bss*HII or *Mlu* I, electrophoresed on a standard 0.75% agarose gel with λ/*Hin*dIII (lane λ) and 1-kb ladder (lane L) standards.

4 min would suffice for 12 kb. In practice, yields were greater if longer extension times were used (Fig. 2*A*). In these r*Tth* polymerase reactions with 5-min extensions, the yield of 9.4-kb product (lane 1) was high, but yield of the 12.7-kb product (lane 2) was low and no 16.7-kb product was detected in lane 3. With a 10-min extension step, the yield of 12.7-kb product (lane 4) was improved, and 14.3- and 16.7-kb products (lanes 5 and 6, respectively) were detectable.

An annealing and extension time of at least 10 min, but no more than 22 min in any cycle, was used for targets longer than 20 kb. Runs using the thermal cycler's autoextension feature to add 15–20 sec per cycle yielded fewer nonspecific amplification products than did those using constant extension times of 15 min or more from the start.

**Cosolvents.** Certain cosolvents that facilitate standard PCR (e.g., refs. 26–29) also facilitate long PCR. Fig. 2*B* illustrates that adding 8% glycerol to standard r*Taq* PCR buffer enabled the amplification of nearly 10 kb more, resulting in a detectable 19-kb band (lane 8) with significantly increased yields of shorter targets (lanes 5–7). Glycerol may influence long amplifications by (*i*) increasing the thermal stability of the polymerase, as much as 2-fold at 95–97.5°C for r*Taq* polymerase and (*ii*) effectively lowering melting and strand separation temperatures (2.5–3°C per 10% glycerol), thus facilitating denaturation of the template and increasing the specificity of primer annealing (P. Landre and R. Watson, personal communication; also refs. 29 and 30).

Combinations of glycerol and DMSO (as in Figs. 1 and 5) were often more effective than glycerol alone. Amplifications of λ targets 25–34 kb long were enhanced by adding 1–3% DMSO, 8% glycerol, or both cosolvents, each at 5%. For 35- to 42-kb targets, 5% DMSO with 6–7% glycerol was more effective. Furthermore, if extension times were reduced to a constant 10 min, 3% DMSO with 8% glycerol enabled amplification of 34 kb, while 1% DMSO with 8% glycerol allowed only 26 kb. Since DMSO—unlike glycerol—reduces the thermal stability of r*Taq* polymerase (P. Landre and R. Watson, personal communication), the functional lowering of melting and strand-separation temperatures [5.5–6°C per 10% DMSO (31)] may be the dominant effect of cosolvents in long PCR. Effective combinations of glycerol and DMSO were generally consistent with the estimated additive reduction in melting and strand-separation temperatures, although the enhancement of yields was not readily duplicated by simply raising the denaturation or annealing temperature. DMSO has been reported to accelerate strand renaturation and may give DNA thermal stability against depurination and/or chain scission (32). Although useful for short G+C-rich targets (27), formamide was not helpful for this system. Glycerol was not tolerated by the Klentaq1 and *Pfu* polymerase combination (W.M.B., unpublished data).

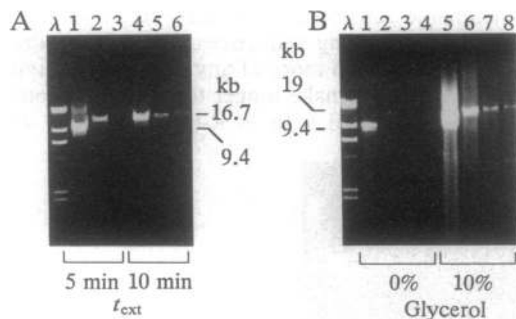


FIG. 2. Effect of increasing extension time or adding glycerol. (*A*) A 5-min extension time ($t_{ext}$) for 9.4-, 12.7-, and 16.7-kb products (lanes 1–3, respectively), compared to a 10-min extension time for 12.7-, 14.3-, and 16.7-kb products (lanes 4–6). Reaction mixtures (50 μl) contained 25 mM Tricine (pH 8.7), 85 mM KOAc, 9% glycerol, 1.7 mM $Mg(OAc)_2$, 0.5 ng ($10^7$ copies) of λ DNA, 0.4 μM each primer, and 2.5 U of r*Tth* polymerase (28 cycles). Aliquots of 9 μl were electrophoresed on a 0.6% agarose gel with λ/*Hin*dIII (lane λ). (*B*) Yields of 9.4-, 14.3-, 16.7-, and 19-kb products in the absence (lanes 1–4) or presence (lanes 5–8) of 10% (wt/vol) glycerol. All reaction mixtures (50 μl) contained Perkin–Elmer r*Taq* PCR buffer, 2 mM $MgCl_2$, 5 ng of λ DNA, 0.3 μM each primer, and 2.5 U of r*Taq* polymerase (25 cycles). Aliquots of 8 μl were electrophoresed on a 0.6% agarose gel with λ/*Hin*dIII (lane λ).

**Polymerase Activity.** r*Taq* and r*Tth* polymerases gave higher yields of longer products than did Hot Tub (limited to 19–21 kb), Vent ($exo^-$) (limited to 12–16 kb), or Vent (no yield at 9 kb) polymerase. r*Tth* polymerase was the most reliable, a result consistent with a previous report (8). For high-copy (target ≥ $10^7$ copies) reactions, yields were highest with 2–2.5 U of polymerase per 50-μl reaction mixture; nonspecific products accumulated at higher enzyme levels. For low-copy (≤$10^4$ copies) reactions, specificity was maximized by using 0.8–1 U per 50-μl reaction mixture—less than the standard ≈1.25 U. With r*Taq* or r*Tth* polymerase alone, however, the longest products obtained reproducibly were 23 kb from high-copy λ reactions and 10–12 kb from low-copy reactions.

**The 3′-to-5′ Exonuclease Activity.** As discussed in the Introduction, nucleotide misincorporations can interfere with strand synthesis. That low levels of a thermostable proofreading DNA polymerase can remove these misincorporations and facilitate PCR was shown with r*Taq*, Klentaq1, or *Pfu* ($exo^-$) mutant as the major DNA polymerase and *Pfu*, Vent, or Deep Vent as the minor editing polymerase. KlenTaq1 with *Pfu* polymerase amplified up to 35 kb from a high-copy λ template but was not applied to targets within more complex genomic DNA (15).

Vent, Deep Vent, *Pfu*, and r*Tma* polymerases were therefore tested for long PCR activity with r*Tth* polymerase. The r*Tth* and Vent polymerase combination was the most reliable: 0.015–0.15 U of Vent per 2–2.5 U of r*Tth* polymerase for high-copy PCRs or per 0.8–1 U of r*Tth* for low-copy PCRs (50-μl reaction mixtures). With limited optimization, combinations of r*Tth* and Deep Vent or r*Tma* polymerase or of r*Taq* and *Pfu* polymerase also facilitated long PCR but were less effective for amplifications of greater than 30–34 kb. Fig. 3 illustrates that the addition of Vent polymerase alone may not sufficiently enhance longer amplifications; both cosolvents and Vent were required.

**Template Integrity.** Longer templates have more sites susceptible to depurination and deamination at elevated temperatures (12, 14). Glycerol and DMSO allow use of lower

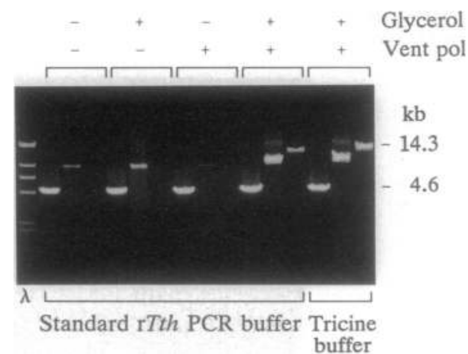


FIG. 3. Effect of adding 4% glycerol, 0.008 U of Vent polymerase (Vent pol), or both on yields of 4.6-, 9.4-, and 14.3-kb products (left to right in each set of lanes). Reaction mixtures (50 μl; 26 cycles) contained Perkin–Elmer r*Tth* PCR buffer (5% glycerol) or 20 mM Tricine (pH 8.7) and 85 mM KOAc, as indicated, 0.5 ng of λ DNA, 0.4 μM each primer, and 2.5 U of r*Tth* polymerase. $Mg(OAc)_2$ was 1.5 mM in the absence of Vent polymerase, 1.4 mM in its presence, or 1.2 mM in Tricine buffer. Aliquots of 4 μl were electrophoresed on a 0.6% agarose gel with λ/*Hin*dIII (lane λ).

denaturation temperatures and a shorter denaturation step, which may minimize damage during PCR. A higher pH for PCR is also protective, since depurination increases with lower pH (12). Tricine has a lower temperature coefficient ($\Delta pK_a$per °C = −0.021) than Tris (−0.031) (33) and has been recommended previously (9). For r*Tth* polymerase, 20–25 mM Tricine at pH 8.5–8.7 (25°C) was best; for r*Taq* polymerase, 25 mM Tris·HCl or Tris acetate at pH 8.9–9 was better. Enhancement of long PCR with a Tricine buffer is illustrated in Fig. 3.

**Salts.** $K^+$ concentrations reduced by 10–40% were more favorable to long PCR than standard levels (50 mM KCl for r*Taq* or 100 mM for r*Tth* polymerase). Lower KCl levels increase the processivity of these enzymes (R. Abramson, personal communication) and enhance the activity of r*Taq* polymerase (34) but may also reduce specificity. Fewer nonspecific products were observed with KOAc than with KCl. In the absence of added 3′-to-5′-exonuclease activity, long PCR was enhanced at total $Mg^{2+}$ levels of 1.7–2 mM. In the presence of proofreading activity, specificity was best at 1.0–1.25 mM total $Mg^{2+}$.

**Primers.** We found 20- to 23-mers of 52–60% G+C content to be successful primers with 68°C annealing and extension. Slightly longer primers (28- to 30-mers) were not advantageous for the λ system. To minimize yields of nontarget products, primers were at 0.4–0.5 μM for high-copy reactions and 0.15–0.2 μM for low-copy reactions.

**Amplification of λ Clones from Plaques.** One use for long PCR is amplification of inserts (typically 9–23 kb) from λ clones without prior labor- and time-consuming DNA isolation. Primers were therefore chosen at the end of the *J* gene and within the *cro* gene of λ, regions flanking the cloning site of most λ vectors. Phage were eluted from 100 randomly selected plaques of a human genomic library in λ FIX II and used directly for PCR. Fig. 4 shows analysis of PCR products from 30 such plaques. Digestion of each product with *Not* I separates the insert from flanking vector sequences, and the presence of both vector fragments confirms that the entire insert was amplified. Yields were variable, but inserts were successfully amplified from 91 of the 100 plaques. The negative results may be due to deletion of the flanking vector sequences, a low level of PCR-resistant sequences within the human genome, or otherwise inhibited reactions. Multiple bands obtained in 5 of the 91 positive clones were attributed to multiple clones in the plaque region selected. Nearly half of the inserts were >20 kb, and only four were <15 kb.

**Amplifications of Targets Within the Human β-Globin Gene Cluster.** For the human β-globin gene cluster, a fixed right-hand primer (5′ to the β-globin gene) was paired with a series of left-hand primers that extended upstream across the δ-globin gene and into the second intron of the $^A\gamma$-globin gene. Fig. 5 shows that up to 22 kb (lane 4) of the β-globin gene cluster could be amplified. Restriction analyses with *Bcl* I and *Eco*RI confirmed these products and suggested that the products would be suitable for sequencing. [A 400-bp region has been sequenced directly from PCR products of this globin series (Fred Reichert and S.C., unpublished data).] Notably, globin targets were amplified less efficiently than λ sequences at a single-copy level in a background of human placental genomic DNA, either at the same overall concentration as the globin target (data not shown) or at a 10-fold lower concentration—for example, compare lanes 3 (19.6-kb globin target) and 7 (20.8-kb λ target) of Fig. 5.

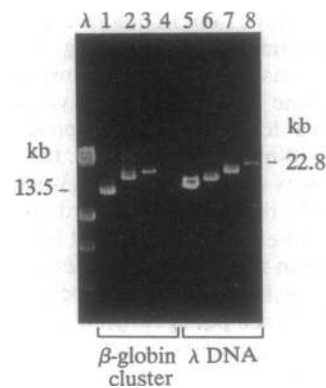


FIG. 5. Amplification of targets within the human β-globin gene cluster. Targets of 13.5, 17.7, 19.6, and 22 kb (lanes 1–4, respectively) were amplified from 37 ng (≈$10^4$ copies) of total human genomic DNA (36 cycles). Targets of 16.5, 18.8, 20.8, and 22.8 kb (lanes 5–8, respectively) were amplified under the same conditions from 0.05 pg ($10^3$ copies) of λ DNA in a background of 3.7 ng of human placental genomic DNA. Aliquots of 12.5 μl were electrophoresed on a FIGE gel with λ/*Hin*dIII (lane λ).

## DISCUSSION

The work presented here demonstrates the ability of PCR to amplify much greater lengths of genomic DNA than was previously possible. This ability to amplify sequences of 10–40 kb has many applications. For example, in genome maps, unclonable gaps of this size may be closed by amplification between flanking sequences. When mapping from cDNA sequences, much more of any gene can be amplified at once. The ability to make longer templates without laborious cloning, combined with PCR-based genome walking

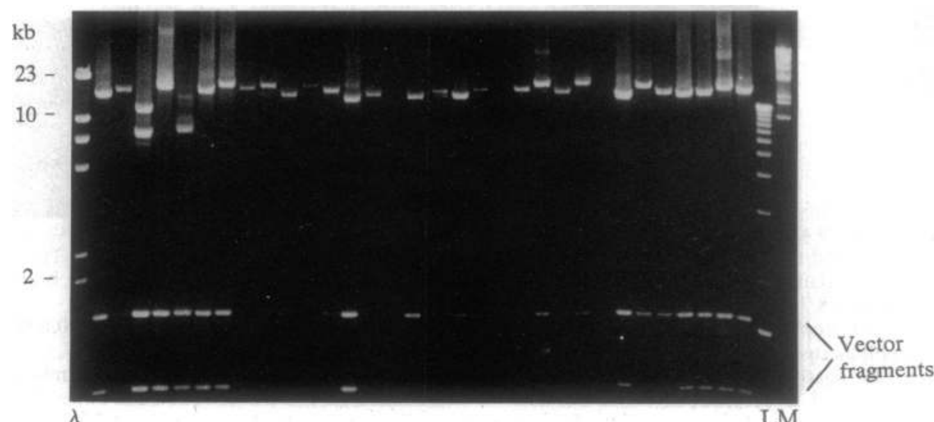


FIG. 4. Representative PCR products from a human genomic library cloned in λ FIX II. Plaques were amplified, digested with *Not* I to separate the insert from the flanking vector sequences, and analyzed by FIGE. Marker lanes are λ/*Hin*dIII (lane λ), 1-kb ladder (lane L), and λ/mono cut mixture (lane M).

(35, 36) and rapid sequencing—as by random hexamer primer walking (37)—may lead to automated genome sequencing. In medical genetics, PCR-based characterization of carriers of clinically relevant large insertions or deletions may be possible. For gene-transfer studies, whole-vector recombinant constructions can be made using PCR-based mutagenesis (38) and transfected without an intermediate cloning step. For cloned DNA, *J* and *cro* gene primers can be used to amplify inserts in nearly all λ-based vectors; cosmid inserts may eventually be amplifiable.

The reaction conditions described for the β-globin gene cluster are a useful starting protocol for amplifying long genomic targets. For optimization, annealing temperature and cosolvent, KOAc, or $Mg(OAc)_2$ levels may need adjustment. Higher r*Tth* polymerase levels may increase yields, unless specificity is adversely affected, and a Vent polymerase titration is recommended. Problems that we encountered within the β-globin gene cluster included secondary priming sites (due to clustered members of the gene family) and interspersed repetitive sequences such as *Alu* (39, 40). These elements of eukaryotic genomic DNA may explain why λ DNA targets were more efficiently amplified, even in a background of human genomic DNA. Amplifications of up to 16 kb from the human neurofibromatosis 1 gene also suggest that primer specificity will be a key to longer genomic PCR amplifications (S.C., unpublished data).

For amplifying even longer genomic regions, increased primer specificity, as through better primer design or nested primers (1), and the role of template damage need to be examined. Other issues requiring further study include the fidelity of DNA replication under these conditions and length-dependent preferential amplifications. The combination of Klentaq1 and *Pfu* polymerases gave a ≈13-fold increase in fidelity over that seen with r*Taq* polymerase alone (15); thus, there is reason to expect that the long PCR conditions reported here will have high fidelity. Length-dependent preferential amplifications are a concern with mixtures of target sizes, as in the case of differently sized alleles (e.g., variable number tandem repeats used for human identity; ref. 41); all sequences must be amplified without the more efficiently amplified shorter sequences predominating.

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