MOLECULAR BEACONS
Spectral Genotyping of Human Alleles

Leondios G. Kostrikis, Sanjay Tyagi, Musa M. Mhlanga, David D. Ho, Fred Russell Kramer

Accelerated by the human genome project, an increasing number of genetic variations, many as small as a single nucleotide substitution, have been found to play a significant role in human disease. Although direct sequencing is adequate for their detection, simpler and faster automated methods are necessary for population studies and clinical diagnostics. Alternative methods currently in use (1) selectively amplify a region of DNA and then examine it for the presence of a mutation, either by gel electrophoresis (2, 3) or by hybridization with nucleic acid probes (4). However, electrophoretic techniques miss some mutations, and additional steps must be taken after hybridization, such as isolation of the hybrids, removal of nonhybridized probes, and the generation of detectable signals in complex enzymatic reactions. Moreover, the manipulations required for both electrophoresis and hybridization are difficult to automate, and the handling of the amplified DNA creates a risk of contaminating untested samples.

We have developed an automated method for detecting mutations, called “spectral genotyping,” in which alleles are identified by fluorescent colors generated in sealed amplification tubes. In this technique, amplification is carried out in the presence of molecular beacons, which are probes that become fluorescent when they hybridize to their target (5). Molecular beacons are hairpin-shaped, single-stranded oligonucleotides consisting of a probe sequence embedded within complementary sequences that form a hairpin stem. A fluorophore is covalently attached to one end of the oligonucleotide, and a nonfluorescent quencher is covalently attached to the other end. In the absence of a target, the fluorophore is held close to the quencher and fluorescence cannot occur. When the probe binds to its target, the rigidity of the probe-target helix forces the stem to unwind, resulting in the separation of the fluorophore and quencher, and restoration of fluorescence. These probes can detect a number of different targets in the same solution (6). This is accomplished by constructing a different molecular beacon for each target and attaching a differently colored fluorophore to each. The probes are placed in the same amplification tube, and the color that develops indicates which targets were present. For genotyping alleles, two molecular beacons are used, one specific for the wild-type allele and labeled with a green fluorophore and the other specific for the mutant allele and labeled with a red fluorophore. The appearance of green fluorescence during amplification indicates homozygous wild-types, red fluorescence indicates homozygous mutants, and both green and red fluorescence indicates heterozygotes.

We used this procedure to distinguish alleles of the β-chemokine receptor 5 (CCR5) gene that determines susceptibility to infection by the human immunodeficiency virus (HIV-1). Individuals homozygous for a 32-nucleotide deletion in this gene (CCR5Δ32) are largely resistant to HIV-1 infection, despite multiple sexual contacts with HIV-infected individuals (7, 8), and heterozygotes are partially protected against disease progression (9–11). To understand the susceptibility of human populations to the spread of HIV-1, large-scale epidemiological studies of the distribution of this mutant allele are needed, necessitating high-throughput assays. Therefore, we developed an automated spectral genotyping assay that identifies CCR5 alleles. For the detection of the wild-type allele, we synthesized a fluorescein-labeled molecular beacon whose probe sequence was complementary to the region that is deleted in the mutant; for the detection of the mutant allele, we synthesized a tetramethylrhodamine-labeled molecular beacon that was complementary to the sequences flanking the region of the deletion, which are brought together in the mutant (12). Human DNA samples were used as templates for polymerase chain reactions (PCRs) in which the region of the CCR5 gene that encompasses the site of the Δ32 mutation was amplified in the presence of both molecular beacons (13).

Figure 1 shows the results from three PCRs that illustrate the principle of spectral genotyping. In a reaction initiated with a DNA sample from a homozygous wild-type individual, only the fluorescein-labeled molecular beacons are able to bind to the amplified regions (amplicons), and only green fluorescence develops during the amplification. In a reaction initiated with DNA from a homozygous mutant individual, only the tetramethylrhodamine-labeled molecular beacons bind to the amplicons, and only red fluorescence develops. However, in a reaction initiated with DNA from a heterozygous individual, both molecular beacons bind to their target amplicons, and both red and green fluorescence develops. In control reactions in which no DNA template is present, fluorescence does not develop.

We carried out spectral genotyping assays in parallel, in a 7700 Prism spectrofluorometric thermal cycler (Applied Biosystems, Foster City, CA) that monitors changes in the fluorescence spectrum of each reaction.
tube, while simultaneously carrying out programmed temperature cycles. The length of the probe sequence in each molecular beacon was designed so that it hybridizes to the amplicons at the same temperature as the primers, and fluorescence measurements were taken during the annealing stage of each thermal cycle. The instrument decomposes the spectrum into its fluorosecin and tetramethylrhodamine components (or into the spectral components of any other fluorophores that are chosen as labels). During the early stages of PCR, the concentration of amplicons is insufficient to generate a detectable signal; however, a point is eventually reached at which the signal becomes significant (the "threshold cycle"). The more template DNA that is in a sample, the sooner the threshold cycle occurs (14).

We determined the genotype of 179 coded human DNA samples that had been characterized previously by the electrophoretic mobility of their CCR5 amplicons (10). Spectral genotyping was performed in sealed 96-well plates. Both molecular beacons were present in each reaction tube. The results showed that 24 samples generated a positive signal for both fluorosecin and tetramethylrhodamine fluorescence (heterozygotes), 151 samples generated a positive signal for fluorosecin fluorescence and remained negative for tetramethylrhodamine fluorescence throughout the course of the reaction (homozygous wild-types), and 4 samples generated a positive signal for tetramethylrhodamine fluorescence and remained negative for fluorosecin fluorescence (homozygous mutants). The ease with which the three genotypes could be distinguished from one another is shown in Figure 2. The results from each sample are plotted according to how many thermal cycles it took before a signal appeared in each color. For the heterozygous samples, similar threshold cycles were seen for both colors. These results were in complete agreement with the previously determined genotypes. Thus, this assay provides a rapid and accurate method for genotyping human CCR5 alleles and should accelerate current efforts to understand the global distribution of the CCR5A32 allele, which plays an important role in both HIV-1 transmission and disease progression, as well as in the natural history of the HIV-1 epidemic.

The difference between the two CCR5 alleles is so large that alternative methods (15), such as those based on the 5'-endonucleolytic cleavage activity of Taq DNA polymerase (14), could also have been used. However, when the difference between the alleles is a point mutation, spectral genotyping offers a particular advantage because molecular beacons are significantly more specific than conventional oligonucleotide probes (6). Only perfectly complementary molecular beacons form hybrids. The presence of a single nucleotide mismatch destabilizes the probe-duplex target to such an extent that they cannot overcome the stability of the stem in the molecular beacons (16). For example, we determined the distribution of the CCR2-641 chemokine receptor allele (17), which is a G-to-A substitution associated with delayed disease progression in HIV-infected individuals (18). We analyzed 974 human DNA samples in a single week. We sequenced 75 of these samples, and the results were in complete agreement with the results from spectral genotyping. Thus, spectral genotyping may be applied to any allelic pair, even those that differ from each other by only a single nucleotide substitution.

Even though we monitored the progress of these reactions with a sophisticated instrument that performs PCRs while simultaneously monitoring fluorescence, the intensity of the fluorescence at the end of the amplifications is so large that genotypes can be identified by visual inspection of the tubes under ultraviolet illumination (heterozygotes appear yellow). It is even possible to dispense with the thermal cycle by using isothermal amplification reactions (19). For the future, the most intriguing challenge is to eliminate amplification and detect targets directly. One way to achieve direct detection is to localize the targets in a small volume. For example, molecular beacons have been used to directly detect specific messenger RNAs in living cells (20). Another direction that is being explored is the simultaneous determination of a large number of genetic variations, using an array of different molecular beacons tethered to unique locations on a surface. For example, green and red molecular beacons specific for each allele in a pair may be present at each location. Unlabeled target nucleic acids can then be hybridized to the array, and the color of the fluorescence at each position will indicate the alleles that are present. Spectral genotyping should be widely applicable for the characterization of alleles.

References and Notes

12. The sequence of the wild-type-specific molecular beacon was fluorosecin-5'CGCGTTGT-GAATTCCTCAAGTATCGACCCG-3'DABCYL, and the sequence of the mutant-specific molecular beacon was tetramethylrhodamine-5'CGCGTTTCTTAAATGAAAAATGACTGCGG-3'DABCYL, where underlines identify the complementary sequences that form the stem and DABCYL is the quencher 4-(6-dimethyl-aminophenylazo)benzoic acid. A protocol for synthesizing molecular beacons is available on the World Wide Web at www.chil.nyu.edu/molecular_beacons.
13. Each 50 μl reaction contained 1 μg of genomic DNA, 0.25 μM of each molecular beacon, 0.5 μM of each primer, 2.5 units of AmpliTag Gold DNA polymerase (Perkin-Elmer), 50 mM KCl, 3.5 mM MgCl2, and 10 mM tris-HCl (pH 8.3). Forty cycles of amplification (94°C denaturation for 30 s, 62°C annealing for 60 s, and 72°C polymerization for 30 s) were performed in a spectrophotofluorometric thermal cycler possessing a 488-nm laser light source.
21. Supported by the Aaron Diamond Foundation and NIH (grant HL-33521). We thank W. Paxton, Y. Huang, and L. Zhang for providing samples of human DNA. L. Kostriks is an Aaron Diamond Fellow.

TechWire Forum: www.sciencemag.org/dmmail.cgi?53541