APPLICATION OF NON-RADIOACTIVE METHODS OF DNA DETECTION IN ANALYSIS OF HUMAN GENETIC DISORDERS*

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We have applied two non-radioactive methods for detection of unique sequences in human genome: 1/polymerase chain reaction, 2/hybridization with digoxigenin-deoxyuridine 5′-triphosphate labeled probes. With the polymerase chain reaction technique we were able to amplify short segments of genes coding for coagulation factors VIII and IX. Electrophoretical analysis of products of polymerase chain reaction enabled us to detect deletions causing hemophilia A or B. To analyse deletions in dystrophin gene, the most frequent cause of Duchenne muscular dystrophy, we have amplified several different fragments of this gene simultaneously. We have studied restriction fragment length polymorphism closely linked to the cystic fibrosis locus with digoxigenin-deoxyuridine 5′-triphosphate labeled probe p3.11 with sensitivity comparable to methods involving the use of radioisotopes.

DNA analysis is an increasingly important source of information in modern medicine. In the last few years analysis of restriction fragment length polymorphisms (RFLPs) has been widely used to diagnose genetic disorders at the DNA level [1, 2]. The method commonly applied for identification of DNA fragments bearing the sequence of interest is hybridization of radioactively labeled molecular probes with DNA digested with

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1 Abbreviations: PCR, polymerase chain reaction; digoxigenin-dUTP, digoxigenin-11-deoxyuridine 5′-triphosphate; RFLP, restriction fragment length polymorphism; Taq DNA polymerase, DNA polymerase from Thermus aquaticus; SDS, sodium dodecyl sulphate, kb, kilobase pair.

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an appropriate restriction enzyme and transferred onto a nitrocellulose membrane. Direct analysis of DNA presents distinct advantages over classical methods, especially in diagnosis of genetic disease before its clinical appearance, prenatal diagnosis, detection of carriers, and discrimination among phenotypically similar genotypes [3-5]. However, the radioisotope-labelled probes strictly limit the use of the technique to specially equipped laboratories. Therefore many efforts have been made to develop non-radioactive methods enabling detection of, and studies on, specific DNA sequences.

We describe here improvements of procedures which permitted analysis of single copy genes responsible for genetic disorders — without the use of radioisotopes — i.e. by polymerase chain reaction (PCR)\(^1\) and hybridization with digoxigenin-dUTP labeled probe followed by colorimetric detection.

PCR is an \textit{in vitro} technique of amplification of short segments of human genome. In this method, a pair of oligonucleotide primers, complementary to sequences flanking a particular region of interest, is used to indicate and direct DNA synthesis in opposite and overlapping directions. With repeated cycles of DNA denaturation, primer reannealing, and DNA synthesis, human DNA sequences can be faithfully amplified several hundred thousand times [6]. The enzyme used in this procedure is a heat-stable DNA polymerase purified from bacterium \textit{Thermus aquaticus} (Taq DNA polymerase) [7]. The relatively high temperature (63 - 70\(^\circ\)C) at the elongation step ensures specific DNA synthesis. Taq DNA polymerase is heat-stable and the addition of fresh enzyme after each denaturation step is not required. In this paper application of PCR for diagnosis of hemophilias A and B, and Duchenne muscular dystrophy (DMD) is described.

Hemophilia A, one of the most common inherited blood coagulation disorders in humans, is caused by a defect in coagulation factor VIII. The wide range in the clinical severity of hemophilia A has been demonstrated to result from accumulation of heterogeneous mutations in the factor VIII gene [8]. With the recent molecular cloning of this gene, intragenic polymorphic DNA sequences have been described [9]; these sequences are currently used for accurate identification of carriers and for prenatal diagnosis. These polymorphism involve recognition sites for the restriction enzymes \textit{BclI}, \textit{BglII} and \textit{XbaI}; the presence or absence of the sites provides a tag for the hemophilia mutation in a pedigree analysis. In studies of these polymorphisms, DNA fragments containing \textit{BclI}, \textit{BglII} and \textit{XbaI} target sequences may be amplified, and PCR products are then subjected to digestion with enzymes and studied electrophoretically.

Hemophilia B (Christmas disease) is caused by mutations in the coagulation factor IX gene [10]. The factor IX gene is about 34 kb long. It contains 8 exons which cover only 8\% of its total length [10]. Deletions in exons of the factor IX gene can be also determined by means of PCR. Exons are first amplified and after electrophoresis the absence of a specific
band may be identified as due to deletion of a particular segment of the gene. A similar procedure permits to study deletions causing DMD. This chromosome X-linked disease is caused by deletions in one of the longest eukaryotic genes, the one coding for the peptide dystrophin. Recently cDNA of dystrophin has been sequenced [11] and this enabled designing of oligonucleotide primers for amplification of specific fragments of the gene. It is possible to amplify several DNA sequences simultaneously. After electrophoresis several bands of different length may be visualized each corresponding to a specific region of cDNA. Deletions may be easily identified by the absence of one or more of the bands in comparison with the DNA from healthy donor.

Although the introduction of the PCR technology permitted to analyse DNA without the use of radioisotopes, it did not replace Southern blot analysis. RFLPs within the factor VIII gene or a locus closely linked to cystic fibrosis can be identified using amplified DNA. If these polymorphisms are not informative, hybridization still has to be performed. We have applied a non-radioactive technique for detection of RFLPs closely linked to cystic fibrosis locus which is unique in human genome, with a sensitivity comparable to that of methods involving the use of radioisotopes. The molecular probe is labeled with digoxigenin-dUTP, and bands after hybridization are visualised by means of colorimetric reaction.

MATERIALS AND METHODS

Preparation of DNA samples. DNA from patients with hemophilia A or B, cystic fibrosis or DMD, and their family members were used in experiments. DNA from blood collected into 0.1% EDTA was prepared by urea extraction or by the method of Kunkel [12] with minor modifications.

The whole peripheral blood sample was diluted with 9 vol. of buffer composed of 10 mM Tris/HCl, pH 7.6, 0.32 M sucrose, 5 mM MgCl₂ and 1% Triton X-100. Leucocyte nuclei were collected by centrifugation (2500 g, 15 min). Pellets were washed with PBS and suspended in lysis buffer (75 mM NaCl, 24 mM EDTA, 1% SDS and 100 μg/ml proteinase K). Mixtures were incubated overnight at 37°C and then extracted at least three times with phenol. DNA after precipitation with ethanol was collected with a glass rod and dissolved in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

Alternatively, DNA was prepared by extraction in the presence of urea as follows: 20 ml of blood was centrifuged (3000 g, 10 min). Cell pellets were suspended in 10 ml of buffer containing 0.8% Triton X-100 and 0.9% NaCl and centrifuged again (27000 g, 20 min). The resulting pellets which
contained cell nuclei were suspended in 10 ml of buffer composed of 10 mM Tris/HCl, pH 7.5, 5.6 M urea, 1% SDS, 0.35 M NaCl and 1 mM EDTA, and then 30 ml of buffer composed of 10 mM Tris/HCl, pH 7.5, 0.35 M NaCl and 1 mM EDTA were added. Mixtures were extracted with phenol-chlorophorm three times. DNA after precipitation with equal volume of isopropanol was incubated for 1 h at −70°C, then DNA was pelleted, washed with 70% ethanol and dissolved in TE buffer.

**Synthetic oligonucleotides.** Oligonucleotide primers were designed to direct synthesis of human coagulation factor IX gene exons, a fragment of human coagulation factor VIII gene containing polymorphic BclI recognition site and fragments of the gene coding for dystrophin.

**Sequence amplification with Taq DNA polymerase.** Amplifications with Taq DNA polymerase (New England Biolabs) were performed according to a modification of the procedure described by Saiki et al. [6]. Target sequences were amplified in 50 µl of the reaction mixture containing 0.5 µg of genomic DNA, 1.5 mM each dATP, dCTP, dGTP and dUTP, 50 pmoles of each oligonucleotide primer and 10% dimethyl sulfoxide—all in 1× reaction buffer (16.6 mM ammonium sulfate, 67 mM Tris/HCl, pH 8.8, 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 6.7 mM EDTA and 170 µg/ml BSA).

To prevent evaporation, 50 µl of paraffin oil was loaded over each sample. Samples were heated at 97°C for 7 min to denature the DNA and cooled in an ice-water bath. One unit of Taq polymerase was added to each sample, which was then transferred to 63°C for 1.5 min for primer-directed DNA synthesis. Subsequent cycles consisted of 30-second denaturation step at 90°C, 40 second annealing of primers to the template at temperatures optimal for each pair of primers, and 1.5 min extension step at 63°C. A total of 40 cycles were performed, with the addition of 1 unit of Taq polymerase in cycle 21.

**Analysis of samples in amplified DNA.** The samples (10 µl) were subjected to electrophoresis in agarose (1.2 or 1.8%) or acrylamide gels (12%) without previous treatment of after digestion with BclI for 2 h at 37°C. Bands containing the PCR products were visualised by ultraviolet fluorescence after staining with ethidium bromide.

**RFLP analysis using digoxigenin-labeled probe.** Approximately 5 µg of genomic DNA was digested with restriction enzymes and subjected to electrophoresis in 0.8% agarose gel at 22 volts for 16 h. Gels were blotted according to Southern [13].

Molecular probe p3.11 [14] was labeled with digoxigenin-dUTP and nitrocellulose filters were processed further according to manufacturer's protocol (Boehringer, Manheim, F.R.G.) with the following modifications: 1. Probes were labeled for 16 h at 37°C, under paraffin.
2. Labeled probes were purified by ethanol precipitation and column centrifugation.
3. Filters after hybridization were incubated for 3 h with blocking reagents, before antibodies conjugated with alkaline phosphatase were added.
4. For the color reaction, the filters were placed, the DNA side down, between two sheets of Whatman 3 MM paper and then substrates were added.

RESULTS

We have applied two non-radioactive methods for detection of unique sequences in human genome—the polymerase chain reaction and hybridization with digoxigenin-labeled probes. PCR primers were designed to amplify specific fragments of human coagulation factor VIII and IX genes, as well as fragments of the gene coding for dystrophin. Exons a, b+c, d, g and h of factor IX gene were amplified. The length of PCR products (511, 635, 234, 327 and 702 bp, respectively) corresponded to the distance framed by primers (see Fig. 1).

We have applied the PCR technique for diagnosis of hemophilia B. Figure 2 shows electrophoretical analysis of amplified DNA from a patient with hemophilia B and his mother as well as from a normal unrelated subject. Synthesis of a 234 bp fragment (exon d of factor IX gene) occurred only with genomic DNA from normal donors as a template for primers.

Fig. 1. Electrophoretic analysis in 1.8% agarose gel of products of polymerase chain reaction containing exons of the human coagulation factor IX gene. Lanes: 1, 2, exon d (234 bp); 3, exon h (702 bp); 4, 5, exon a (511 bp); 6, 7, exons b+c (635 bp); 8, 9, exon g (327 bp); 10, 11, controls, no DNA added; 12, λDNA digested with EcoRI and HindIII
d1 and d2. This band was not observed after amplification of genomic DNA from a boy with hemophilia B.

For detection of carriers of hemophilia A, a DNA fragment of the factor VIII gene containing the BclI polymorphic recognition site was synthesized using the PCR technique. Amplified sequences were further analyzed by means of digestion with BclI and electrophoresis. PCR products from normal subjects were completely digested giving rise to two fragments of 99 and 43 base pairs. PCR products from the carriers of hemophilia A were only partially digested, which resulted in three fragments (of 142, 99 and 43 bp; Fig. 3). For the analysis of deletions in DNA from patients with DMD several DNA fragments were amplified simultaneously using several pairs of primers. After electrophoresis some PCR products were not observed in patients in comparison with the positive controls, and therefore the deleted region can be easily identified (Fig. 4).

As a second method of non-radioactive detection of changes in DNA we applied digoxigenin labeled probes for analysis of the RFLPs closely linked to the cystic fibrosis locus. After modifications we were able to detect single copy sequences in human genome by this method. Figure 5 shows results of hybridization of the genomic DNA digested with MspI with the digoxigenin-dUTP labeled molecular probe p3.11. After hybridization to the target DNA the hybrids were detected by enzyme-linked immunoassay using an antibody-conjugate and subsequent enzyme-catalysed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT), reaction was sensitive enough to detect single copy sequences in the human genome.

**DISCUSSION**

Two non-radioactive methods for detection of unique sequences in human genome: the polymerase chain reaction and hybridization with digoxigenin-labeled probes were applied for rapid diagnosis of some genetic disorders. Enzymatic amplification was used for rapid production of large quantities of DNA from the regions containing variations linked to three genetic disorders: polymorphic restriction sites linked to hemophilia A, and deletions causing hemophilia B and Duchenne muscular dystrophy. Restriction site polymorphism can be analyzed by digestion of amplified samples. This approach is applicable to diseases in which the mutation alters a restriction site, such as sickle cell anemia. We have studied the sequence recognized by BclI which is one of the most informative intragenic factor VIII polymorphisms. After amplification, the 142 bp BclI fragment is partially or completely cleaved to 99 and 43 bp in persons who are hemizygous or heterozygous for the presence of this site (Fig. 3). Although some background
Fig. 2. Diagnosis of hemophilia B by electrophoretic analysis of products of amplification in vitro in 1.2% agarose gel. Denaturation at 90°C for 30 s, annealing at 20°C for 40 s, elongation at 63°C for 90 s. Lanes: 1, KBL DNA marker; 2, DNA from mother; 3 DNA from son with hemophilia B, deletion of exon d; 4, control DNA; 6, pBR322 digested with restriction enzyme MspI; 9, λDNA digested with restriction enzyme HindIII.

Fig. 3. Detection of carriers of hemophilia A in family by analysis of the BclI polymorphism in amplified sequences. PCR products were digested with restriction enzyme BclI and subjected to electrophoresis in 12% polyacrylamide gel. Lanes: 1, DNA from father (normal); 2, DNA from mother (carrier of hemophilia A); 4, 5, DNA from two daughters (normal); 6, KBL DNA marker; 7, 8, controls, no DNA added; 9, 10, PCR products not digested with BclI from mother and father.
Fig. 4. Analysis of deletions in DNA from patients with Duchenne muscular dystrophy. Several fragments of the gene encoding dystrophin were amplified simultaneously and subjected to electrophoresis in 1.5% agarose gel. Lanes 1, positive control, healthy male; 2, DMD patient, deletion cDNA 7; 3, DMD patient, deletion cDNA 7; 4, DMD patient, deletion cDNA 7/8; 5, DMD patient, no deletion; 6, DMD patient, deletion cDNA 7/8; 7, DMD patient, no deletion; 8, DMD patient, deletion cDNA 8; 9, DMD patient, no deletion; 10, control, no DNA added; 11. KBL DNA marker

bands are present, the heterozygote (lane 2) is clearly distinguishable from persons who are homozygous for the site (lanes 1, 4, 5).

In the case of disorders caused by gene deletions, such as alpha-thalassemia, diagnosis can be made simply by observing the presence or absence of the sequences in amplified samples. We have demonstrated this approach for hemophilia B and Duchenne muscular dystrophy. Analysis of PCR products from a patient with hemophilia B (Fig. 2) revealed a deletion of exon d in his factor IX gene (lane 3). The corresponding fragment was present in amplified sample from his mother (lane 2).

To analyse deletions causing DMD several sequences were amplified simultaneously in DNA samples from DMD patients (Fig. 4) with the use of several primer pairs. We observed the absence of one or two amplified DNA fragments due to deletions in dystrophin gene (lanes 2, 3, 4 6 and 8). In some cases no deletions could be identified with this set of primers (lanes 5, 7 and 9).

PCR enabled us to study these two kinds of variations in human genome without the need of Southern blotting and hybridization. This simple non-radioactive method can readily and directly diagnose genetic disorders caused by gene deletions and mutations which alter restriction endonuclease cleavage sites. DNA synthesis with Taq DNA polymerase is performed at
Fig. 5. Detection of unique gene sequence in MspI-digested DNA samples using the molecular probe p3.11 labeled with digoxigenin. Lanes: 1, homozygote 4.2/4.2 kb; 2, heterozygote 1.8/4.2 kb; 3, homozygote 4.2/4.2 kb; 4, homozygote 1.8/1.8 kb

a high temperature; this increases the specificity of the oligonucleotide priming, and the amplified sequence can be directly visualised after staining as a discrete band in agarose gel, circumventing the need for DNA hybridization. This procedure may be readily applied to prenatal diagnosis of genetic disorders, which is reduced to the following steps. Fetal cells are lysed and 30 cycles of polymerase chain reaction are performed. For diagnosis of gene deletions an aliquot of the reaction mixture is immediately fractionated by gel electrophoresis, while for studies of restriction site polymorphisms an aliquot is first digested with an appropriate restriction enzyme. After staining of DNA in gels, the mutations can be directly detected. Diagnosis can be completed in one day. For single locus analysis in single cells the probability of erroneous diagnosis due to replication errors in PCR is of the order of 1 [15].

Sometimes southern blot analysis can not be substituted by the polymerase chain reaction. If an exact upstream and downstream sequence of the polymorphic restriction site is not known there is no possibility of designing specific oligonucleotide primers for PCR. Therefore hybridization
with a molecular probe still has to be performed. However, the use of radioisotopes may also be substituted by non-radioactive markers, for example digoxigenin.

We have applied digoxigenin labeled probe p3.11 for analysis of the RFLP closely linked to the cystic fibrosis locus, which is unique in human genome. Sensitivity of the modified method is similar to that of methods involving the use of radioisotopes. Additionally, the use of probes labeled with digoxigenin-dUTP simplifies the DNA analysis for diagnostic purposes. Labeled probes can be stored for several months and the frequent labeling is no longer necessary.

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REFERENCES

