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 $^{87}Sr/^{86}Sr$ 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 $^{87}Sr/^{86}Sr$ across the Permian-Triassic boundary (*9*), the time of the most extreme mass extinction in the Phanerozoic 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 $^{87}Sr/^{86}Sr$ 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.

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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 10^5 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 thermolability 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* (*Taq*), that can survive extended incubation at 95°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 amplifi-

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cation with optimal amounts of either Klenow or *Taq* DNA polymerase and analyzed by agarose gel electrophoresis (Fig. 1A) and Southern hybridization (Fig. 1B). The PCR primers direct the synthesis of a 167-bp segment of the human β-globin gene (*13*). Electrophoretic examination of the reactions catalyzed by the Klenow polymerase 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° 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 3×10^5-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 4×10^6 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 increases 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° 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

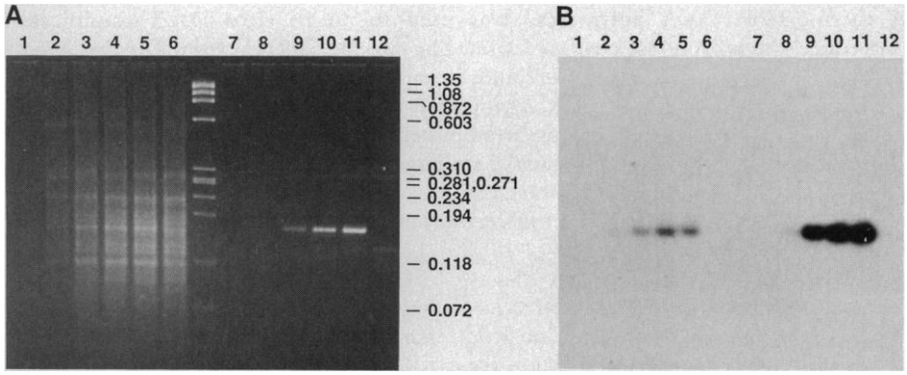

Fig. 1. Comparison of Klenow and *Taq* DNA polymerase–catalyzed PCR amplification products of the human β-globin gene. (**A**) Electrophoretic analysis of the PCR products obtained with Klenow polymerase (lanes 1 to 6) and *Taq* polymerase (lanes 7 to 12) after 0 cycles (lanes 1 and 7), 20 cycles (lanes 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 φX174 replicative form (RF) DNA (New England Biolabs). (**B**) Southern analysis of the gel with a ^{32}P-labeled oligonucleotide probe. Compared against standards, the intensities of the bands in the Klenow amplifications were estimated to be equivalent to increases of 2.8×10^5, 1.1×10^6, 2.2×10^6, and 2.2×10^6 (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 2.8×10^5, 4.5×10^6, 8.9×10^6, and 1.7×10^7 (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*). Briefly, 100-μl reactions containing 1 μg of genomic DNA in 50 m*M* NaCl, 10 m*M* tris (*p*H 7.6), 10 m*M* $MgCl_2$, 10% dimethyl sulfoxide, 1 μ*M* each primer (PC03 and KM38), and 1.5 m*M* each of the deoxyribonucleotide triphosphates (dNTP: dATP, dCTP, TTP, dGTP). The reactions were performed by 20 to 35 repetitions (cycles) as follows. The samples were heated from 37° 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 m*M* KCl, 10 m*M* tris (*p*H 8.4), 2.5 m*M* $MgCl_2$, each primer (PC03 and KM38) at 1 μ*M*, each dNTP (dATP, dCTP, TTP, dGTP) at 200 μ*M*, 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° 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 deoxyribonucleotide 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° or 70°C to ensure that the final extension step was complete. After precipitation with ethanol and resuspension in 100 μl TE buffer (*20*), each sample (8 μl) was resolved on a composite gel of 3% NuSieve and 1% SeaKem agaroses (FMC) in tris-borate buffer and stained with ethidium bromide (*20*). Southern transfers were performed essentially as described (*23*) onto Genetrans-45 nylon membranes (Plasco). A 19-base oligonucleotide probe specific for the amplified β-globin fragment, 19A, was 5′ end-labeled with ^{32}P and hybridized to the filter (*3*). The autoradiogram was exposed for 3 hours with a single intensification screen.

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 10^5 to 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 of the same 10^{-6} dilution were amplified for 60 cycles with annealing at 55°C and analyzed for the presence of a β-globin amplification product by Southern hybridization. Each of these samples of genomic DNA (equivalent to that in 150,000 cells) should have, on average, 0.3 copy of the β-globin gene. The fraction of those samples that contain at least one copy is expressed as

$$1 - e^{-0.3} = 0.26$$

or 4 out of 15; the remaining 11 should contain no β-globin templates. The observed frequency was 9 of 15 (Fig. 3). Although this is twice as high as the expected value, it does not significantly affect the interpretation of the results. The distribution of successful amplifications is consistent with a concentration of approximately one β-globin gene per 500,000 cells which indicates that of the nine positive samples, most, if not all, originally contained a single copy of the β-globin gene. If only one target molecule was present initially, the intensities of the PCR products in the 10^{-6} samples are equivalent to amplifications of about 10^9 to 10^{10}. This experiment supports the conclusion that *Taq* polymerase–mediated PCR performed with primer annealing at 55°C is able to successfully amplify a single target molecule in a DNA background of 10^5 cells. Subsequent investigations have demonstrated that the reaction can detect a single copy of target DNA in 10 μg of DNA, the equivalent of 1.5 million diploid cells (*15*).

The variation in the electrophoretic pattern of PCR products (Fig. 3A) probably reflects the stochastic nature of nonspecific priming events. The stringency of the primer annealing step is sufficiently high that the priming of a nontarget sequence is rare and does not always occur in every sample. Only when such a priming event occurs during the first few cycles of amplification is there an opportunity for an electrophoretically visible band to accumulate. Similarly, the variability of the intensities of the specific β-globin signal (Fig. 3B) is presumably the result of the failure of the polymerase to locate and extend all template strands during the first few cycles of the reaction. Because there is initially only one target molecule, any inefficiency of this sort would strongly effect the final yield of amplification product.

Three pairs of primers which, in various combinations, define segments of 110 to 400 bp were used to examine the relative abilities of the Klenow and *Taq* polymerases to synthetize longer PCR products. Southern analysis of the reaction products after 25 cycles of amplification reveals that the Klenow polymerase does not sustain the exponential accumulation of DNA sequences much greater than 250 bp (Fig. 4A). The *Taq* polymerase, in contrast, can readily effect the synthesis of segments longer than 400 bp. With other primers and longer extension times, this enzyme can amplify genomic target sequences of up to 2.0 kb (Fig. 4B); longer fragments can be synthesized but with reduced efficiency and yield.

In an application exploiting this capability of *Taq* polymerase, complementary DNA (cDNA) inserts in the phage λgt11 cloning vector were amplified from crude phage suspensions with primers that flank the Eco RI insertion site of the vector. We chose 16 plaques at random—15 with inserts and 1 without—and subjected them to 25 cycles of amplification. A single band corresponding to the amplified insert DNA was observed for each phage isolate; these fragments were 400 to 2000 bp in length (Fig. 5). The phage that did not contain an insert produced an 87-bp fragment, the distance between the primers on the uninterrupted vector. Between 0.5 and 1.0 μg of each cDNA insert fragment was synthesized, which, on the basis of 10^6 phage per plaque, represents an amplification of approximately 10^6. The extent and purity of these amplifications suggest that PCR with *Taq* DNA polymerase may be an effective and efficient way to isolate the inserts of plasmid or

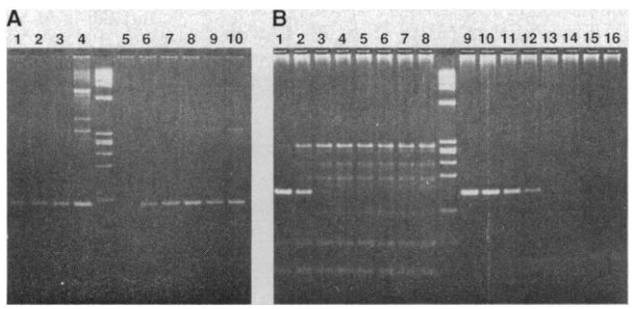

Fig. 2. Factors that influence the specificity of *Taq* polymerase–catalyzed amplifications. (**A**) Electrophoretic analysis of specificity as affected by increasing extension times: 0, 0.5, 2, and 8 minutes (lanes 1 to 4, respectively); and by increasing amounts of enzyme: 0.25, 0.5, 1, 2, 4, and 8 units (lanes 5 to 10, respectively). (**B**) Electrophoretic examination of the effect of annealing temperature on specificity at 40° (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 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 40° 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.

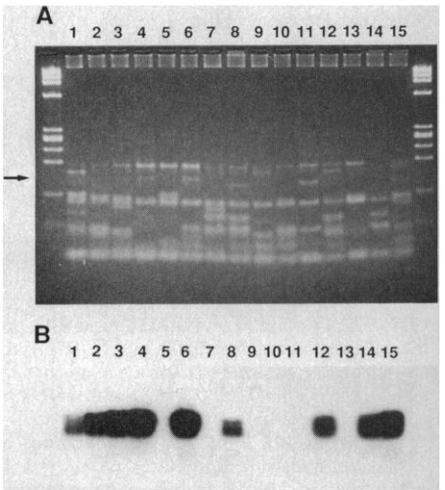

Fig. 3. Poisson distribution of single target sequences in samples of 10^5 cells. (**A**) Electrophoretic analysis of PCR products in a set of 15 samples (lanes 1 to 15). The arrow indicates the position of the 150-bp amplification product that is visible in some samples (lanes 3, 4, and 6). Molecular 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 ^{32}P-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-DPβ 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 DPβ genomic DNA, 17 misincorporated bases were identified representing an overall error frequency 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 cumulative and a 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 2×10^{-4} (*17*).

Although this value is somewhat greater than the 8×10^{-5} misincorporation rate observed 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

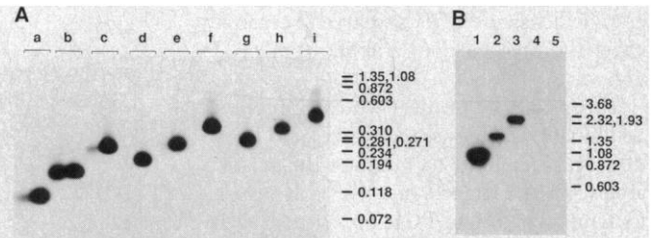

Fig. 4. Examination of the ability of Klenow and *Taq* DNA polymerases to amplify longer target segments. (**A**) Autoradiogram of Southern filter comparing relative efficiencies of Klenow and *Taq* polymerase at amplifying fragments of increasing size. The lanes are grouped in pairs, Klenow polymerase amplifications on the left and *Taq* polymerase amplifications on the right, according to the primers used: 110 bp, primers PC03 and PC04; 167 bp, PC03 and KM38; 250 bp, PC03 and GH21; 205 bp, KM29 and PC04; 262 bp, KM29 and KM38; 345 bp, KM29 and GH21; 268 bp, GH20 and PC04; 325 bp, GH20 and KM38; and 408 bp, GH20 and GH21 (lane pairs a to i, respectively). Molecular markers as described (Fig. 1). (**B**) Autoradiogram of Southern blot for examining capacity of *Taq* polymerase to synthesize products greater than 1 kb: 989 bp, RS40 and RS80; 1390 bp, GH20 and RS80; 1988 bp, GH20 and RS114; 2489 bp, GH20 and RS115; and 3015 bp, GH20 and RS116 (lanes 1 to 5, respectively). Molecular marker is a combination of BstE II–digested λ DNA and Hae III–digested φX174 RF DNA. Amplifications were performed for 25 (A) or 30 cycles (B) with the indicated primers and 2 minutes (A) or 10 minutes (B) primer extension steps. Electrophoretic resolution and Southern analysis were performed on 10 μl of each sample.

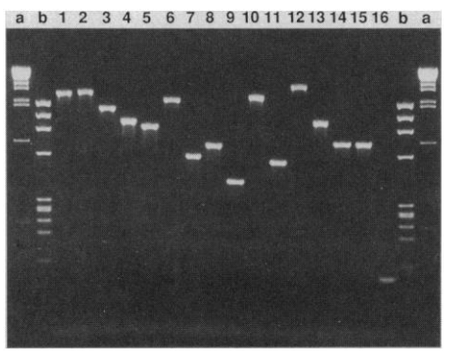

Fig. 5. Amplification of inserts in a phage λ cDNA library. Lanes 1 to 15, phages containing inserts; lane 16, phage without a detectable insert. Molecular markers are either 500 ng of BstE II–digested λ DNA (lanes a) or 250 ng of Hae III–digested φX174 RF DNA (lanes b). A λgt11 human fibroblast cDNA library (Clontech) was plated on X-gal 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.

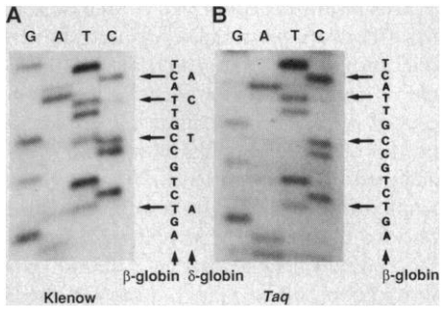

Fig. 6. Direct sequencing of a PCR-amplified human genomic target. Autoradiograms of sequencing gels derived from (**A**) Klenow polymerase–catalyzed and (**B**) *Taq* polymerase–catalyzed amplification reactions. Nucleotide sequences determined from the films are listed beside the autoradiograms, including the ambiguous bases ascribed to δ-globin. A 110-bp region of β-globin was amplified in samples of MOLT4 DNA with the primers PC03 and PC04 and either Klenow or *Taq* polymerase (Fig. 1). The sequencing of the PCR products was done as described (*8*) with modifications to permit the substitution of reverse transcriptase for the Klenow fragment of DNA polymerase I (*24*). Approximately 0.1 to 1.0 pmol of microconcentrator-purified PCR product and 3 to 5 pmol of ^{32}P-labeled sequencing primer, RH09, were combined in 10 μl of 50 m*M* KCl, 50 m*M* tris (*p*H 8.0), 5 m*M* $MgCl_2$, and 10 m*M* 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: "dddG" is 20 μ*M* dGTP, 10 μ*M* dATP, 100 μ*M* TTP, 100 μ*M* dCTP, and 6 μ*M* ddGTP; "dddA" is 100 μ*M* dGTP, 10 μ*M* dATP, 100 μ*M* TTP, 100 μ*M* dCTP, 2.5 μ*M* ddATP; "dddT" is 100 μ*M* dGTP, 10 μ*M* dATP, 20 μ*M* TTP, 100 μ*M* dCTP, 15 μ*M* ddTTP; "dddC" is 100 μ*M* dGTP, 10 μ*M* dATP, 100 μ*M* TTP, 20 μ*M* dCTP, 5 μ*M* ddCTP [each in 50 m*M* KCl, 50 m*M* tris (*p*H 8.0), 5 m*M* $MgCl_2$, and 10 m*M* dithiothreitol]. Five units of AMV reverse transcriptase (Life Sciences) was added to each reaction and incubated at 37°C for 15 minutes. The reactions were held for an additional 15 minutes after the addition of 1 μl of a solution containing 1 m*M* 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 (*25*). Electrophoresis and autoradiography were by standard techniques (*20*).

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 ^{32}P 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 δ-globin gene sequences. The δ-globin gene is closely related to β-globin, and both of the PCR primers match δ-globin at 18 out of 20 positions (*2*). Because of the relative nonspecificity of the Klenow-mediated amplifications, δ-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 δ-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-DQα 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 10^6 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.

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13. Names and sequences of the synthetic oligonucleotides used in this report. GH20: GAAGAGCCAAGGACAGGTAC, GH21: GGAAAATAGACCAATAGGCAG, KM29: GGTTGGCCAATCTACTCCCAGG, KM38: TGGTCTCCTTAAACCTGTCTTG, PC03: ACACAACTGTGTTCACTAGC, PC04: CAACTTCATCCACGTTCACC, RH09: AACCTCAAACAGACACC, RS40: ATTTTCCCACCCTTAGGCTG, RS79: CATGCCTCTTTGCACCATTC, RS80: TGGTAGCTGGATTGTAGCTG, RS81: CTGGGTTAAGGCAATAGCA, RS114: CCTCCAAATCAAGCCTCTAC, RS115: ATCCTGAGGAAGAATGGGAC, RS116: GTTTGATGTAGCCTCACTTC, 19A: CTCCTGAGGAGAAGTCTGC, 1218: GGTGGCGACGACTCCTGGAGCCCG, 1222: TTGACACCAGACCAACTGGTAATG.
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 enzyme, and the length of extension time at 70°C that defines the conditions under which the activity of the enzyme becomes limiting. Sufficient molar excesses of deoxyribonucleotide triphosphates 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 purine 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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Genomic Amplification with Transcript Sequencing

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A sequencing method called genomic amplification with transcript sequencing (GAWTS) is described that is based on amplification with the polymerase chain reaction (PCR). GAWTS bypasses cloning and increases the rate of sequence acquisition by at least fivefold. The method involves the attachment of a phage promoter onto at least one of the PCR primers. The segments amplified by PCR are transcribed to further increase the signal and to provide an abundance of single-stranded template for reverse transcriptase–mediated dideoxy sequencing. An end-labeled reverse transcriptase primer complementary to the desired sequence generates the additional specificity required to generate unambiguous sequence data. GAWTS can be performed on as little as a nanogram of genomic DNA. The rate of GAWTS can be increased by coamplification and cotranscription of multiple regions as illustrated by two regions of the factor IX gene. Since GAWTS lends itself well to automation, further increases in the rate of sequence acquisition can be expected.

IN CONTRAST TO AUTOSOMAL RECESsive mutations, deleterious X-linked mutations are eliminated within a few generations because the affected males reproduce sparingly if at all. Thus, each family in an X-linked disease such as hemophilia B represents an independent mutation. From the perspective of efforts to understand the

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