PERSPECTIVE: ANALYTICAL BIOTECHNOLOGY

DNA Amplification by the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) is a technique involving enzymatic amplification of nucleic acid sequences via repeated cycles of denaturation, oligonucleotide annealing, and DNA polymerase extension. PCR has revolutionized the practice of DNA technology as it allows virtually any nucleic acid sequence to be readily generated in vitro in relatively great abundance, so that subsequent analyses are not confounded by the presence of other DNA fragments or a lack of material with which to work. PCR also enables the sequence of individual DNA fragments to be altered. The method has advantages over conventional procedures for DNA cloning and analysis in many circumstances because it is faster, simpler, and more flexible. The total range and number of applications that have evolved in the short time since the first report of PCR are enormous. This review describes some of the history of PCR, the principle of the method, practical considerations for performing PCR, and a variety of applications.

I. INTRODUCTION

The polymerase chain reaction (PCR) is a recently developed procedure for the in vitro amplification of DNA sequences that has gained widespread acceptance in many areas of molecular biology (*1-5*). The method is so simple and useful that it has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed. Since the first description of the method, more than 800 publications involving PCR and its applications have been reported (*6*). Variations and developments of the basic procedure continue to be described an an increase rate, which can provide considerable confusion for those wishing to adapt PCR for their own studies. It appears likely that the method will be a part of the tools of the molecular biologist for some time to come, and this review aims both to update those familiar with PCR with some recent developments and to provide the novice with some insight into the potential of this procedure.

PCR was conceived and demonstrated to be a practical method for manipulating nucleic acid fragments by Dr. Kary Mullis in 1983 while working at the Cetus Corporation in Emeryville, CA (*7, 8*). The invention grew from a theoretical scheme to perform limited dideoxynucleotide sequencing of unique human genes using synthetic oligonucleotides for the purpose of diagnosing common human disease mutations. The high complexity of the human genome (3.3×10^9 base pairs) was an obvious obstacle to such a direct sequencing strategy, and so a second oligonucleotide was added to the scheme. The additional primer was initially conceived to block the progression of the synthesis of the first primer, but later it was included to bind to the other DNA strand, so that each strand of the mutant allele would contribute to the eventual signal. If the scheme involving simultaneous hybridization of primers to each strand was modified by heating the mixture and then repeating the annealing and extension steps, then the primary signal would be increased even further. Repeating the steps would enable the products of the first round to be duplicated in the second cycle, to yield two copies. Repeat again and there would be four copies, and so on.

Like many great ideas, this one languished in the back of its inventor's mind for several weeks before it was finally attempted. Two primers were synthesized to be perfectly complementary to each end of a 110 base pair region of a cloned segment of the human β-globin gene, the amplification was performed, and the products were identified by acrylamide gel electrophoresis. The result was the anticipated 110 base fragment and the beginning of the development of PCR as a basic technique in molecular biology (*1*).

II. PRINCIPLES OF THE POLYMERASE CHAIN REACTION

A. The PCR Amplification Scheme. An examination of the details of the PCR amplification scheme reveals that the method is as simple as it is elegant (Figure 1). Oligonucleotide primers are first designed to be complementary to the ends of sequence to be amplified and then mixed in molar excess with the DNA template and deoxyribonucleotide triphosphates in an appropriate buffer. Following heating to denature the original strands and cooling to promote primer annealing, the oligonucleotides each bind to a different strand of the target fragment. The primers are positioned so that when each is extended by the action of a DNA polymerase, the newly synthesized strands will overlap the binding site of the opposite oligonucleotide. This is illustrated as the first PCR cycle in Figure 1. As the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies. The end result is an exponential increase in the total number of DNA fragments that include the sequences between the PCR primers, which are finally represented at a theoretical abundance of 2^n, where n is the number of PCR cycles performed.

An important feature of this scheme is that the majority of the amplification products that are present following many PCR cycles are double-stranded DNA fragments of discrete length. The strands that are synthesized as copies of the original template are bounded at the 5′ terminus by the oligonucleotide primer while the 3′ terminus is determined by the position at which the DNA polymerase finishes its synthesis. In contrast, the products of polymerase extension that result from priming of DNA strands that were produced

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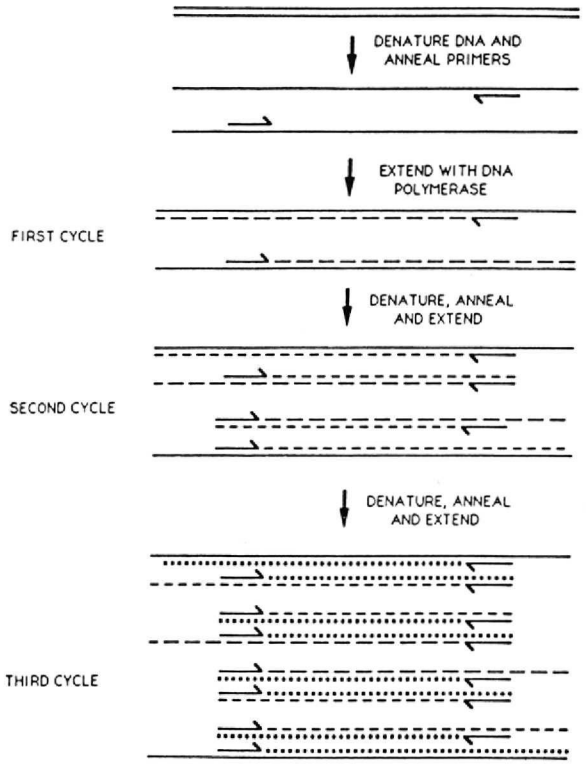

Figure 1. Principle of the polymerase chain reaction (PCR) amplification scheme. The DNA to be amplified is denatured to single strands and annealed to oligonucleotide primers that are oriented so that when each is extended by the action of a DNA polymerase, the newly synthesized strands will overlap. The process of denaturation, annealing, and polymerase extension is repeated many times, resulting in an exponential accummulation of strands that contain the sequence between the priming sites. In the first cycle the products are defined at the 5′ terminus by the oligonucleotide, but the 3′ terminus is not defined. After the third cycle the majority of products will be DNA strands that are defined at both termini, and after many cycles the predominant product will be double-stranded, blunt-ended DNA.

during the PCR will have both their 5′ and 3′ termini defined by the position of the oligonucleotide primers as a consequence of "run off" syntheses. This point is most easily appreciated by following the fate of individual strands and their copies in Figure 1. As the strands with indeterminate 3′ termini can only be generated by the copying of the original template, their abundance (A) is described by the simple arithmetic progression $A = 2n$. The overall consequence is that the fragments without discrete length are relatively rare compared to those with defined termini, and when a PCR product is analyzed by agarose gel electrophoresis, it appears as a double-stranded DNA fragment (Figure 2).

In addition to the production of double-stranded, blunt-ended DNA fragments two other features of the scheme contribute greatly to the utility of PCR. First, the position of binding of the primers defines the boundaries of the amplified fragment and so the usual requirement for restriction endonuclease recognition sites is obviated. As only a limited number of DNA sequences are restriction endonuclease recognition sites, PCR greatly increases the flexibility of choice of fragments size and composition. Second, the PCR oligonucleotides need not be exactly complementary to the template DNA. "Tails" may be added at the 5′ terminus to introduce sequences to the ultimate PCR products, or mismatches may be introduced within the priming sites (Figure 3) (*1*). Either strategy can be exploited to introduce restriction endonuclease recognition sites or other useful sequence motifs into the amplified DNA sequences.

B. The Ingredients for Performing PCR. PCRs can now be performed enabling the amplification of DNA fragments up to several kilobases in length by more than one million times their initial abundance. The procedure is highly automatable and requires just a few hours from beginning the thermocycling to the product analysis. This was not always the case, and the practical requirements for performing a PCR have been grately simplified since the first published reports of the method. The single most important development has been the purification and commercial distribution of a heat-resistant DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (*Taq*) (*9*). Prior to 1988 anyone wishing to perform the procedure was obliged to sit patiently by a series of water baths or heat blocks and add a fresh aliquot of *E. coli* DNA polymerase after each denaturation step, which was typically carried out by immersing the reaction vessel in boiling water for 0.5–2.0 min. This odious task can now be circumvented by adding the *Taq* polymerase once, at the beginning of the reaction cycles (Figure 4) (*10, 11*). The enzyme can withstand repeated heating to 94 °C, and so each time the mixture is cooled to allow the oligonucleotide primers to bind, the catalyst for the extension is already present. The availability of *Taq* polymerase has also greatly simplified the automation of the reaction as it is a much easier task to construct an apparatus that will cycle a reaction tube through different temperatures than to manufacture a device that

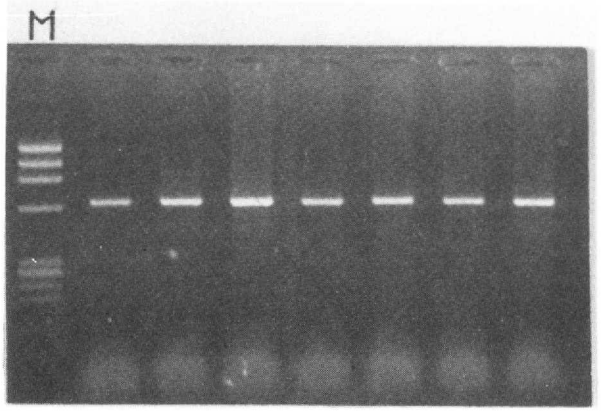

Figure 2. Agarose gel electrophoresis of PCR amplified DNA. Oligonucleotide primers specific for the amplification of an approximately 600 base pair region of the human β-globin gene were constructed (sense primer 5′ GACACAACTGTGTTCACTAGCAACC3′, antisense primer 5′ ATCCTGAGACTTCCACACTGATGCA3′). Each PCR was performed with 250 ng of human DNA and 25 cycles of 94 °C, 30 s; 59 °C, 30 s; and 68 °C, 1 min. Lane B contains molecular weight markers. Other lanes contain approximately 10% of the products of PCRs from seven different DNA samples.

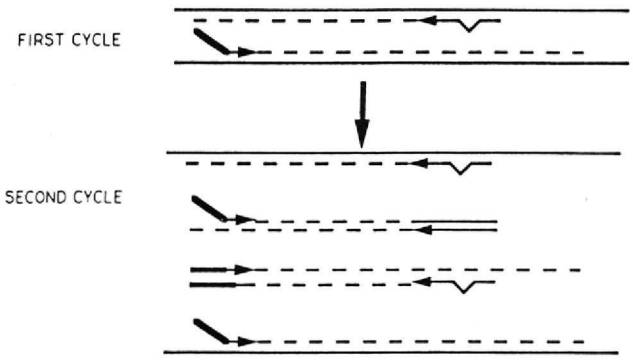

Figure 3. Incorporation of new sequences via PCR primers. The primer on the left has additional sequences at the 5′ terminus while the primer on the right has internal bases that are mismatched with the DNA template. After the first PCR cycle the new sequences become the new DNA template and are copied into subsequent products.

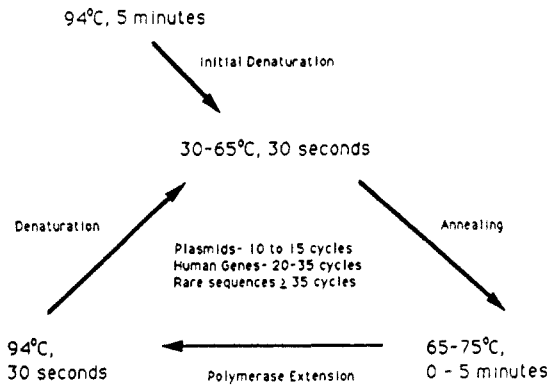

Figure 4. Typical thermocycling profile for PCR. The reaction mix may be assembled and then initially heated for several minutes to ensure that the template strands are effectively denatured. The reaction is cooled to a low enough temperature for the oligonucleotide primers to bind and then the temperature adjusted to the optimum temperature for the DNA polymerase to function. Generally the polymerase extension is carried out for 0.5–5 min to amplify fragments from 100 base pairs to 4.0 kb. The mix is again heated to 94 °C to denature the newly synthesized duplexes, and the reaction steps are repeated.

would perform both the thermocycling and the addition of enzyme aliquots. Currently there are at least seven different commercially available thermocycling instruments suitable for PCR and a number of published descriptions of homemade apparatuses (e.g. ref 12).

One of the least appreciated contributions to the widespread application of PCR has been the development of reliable automated chemistry for oligonucleotide synthesis (*13*). Until a few years ago the construction of a single oligonucleotide was a substantial task that could only be performed by a skilled organic chemist. Now it is possible to purchase either an oligonucleotide synthesizer that can be operated by a technician or the oligonucleotides themselves from a commercial vendor. Multiplex oligonucleotide synthesis machines have been constructed with the aim of reducing the overall cost of synthesis, which will be welcomed by PCR users (*14*). As the oligonucleotides define the eventual PCR products, there is little doubt that in the absence of their ready supply, PCR would not enjoy the wide acceptance that it has gained today.

A broad range of nucleic acid sources are suitable templates for PCR amplification. Purified DNAs from all parts of the evolutionary scale have been amplified, and many abbreviated protocols for the DNA purification are now reported (*1*, *10*, *15*). In extreme cases, cells may be simply boiled to release nuclear material and then used directly in the reactions. Paraffin-embedded samples containing DNA that may be substantially degraded (*16*) have also been amplified efficiently. In general the protocols for PCR amplification of crude DNA extracts have had the greatest success when the target fragments are relatively abundant. However the overall large number of reports of amplification from crude extracts shows that when the procedure is carefully standardized, simple methods can be reliably used for the sample preparation.

The remaining ingredients for PCR are standard laboratory items: deoxyribonucleotide triphosphates and a magnesium source in an appropriate buffer. Many investigators report that both the efficiency and specificity of PCRs can be affected by variations in the concentration and ratio of free magnesium, deoxyribonucleotide triphosphates, and primers, and these may need to be carefully titrated to optimize the individual reactions. Others have included dimethyl sulfoxide (10%) in the *Taq* polymerase buffer (*10*, *17*) and have reported an increase in the efficiency of reaction that use multiple primer sets (*18*). The necessity or value of this ingredient is a controversial issue, as DMSO can inhibit *Taq* polymerase activity (*19*) and yet many successful studies have been carried out in its presence (*17*).

C. The Specificity of PCR. PCR can be easily used to amplify single DNA fragments from complex nucleic acid sources to apparent homogeneity when analyzed by agarose gel electrophoresis. When contrasted with the difficulties that are usually associated with oligonucleotide hybridization to complex DNA mixtures, this high specificity is remarkable. When a 20 base oligonucleotide, for example, is hybridized to human genomic DNA in the Southern analysis procedure, the result is frequently a radioactive smear even when the probe is perfectly complementary to a unique site in the genome. Presumably the hybridization specificity cannot be easily maintained at the stringency necessary to provide sufficient signal for efficient detection, and at lower stringency the oligonucleotide binds at many sites with related sequence. By analogy, during PCR of a unique human gene fragment, each oligonucleotide primer probably binds at first to many sites and yields a variety of DNA extension products. In this case the specificity of the PCR arises from the frequency at which two such priming events will occur at positions that are close to one another and in the orientation that will allow the two products to overlap. Thus, while the probability of priming occurring at more than two sites in the genome during PCR of human DNA may be high, the likelihood of the nonspecific events occurring close together is very low. It is the coordination of these independent events that gives PCR its great specificity. Following the first few cycles of amplification the abundance of the "correct" DNA template increases exponentially, and this further increases the probability of the amplified fragments having the correct identity.

To a limited extent the specificity of PCR can be manipulated by variation of the lowest temperature of the reaction at any time during the thermocycling. The *Taq* DNA polymerase has an optimum activity around 70 °C and is not inactivated by short incubations at temperatures at which PCR generated fragments will denature (usually 90–95 °C) (9, 19) and so a wide range of annealing temperatures may be employed. In practice, when it is desired to amplify a single fragment, it is possible to choose an arbitrary annealing temperature with which to test the behavior of a set of oligonucleotide primers. If the temperature is lower than the optimum, the reaction products will frequently include additional DNA fragments that can be visualized by agarose gel electrophoresis and ethidium bromide staining. Increasing the annealing temperature will reduce the likelihood of the nonspecific priming but will not reduce the efficiency of the correct priming events. As the temperature is further increased the oligonucleotides cannot bind to even perfectly matched sites and no product is obtained. A final annealing temperature is usually chosen by this empirical approach, using the agarose gel electrophoresis profile of the reaction products as the indicator of when the annealing optimum has been found.

"Nested" oligonucleotide PCR primers have been employed to improve the specificity of reactions that do not otherwise yield homogeneous products (*1*) (Figure 5). These protocols use a two-step reaction scheme beginning with amplification by the outer primer set, followed by initiation of a second reaction with the internally binding primers. The overall lower complexity of the template for the second PCR ensures a more homogeneous final product. Nested primers were widely used to overcome problems associated with the relatively low specificity of the *E. coli* DNA polymerase catalyzed reactions but has since been less favored when using *Taq* DNA polymerase. The applications for nested priming are now almost primarily for amplification of rare sequences, or PCR using

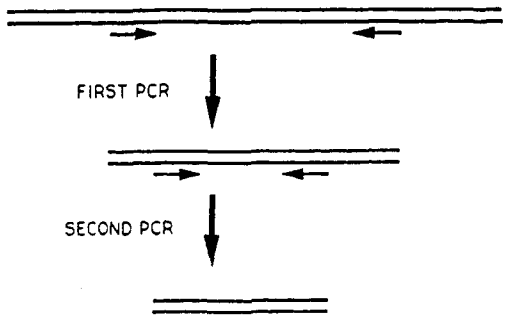

Figure 5. Nested PCR priming for improved specificity. Several PCR cycles are carried out in the usual fashion and then a second primer set is used to initiate amplification from within the first fragment. This stratergy has previously been used to overcome problems associated with the generation of spurious amplification products, but should not usually be necessary.

redundant priming sites (see below).

It is sometimes desirable to perform low stringency PCRs in order that primers may bind to sites with less than perfect homology. Usually this is to introduce modified bases into a DNA fragment or to allow recovery of related sequences such as members of gene families or fragments that code for functionally related proteins. Some of these applications are discussed below.

D. The Sensitivity of PCR. The frequent question asked about PCR of "how *sensitive* is the assay?" is usually confused with the related but different statistic of "what is the *lower limit of detection* of the method?". The first question cannot yet be generally answered, as the ability to amplify individual fragments under conditions of reduced template concentration is affected greatly by the individual primers used, the purity of the template material, and the relative abundance of the nonspecific DNA. In these cases the *sensitivity* varies as the measure of the amount of target that can be reliably identified above a background signal.

In contrast the minimum amount of material that can be identified is clearly established. Saiki et al. (*11*) have amplified single DNA molecules from dilutions of cells containing a single copy of human β-globin sequence, while others have reported PCR amplification of unique sequences from single human spermatozoa (*20*). There may be difficulties in obtaining high reproducibility of such assays, but it is clear that PCR can be initiated from a single template molecule and that the products can be recovered for further analyses.

E. Efficiency of PCR. (*i*) *Theoretical vs Actual Efficiency.* If PCR could be performed with 100% of its theoretical maximum efficiency, then it would take only 25 PCR cycles to generate more than 100 μg of a 1 kb unique human DNA fragment from 100 ng of total human DNA. Although there have been relatively few published studies that attempt to rigorously address the actual efficiency of any PCRs, in practice only a few micrograms are usually generated in this kind of experiment indicating that the scheme is operating with less than its maximum potential (*1*, *21*) (Figure 6). Factors that can explain the difference between the observed and expected amplification efficiencies involve both the initial (lag) phase, the maximum rate of doubling, and the eventual saturation of the reaction. First, some of the template may never be available due to the presence of strand breaks or failure of the DNA to dissociated from other macromolecules during purification and the initial thermocycles. In addition some of the initial template may hybridize to primers with lowered efficiency because of structural constraints or a tendency of the long parental strands to quickly snap back to re-form the original duplex. A low efficiency during the first few PCR cycles will greatly influence the overall yield of the reaction.

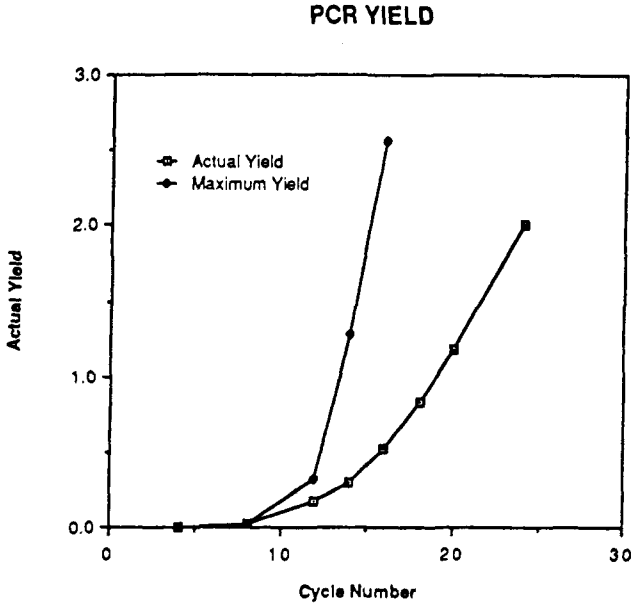

Figure 6. Theoretical vs actual yield of a PCR. Data describing the actual yield (picograms of product) were taken from ref 21. The theoretical (maximum) yield was calculated by assuming each cycle doubled the amount previously present.

Following the lag phase the reactions may proceed at as much as 80% of their possible rate (*2*). This suggests that the intrinsic ability of primers to bind to the desired target and be efficiently extended under optimum conditions is not likely to limit the amplification. In contrast the senescence of PCRs has been frequently observed where more cycles are performed without yielding more product. At saturation the increase in concentration of the amplified frgments presumably increases the subsequent competition between primer/template and template/template annealing. The amount of enzyme may also become a limiting factor as *Taq* is usually added at a very low molar concentration which reduces the potential for adequate numbers of polymerization events after many cycles. The primers and deoxyribonucleotide triphosphates should not be exhausted and should not limit the final reactions.

(*ii*) *Difficult PCRs.* Some DNA may be difficult to amplify because of intrinsic features of the sequence, which has prompted the development of useful reaction condition modifications. In particular the inefficient amplification of sequences of high GC content, especially those with potential to form stable stem loop structures, has been reported (*22*). A simple solution can be the inclusion of 10% dimethyl sulfoxide in the PCR buffer which may function by lowering the melting temperature of regions with extensive secondary structure (*23*). Other modifications include the use of nucleoside analogues and mismatched oligonucleotides in the reaction mix. The substitution of 7-deaza-2′-deoxyguanosine for dGTP, for example, was shown to improve the efficiency of amplification of a high GC content region of the murine ornithine decarboxylase gene (*22*). Vrieling et al. (*24*) used a base mismatch in an oligonucleotide primer directed toward the 5′ terminus of the human hypoxanthine phosphoribosyltransferase cDNA that possessed a 9 base inverted repeat. When this primer was incorporated into the PCR product, the mismatch destroyed a potential hairpin structure and enabled efficient amplification.

III. VARIATIONS ON THE BASIC PCR

A. RNA PCR. RNA can also be used as template for PCR following reverse transcription (*25–27*) (Figure 7). This is a useful procedure for the study of expressed gene sequences and retroviruses. In many cases the PCR primers can be

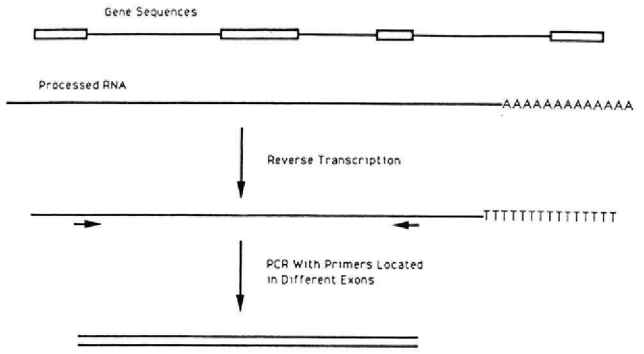

Figure 7. RNA sequences may be amplified by PCR following a reverse transcription reaction. When the target sequence is a processed gene from a higher eukaryote, it is useful to choose primer sequences that span intronic regions. Spurious amplification from contaminating endogenous DNA can then be distinguished by size.

designed to specifically amplify the cDNA sequences by choosing fragments spanning intronic regions. Contamination of RNA preparations by genomic DNA therefore will not generate spurious amplification products or will result in products of a different size that can be distinguished from the cDNA fragment. Protocols for RNA preparation and cDNA synthesis prior to PCR amplification have varied greatly in the details of cDNA priming and in the initial sample preparation, but all follow the same basic scheme (*27*, *28*). In general the quality of the cDNA syntheses need not be as high as that usually required for the construction of cDNA libraries, as incomplete cDNA strands will be lost during the PCR amplification. The "full length" cDNA strands that may be present at low abundance will be the fragments that are eventually recovered by the DNA amplification. It is not necessary to generate a second cDNA strand by a controlled synthesis reaction, as it will be generated during the first PCR cycle by the *Taq* DNA polymerase.

B. Quantitative PCR. PCR amplifications have been performed with sufficient reprodubility to provide a quantitative estimate of DNA templates (*29–31*). In most examples the relative amount of the fragment of interest is determined by comparison with a standard generated by a second primer pair that is included in the same reaction. The reliability of the quantitative measurements can be easily tested by performing reconstruction experiments that examine variables including the reaction ingredients, number of cycles, effect of different primer sequences, relative target abundance, and the ratio of abundance of the test and reference fragments. In general the accuracy of the quantitative determinations may be directly correlated with the efforts to provide a good measure of PCR reproducibility. As many quantitative PCR studies have focused upon levels of gene expression the reproducibility of cDNA syntheses has also been examined (*30*).

An innovative approach to control some of the intrinsic variation in the efficiency of different primer sets at different template concentrations has been described by Gilliland et al. (*31*). Reference fragments were constructed to be identical with a test DNA, except for the addition of a short internal linker sequence that enable the two species to be resolved by agarose gel electrophoresis. As the two different fragments could be simultaneously amplified by the same pair of oligonucleotides, possible variation in the performance of different primers was overcome. The reference template was titrated against the total amount of the complex nucleic acid mixture containing the fragment of unknown quantity, and a concentration of reference that provided equal amplification signal to the test sequence was identified. The amount of the reference added at this point corresponded to the amount of test material in the reaction.

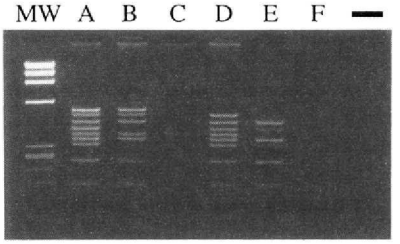

Figure 8. Multiplex PCR. The simultaneous amplification of as many as nine different PCR primer sets in a single reaction has been demonstrated. The normal products of a "nineplex" amplification used in the diagnosis of gene deletions that lead to Duchenne muscular dystrophy are shown (see refs 18 and 32).

C. Multiplex PCR and DNA Deletion Detection. PCR provides a conceptually very simple assay for the presence or absence of DNA sequences: If the fragments are present, then it should be possible to amplify them and detect the expected PCR product. If the sequences are absent, then PCR will not be possible. The demonstration of PCR as a practical and reliable method for DNA deletion detection was by application to the diagnosis of DNA deletions leading to the human genetic disease Duchenne muscular dystrophy (DMD) (*18*, *28*, *32*). DMD is one of the most common human genetic disorders and results from mutations at the X-chromosome linked *Dystrophin* gene locus. The *Dystrophin* gene is the largest human gene yet discovered, spanning more than 2 Mb of DNA. Approximately 50% of DMD cases arise as a consequence of partial gene deletions at this locus, and prior to the application of PCR, the diagnosis of these lesions was performed by a laborious series of Southern analyses. The cloning and sequencing of selected regions of the locus allowed the construction of a series of PCR primers that were specific for nine regions frequently involved in the DNA deletion events. Next suitable conditions for the simultaneous amplification of the nine fragments were identified. Through amplification of different length fragments, all nine PCR products could be simultaneously visualized by agarose gel electrophoresis.

The normal DMD multiplex PCR profile is therefore a series of nine bands on an agarose gel (Figure 8). Loss of one of the regions due to a DNA deletion mutation is visualized by the loss of corresponding bands on the gel. Extensive testing using previously characterized DMD patient material has proved multiplex deletion detection to be a reliable alternative to Southern analysis (*33*). The multiplex deletion assay is sensitive to the problem of PCR contamination as the possible introduction of amplifiable sequences from exogenous sources can lead to erroneous diagnoses. The general problem of PCR contamination is discussed further below, and the methodological approach to maintaining accurate DMD diagnoses has been discussed elsewhere (*28*, *32*).

Multiplex PCR assays for DNA deletion detection has also been established for the human (*34*) and hamster (*35*, *36*) hypoxanthine phosphoribosyltransferase genes (8-plex) and the human steroid sulfatase locus (*37*) (3-plex). In each case the limiting factor in the development of the assay was the availiability of sufficient DNA sequence information to construct sets of oligonucleotide primers that would function under identical conditions and produce fragments that could later be resolved on the basis of their size. As the rate of generation of new DNA sequence information from the human and other genomes increases, it is envisioned that standard multiplex DNA primer sets for other interesting regions will be established.

In addition to simplifying screening for DNA deletions, the multiplex PCR approach can facilitate other DNA analyses by reducing the overall number of manipulations necessary

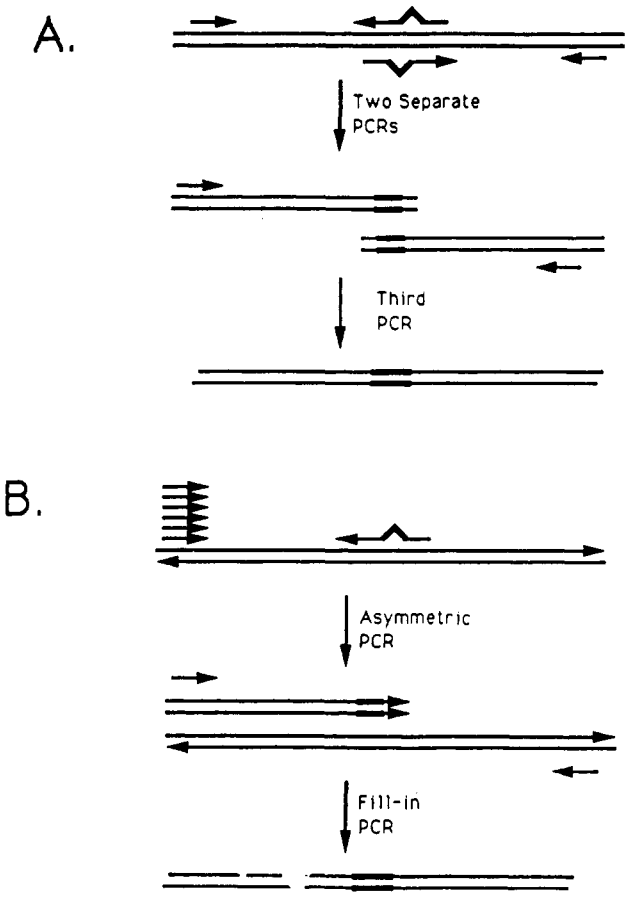

Figure 9. Two schemes for mutagenizing DNA fragments in regions that are internal to PCR primers. The sequences to be introduced are represented as thick lines. (A) Two separate PCRs are carried out, each using an oligonucleotide complementary to the template and containing the new sequence in an opposite orientation to one of the flanking primers. The products of the first two PCRs are mixed and a PCR carried out where each of the two shorter products primes the other. The resulting full length strands are further amplified by the original flanking primers in the third PCR. (B) An alternative scheme uses just one internal oligonucleotide and an "asymmetric PCR" to generate an abudance of one strand containgthe new sequences. The second "fill-in PCR" uses the single strands from the first reaction, a small amount of the original template, and the two flanking primers.

for the amplification of multiple fragments. Many of the methods described below for the analysis of PCR products can be as easily applied to the products of a multiplex reaction as to a single DNA fragment. All the exons from a single gene, for example, can be amplilfied simultaneously and then DNA sequencing reactions carried out on each of the individual fragments. Alternatively other procedures described below for the analysis of PCR products may be applied.

D. Mutagenizing DNA Sequences by PCR. The usual methods for altering the sequence of DNA fragments involve either chemical mutagenesis or construction of oligonucleotides that may be incorporated into cloned copies of the normal sequence. These in vitro mutagenesis strategies are limited by the relatively long period required to grow bacteria containing the new constructs and the need to examine many candidate clones by DNA sequencing. PCR can be applied for the alteration of DNA sequences in at least two ways. The first method exploits the natural error frequency of the *Taq* polymerase (*38*) and involves cloning and sequencing many PCR generated fragments. A limitation of this approach is that the rate of certain base substitutions is significantly higher than others, with a predominance of G to A transitions (*39*). To enhance the mutation frequency, the error frequency of the polymerase may be manipulated by altering the reaction conditions (*17*).

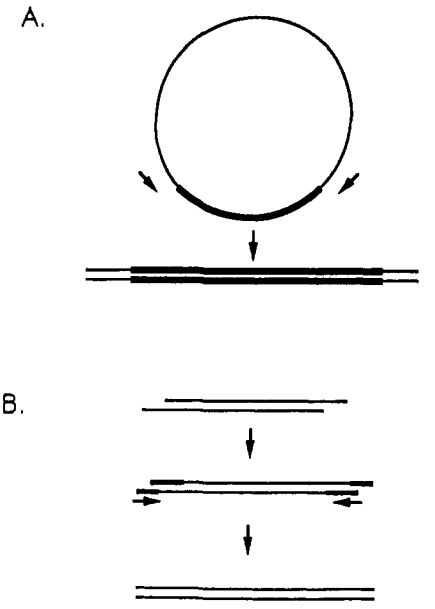

Figure 10. Two strategies for amplifying unknown DNA sequences. (A) The sequences inserted into a DNA cloning vector may be amplified by constructing oligonucleotide primers that hybridize to vector sequences on either side of the insertion site. (B) When the fragment has not been cloned, then it may be amplified by the ligation of DNA linkers containing the primer recognition site at each end.

Directed PCR mutagenesis strategies employ oligonucleotides that are synthesized with the new sequence, but retain sufficient homology to the normal template to enable PCR priming. Incorporation of these alterations into the terminal ends of PCR fragments is straightforward provided the eventual construct can be reconstituted with appropriate restriction endonuclease recognition sites. Alternatively, DNA base alterations can be introduced into regions between PCR primers via a third oligonucleotide. In these schemes it is sometimes necessary to perform several PCRs so that the new sequence can first be incorporated onto the end of a short fragment, which can then be spliced to a larger segment by polymerase extension. PCR with primers at the ends of the ultimate fragment can be used to rescue the full length material (Figure 9) (*40–42*). A similar approach can also be used to rescue the products of multiple simultaneous ligations of synthetic oligonucleotides.

IV. PCR FROM INCOMPLETE DNA SEQUENCE INFORMATION

Frequently the DNA sequence of a region to be analyzed is not entirely or exactly known. In these cases PCR may be applied if there is sequence information from flanking regions or related DNA templates. A number of applications have evolved to improve the versatility of PCR in these circumstances.

A. Vector PCR. DNA fragments that have been inserted into vectors for the purpose of clonal propagation, DNA sequencing, or functional expression may be rescued by PCR using oligonucleotides that are directed toward sequences adjacent to the cloning sites (Figure 10) (*11*). This provides a useful alternative to the usual procedure for "minipreps" that were previously used to screen bacterial colonies or phage plaques to identify and characterize recombinant molecules. The savings in the time and labor necessary for the generation of the DNA fragments by PCR relative to microbial inoculation, growth and purification greatly increases the scale upon which experiments may be carried out. In addition the method can be used for the in vitro amplification of entire cloned

libraries, although the relative recovery of inserts of different size has not been rigorously studied.

B. Linker PCR. In a related application, synthetic oligonucleotides have been ligated at each end of restriction endonuclease digested fragments, to act as PCR priming sites (Figure 10). Linker PCR differs from vector PCR, primarily because the same priming sequence is at each end of the DNA to be amplified. The amplification proceeds efficiently from the linker sequences, even when the initial DNA sample is a complex mixture. Linker PCR has been adapted to schemes for subtractive DNA hybridization (*43*), rescue of DNA sequences taken directly from microdissected human chromosomes (*44*), and recovery of DNA sequences that bind to specific proteins (*45*). In each case the addition of the linker sites and the subsequent PCR have overcome inherently inefficient steps that often made these procedures either impractical or impossible.

C. PCR from Repeated Sequences. The identification of species-specific highly reiterated sequences in many genomes has been exploited to provide markers for the rescue of human DNA from cell hybrids by DNA hybridization. PCR has now been applied in these studies via the construction of oligonucleotides that are homologous to the consensus sequence of the human *Alu* repeat units (*46*). As a result of the different distribution of the repeats in various regions of the human genome, and the absence of complementary sequence in the Chinese hamster genome, "*Alu*" PCR has enabled the rapid generation of human specific "PCR fingerprints" directly from human–hamster hybrid cell lines. When these PCR products were isolated, radiolabeled, and used as probes against DNA mapping-reference panels, the *Alu* products were shown to be predominantly of human origin. This PCR-mediated method of cell hybrid characterization may be contrasted with the efforts of genomic DNA library construction and screening that would normally be required to rescue a human sequence from a cell hybrid.

Other examples of PCR using oligonucleotide primers that are complementary to repeated sequences have focused on the development of DNA fingerprints that can be used to distinguish individuals (*47*) or substrains of single species (*48*). The principle of this application is that individuals will exhibit sufficient natural variation in their genomic organization for the efficiency of amplification of different fragments between repeated elements to vary. The end result is a distinct pattern that is discernible by agarose gel electrophoresis and is specific to the individual from whom the DNA was first obtained.

D. Redundant Primers. Redundant or degenerate oligonucleotide primers were first used in PCR to solve a practical problem that has long been regarderd as an intrinsic difficulty of screening cDNA libraries using mixed oligonucleotide probes constructed from amino acid sequence information. The difficulty of this procedure is a direct consequence of the redundancy of the genetic code. In order to be certain that a perfectly complementary probe is used to screen for a cDNA, many different possible oligonucleotides must be synthesized. The complexity of the probe mixture complicates the cDNA screening and frequently results in either many falsely positive hybridization signals or, at higher stringency, no signal at all. To overcome this problem while attempting to clone the porcine urate oxidase cDNA, redundant PCR primer families were syntheszed as an alternative to redundant hybridization probes (*49*). The two oligonucleotide sets were then used in PCR with a freshly synthesized porcine liver cDNA and the products of the reaction rescued by cloning into a DNA sequencing vector. Individual recombinant colonies were screened by using a hybridization probe that was internal to the two PCR priming sites and positive clones were sequenced. The bona fide cDNA synthesis products were then identified by the homology between their possible translation products and the original porcine urate oxidase amino acid sequence data.

This study both demonstrated that redundant oligonucleotides could be successfully used as PCR primers and established a novel method for cDNA cloning. In a variation of this approach, other investigators have designed redundant PCR primers based upon amino acid sequence that appear to be conserved in different species and in functionally related proteins (*50*). In some cases every possible codon that would yield the conserved amino acid sequence was used in the priming mixture, but elsewhere the primers have not been complex mixtures and the difference in the sequence of the priming sites has been compensated by a relaxed reaction stringency. Combinations of redundant and low stringency priming have now been used to define novel retroviruses (*50*) and to isolate members of gene families (*51*). A common feature of these studies has been the recovery of oligonucleotides with mismatches to the priming sites and the need to carefully screen candidate clones that are isolated by the procedure, to ensure that they are related to the expected sequences.

E. Anchor PCR. The characterization of DNA fragments that are adjacent to known sequences has provides a considerable challenge to the PCR method which normally requires the knowledge of sequence from each end of the element to be amplified. In applications such as the isolation of as viral integration sites or the sequencing of a gene deletion, only one side of the junction is usually known. Variations on the theme of "anchor PCR" have been developed for this purpose. Different anchor PCR schemes have in common a unique primer which is to function opposite a highly redundant priming site (Figure 11). In some applications the redundant priming site may be naturally present, such as the oligo.d(T) that results from copying the polyadenylated tail at the 3′ terminus of most mammalian mRNAs (*52*). Alternatively the redundant site may be introduced by a terminal transferase catalyzed "tailing" reaction or ligation of a linker/primer moiety (*53*, *54*). In each scheme the aim is to recover a product that is generated by PCR using the unique primer opposite the redundant priming site. Despite the fact that these strategies result in products with considerable complexity, the net result is a enrichment for the fragment of interest and a great increase in the ease of its recovery, relative to conventional methods of library construction and screening.

A recent development that utilizes a cloned DNA binding protein has great potential for improving anchor PCRs. Lew and Kemp (*55*) have used the yeast GCN4 gene product that has a strong affinity for a 10 base motif in double-stranded DNA, but not single-stranded DNA, to facilitate the isolation of a single fragment from cDNA library extracts. The recognition signal for the protein was attached at the 5′ terminus of the unique PCR primer and several cycles of PCR were performed using this oligonucleotide and a redundant primer that was homologous to one of the vector arms. Next the GCN4 protein was used to capture DNA fragments in the mixture that had incorporated the protein binding motif into double-stranded DNA. The captured material was separated from all the other ingredients of the initial reaction and used to reinitiate PCR. In the second amplification reaction the desired fragment bearing the unique primer at one end and the redundant primer at the other constituted the majority of the available template and was amplified to apparent homogeneity.

F. PCR and Circularization. A second scheme for the characterization of fragments adjacent to known sequences is PCR following ligation-mediated circularization. This technique was reported 3 times from independent sources

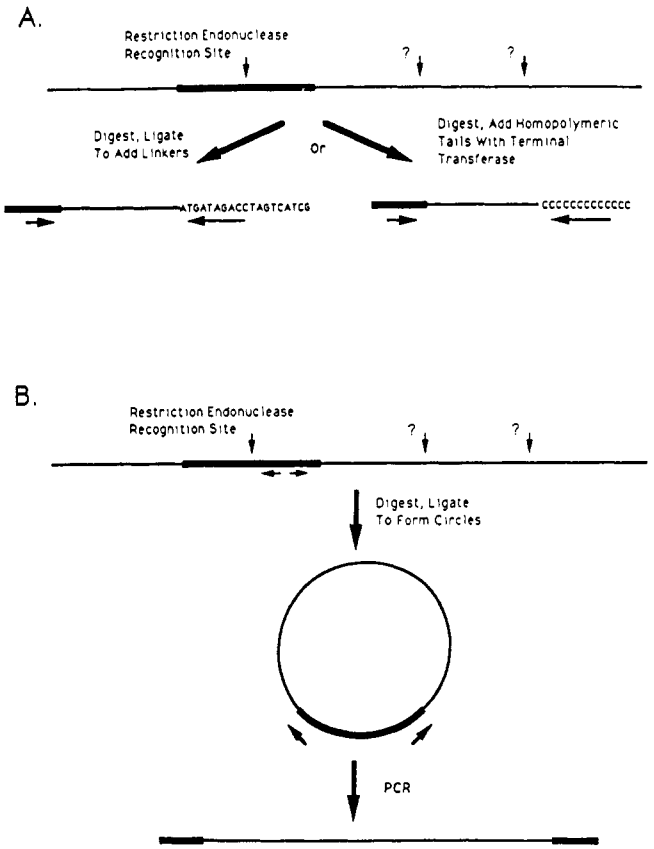

Figure 11. Strategies for amplifying unknown DNA sequences (narrow lines) that are adjacent to known sequences (thick lines). Known restriction endonuclease recognition sites are indicated by an arrow, and query marks denote possible but not certain enzyme sites. (A) Anchor PCR. The DNA mixture containing the sequences to be amplified is digested via a restriction endonuclease and a PCR priming site added to the unknown sequence by either ligation or terminal trasnsferase. A unique primer is then used in conjunction with the redundant primer to amplify the specific sequence. This method has the disadvantage that fragments with the redundant priming sites at each end will be amplified in competition with the desired sequence. (B) Circular PCR. The DNA mixture is digested and ligated at very low concentration in order to promote circle formation. Primers are constructed from known sequence information to amplify around the circle and include new sequences.

during 1988 (*56–58*) and involves the digestion of a DNA fragment by a restriction endonuclease and its subsequent ligation at very low concentration in order to promote the formation of circular products. Judicious choice of the restriction endonuclease leads to the generation of a circle that contains the previously known sequence on a contiguous circular molecule with the unknown sequence. PCR primers are synthesized from the known sequence in an orientation that enables amplification around the circle and includes the unknown sequence. Once amplified the fragment is recovered and analyzed (Figure 11). Aside from the initial descriptions there have been few reports of the successful application of the circularization PCR method. However the procedure has enormous potential for the simplification of junctional fragment mapping, gene walking and jumping, and mutation characterization. Therefore it is very likely that many examples of PCR circularization will be forthcoming.

V. IDENTIFYING SUBTLE SEQUENCE VARIATION IN PCR PRODUCTS

A. Sequencing PCR Products. The high resolution of DNA sequence has generated a considerable interest in DNA sequencing protocols for the analysis of PCR products. The sequencing strategies are broadly divided into two approaches: cloning and direct sequencing. The cloning of PCR products

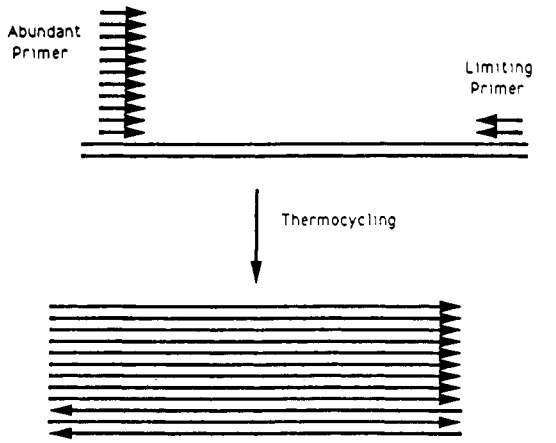

Figure 12. Asymmetric PCR for the production of single DNA strands for sequencing or as hybridization probes. PCR is performed with a limiting amount of one of the primers. After several cycles the supply of the limiting primer will be exhausted and further rounds of heating and cooling will generate an increase in the products of extension of the remaining oligonucleotide. The result is a mixture of single- and double-stranded products.

is straightforward using standard subcloning protocols and can be facilitated by addition of artificial restriction endonuclease recognition sites at the 5′ terminus of the PCR primers or can use natural sites or blunt ended ligations. The cloning of the PCR products prior to sequencing has the advantage that standard sequencing methods can be used and when complex alleles are analyzed from a heterozygote the linkages of different base substitutions can be determined. The disadvantages of the cloning and sequencing mainly arise as a consequence of the frequency of errors that are introduced by the *Taq* polymerase during amplification. During PCR between $1/10^4$ and $1/(5 \times 10^4)$ base additions will involve the incorporation of an incorrect nucleotide, which will result in an average of approximately one incorrect base in every clone derived from the amplification of a 1 kb fragment that has been amplified in 30 PCR cycles (*11, 17, 38, 39*). To overcome this difficult it is necessary to analyze several independent isolates to derive a consensus sequence. In addition to the polymerase errors, the cloning method has the disadvantage of being relatively time-consuming and labor intensive when compared to direct DNA sequence analysis.

Direct sequencing can rarely be carried out using standard sequencing protocols, presumably because of the presence of low levels of leftover primers and nonspecific PCR products present in the reactions, and also as the second strand of the PCR product can compete with the sequencing primer for the DNA template strand. Approaches have been devised to overcome these problems, by either partially purifying the double stranded PCR products or converting the double-stranded fragments to single DNA strands. The first report of single-strand production by PCR was achieved by using an unbalanced ratio of the two oligonucleotide primers in "asymmetric PCR" (*59*). During asymmetric amplification the supply of one of the PCR primers is exhausted and the subsequent extension reactions are primed in one direction only. The result is a linear increase in the amount of one strand relative to the other, and a template mixture that is much more suitable for dideoxynucleotide DNA sequencing than is a double-stranded PCR product (Figure 12). Alternative approaches to single-stranded DNA production by PCR and direct sequencing are reviewed elsewhere (*60*).

The majority of direct DNA sequencing studies have been carried out using radioactive labels, but recently fluorescent DNA sequencing procedures have been adapted for the analysis of PCR products (*26, 61*). The fluorescent protocols take advantage of automated devices for the direct on-line

monitoring of the sequencing gel electrophoresis and computer-directed data entry. As PCR is used for the preparation of the templates there is potential for complete automation of almost all the steps for DNA sequencing. Beginning with either cloned DNA sequences in bacteria or fragments in more complex mixtures, a DNA template can be generated by PCR and then transferred to the sequencing section vessel with the aid of a robot. The sequencing reactions are performed and the products loaded onto the fluorescent sequence device. It is envisioned that subtle refinements in the individual steps described here and the development of suitable hardware will result in PCR-based sequencing being an integral feature of all large scale DNA sequencing facilities.

A likely feature of PCR-based fluorescent sequencing protocols will be the use of solid supports for the template strand (*62, 63*). A biotin residue can be introduced into a PCR product via the 5′ terminus of an oligonucleotide primer and the strand efficiently captured by using streptavidin-coated magnetic beads. The beads can be easily manipulated by rare-earth magnets which will greatly simplify the automation and hardware construction. In addition, the beads can be vigorously washed to remove salt and other potential contaminants from the PCR which results in an ultimately superior DNA sequence profile (*34*).

B. Scanning PCR Products for Mutations. When it is not desired to know the precise DNA sequence of PCR products, or when they are too long for DNA sequencing to be practical, there are a variety of other possible analytical methods that may be applied. The final choice of method will be determined by many factors, including the number of fragments to analyze, how much is known about the normal sequence, whether the anticipated changes involve one or many DNA bases, and if the sample is homogeneous or contains a mixture of closely related sequences.

In the simplest case the length of each PCR fragment may be determined by agarose or acrylamide gel electrophoresis. More subtle variation can be identified by restriction endonuclease digestion, melting analysis, or heteroduplex mapping. The restriction endonuclease digestion analysis has the advantage that gross differences between two fragments can be easily identified, and some single DNA base substitutions will be detected. However, most base substitutions will not involve restriction endonuclease recognition sites and therefore a large fraction of the possible variation will be unrecognized.

The heteroduplex mapping procedures that have been used to analyze PCR products include ribonuclease A (RNase A) cleavage (*64*) and chemical detection of mismatches (*65*). In each case a radioactive probe is hybridized to the amplified fragments and then digested at sites of base mismatches using RNase A, for RNA probes, or a combination of hydroxylamine and osmium tetroxide, for DNA probes. The digested probe is then analyzed by denaturing acrylamide gel electrophoresis and autoradiography to identify the site of cleavage which indicates the approximate position of the mutation.

The RNase A digestion method has been used to analyze a range of PCR products and has the advantage of being simple to perform (*66, 67*). However, not all base mismatches between the probe and analyte fragments will be cleaved by the enzyme, and therefore the chemical cleavage technique may be favored. All mismatched thymidine and cytosine residues will be identified by the hydroxylamine and osmium tetroxide modification reactions and so the examination of both strands of a heteroduplex by chemical cleavage will lead to the detection of all possible mismatches (*68, 69*). There are now several examples of the application of both of these procedures to the analysis of PCR products.

PCR can further simplify heteroduplex mapping by facilitating the production of radiolabeled probes. The DNA probes may be generated by a variation of the asymmetric PCR protocol for preparing DNA sequencing templates. The starting material can be either a cloned DNA segment or the same region that is defined by PCR primers for the analyte fragments, but amplified from a different source. A unique human gene segment, for example, could be amplified from a normal individual and single DNA strands generated by further asymmetric amplification in the presence of a radioactive nucleotide. The single strands may then be hybridized to material amplified with the same primers from an individual with a suspected mutation in the region, and the heteroduplex analyzed by chemical cleavage (*68*).

RNA probes can be prepared in a similar fashion by the inclusion of a DNA sequence motif on the 5′ terminus of one of the PCR primers that will be recognized as a transcription initiation site by a bacterial RNA polymerase. The promoter will be incorporated by the PCR and the eventual transcription product will be a single-stranded RNA that extends the length of the amplified fragment. The attachment of the RNA promoters was initially exploited to simplify DNA sequencing of PCR fragments (*70, 71*), but that method has not enjoyed widespread popularity probably because of the total number of manipulations involved and the extra length of oligonucleotide that must be synthesized. Nevertheless, the incorporation of the RNA promoters provides a simple approach for the preparation of single-stranded radiolabeled RNA for heteroduplex mapping studies.

Denaturing gradient gel electrophoresis provides a method for the analysis of PCR products by exploiting differences in melting temperatures of closely related sequences (*72*). The method was first developed by studying the electrophoretic behavior of cloned DNA sequences in acrylamide gels containing a gradient of formamide and urea. As the fragments migrate into a region where partial denaturation begins, the electrophoretic movement stops abruptly. The temperature at which a fragment begins to melt is altered by single DNA base substitutions, and therefore the precise position in the gel where the electrophoretic migration changes is determined by the DNA sequence. Denaturing gradient gel electrophoresis constitutes a method for single DNA base substitutions in DNA fragments from 100 to 1000 bases in length and so is useful for PCR products. A limitation of the assay is that in general only those DNA base alterations that arise in regions which have a relatively low local melting temperature and are adjacent to high melting "domains" will be resolved.

The introduction of PCR has enhanced the utility of the assay, primarily because it is now possible to manipulate the overall melting profile of fragments by judicious positioning of the oligonucleotide primers (*73, 74*). In this manner DNA base changes that are in regions that melt only under strongly denaturing conditions can be amplified independently of regions that melt at a lower temperature, even when there is no convenient restriction endonuclease recognition site to separate the two fragments. Single base changes in even the highest melting domains can be detected by the inclusion of GC-rich high-temperature melting "clamps" at the 5′ terminus of one of the oligonucleotide primers (*75*). In this approach the PCR will simultaneously increase the abundance of the fragment so that they can be detected without radioisotopes and covalently link a high melting region that will ensure that all changes in the fragment can be detected.

The relatively high error frequency of the *Taq* polymerase (see above) provides a possible obstacle to the use of denaturing gradient gel electrophoresis. In an example discussed above (section V A) sufficient amplification is performed so that it is probable that each 1 kb double-stranded DNA fragment will contain a single base substitution. These misincorporated bases are usually not problematic as they are

randomly distributed throughout the amplified fragment and would not normally be observed by direct DNA sequencing or heteroduplex mapping. In denaturing gradient gel electrophoresis one single DNA base substitution per molecule would be expected to result in smear on the final autoradiograph due to subtle but different electrophoretic shifts in each molecule. In practice when fragments of less than 1 kb are analyzed, and the number of cycles of amplification is minimized, this is rarely a problem. In circumstances where the initial target abundance is very low, an enzyme with a higher fidelity than the *Taq* DNA polymerase may be substituted for the first few cycles of PCR. Keohavong et al. have shown that when T7 DNA polymerase is used in this manner they are able to markedly improve the overall electrophoretic profile (*73*).

C. Rapid Detection of Known Sequence Differences. Frequently there is prior knowledge of a possible DNA base alteration in a PCR-amplified DNA fragment, such as when a common disease allele or polymorphic DNA marker is being analyzed. Two-standard approaches have been developed for the analysis of known single DNA base differences in PCR products, the first requiring restriction endonuclease digestion and the second using allele specific oligonucleotides (ASO's) (*76*). Unfortunately the majority of natural DNA variation does not occur in restriction endonuclease recognition sites and so the technique of allele specific oligonucleotide (ASO) hybridization is more usually used. ASO hybridization is generally carried out by first tethering the target fragment to a solid support and then sequentially hybridizing radioactive oligonucleotides that are complementary to each possible DNA sequence. Mismatches between the probe and the target fragment are identified by stringent washing, so that only perfectly matched oligonucleotides remain bound. There are abundant reports of mutation detection by restriction endonuclease digestion and ASO probing, reflecting the utility and simplicity of the methods.

Saiki et al. (*77*) have demonstrated that the ASO hybridization may be further simplified by reversing the role of the probe and analyte fragments. A series of ASOs were attached to a membrane support and hybridized to the products of a single PCR. Allele-specific binding was detected by a peroxidase-based colorimetric reaction that was linked to a biotin moiety on one of the PCR primers. This approach simplifies the routine screening for common human disease alleles as each individual sample is developed on a single filter.

While ASO probing and restriction endonuclease digestion identification of mutations have been greatly simplified by PCR, they are not inegrally dependent on the actual amplification reaction. In contrast, two recently developed methods have used the amplification reactions themselves to detect mutant alleles. Each method uses oligonucleotides directed toward the predicted DNA sequences as PCR primers, rather than as ASO probes. In one variation stringent PCR conditions are identified where a PCR primer will not function when there is a single DNA base mismatch in the usual priming binding site (*78*, *79*). The mismatch has typically been placed at the 3′ terminus of the oligonucleotide, so that even if primer binding occurs the extension reaction will be inhibited. These reactions depend upon the absence of a 3′ to 5′ exonucleolytic activity in the *Taq* DNA polymerase (*80*). The alternative scheme uses two differentially labeled oligonucleotides in a single amplification reaction that may be performed at low stringency (*81*, *82*). In this case the perfectly matched primer competes with the mismatches primer for the binding site. In the competition method the base mismatches may be placed at the 3′ terminus or within the oligonucleotides. Both schemes can be enhanced by the adaptation of fluorescent oligonucleotide labels and can be adapted for the detection of variants that may be present at low abundance in a complex mixture.

VI. APPLICATIONS OF PCR

A. Diagnosis and Characterization of Human Genetic Diseases. Spectacular progress has been made in the characterization of mutations leading to human genetic diseases due to PCR. Previously only a handful of the molecular changes leading to the diseases that frequently result from de novo mutation events had been identified but now several hundred altered sequences have been characterized from patients with hemophilia, Duchenne muscular dystrophy, HPRT deficiency, ornithine transcarbamylase deficiency, and delta amino transferase deficiency. This list is by no means exhaustive and many other loci have been similarly studied. Previously these diseases provided a difficult diagnostic problem as there was no way to predict where in a gene a mutation might occur, but it is currently possible to survey entire genes from each patient in order to identify the altered bases and then to use that information to design further PCR based assays to diagnose other family members or to determine carrier status. The precise combination of analytical techniques may vary from locus to locus, depending mainly upon the size of the gene and the number of exons, but PCR based approaches now ensure that alterations in most "average" hemizygous (i.e. X-chromosome linked) genes can be quickly characterized. Mutations in autosomal genes and the detection of heterozygous loci provide a more difficult task due to the mixture of normal and altered sequences in the same sample. Nevertheless successful examples of mutational characterization abound (*3*, *4*, *28*).

The sensitivity of PCR has been exploited to detect sequences that are expressed at very low levels in tissues that are more easily obtained from patients than samples that are relatively abundant in the specific mRNA (*83*). *Dystrophin* mRNA, for example, can be amplified from lymphoblastoid cell lines for further analysis even though the sequences cannot be detected there by conventional filter hybridizations. Amplification of these rare sequences therefore has potential to obviate the need for painful muscle biopsies in DMD patients and invasion of different deep tissue in other diseases (*84*). The potential for rapidly identifying mutant sequences in early embryos has also been explored (*85*).

Simple PCR tests have been designed for the rapid detection of mutant alleles that are commonly associated with hemoglobinopathies, thalassemias, phenyketonuria, Tay-Sachs disease, and α-1-antitrypsin deficiency. The recent report of the molecular basis of the most common cystic fibrosis mutation has led to an allele-specific PCR assay (*86*). In general the only limitation on the development PCR based assays for other common alleles is the knowledge of the precise DNA sequence changes. New candidate mutations are being added to this list an an increasing rate. Finally, PCR has enabled the analysis of more complex human genetic traits. In particular the molecular dissection of the human HLA locus and its associated disease predisposition has been greatly facilitated by PCR-based sequencing studies (*59*, *87*, *88*).

B. Diagnosis and Characterization of Infectious Diseases. The lengthy period required for microbial growth and biochemical characterization of human pathogens can be circumvented when sufficient DNA sequence of a microorganism is known to enable a PCR primer set to be constructed.

Only a few of the many microorganisms that influence human health have been extensively analyzed at the DNA sequence level and currently the routine detection of most of these is by biochemical or immunological methods. The exceptions are mostly in the category of organisms that are difficult to culture and those for which detection at a minimum level is desirable. The human immunodeficiency virus (HIV)

is foremost among these because of the current epidemic of the acquired immunodeficiency syndrome. PCR has been applied for routine diagnosis of HIV infection (*89*, *90*) and has identified viral sequences prior to serological conversion (*91*), and in situations where the host has serologically reverted (*92*). The cellular distribution of HIV reservoirs in infected hosts has been studied by PCR (*93*) and DNA sequence analysis of the PCR amplified viral sequences has revealed the underlying diversity of the virus and aided the study of the evolution of HIV molecular heterogeneity (*94*, *95*).

The human papilloma virus has also been extensively studied by DNA amplification, overcoming the difficulties that arise because no in vitro culture system is available. Broker, Chow, and co-workers have used PCR to derive an extensive map of the transcriptional activity of related HPV strains and subsequently determined the functional significance of the different transcripts (*96*). In addition to vastly increasing the knowledge of the HPV lifecycle and pathogenicity, these studies provide an excellent example in demonstrating that a predominantly molecular biology approach can be used to dissect the functional organization of an organism, even with little prior knowledge of its overall biology.

An increase in the application of PCR in the characterization of infectious disease is predicted as more investigators become familiar with the methodology and the DNA sequence of more interesting organisms is derived. In addition the technique will find increasing application in diagnostic settings, particularly as more simple detection systems are devised.

C. Diagnosis and Characterization of Neoplasia. PCR applications in cancer have focused upon the characterization oncogene mutations and the detection of residual disease in human leukemia. Studies of oncogenic variation include both the correlation of molecular changes with tumorogenesis and the identification of mutations in recessive oncogenes. Commonly found alterations in human *ras* sequences are now known to correlate with formation of certain tumor types (*97*). Germ line and somatic mutations in the human retinoblastoma gene have been detected, which has both verified the involvement of the gene product in the disease and provided molecular diagnosis for individuals at risk (*98*, *99*).

Detection of residual disease takes advantage of molecular rearrangements associated with common heamatopoetic neoplasias including follicular lymphoma and chronic myelogenous leukemia (*100*, *101*). In each case PCR primers are positioned to amplify the rearranged sequences, but not normal DNA. The sensitivity of this assay vastly exceeds previous cytogenetic tests and allows rapid monitoring of patients undergoing remission to detect early sign of recurring disease and to direct subsequent chemotherapeutic strategies.

D. Studies of Somatic Mutations. The considerable interest in generation of mutagen fingerprints by the analysis of multiple mutations induced by a single agent has prompted use of PCR-based direct DNA sequencing methods to examine individual mutagenic events. The underlying principle of these studies is that even weak mutagens may be distinguished by the kinds of molecular alterations that they cause. Several hundred individual clones have now been examined in various laboratories, which could not have been achieved by conventional methods.

The construction of recombinant animal disease models has also been facilitated by the procedure. A popular strategy is to construct PCR primers that will only function as a primer pair following the incorporation of a new sequence or induction of a directed genetic event. Thus candidate murine embryo cells have been screened by sib selection to identify homologous recombinants and to increase the efficiency of subsequent reinitiation of the cells into chimeric whole mice. The offspring of the recombinant mice can also be monitored by the procedure to ensure that the correct genotype is recovered (*102*).

E. Evolutionary Studies. One of the first demonstrations of the versatility of PCR was the direct DNA sequence analysis of mitochondrial DNA from different humans and other species. The sequence data base was then used to construct phylogenetic relationships for comparison with established evolutionary trees (*104*). These studies have produced one the most interesting PCRs, by recovering sequence from DNA from a 7000 year old brain (*105*). Evidence for the controversial "Eve" hypothesis, that human ancestry could be traced to a single African women from approximately 6000 years ago, has also been generated. The overall range of evolutionary issues that are addressed by these studies is much wider than this and currently a very active area of investigation.

F. The Human Genome Project. The proposal to sequence the entire human genome has gained considerable momentum and it has been generally accepted that PCR will play an integral role in its outcome. PCR will be used in at least three ways. First, simplified DNA sequencing template production protocols may be adapted for the routine preparation of the enormous number of individual sequencing reactions to be carried out (*61*). Second, sequence tag segments (STSs) will be isolated by PCR (*106*). These proposed elements will be short dispersed regions of DNA sequence information that will provide both DNA mapping data and a signature for macromolecular DNA clones that are isolated during the initial phase of human genome mapping. Third, PCR and direct DNA sequencing will provide the simplest means for selected verification of interesting regions of sequence and for the joining of contiguous elements that cannot be recovered by alternative strategies. Overall, the proposed endeavor requires many steps to be simplified and automated, which are key features of PCRs contribution to other areas of study.

VII. PCR CONTAMINATION

The extreme sensitivity of PCR makes the procedure very vulnerable to the consequences of contamination with minute amounts of material. In general, it is the products of previous PCRs that constitute the most likely source of spurious amplification and users of the method have quickly learned to practice careful laboratory techniques. Most of the precautions that must be taken to ensure that there is no cross contamination of reactions are a matter of common sense and have been elaborately discussed elsewhere (*17*, *107*). Nevertheless anyone planning to use PCR is well advised both to consult these references and to perform some simple calculations to reinforce the magnitude of the problem. DNA plasmid contamination has been widely recognized as a common problem while performing Southern analysis for the detection of single copy human gene sequences. In this case about 1 pg of a contaminating 5 kb sequence can generate a signal that is not distinguishable from the real target. By comparison, a contaminating PCR template present at a 100-fold lower abundance would be interpreted as a real signal if the number of reaction cycles was taken to six or seven more than the absolute minimum usually used for amplification. The problem is greatly exacerbated when rare sequences are to be amplified (e.g. a single human spermatozoon) and these kinds of experiments require elaborate physical isolation procedures. However, despite the magnitude of the contamination problem, rigorous laboratory practice and an abundance of control reactions will ensure that few PCRs yield spurious data.

VIII. ALTERNATIVE AMPLIFICATION SCHEMES

Three alternative schemes for the amplification of specific

nucleic acid sequences have been described. The first scheme employs a DNA ligation cascade as the mechanism of amplification (*108*). Synthetic oligonucleotides are hybridized so that the 3′ terminus of one strand is directly adjacent to the 5′ terminus of the next. The two fragments are joined by DNA ligase provided the template for their head-to-toe annealing is present. When both strands of the target fragment are included (i.e. four oligonucleotides are synthesized) and the process is repeated many times there is an exponential increase in the abundance of the oligonucleotide dimers. This scheme has the disadvantage of not generating any new sequence information between the priming sites and being relatively slow to perform. Nevertheless it is sensitive to the presence of subtle difference in the template sequence and the possibility for isolation of a thermostable DNA ligase would make this scheme useful for the routine detection of rare sequences.

The transcription-based amplification scheme (TAS) employs an RNA polymerase promoter at the 5′ terminus of one oligonucleotide primer and uses sequential steps of cDNA synthesis and RNA transcription (*109*). During the first strand of cDNA synthesis the reverse transcriptase incorporates the promoter recognition sequence which is then copied into a blunt-ended, double-stranded DNA product. T7 or SP6 RNA polymerase is then used to generate from 10 to 1000 RNA copies of each cDNA strand. The process is repeated to produce the overall amplification. Conceptually this process is similar to that described by Sommer and co-workers (*70*, *71*) for the production of RNA from PCR products to facilitate DNA sequencing. However the TAS scheme is distinct as it uses multiple cycles of the cDNA/RNA syntheses, and does not require DNA polymerase.

The third scheme is distinct from the other methods in that a hybridization is carried out prior to amplification. RNA probes are first annealed to the target sequence and the complex is then amplified through the action of the bacterial enzyme Qβ-Replicase (*110*). The requirement for the specific hybridization event to occur prior to the amplification ensures the overall specificity of the reaction.

Each of these schemes is potentially useful, but so far none has been widely used. It is interesting that the incentive to develop alternative amplification schemes is largely generated by the restrictions on commercial use of PCR and the potential return upon the invention of a suitable replacement.

IX. CONCLUSIONS AND FUTURE PROSPECTS

PCR has clearly been established as an important method for the analysis and manipulation of DNA. It is likely that every molecular biology laboratory that has already used recombinant DNA technology will be able to find use for the procedure and that this trend will increase in the future. The wide range of applications described here is not exhaustive and many new applications and variations of the method may be anticipated.

Future prospects will include the wider availability of low cost synthetic oligonucleotides, development of simplified methods for detection of PCR products, and a reduced reliance on gel electrophoresis for the analysis of the reaction products. The precise format of eventual PCR detection systems is difficult to predict, but the success discussed here of avidin/biotin capture schemes, fluorescent primers, and enzyme-based colorimetric assays points toward ultimate simplicity and convenience. Whatever new schemes evolve to add to the power and versatility of PCR, there should be no surprises, given the great changes in methodological approaches that have already been seen.

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