If the K-T boundary isotopic spike is indeed the result of impact-related acid rain, the oceanic strontium isotope record may reveal other large impacts. The seawater strontium curve of Burke et al. (9), which spans the past 500 million years, shows at least two other prominent high spikes in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio, one in the mid-Cretaceous, at ~100 million years, and the other in the Pennsylvanian, at ~290 million years. The first appears to precede by a few million years the mass extinction event at the Cenomanian-Turonian boundary. There is also a large increase in $^{87}\text{Sr}/^{86}\text{Sr}$ across the Permian-Triassic boundary (9), the time of the most extreme mass extinction in the Phanerzoic record (17). However, the increase appears to be rather gradual, extending over 20 million to 25 million years, and is thus quite different in character from the K-T spike. Nevertheless, data are sparse for this interval, and more work will be required to determine the exact nature of the increase.

The occurrence of a spike toward higher values in the seawater $^{87}\text{Sr}/^{86}\text{Sr}$ record at the K-T boundary is tantalizing evidence for enhanced continental weathering, possibly due to impact-related acid rain. Detailed strontium isotopic studies through this and other intervals where such spikes appear are required to determine precisely the nature of the isotopic variations with respect to stratigraphy, and particularly with respect to mass extinctions.

REFERENCES AND NOTES

8. Since strontium is removed from the oceans mainly by biogenic carbonate precipitation, removal rates may have been much less for some period after the K-T boundary event, leading to higher strontium concentrations in seawater and a temporary increase in “residence time.”
15. G. W. Bras, Geochim. Cosmochim. Acta 40, 721 (1976); this paper provides data for both strontium isotopic ratios and strontium-to-calcium ratios for limestones.
18. I thank many colleagues at Scripps for comments on the ideas expressed in this report, in particular G. Arrhenius, S. Gailer, J. Gieskes, M. Kastner, D. Lai, G. Lugmair, and H.-G. Stosch. Comments from two anonymous reviewers also improved the original manuscript. I thank P. Hey for preparation of the manuscript. This work was supported in part by grants from the National Science Foundation and the National Aeronautics and Space Administration.
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Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase

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A thermostable DNA polymerase was used in an in vitro DNA amplification procedure, the polymerase chain reaction. The enzyme, isolated from *Thermus aquaticus*, greatly simplifies the procedure and, by enabling the amplification reaction to be performed at higher temperatures, significantly improves the specificity, yield, sensitivity, and length of products that can be amplified. Single-copy genomic sequences were amplified by a factor of more than 10 million with very high specificity, and DNA segments up to 2000 base pairs were readily amplified. In addition, the method was used to amplify and detect a target DNA molecule present only once in a sample of $10^8$ cells.

THE ANALYSIS OF SPECIFIC NUCLEOTIDE SEQUENCES, like many analytic procedures, is often hampered by the presence of extraneous material or by the extremely small amounts available for examination. We have recently described a method, the polymerase chain reaction (PCR), that overcomes these limitations (1, 2). This technique is capable of producing a selective enrichment of a specific DNA sequence by a factor of $10^6$, greatly facilitating a variety of subsequent analytical manipulations. PCR has been used in the examination of nucleotide sequence variations (3-5) and chromosomal rearrangements (6), for high-efficiency cloning of genomic sequences (7), for direct sequencing of mitochondrial (8) and genomic DNAs (9, 10), and for the detection of viral pathogens (11).

PCR amplification involves two oligonucleotide primers that flank the DNA segment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridize to opposite strands of the target sequence and are oriented so DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Moreover, since the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific target fragment, approximately $2^n$, where $n$ is the number of cycles.

One of the drawbacks of the method, however, is the thermostability of the Klenow fragment of *Escherichia coli* DNA polymerase I used to catalyze the extension of the annealed primers. Because of the heat denaturation step required to separate the newly synthesized strands of DNA, fresh enzyme must be added during each cycle—a tedious and error-prone process if several samples are amplified simultaneously. We now describe the replacement of the *E. coli* DNA polymerase with a thermostable DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus* (Tag), that can survive extended incubation at 55°C (12). Since this heat-resistant polymerase is relatively unaffected by the denaturation step, it does not need to be replenished at each cycle. This modification not only simplifies the procedure, making it amenable to automation, it also substantially improves the overall performance of the reaction by increasing the specificity, yield, sensitivity, and length of targets that can be amplified.

Samples of human genomic DNA were subjected to 20 to 35 cycles of PCR amplification.

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The PCR primers direct the synthesis of a cation with optimal amounts of either genomic sequences under what are essential-merase reveals a broad molecular size distribution of amplification products (7) that is presumably the result of nonspecific annealing and extension of primers to unrelated genomic sequences under what are essentially nonstringent hybridization conditions: Klenow polymerase reaction buffer at 37°C.

Nevertheless, Southern blot analysis with a β-globin hybridization probe reveals the β-globin amplification fragment in all samples in which the β-globin target sequence was present.

A substantially different electrophoretic pattern is seen in the amplifications performed with Taq DNA polymerase, where the single predominant band is the 167-bp target segment. This specificity is evidently due to the temperature at which the primers are extended. Although the annealing step is performed at 40°C, the temperature of Taq polymerase-catalyzed reactions was raised to about 70°C, near the temperature optimum of the enzyme. (12). During the transition from 40°C to 70°C, poorly matched primer-template hybrids (which formed at 40°C) dissociate, and only highly complementary substrate remains as the reaction approaches the temperature at which catalysis occurs. Furthermore, because of increased specificity, there are fewer nonspecific extension products to compete for the polymerase, and the yields of the specific target fragment are higher.

As the values for extent of amplification and overall efficiency indicate (Fig. 1), the exponential accumulation of PCR amplification products is not an unlimited process. Eventually, a level of amplification is reached where more primer-template substrate has accumulated than the amount of enzyme present is capable of completely extending in the allotted time. When this occurs, the efficiency of the reaction declines, and the amount of PCR product accumulates in a linear rather than an exponential manner. Under the conditions described (Fig. 1), the Klenow polymerase reaction begins to “plateau” around the 20th cycle, after a 3 x 10^6-fold amplification of the β-globin target sequence. The higher specificity of the Taq polymerase-catalyzed reaction, however, permits it to proceed efficiently for an additional five cycles, to an amplification level of 4 x 10^6 before the activity of the polymerase becomes limiting (14).

The specificity of the Taq DNA polymerase-mediated amplifications can be affected by the time allowed for the primer extension step and by the quantity of enzyme used in the reaction. The electrophoretic patterns of PCR products obtained with different extension times and units of Taq DNA polymerase on otherwise identical samples indicate that the amount of nonspecific DNA decreases as the extension times become longer or the number of Taq polymerase units increases (Fig. 2A).

A significant improvement in specificity is obtained when the temperature of the primer annealing step is raised from 40°C to 55°C. This effect is demonstrated by the amplification of the β-globin gene in a set of dilutions of normal genomic DNA into the DNA of a mutant cell line with a homozygous deletion of the β-globin gene (Fig. 2B). These samples, each containing 2 μg of DNA, represent β-globin gene frequencies that range from one copy per genome (two copies per diploid cell) in the undiluted normal DNA sample, to as little as one copy per 10^6 genomes (one copy per 500,000 cells) in the 10^-6 dilution. After 40 cycles of PCR with primer annealing at 40°C, the specific amplification fragment can be seen in the 10^-2 dilution by electrophoretic examination and

Fig. 1. Comparison of Klenow and Taq DNA polymerase-catalyzed PCR amplification products of the human β-globin gene. (A) Electrophoretic analysis of the PCR products obtained with Klenow polymerase (lanes 1 to 6) and Taq polymerase (lanes 7 to 12) after 0 cycles (lanes 1 and 7), 20 cycles (lanes 2 and 8), 25 cycles (lanes 3 and 9), 30 cycles (lanes 4 and 10), and 35 cycles (lanes 5, 6, 11, and 12) of amplification. The DNA samples that were amplified were prepared from the human cell lines MOLT4 (lanes 1 to 5 and 7 to 11) and GM2064 (lanes 6 and 12). MOLT4 is homozygous for the normal β-globin gene (2), GM2064 possesses a homozygous deletion of the entire β-globin gene complex (21). The molecular size marker is 250 ng of Hae III-digested dX174 replicative form (RF) DNA (New England Biolabs). (B) Southern analysis of the gel with a 32P-labeled oligonucleotide probe. Compared against standards, the intensities of the bands in the Klenow amplifications were estimated to be equivalent to increases of 2.2 x 10^6, 1.1 x 10^6, 2.2 x 10^6, and 2.2 x 10^6 (lanes 2 to 5) with corresponding overall reaction efficiencies of 87, 74, 63, and 52% (calculated according to (2)). The values for the intensities of the bands in the corresponding Taq reactions were 2.8 x 10^6, 4.5 x 10^6, 8.9 x 10^6, and 1.7 x 10^6 (lanes 8 to 11) with overall efficiencies of 87, 85, 70, and 61%.

Amplification of genomic targets by PCR with Klenow polymerase was performed as described (7).

**B**

Briefly, 100-μl reactions containing 1 μg of genomic DNA in 50 mM NaCl, 10 mM Tris (pH 7.6), 10 mM MgCl₂, 10% dimethyl sulfoxide, 1 μM each primer (PC03 and KM38), and 1.5 mM each of the four deoxynucleoside triphosphates (dNTP: dATP, dCTP, dTTP, dGTP). The reactions were performed by 20 to 35 repetitions (cycles) as follows. The samples were heated from 37°C to 95°C over a 2.5-minute period (to denature the DNA) and cooled to 37°C (3 minutes) (to anneal the primers); 1 unit of Klenow polymerase (USB) was added to each sample and then incubated at 37°C for 2 minutes (to extend the bound primers). Amplifications with Taq polymerase took place in 100-μl reaction mixtures containing 1 μg of genomic DNA in 50 mM KCl, 10 mM Tris (pH 8.4), 2.5 mM MgCl₂, each primer (PC03 and KM38) at 1 μM, each dNTP (dATP, dCTP, dTTP, dGTP) at 200 μM, gelatin at 200 μg/ml, and 2 units of polymerase. The samples were overlaid with several drops (~100 μl) of mineral oil to prevent condensation and subjected to 20 to 35 cycles of amplification as follows. The samples were heated from 70°C to 95°C over a 1-minute period (to denature the DNA), cooled to 40°C over 2 minutes (to anneal the primers), heated to 70°C in 1 minute (to “activate” the polymerase), and incubated at that temperature for 0.5 minute (to extend the annealed primers). Additional Taq DNA polymerase was not added to the samples during amplification. One unit of enzyme is the amount that will incorporate 10 nmol of total deoxynucleoside triphosphates into acid-precipitable material in 30 minutes at 74°C with activated salmon sperm DNA as template (12). The enzyme was prepared from *T. aquaticus*, strain YT1, by a modification of published procedures (22). Thermal cycling was performed in a programmable heat block (Perkin Elmer–Cetus Instruments). After the last cycle, all samples were incubated for an additional 5 to 10 minutes at 37°C or 70°C to ensure that the final extension step was complete. After precipitation with ethanol and resuspension in 100 μl TEB buffer (20), each sample (8 μl) was resolved at a composite gel of 3% NuSieve and 1% SeaKem agarose (FMC) in tris-borate buffer and stained with ethidium bromide (20). Southern transfers were performed essentially as described (23) onto Genetrans-45 nylon membranes (Pall). A 19-base oligonucleotide probe specific for the amplified β-globin fragment, 19A, was 5′ end-labeled with 32P and hybridized to the filter (3). The autoradiogram was exposed for 3 hours with a single intensification screen.

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in the 10^{-4} dilution by Southern analysis (15). However, in parallel samples amplified with 55°C annealing, much less nonspecific DNA is present and the product band is visible in the original gel in the 10^{-4} dilution and, by Southern analysis, in the 10^{-6} dilution (15). Although annealing at 55°C is usually of limited value when amplifying genomic targets present at one or two copies per cell (Fig. 2B, lane 1 compared to lane 9), the reduction in the amount of nonspecific primer extension products improves the limit of sensitivity by two orders of magnitude. Since 2 μg of the 10^{-6} dilution would contain, on average, only 0.6 copy of the β-globin gene, these data suggest that PCR with Taq polymerase may be able to amplify a single target molecule in 10^{6} cells.

This result was confirmed by demonstrating a Poisson distribution of successful amplifications on limiting amounts of the β-globin template. Fifteen identical 1-μg samples contained 2 μg of DNA and were amplified for 40 cycles with either 400 or 550°C annealing with 60 minutes (lanes 1 to 4, respectively); and by increasing extents of Southern analysis of specificity as affected by increasing extension and units (lanes 5 to 8, respectively); and by increasing amounts of enzyme: 0.25, 0.5, 1, 2, 4, and 8 units (lanes 5 to 10, respectively). (B) Electrophoretic examination of the effect of annealing temperature on specificity at 40°C (lanes 1 to 8) and 55°C (lanes 9 to 16) with serial dilutions of MOLT4 DNA in GM2064 DNA: undiluted MOLT4 (lanes 1 and 9), 10^{-1} (lanes 2 and 10), 10^{-2} (lanes 3 and 11), 10^{-3} (lanes 4 and 12), 10^{-4} (lanes 5 and 13), 10^{-5} (lanes 6 and 14), 10^{-6} (lanes 7 and 15), and undiluted GM2064 (lanes 8 and 16). Molecular size marker was as described (Fig. 1). The amplifications were generally conducted as described (Fig. 1). The reactions for (A) contained 1 μg of DNA and were subjected to 30 cycles with the primers PC03 and PC04 (110-bp product) with the indicated times of extension and units of enzyme. The samples for (B) each contained 2 μg of DNA and were amplified for 40 cycles with either 400°C or 55°C annealing, with the primers RS79 and RS80 (150-bp product). A portion of the reaction, 5 μl, was subjected to electrophoresis on a NuSieve-SeaKem agarose gel.

Fig. 2. Factors that influence the specificity of Taq polymerase-catalyzed amplifications. (A) Electrophoretic analysis of specificity as affected by increasing extension times: 0, 0.5, 2, and 8 minutes (lanes 1 to 4, respectively); and by increasing amounts of enzyme: 0.25, 0.5, 1, 2, 4, and 8 units (lanes 5 to 10, respectively). (B) Electrophoretic examination of the effect of annealing temperature on specificity at 40°C (lanes 1 to 8) and 55°C (lanes 9 to 16) with serial dilutions of MOLT4 DNA in GM2064 DNA: undiluted MOLT4 (lanes 1 and 9), 10^{-1} (lanes 2 and 10), 10^{-2} (lanes 3 and 11), 10^{-3} (lanes 4 and 12), 10^{-4} (lanes 5 and 13), 10^{-5} (lanes 6 and 14), 10^{-6} (lanes 7 and 15), and undiluted GM2064 (lanes 8 and 16). Molecular size marker was as described (Fig. 1). The amplifications were generally conducted as described (Fig. 1). The reactions for (A) contained 1 μg of DNA and were subjected to 30 cycles with the primers PC03 and PC04 (110-bp product) with the indicated times of extension and units of enzyme. The samples for (B) each contained 2 μg of DNA and were amplified for 40 cycles with either 400°C or 55°C annealing, with the primers RS79 and RS80 (150-bp product). A portion of the reaction, 5 μl, was subjected to electrophoresis on a NuSieve-SeaKem agarose gel.

Fig. 3. Poisson distribution of single target sequences in samples of 10^{6} cells. (A) Electrophoretic analysis of PCR products in a set of 15 samples (lanes 1 to 15). The arrow indicates the position of the 150-bp amplification product that is visible in some samples (lanes 3, 4, and 6). Molecular markers as described (Fig. 1). (B) Southern analysis of the gel with a β-globin-specific oligonucleotide probe. Fifteen 1-μg samples of the 10^{-6} dilution of MOLT4 DNA in GM2064 DNA prepared previously (Fig. 2) were amplified for 60 cycles with 55°C annealing, and the primers RS79 and RS80. The amplified samples (5 μl) were resolved in a NuSieve-SeaKem gel and transferred onto a nylon membrane. The filter was probed with a 32P-labeled (5′ end) oligonucleotide probe, RS81, specific for the 150-bp β-globin amplification product. Methods were essentially as described in Fig. 1 and 2.
phage recombinants, eliminating the need for plasmid or phage growth, vector purification, and insert isolation.

The fidelity of the thermostable Taq polymerase in the amplification reaction was assessed by cloning and sequencing individual amplification products (7) with primers that define a region of the HLA-DPB gene (16). The products of 30-cycle PCR amplifications were cloned into an M13 vector, multiple isolates of the same allele obtained, and their sequences compared. In 28 separate clones, each with 239 bp of amplified DPB genomic DNA, 17 misincorporated bases were identified representing an overall misincorporation rate of 0.25% (17). These misincorporations occurred throughout the amplified product and no deletions or insertions were detected. Because each misincorporation event is retained and propagated through succeeding cycles of amplification, the frequency of errors observed in the cloned products is a direct function of the number of doublings; the actual rate of misincorporation is lower. If constant over the 30 PCR cycles, the misincorporation rate per nucleotide per cycle for Taq polymerase is estimated at $2 \times 10^{-4}$ (17).

Although this value is somewhat greater than the $8 \times 10^{-5}$ misincorporation rate composed with Klenow polymerase–catalyzed PCR (7, 18), the errors made by Taq DNA polymerase should not be a problem. Analytic procedures that use a significant portion of the reaction product, such as direct sequencing (see below) or filter hybridization with allele-specific oligonucleotide probes (3), are not affected by the small fraction of misincorporated bases. Cloning and sequencing individual amplification fragments may include these errors, but they are readily identified by analyzing several isolates and establishing a consensus. A difficulty could arise if PCR were attempted on a sample initially containing only a few copies of the target template. In that situation, a misincorporation during the early stages of the reaction would represent a substantial fraction of the molecules present and could complicate the analysis of the amplification product. However, the amplification and comparison of several samples would reveal and resolve any inconsistencies.

Even though the Klenow DNA polymerase has better fidelity in PCR amplification, it is more likely to produce a different type of sequence artifact. Some PCR products made by the Klenow enzyme were composed of a mosaic of the different alleles being amplified. These “shuffled” clones apparently arise from incomplete extension of the annealed primer during one cycle. In later cycles, these incomplete products may hybridize to other allelic templates and be extended, thus producing the mosaic (18). Few shuffled clones have been observed with the Taq DNA polymerase, which may be the result of higher processivity.

The higher specificity of Taq polymerase–mediated amplifications can facilitate the direct sequencing of single-copy human genes, particularly those that may be members of a gene family (10). A 110-bp fragment of the β-globin gene was amplified

![Fig 4. Examination of the ability of Klenow and Taq DNA polymerases to amplify longer target segments.](image)

**A** Autoradiogram of Southern filter comparing molecular markers as described (Fig. 1). (A) Klenow polymerase–catalyzed and (B) Taq polymerase–catalyzed amplification reactions. Nucleotide sequences determined from the cloned products were compared to the appropriate fragments of human genomic DNA (19). Approximately 0.1 to 1.0 pmol of microconcentrator-purified PCR product and 3 to 5 pmol of synthetic sequencing primer, RH909, were combined in 10 μl of 50 mM KCl, 50 mM Tris (pH 8.0), 5 mM MgCl₂, and 10 mM dithiothreitol. The reaction was heated at 95°C for 10 minutes then quenched on ice, and 2.2 μl was added to each of four 2-μl solutions: "ddG" is 20 μM dGTP, 10 μM dATP, 100 μM TTP, and 100 μM dCTP; "ddA" is 100 μM dATP, 10 μM dTTP, 100 μM dCTP, and 2.5 μM dGTP; "ddT" is 100 μM dGTP, 10 μM dATP, 100 μM dCTP, and 2.5 μM dTTP; "ddC" is 100 μM dGTP, 10 μM dATP, 100 μM dCTP, and 2.5 μM dTTP. The reaction mixtures were heated for 5 minutes at 95°C before loading 2.5 μl on an 8% polyacrylamide sequencing gel (25). Five units of AMV reverse transcriptase (Life Sciences) was added to each reaction and incubated at 37°C for 15 minutes. The reactions were then held for an additional 15 minutes after the addition of 1 μl of a solution containing 1 mM each dNTP. Five microliters of formamide-dye stop mix was added to each reaction and heated for 5 minutes at 95°C before loading 2.5 μl on an 8% polyacrylamide sequencing gel. Electrophoresis and autoradiography were by standard techniques (20).

![Fig 5. Amplification of inserts in a phage λ cDNA library. Lanes 1 to 15, phages containing inserts; lane 16, phage without a detectable insert. Molecular markers are either 500 ng of BstE II-digested λ DNA (lanes a) or 250 ng of Hae III–digested χ174 RF DNA (lanes b). A λgt11 human fibroblast cDNA library (Clontech) was plated on Xgal plates at high dilution by standard techniques (20). Well-isolated plaques were selected at random, 15 clear (with insert) and 1 blue (without insert), and excised with the tip of a Pasteur pipette. The agarose plugs were eluted in 0.2 ml of deionized water for 30 minutes, and 50 μl of the eluates was subjected to 25 cycles of amplification as described (Fig. 1). The primers used were two 24-base sequencing primers, 1218 and 1222 (New England Biolabs) that flank the Eco RI insertion site of the vector. Each of the amplified samples (10 μl) was resolved on a 1.4% SeaKem agarose gel.
with either Klenow or Taq polymerases. A third primer, complementary to a region of the DNA between the two PCR primers, was end-labeled with 32P and used in the chain-termination sequencing reaction (19). The sequence of the Klenow polymerase–catalyzed amplification product displays base pair ambiguities at several positions (Fig. 6). The origin of these extra bands is attributed to the presence of β-globin gene sequences. The β-globin gene is closely related to β-globin, and both of the PCR primers match β-globin at 18 out of 20 positions (2). Because of the relative nonspecificity of the Klenow-mediated amplifications, β-globin is coamplified to at least 10% of the level of β-globin (7). However, the higher specificity of Taq polymerase reactions performed with 55°C annealing does not permit the primers to anneal to β-globin and only the β-globin segment is amplified (Fig. 6).

The amplification of RNA transcripts can also be performed with Taq polymerase PCR. After conversion of the messenger RNA (mRNA) to first-strand cDNA with oligo(dT) primers and reverse transcriptase by standard methods (20), the resulting single-stranded cDNA can be directly amplified by PCR. With the HLA-DQA PCR primers reported previously (7), mRNA transcripts present at about 0.01% in 100 ng of cDNA prepared from lymphoblastoid polyadenylated [poly(A) + ] RNA could be easily amplified to generate approximately 1 μg of the specific 242-bp amplification fragment.

Our data demonstrate the highly specific nature of Taq polymerase–mediated PCR and its effect on the efficiency and sensitivity of the reaction. The amplification of both DNA and RNA targets was readily accomplished by means of this thermostable enzyme, often with yields and purities comparable to fragments prepared from clonally isolated recombinants. This facilitates rapid sequence analysis of mutants and variants at a known locus by allowing the PCR product to be sequenced directly. Similarly, the analysis of unknown sequences could be expedited by PCR amplification of the cloned segments with vector-specific primers that flank the insertion site. The ability to amplify and manipulate a target sequence present only once in a sample of 10^5 to 10^8 cells should prove valuable in many areas of molecular biology. Clinical applications include the diagnosis of infectious diseases and of rare pathologic events such as chromosomal translocations. Moreover, the sensitivity of the procedure should enable the analysis of gene expression or rearrangement in single cells. By virtue of the exponential accumulation of literally billions of copies derived from a single progenitor sequence, PCR based on Taq DNA polymerase represents a form of “cell-free molecular cloning” that can accomplish in an automated 3- to 4-hour in vitro reaction what might otherwise take days or weeks of biological growth and biochemical purification.

REFERENCES AND NOTES
14. The “plateau” effect is not directly determined by the number of cycles or degree of amplification. Rather, it is the concentration of total PCR product, the concentration of the primer, and the length of extension time at 72°C that defines the conditions under which the activity of the enzyme becomes limiting. Sufficient molar excesses of deoxynucleotidetriphosphates and primers were present in the reactions so the consumption of these reagents was not a factor.
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17. Thirteen of the errors involved transitional changes (where one nucleotide replaces the other), ten of them resulting in a G-C pair. Of the four transversional misincorporations (where a purine nucleotide replaces a pyrimidine), two were A-T to T-A and two were G-C to T-A. The formula used to calculate the misincorporation rate is m = 2(f/d), where f is the observed error frequency in the PCR product and d is the number of doublings. [W. Hayes, The Genetics of Bacteria and Their Viruses (Wiley, New York, 1965).]
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26. We thank T. L. Bugawan and C. Long for assistance with cloning and sequencing of PCR products; L. Johnson, R. Leath, D. Jones, and J. Widsanos for engineering and instrument support; D. Carl in for statistical interpretation; S. Nison and E. Ladner for graphic services; A. Wilson for the phrase “cell-free cloning”; and J. Sninsky and T. White for advice and encouragement. 9 October 1987; accepted 17 December 1987.