Nucleic Acids Research

Mutation detection using nucleotide analogs that alter electrophoretic mobility

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ABSTRACT

A simple primer extension assay has been developed to distinguish homologous DNA segments differing by as little as a single nucleotide. DNA strands are synthesized with one of the four natural nucleotides replaced with an analog that affects electrophoretic mobility. DNAs that are the same length but differ in the number of analog molecules per strand exhibit different mobilities on a sequencing gel. In combination with the polymerase chain reaction (PCR; 1, 2), this method has been used to distinguish mutant and normal alleles of the human insulin receptor gene that differ by a single-base substitution. The method appears to be generally applicable to the detection of any nucleotide polymorphism in any segment of DNA.

INTRODUCTION

Genetic mapping of nucleotide polymorphisms (3) has become a powerful approach for characterizing the human genome as well as other complex genomes. These genetic maps are a critical component in the identification of genes associated with human disease as well as other genes of importance. Furthermore, detection of specific mutations is the basis for the emerging field of DNA diagnostics (4, 5). For genetic mapping, nucleotide polymorphisms are most commonly assayed by analysis of restriction fragment length polymorphisms (RFLP's, 3). Many nucleotide changes are not detected by RFLP analysis because these changes do not affect cleavage with a restriction enzyme. Hybridization with oligonucleotide probes (6, 7) can be used to assay for specific, known mutations, but these techniques cannot be used generally to identify previously undetected mutations. Thus, there is a need for techniques that can detect any single-base change that might occur and that can be used where the site of mutation is not known in advance. Several approaches to this problem have been pursued, including nuclease (8, 9) or chemical (10, 11) cleavage at mismatches in heteroduplexes and electrophoresis through denaturing gradient gels (12−14).

We have developed a simple method for detecting nucleotide differences based on the fact that the incorporation of certain nucleotide analogs into DNA causes a detectable shift in electrophoretic mobility. A DNA polymerase is used in a primer extension reaction to synthesize DNA strands of defined length. As substrates for this synthesis, one of the four natural deoxynucleoside triphosphates is replaced with a mobility-shifting analog. When DNAs are compared by electrophoresis in a denaturing gel, strands that are the same length but differ in the number of analog residues will migrate at different rates. This difference in migration can be used to distinguish homologous DNAs differing by nucleotide substitutions.

MATERIALS AND METHODS

PCR reactions contained approximately 200 ng human genomic DNA, 200 ng each of primers A and B (see Figure 1), 10 nmol of each deoxyribonucleoside triphosphate, and 5 units Taq DNA polymerase (Perkin-Elmer Cetus) in 20 μl PCR buffer (67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 4.5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 170 μg/ml bovine serum albumin). PCR reactions were performed under mineral oil in a DNA Thermal Cycler (Perkin-Elmer Cetus) as follows: 94°C (7 min), 55°C (3 min), 72°C (4 min) for one cycle and then 30 cycles of 94°C (2 min), 55°C (3 min), 72°C (4 min) with one second transition steps. Each amplified DNA sample was extracted with phenol:chloroform:isoamyl alcohol (50:49:1), passed over a spun G-50 column, precipitated with ethanol, dissolved in 20 μl TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA), and stored at 4°C. Approximately 1/80th of this amplified material was used as template in each primer extension reaction. Primer extension reactions (5μl) contained denatured PCR-amplified DNA, 50nM primer C (see Figure 1) end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase, 10 μM TTP or biotin-11-dUTP (Enzo Biochem), 10 μM dCTP or 5-(bio-AC-AP3)dCTP (gift from George Trainor), 10 μM dATP, 10μM dGTP, 50 mM Tris-HCl, pH 9.0 at 25°C, 5 mM $MgCl_2$, and 0.4 units/μl Taq DNA polymerase (Perkin-Elmer Cetus or Promega). After incubation at 70°C for 5 min, the samples were dried under vacuum in a Speed-Vac concentrator (Savant) for 5 min, resuspended in formamide-stop mix (95% formamide; 12.5 mM EDTA; 0.3% bromophenol blue; 0.3% xylene cyanol), boiled for 5 min, and electrophoresed on a 8 M urea/6% acrylamide gel at 65 watts for 3 h. The dried gel was exposed to X-ray film.

RESULTS AND DISCUSSION

Langer et $al.$ (15) noted that incorporation of biotinylated nucleotides into DNA causes a retardation in mobility when the DNA is electrophoresed in an agarose gel. A more striking finding is that the addition of a fluorescence-tagged dideoxynucleotide to a DNA strand consistently causes that DNA to migrate two bases slower than expected in a sequencing gel (16). Prompted by these observations, we have found that incorporation of biotin-11-dUTP, a commercially available analog of TTP, into a DNA strand causes a one nucleotide mobility shift when the DNA is fractionated on a sequencing gel. This means that, for each biotinylated residue incorporated, the biotin-containing DNA strand migrates at a position approximately one nucleotide slower than is expected based on the length of the DNA strand. This mobility-shifting property of nucleotide analogs can be used as the basis for a method to detect mutations because it makes base composition a major factor affecting migration through a sequencing gel.

The utility of mobility-shifting nucleotide analogs is illustrated by the analysis of a segment of the human insulin receptor gene. Kadowaki et $al.$ (17) describe a nonsense mutation in the insulin receptor gene that causes premature termination after amino acid 671 in the α subunit. In Figure 1 the sequence of a segment of the insulin receptor gene shows the location of the mutation consisting of a C to T change in the coding strand. We have analyzed genomic DNA from a patient heterozygous for this deleterious mutation and from an unaffected individual. Using the two flanking oligonucleotide primers shown in Figure 1 and PCR (1, 2), a 140-base pair DNA segment containing the mutational site was amplified in each of the genomic DNA samples. These amplified DNA fragments were used as templates for a series of primer extension reactions. The amplified DNA made from the heterozygote contains templates derived from both the mutant and normal alleles of the
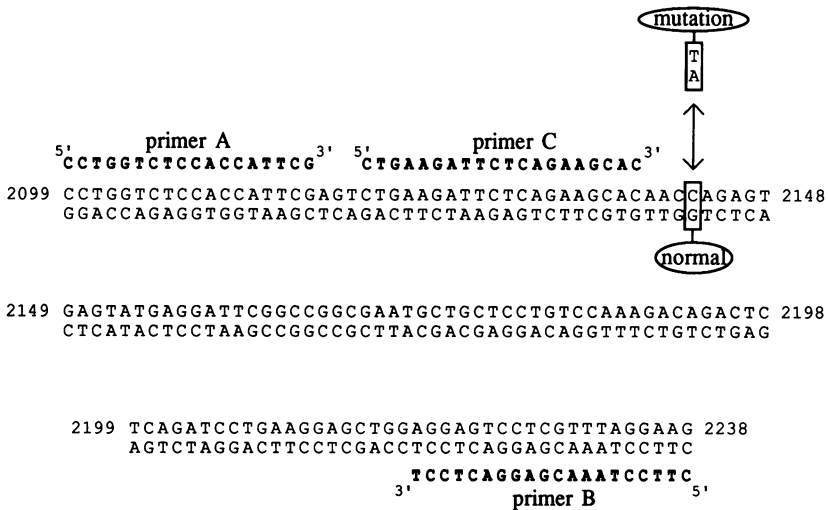
Figure 1 *Sequence of a segment of the human insulin receptor gene.* This sequence is taken from the cDNA sequence reported by Ullrich *et al.* (21) and the numbers refer to their numbering scheme. The location of the C to T change that creates a TAG nonsense mutation is marked. Primers A and B were used for PCR amplification of human genomic DNA; primer C was used for the primer extension reactions displayed in Figure 2.

insulin receptor gene, while the amplified DNA from the normal individual contains only the template derived from the normal allele. An oligonucleotide that hybridizes internally on the amplified DNA was used to prime the extension reactions in order to reduce the likelihood of detecting spurious amplification products (18). Using ^{32}P-labeled primer C (see Figure 1), separate extension reactions were performed using the four natural deoxynucleoside triphosphates, using biotin-11-dUTP in place of TTP, and using 5-(bio-AC-AP3)dCTP, a biotinylated dCTP analog, in place of dCTP. Figure 2 shows the primer extension products fractionated on a standard sequencing gel. All extension products are expected to be 119 nucleotides long. Comparison of lanes 3−6 with lanes 1 and 2 demonstrates that the mobility of DNA strands containing either analog is retarded relative to strands containing only natural nucleotides. For the reactions using biotin-11-dUTP, extension products synthesized on the normal template should contain 20 biotin-11-deoxyuridine residues, while products synthesized on the mutant template should contain 21 analog residues. Thus, in the mutant heterozygote sample in lane 4, two bands are observed—the extension products synthesized on the normal and mutant templates. The mutant product is expected to migrate slower than the normal product because the mutant product contains one additional biotin-11-deoxyuridine residue. Comparison with the normal sample in lane 3 shows that the mutant product does indeed migrate slower. Similar results are observed in lanes 5 and 6 for the reactions using 5-(bio-AC-AP3)dCTP. In this case, the normal product contains 22 5-(bio-AC-AP3)-deoxycytidine residues and the mutant product contains only 21 analog residues, so the normal product migrates slower. These results demonstrate that a single-base substitution can be identified using mobility-shifting nucleotide analogs and that a heterozygote for a point mutation can be distinguished from a homozygote.
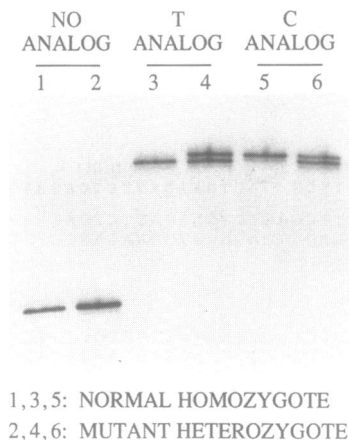
Figure 2 *Primer extension reactions on human insulin receptor gene templates using mobility-shifting nucleotide analogs.* The ^{32}P-labeled primer extension products shown in this autoradiogram were synthesized using the PCR-amplified templates prepared from the homozygous normal individual (lanes 1, 3, 5) and the heterozygous mutant patient (lanes 2, 4, 6). The primer extension reactions contained no mobility-shifting nucleotide analogs (lanes 1, 2), biotin-11-dUTP in place of TTP (lanes 3, 4), or 5-(bio-AC-AP3)dCTP in place of dCTP (lanes 5, 6). The dCTP analog 5-(bio-AC-AP3)dCTP is a close structural analog of biotin-11-dUTP in which the olefinic group in the linker-arm has been replaced with a functionally equivalent acetylenic group. For details on this acetylenic linker-arm see Prober *et al.* (16) and Hobbs (22).

Longer exposure of the autoradiogram displayed in Figure 2 shows that, in all six lanes, there are minor bands migrating approximately one nucleotide slower ($+1$) and one nucleotide faster (-1) than the major bands. After 30 cycles of PCR amplification using *Taq* DNA polymerase, the overall error frequency is estimated to be 0.25% (19, 20). With this magnitude of error frequency, a small amount of $+1$ and -1 product would be expected. Whether due to an inherent property of the enzyme, reaction conditions, enzyme purity, or some other factor, the extraneous bands are not abundant enough to cause ambiguous interpretation of the data for fragments of the size being analyzed in Figure 2. We are currently analyzing larger DNA segments in order to determine the size limit for detection of single nucleotide substitutions. This will depend on the resolving power of the gel and on the incremental mobility shift caused by each additional analog residue in larger DNA fragments. With regard to extraneous bands, it should also be noted that the nucleotide analogs used in this procedure must be of high purity. Even a small amount of contamination with unsubstituted nucleotide will lead to series of fragments that migrate faster than the major band because they do not have a mobility-shifting analog at every available position.

There are a number of features that should make the use of mobility-shifting nucleotide analogs an attractive method for detecting mutations and polymorphisms. All types of nucleotide changes can be detected by this technique. For the analysis of double stranded DNA, all single nucleotide substitutions can be studied with just two analogs by using each of the two strands as template strand in separate primer extension reactions. C and T analogs will probably prove the most useful because it is easier to synthesize pyrimidine analogs. Of course, small insertions and deletions are also detected because they alter the

total number of nucleotides in the extension products. The analysis is simple and rapid. The reactions and gels are basically the same as those used in standard dideoxynucleotide sequencing. Currently under investigation is the possibility of including mobility-shifting nucleotide analogs directly in the polymerase chain reaction, which would make this type of analysis even more rapid. The technique can be readily adapted to nonradioactive detection schemes. If biotin-containing analogs are used, there are a variety of reagents that can be used to visualize the biotinylated DNA. In order to detect the biotin, it might be best to first transfer the DNA fragments from the gel to a nylon or nitrocellulose support. Our current approach to nonradioactive labeling is to use fluorescence-tagged primers that can be detected using Du Pont's GENESIS™ 2000 system (16). In addition to more rapid detection, the use of different fluorescent tags makes it possible to include an internal standard in each lane. Another attribute of this method is that, for any specific segment being tested, all of the detected fragments are confined to a narrow region of the gel. Thus, by analyzing segments of different length or by staggering loading times, multiple samples can be electrophoresed in a single lane of the gel.

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