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Direct Cloning and Sequence Analysis of Enzymatically Amplified Genomic Sequences

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A method is described for directly cloning enzymatically amplified segments of genomic DNA into an M13 vector for sequence analysis. A 110–base pair fragment of the human β-globin gene and a 242-base pair fragment of the human leukocyte antigen DQα locus were amplified by the polymerase chain reaction method, a procedure based on repeated cycles of denaturation, primer annealing, and extension by DNA polymerase I. Oligonucleotide primers with restriction endonuclease sites added to their 5′ ends were used to facilitate the cloning of the amplified DNA. The analysis of cloned products allowed the quantitative evaluation of the amplification method's specificity and fidelity. Given the low frequency of sequence errors observed, this approach promises to be a rapid method for obtaining reliable genomic sequences from nanogram amounts of DNA.

UNDERSTANDING THE MOLECULAR basis of genetic disease or of complex genetic polymorphisms, such as those in the human leukocyte antigen (HLA) region, requires detailed nucleotide sequence information from a variety of individuals to localize relevant variations. Currently, the analysis of each allelic variant requires a substantial effort in library construction, screening, mapping, subcloning, and sequencing. We report here a method for the enzymatic amplification of specific segments of genomic DNA and their direct cloning into M13 vectors for sequence analysis, using modifications of the polymerase chain reaction (PCR) (*1, 2*). This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by the Klenow fragment of DNA polymerase, and results in an exponential increase in copies of the region flanked by the primers. This method greatly reduces the number of cloned DNA fragments to be screened, circumvents the need for full genomic libraries, and may allow cloning from nanogram quantities of genomic DNA. In addition, the cloning and sequencing of PCR-amplified DNA is a powerful analytical tool for the study of the specificity and fidelity of this newly developed technique.

To develop this technique for genomic sequence determination and to analyze the individual products of PCR amplification, we chose the oligonucleotide primers and probes previously described for the diagnosis of sickle cell anemia (*2*). These primers amplify a 110-bp segment of the human β-hemoglobin gene containing the Hb-S mutation (Fig. 1). They were modified near their 5′ ends (Fig. 1) to produce convenient restriction sites (linkers) for cloning directly into the M13mp10 sequencing vector (*3*). These modifications did not affect the efficiency of PCR amplification of the specific β-globin segment (Fig. 2B), even though the overall pattern of PCR-amplified products is different (Fig. 2A). After amplification, the PCR products were cleaved with the appropriate restriction enzymes and dialyzed to remove inhibitors of ligation. These fragments were ligated into the M13 vector, transformed into the JM103 host, and plated out, and the resulting plaques were screened by hybridization with a labeled oligonucleotide probe to detect the β-globin clones. The plaques were also screened with the labeled PCR oligonucleotide primers to identify all of the clones containing amplified DNA. Individual clones were then sequenced directly by using the dideoxy primer-extension method (*4*).

The incorporation of different restriction sites at the termini of the amplified product allows digestion of the vector with two restriction enzymes, thereby reducing the background of blue plaques from vectors without inserts to less than 11% (Table 1). More than 80% of the clones contained DNA inserts with the PCR primer sequences but only about 1% of the clones hybridized to the internal β-globin probe. These nonglobin fragments presumably represent amplifications of other segments of the genome. This observation is consistent with the gel and Southern blot analysis of the PCR-amplified DNA from a β-globin deletion mutant and a normal cell line (Fig. 2). The similarity of the observed gel profiles reveals that most of the amplified genomic DNA fragments arise from nonglobin templates. Sequence analysis of two of these nontarget clones showed that the segments between the PCR primer sequences were unrelated to the β-globin gene and contained an abundance of dinucleotide repeats, similar to some genomic intergenic spacer sequences (*5*).

When ten of the clones that hybridized to the β-globin probe were sequenced, nine proved to be identical to the β-globin gene and one contained five nucleotide differences but was identical to the δ-globin gene. The β-globin PCR primers each have two mismatches with the δ-globin sequence (Fig. 1). The preferential PCR amplification of β-globin relative to δ-globin observed in our clonal analysis agrees with the results obtained with the oligomer restriction assay on the PCR reaction (*2, 6*). Each of these ten sequenced clones contains a segment of 70 bp originally synthesized from the genomic DNA template during the PCR amplification process. Since no sequence alterations were seen in these clones, the frequency of nucleotide misincorporation during 20 cycles of PCR amplification is less than 1 in 700.

To analyze the molecular basis of genetic polymorphism and disease susceptibility in the HLA class II loci, this approach has been extended to the amplification and cloning of a 242-bp fragment from the second exon of the HLA DQα locus, which exhibits localized allelic variability. In this case the primers, whose sequence is based on conserved regions of this exon, contain 5′-terminal restriction sites with no homology to the DQα sequence (Fig. 1). The specificity of amplification achieved with these primers is greater than that achieved with the β-globin

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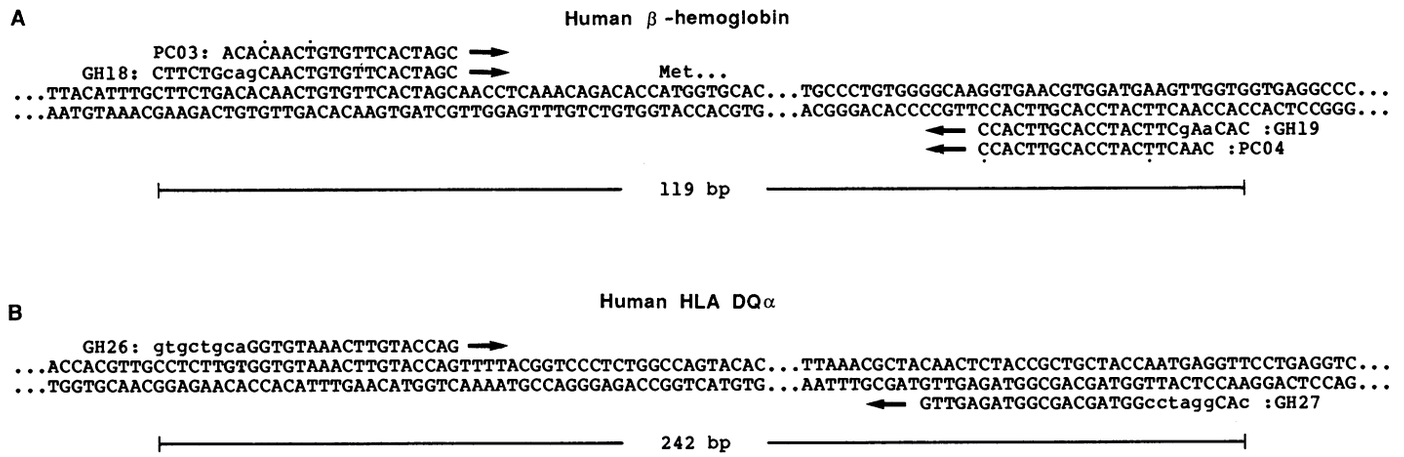

Fig. 1. Oligonucleotide primers for PCR amplification. (A) The two central sequence lines show both strands of the human β-hemoglobin gene (*5*) from 52 bp upstream of the initiation codon (Met) to 84 bp downstream. Above and below this sequence are the oligonucleotide primers used to amplify this segment of the genome. The 3′ end of each primer is marked by an arrow to indicate the direction of extension by DNA polymerase. Dots indicate nucleotide differences between β- and δ-globin. The primers PC03 and PC04 were used in our previous study (*1, 2*) and are completely homologous to the target gene. These sequences were extended and modified to produce the linker-primers GH18 and GH19. The modifications (lowercase letters) create a Pst I site in GH18 and a Hind III site in GH19. The amplified segment is 110 bp with PC03 and PC04, and 119 bp with GH18 and GH19. (B) The central sequences show a segment of the human HLA DQα gene (*10*) from codons 9 to 92 (codons are numbered for the mature protein). This region codes for the highly polymorphic outer domain of the protein. As above, the oligonucleotide linker-primer sequences used to amplify this segment are shown, with lowercase letters designating nonhomologous bases. In this case the desired restriction sites (Pst I for primer GH26 and Bam HI for GH27) were added to the 5′ end of the primers without regard to the target sequence. The length of the amplified product from HLA DQα is 242 bp.

Table 1. Distribution of β-globin PCR clones. An entire PCR reaction was digested at 37°C with Pst I (20 U) and Hind III (20 U) for 90 minutes (for β-globin) or Bam HI (24 U) and Pst I (20 U) for 60 minutes (for HLA DQα). After phenol extraction (*11*), the DNA was dialyzed to remove low molecular weight inhibitors of ligation (presumably the deoxynucleoside triphosphates used in PCR), and concentrated by ethanol precipitation. All (β-globin) or one tenth (DQα) of the material was ligated to 0.5 μg of the cut M13 vector under standard conditions (*11*) and transformed into approximately 6×10^9 cells of freshly prepared, competent JM103 (*3*) in a total volume of 200 μl. Fresh JM103 culture (150 μl) was then mixed with 10 to 30 μl of the transformed cells, plated on IPTG/X-gal agar plates (*3*), and incubated overnight. The plates were scored for blue (parental) plaques and lifted onto BioDyne A filters (*3*). These filters were hybridized (*2*) either with the labeled PC04 oligonucleotide to visualize all of the clones containing PCR-amplified DNA (primer plaques) or with the RS24 oligonucleotide probe [the exact complement of the RS06 (*6*) probe] to specifically visualize the clones containing hemoglobin sequences (globin plaques). Ten clones from the latter category were sequenced by the dideoxy extension method (*4*). Nine were identical to the expected β-hemoglobin target sequence and one was identical to the homologous region of the human δ-hemoglobin gene (*5*).

Category	Number	Frequency (%)
Total plaques	1496	100
White plaques	1338	89
Primer plaques	1206	81
Globin plaques	15	1

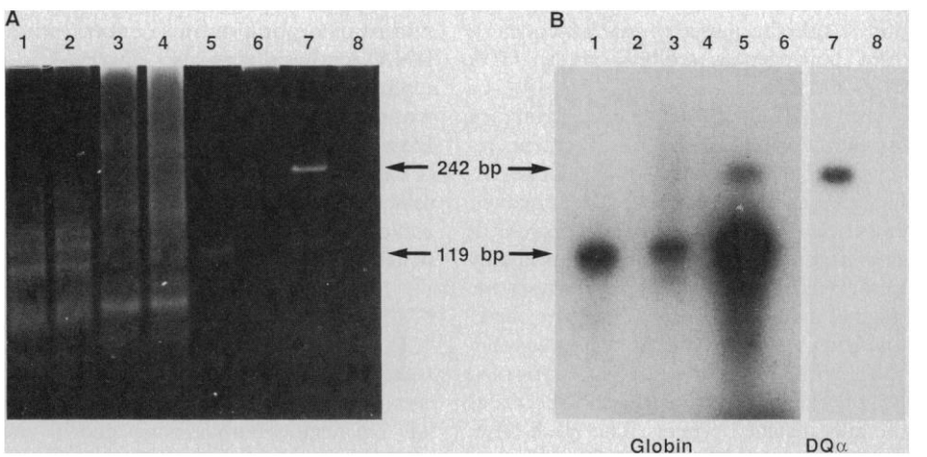

Fig. 2. PCR amplification with oligonucleotide linker-primers. DNA was amplified by mixing 1 μg of genomic DNA in polymerase buffer (*2*) with 100 pmol of each primer. Samples were subjected to 20 cycles of PCR, each consisting of 2 minutes of denaturation at 95°C, 2 minutes of cooling to 37°C, and 2 minutes of polymerization with 1 U of Klenow DNA polymerase. After amplification, the DNA was concentrated by ethanol precipitation and half of the total reaction subjected to electrophoresis on a gel of 4% NuSieve agarose in tris-borate-EDTA buffer. The gel was photographed, and the DNA was transferred to Genatran nylon membrane and hybridized (*2*) to a labeled probe specific for the target sequence. The blot was then washed and autoradiographed. For the amplification of β-globin, the starting DNA was either from the Molt-4 cell line or from the globin deletion mutant GM2064 (*2*). For lane 5, the starting material was 11 pg of the β-globin recombinant plasmid pBR328::β^A (*6*), the molar equivalent of 5 μg of genomic DNA. For lane 6, the reaction was performed as in lane 1 except that no enzyme was added. To increase the efficiency of amplification of the longer HLA DQα segment, dimethyl sulfoxide was added to 10% and the polymerization was carried out at 37°C for 27 cycles. The starting DNA for the amplification of DQα was either from the consanguineous HLA-typing cell line LG-2 or from the HLA class II deletion mutant LCL721.180 (*7*). (A) Ethidium bromide–stained gel showing total amplified products. (Lane 1) Primers PC03 and PC04 on Molt-4 DNA; (lane 2) PC03 and PC04 on GM2064 DNA; (lane 3) GH18 and GH19 on Molt-4; (lane 4) GH18 and GH19 on GM2064; (lane 5) GH18 and GH19 on pBR328::β^A DNA; (lane 6) PC03 and PC04 on Molt-4, no enzyme; (lane 7) primers GH26 and GH27 on LG-2 DNA; (lane 8) GH26 and GH27 on LCL721.180 DNA. (B) Southern blots showing specific amplified products. Lanes are numbered as in (A). Lanes 1 through 6 were hybridized to the labeled RS06 oligonucleotide probe (*2*). Lanes 7 and 8 were hybridized to a cloned DQα cDNA probe labeled by nick-translation (*11*).

primers, since gel electrophoresis of the PCR products reveals a discrete band at 240 bp absent from the HLA deletion mutant (*7*) (Fig. 2). In addition, hybridization screening of the M13 clones from this amplification indicates that about 20% are homologous to the DQα probe, an increase of 20 times over the β-globin amplification. The basis for the difference in the specificity of amplification, defined as the ratio of target to nontarget clones, is not clear. It is likely to reflect the primer sequences and their genomic distribution rather than the different reaction conditions used (Fig. 2). The differences between the sequence of the β-globin primers GH18, GH19 and PC03, PC04 (Fig. 1) may account for the observed change in the gel profile of the PCR-amplified products (Fig. 2, lanes 1 and 2 versus lanes 3 and 4), reflecting some difference in the amplification of nontarget segments.

Three HLA DQα PCR clones derived from the homozygous typing cell LG2 were subjected to sequence analysis (*8*). Two clones were identical to a DQα complementary (cDNA) clone from the same cell line. One differed by a single nucleotide, indicating an error rate of approximately 1/600, assuming the substitution occurred during the 27 cycles of amplification. This procedure has also been used to analyze sequences from polymorphic regions of the HLA DQβ and DRβ loci.

Our rapid method for the cloning of specifically amplified genomic fragments relies on the ability of oligonucleotides to function as PCR primers with unpaired bases near their 5′ ends. In the later cycles of amplification these primers anneal primarily to the amplified products rather than to the original genomic sequences, and are therefore fully complementary. The β-globin linker-primers GH18 and GH19 appear to be approximately as efficient and specific as the fully matched primers. The HLA DQα linker-primers GH26 and GH27, with even more 5′ mismatches, show an amplification specificity 20 times higher than the β-globin primers. A large number of DQα clones were obtained from just 100 ng of this amplified genomic DNA. Our data suggest that the error rate over many cycles of amplification is sufficiently low so that reliable genomic sequences can be determined directly from PCR amplification and cloning.

This procedure offers significant advantages over standard cloning protocols for the analysis of sequence polymorphisms in that it circumvents the construction and screening of full genomic libraries and could potentially be initiated from nanogram amounts of DNA. It is capable of isolating only a limited region of the genome, however, and requires sequence information to identify conserved primer and probe segments. Unlike direct genomic sequencing (*9*), the cloning of amplified DNA allows the separation of related genes and alleles prior to sequencing and does not require a detailed knowledge of adjacent restriction sites. The application of oligonucleotide linker-primers to introduce specific restriction sites into PCR-amplified DNA may also prove useful as a general cloning strategy.

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Inhibition of Endothelial Regeneration by Type-Beta Transforming Growth Factor from Platelets

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Damage to the vessel wall is a signal for endothelial migration and replication and for platelet release at the site of injury. Addition of transforming growth factor-beta (TGF-β) purified from platelets to growing aortic endothelial cells inhibited [^{3}H]thymidine incorporation in a concentration-dependent manner. A transient inhibition of DNA synthesis was also observed in response to wounding; cell migration and replication are inhibited during the first 24 hours after wounding. By 48 hours after wounding both TGF-β–treated and –untreated cultures showed similar responses. Flow microfluorimetric analysis of cell cycle distribution indicated that after 24 hours of exposure to TGF-β the cells were blocked from entering S phase, and the fraction of cells in G_1 was increased. The inhibition of the initiation of regeneration by TGF-β could allow time for recruitment of smooth muscle cells into the site of injury by other platelet components.

THE CONTINUITY OF THE VASCULAR endothelium is lost as atherosclerotic lesions progress (*1*). In contrast, even in large wounds the endothelium rapidly regenerates both in vitro and in vivo (*2*). This raises the possibility that some property of the atheromatous wall or element of the blood interacting with the wall at the developing plaque can act to prevent the normal processes of endothelial regeneration. An obvious source of such an effect at sites of denudation is the platelet. Among the components released from platelets at a site of vascular injury are a group of growth regulatory proteins, including platelet-derived growth factor (PDGF), a growth factor similar to epidermal growth factor (EGF), and transforming growth factor-beta (TGF-β) (*3, 4*).

The endothelium grows as a strictly density-inhibited monolayer. Although endothelial cell growth requires serum or plasma, PDGF and EGF are not required (*5*). In the presence of acid-treated serum, aortic endothelial cells have been shown to form colonies in soft agar (*6*). Similar results were obtained from mouse embryo cells (AKR-2B) or normal rat kidney (NRK) cells and the activity that promotes growth in soft agar was identified as TGF-β (*4*). We report here that TGF-β purified from human platelets can inhibit the endothelial regeneration process by inhibiting both replication and migration.

Transforming growth factors were originally found in viral-transformed rodent cells, and it was postulated that they were involved in neoplastic cell growth (*7, 8*). However, their presence in normal tissues, including kidney, placenta, and platelets, suggests a more general function (*4, 9*). Platelets contain 40 to 100 times as much TGF-β as other nonneoplastic tissues. TGF-

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