DIAGNOSTIC DNA

By JULIE ANN MILLER

Medical diagnosis at its most basic level is often a genetic problem. A patient may be genetically incapable of a normal biological function, or some foreign microorganism, with its own genes, may have established residency at the expense of the patient's health.

What could be more direct than to approach medical diagnosis by going right to the patient's, or the infecting microorganism's, genes? Recently developed methods of cutting, reproducing and selecting lengths of DNA are allowing biologists to do just that. These "DNA probe" techniques are already being used in medical research and are expected soon to reach the diagnostic market. Biotechnology companies anticipate selling to hospital laboratories, and perhaps eventually to physician's offices, simple kits that will allow disease diagnosis more rapidly and with greater accuracy than methods currently available.

"This is a technique under very aggressive development right now," says Dean Engelhardt of Enzo Biochem, Inc., in New York. The method is based on the strong binding between strands of DNA molecules with complementary sequences. Of the four types of DNA subunits that form each strand, adenosine on one strand tends to line up with thymidine on the other strand, and guanosine pairs to cytosine. This binding of strands is called hybridization. It depends on the same forces that hold together the two strands of the DNA helix. Although the match of complementary bases does not need to be perfect for hybridization to occur, the better the match the tighter the binding will be.

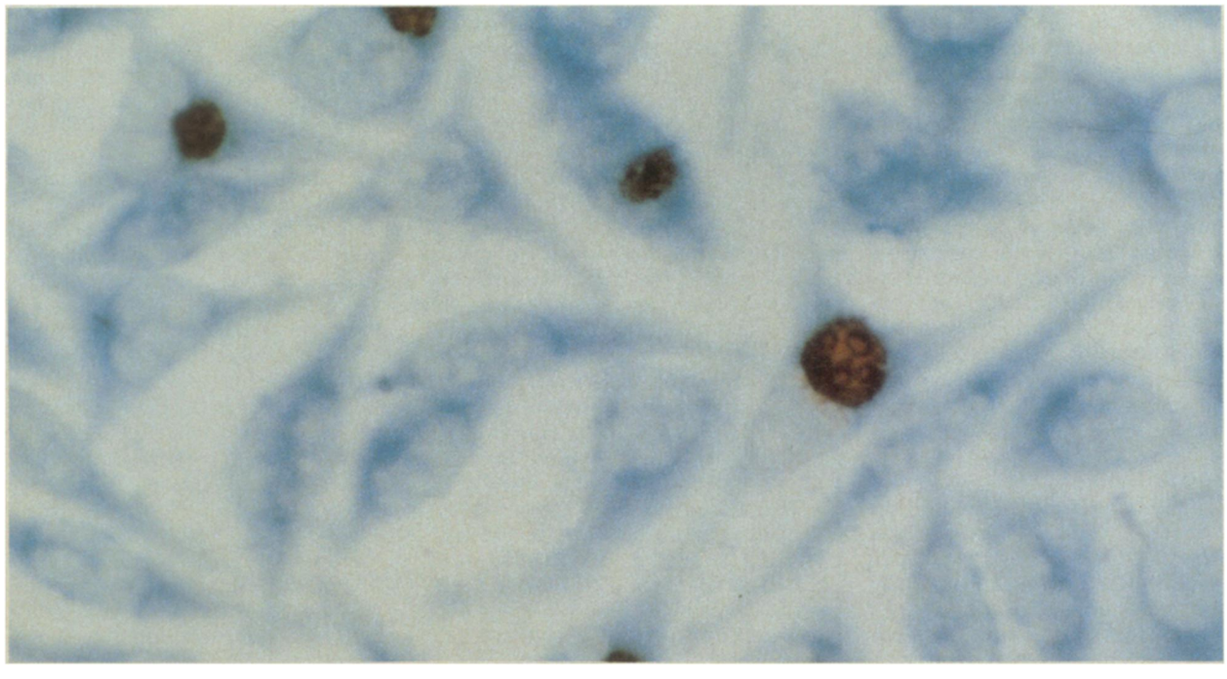
To be a useful tool, a DNA probe must have two components: a detector and a reporter. The detector is a fragment of DNA selected to hybridize to a segment of the gene being sought. The length of the fragment can range from 15 to several thousand nucleotides. The researchers must add to the DNA fragment a reporter or marker component, such as radioactivity or fluorescence, to allow its later identification. Probes can also be made from RNA molecules, which bind to DNA strands by the same principles.

The probes can detect a specific gene or segment of DNA either in its chromosomal location in a cell or in a sample of DNA or DNA segments. An important characteristic of the DNA probe technique is that it does not depend on a gene being active. It can locate a gene that is not currently being expressed in a cell or even one that is in an organism that is dormant.

Probes can be designed to be specific to a particular gene, chromosome or microorganism or to be a more general screening tool for a broad group of species. Probes have already been developed for a variety of viruses and bacteria. For use in prenatal diagnosis of genetic diseases, probes have been made that detect the Y chromosome or specific abnormal human genes.

Scientists use a variety of methods to choose a probe. Where they have identified genes characteristic of an organism, such as the gene for a particular toxin, DNA probes that bind these genes are likely to provide sensitive detection factors.

Detection of drug-resistant bacteria can serve as one example of the power and specificity of the DNA probe technique when a specific gene is targeted. Not only



Enzo Biochem, Inc.

Bacteria responsible for chlamydia, a sexually transmitted infection, are identified by a brown stain in one non-radioactive DNA probe technique.

Soon to hit the medical market is a new technique that can identify disease-causing organisms and detect hereditary disorders

can probes detect the presence of microorganisms, but they can also distinguish between bacteria with different genetically determined characteristics. For example, scientists know what gene is responsible for bacterial resistance to the drug ampicillin. Such resistance is an important factor in the treatment of gonorrhea and other infections.

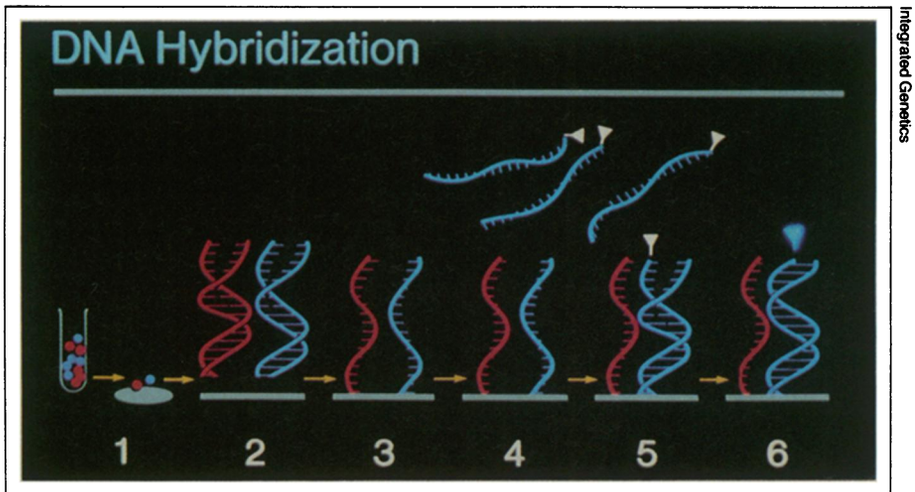
Paula Olsiewski and colleagues at Enzo Biochem made a DNA probe that binds just to the ampicillin-resistance gene. If the probe binds to a sample of bacteria from a patient, then one can conclude the patient is infected with an ampicillin-resistant organism. Olsiewski envisions this probe being used in concert with probes that distinguish different bacteria. "You can simultaneously determine pathogenicity and drug resistance," she says.

But one does not need to have such an identified gene in order to create a probe. A recently developed test for Salmonella, a bacterium responsible for food poisoning, illustrates an alternative approach to the technique.

Renee Fitts and colleagues at Integrated Genetics in Framingham, Mass., used an enzyme to cut Salmonella DNA into about a thousand fragments, and they inserted those fragments into plasmids, rings of DNA, which can be reproduced in laboratory bacteria. They then screened copies of those fragments against the DNA of a series of different Salmonella isolates and against the DNA of other similar intestinal bacteria, including *Escherichia coli*.

The scientists found that the intestinal bacteria are surprisingly similar genetically. There were only ten DNA fragments that were found solely in Salmonella. Together these fragments make up less than 1 percent of the total Salmonella DNA. Of the ten, two fragments bind to all the Salmonella isolates tested, and therefore are good candidates for a diagnostic probe. The fragments have been labeled with radioactivity and used in field trials, where they appear to be faster and more sensitive than the methods in current use.

Because the Salmonella test is used on foods, as well as on clinical samples, special technical problems had to be overcome. "How do you do a hybridization in peanut butter?" Fitts says. She and colleagues have developed a technique that works on "heavily particulate" substances, such as peanut butter or macaroni, as well as stool samples. They are finishing their initial field tests and are planning to do a comparative field study, where laboratories are asked to compare the old and new techniques, to begin by the end of the year.



Identifying microorganisms with the DNA probe techniques is a sequential process. Here, the organisms collected on a filter (1) are disrupted and their DNA strands are separated (2). The single strands of DNA are bound to the filter (3). Then investigators add the DNA probe, which is labeled (white triangles) for later identification (4). The probes bind only to DNA strands that have the appropriate (complementary) sequence (5). Excess probe is washed away and the remaining bound probe molecules are identified by procedures that detect their label (6).

Integrated Genetics

Attempts to screen for a group of microorganisms called mycoplasmas, which are known to cause diseases in animals, ran into quite a different set of problems. Whereas one must differentiate very similar organisms when working with Salmonella and other intestinal bacteria, scientists attempting to make a "generic" probe for mycoplasmas have had trouble finding any common grounds for identification.

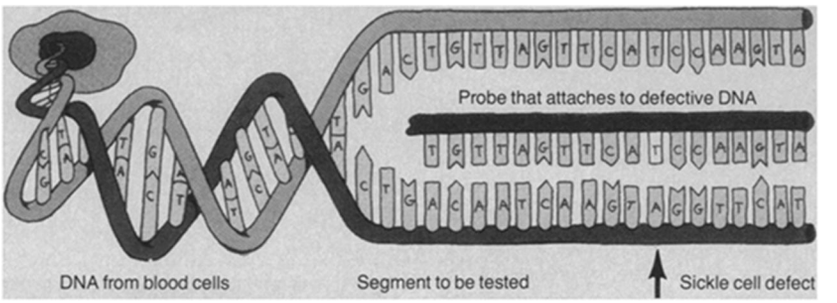
"Any sequence from one mycoplasma is unlikely to recognize a sequence from any other," says David Kingsbury of the University of California at Berkeley. But he and colleagues finally found that the genes for ribosomal RNA tend to be shared by the organisms. Now they have made a DNA probe that binds to all but one of the eight mycoplasmas found in clinical samples. In one of the first applications of this probe, they discovered that many samples of "healthy" human cells used in tissue culture work actually harbor mycoplasma infections.

DNA analyses have also been developed for detecting abnormal human genes, for example those that cause sickle cell anemia and those involved in hemoglobin disorders. A group of British scientists recently reported success in using DNA probes to identify the Y chromosome in first-trimester prenatal testing using chorionic villi biopsy. Fetal sex determination is important in cases at risk for severe X-linked disease where there is either no specific fetal diagnosis for the condition or a specific diagnosis of the condition in male fetuses is only possible later in pregnancy.

"The use of [DNA] probes means that the chromosomal sex of the fetus can be identified more quickly than by chromosome preparation and more accurately than by sex chromatic staining, and has the additional advantage that the same DNA preparation can be used for other diagnostic tests," say J. R. Gosden of Western General Hospital in Edinburgh, Scotland, C. H. Rodeck of King's College Hospital Medical School in London and colleagues. The probe technique does not depend on growing cells in culture, and thus avoids the problems of contamination that often hinder direct analysis of the chromosomes.

Gosden and co-workers have used two probes that bind to the Y chromosome and have correctly determined sex in a series of 77 fetuses. The test requires 1 to 5 micrograms of DNA from the biopsy, which usually yields 5 to 20 micrograms. The test

Sickle cell anemia can be quickly diagnosed with DNA probe techniques. This probe is designed to bind selectively to DNA containing the abnormality characteristic of the disease.



Industrial Biotechnology Association

is not affected by the presence of maternal tissue in the sample. By doing the same test on DNA from the parents of the fetus, the researchers can be assured that the results will not be incorrect due to an inherited abnormality, such as a piece of the normal Y chromosome that has been moved to some other chromosome. They report their work in HUMAN GENETICS (Vol. 66, No. 4, 1984).

Biotechnology companies are working on new approaches to the reporter, as well as the detector, sections of the probe. The most common label for a DNA probe in research laboratory work is radioactivity. But radioactivity is less suitable for clinical diagnostic tests—it decays rapidly and presents handling and disposal problems. Biotechnology companies are now developing alternative labeling procedures that allow fluorescence, a precipitate or a dye to mark the final location of the probe.

For example, the Enzo scientists label their probes with a vitamin called biotin attached to a nucleotide linker. This biotin tag does not interfere with the DNA double helix formation required for hybridization. It binds to several proteins that can be used to generate a useful signal.

In the ampicillin resistance example, the biotin-labeled DNA probe is detected by a complex of molecules — a protein called streptavidin, which binds biotin tightly, and the enzyme horseradish peroxidase, which is commonly used in biology to produce a brown precipitate. Alternatively the streptavidin complex can be prepared with enzymes that produce pink, blue or violet products. Two different probes can be detected in a single procedure by using different tagging methods.

In addition to their use in diagnosing infections, DNA probes are becoming important tools in other areas of medical research. For example, most of the mycoplasmas found in humans have not been linked to any disease, but with sensitive detection methods these organisms may be found to underlie some currently serious and little-understood diseases.

Kingsbury and colleagues are examining mice that have a disease resembling rheumatoid arthritis. A mycoplasma infection early in a mouse's life can later cause symptoms resembling the human disease. Although previous studies had found no sign of mycoplasma infection at the time when the joint problems develop, the DNA probe reveals significant levels of mycoplasma in the affected joints, Kingsbury reports. He also has preliminary evidence of mycoplasma presence in human arthritis patients.

Another medical application of the DNA probe technique is to distinguish between normal and malignant cells. Some cancers are characterized by a specific alteration in any of about 20 genes, called oncogenes (SN: 11/13/82, p. 316). Scientists have already made DNA probes that will identify such an altered gene in a human tissue sample.

The technique is also helping cancer researchers plot the associations between different types of cancer. A biotechnology company, Oncor, Inc., in Gaithersburg, Md., is selling to researchers a set of DNA probes for identifying six oncogenes. These probes are being used to determine whether newly discovered oncogenes are related to known oncogenes and to locate oncogenes on the human chromosomes.

Blood and tissue typing is another area where DNA hybridization is being used to improve diagnostic capabilities. In the decades since the ABO blood groups were discovered, more and more blood and tissue cell surface proteins have been found that contribute to the success or failure of blood transfusions and tissue transplants. But the most recent analyses of the DNA behind these factors has boosted still more the apparent genetic complexity. They have identified both more chromosome locations that are involved and more variations in the genes that fill those loci.

These fine distinctions are not solely academic, says Henry Erlich of the Cetus Corporation in Emeryville, Calif. Recently there have been two cases where the standard blood tests showed the same pattern of cell surface (HLA) markers in a tissue donor and a recipient. But the samples were mismatched on factors detected only by DNA analysis. In each case the transplant was rejected, Erlich says.

Fine genetic differences of the HLA region are expected to become more important in predicting and diagnosing diseases not related to transfusions or transplants. Already about 75 different diseases have been associated statistically with certain HLA types. Some of these associations may reflect the role products of the HLA genes play in body immunity. In other cases the association may simply reflect the proximity on the chromosome of an HLA gene and a gene underlying an unrelated disease. Some HLA genes are expected to prove more useful than others as markers for disease screening.

In addition to chromosomal location, the distribution of gene variations (alleles) in the population is important. According to David Botstein of the Massachusetts Institute of Technology and Integrated Genetics, Inc., for genetic analysis it is best to have five to ten alleles of a given gene, so that a person is likely to inherit a different allele from each parent.

"We are gradually accumulating a library of genetic markers that indicate susceptibility to IDDM [insulin-dependent diabetes mellitus]," Erlich says. Previous studies had associated the disorder with the HLA alleles called DR3 and DR4. But this association is not clinically useful in predicting who will develop the disease. Each person with DR4, for example, only has one chance in 50 of developing the disease. (In the U.S. general population, one person in about 300 has IDDM.) However, Erlich envisions that a DNA probe could be produced that would identify a region of polymorphic DNA where people with a certain sequence would have a 10 to 33 percent chance of developing diabetes. This information would be useful in early detection for more effective therapy, for example the initiation of immunosuppressant therapy (SN: 6/16/84, p. 375).

The DNA probe techniques on the horizon are the second major contribution of biotechnology to diagnostic testing. The first contribution, in use for about two years, is tests employing specific, uniform (monoclonal) antibodies (SN: 5/7/83, p. 296). Among their initial uses are pregnancy testing, HLA typing and diagnosis of sexually transmitted diseases.

Will the DNA probe techniques overshadow monoclonal antibodies? Both techniques depend on a tagged "probe", either an antibody or a segment of nucleic acid, binding to its target. A panel at a recent symposium held in Arlington, Va., by the Health Industry Manufacturers Association argued the relative merits of the two developing methods. Which is better in an individual situation may just depend on whether the most appropriate target is a molecule on the surface of a cell (so that an antibody is the preferred detector) or a piece of DNA (so that a DNA probe is most suitable).

Robert Yolken of Johns Hopkins University Medical School in Baltimore says that although tests employing the newer, DNA-probe technique currently take

DNA probes establish landmarks on chromosomal maps

Scientists using DNA probes recently for the first time were able to locate a disease-causing gene — the gene for Huntington's disease — that could have been anywhere on any of the human chromosomes (SN: 12/24 & 31/83, p. 408). The method employed was similar to the use of HLA genes as chromosomal markers for nearby disease genes (see page 106). The broad application of the DNA probe methods to locating genes is possible because any piece of identifiable DNA, whether or not its function is known, can serve as a chromosomal marker. The newest twist to this approach, used for the Huntington's gene, is a technique called restriction fragment length polymorphism (RFLP). It was developed by scientists including David Botstein of the Massachusetts Institute of Technology and Integrated Genetics, Inc. and Ray White of the University of Utah in Salt Lake City.

The major obstacle in mapping the thousands of human inherited disorders has been the lack of landmarks on the chromosomes. The new technique provides such landmarks. Special enzymes are used to snip the chromosomes at characteristic places into several thousand fragments. Genetic differences between individuals affect where these cuts are made and thus how large the fragments will be. Radioactively labeled DNA probes are used to identify the fragments.

The RFLP technique requires family studies, both to make the original association between a gene and marker fragment and to do individual diagnoses. In every family the gene will be in the same location, but that location won't necessarily be marked by the same fragment pattern.

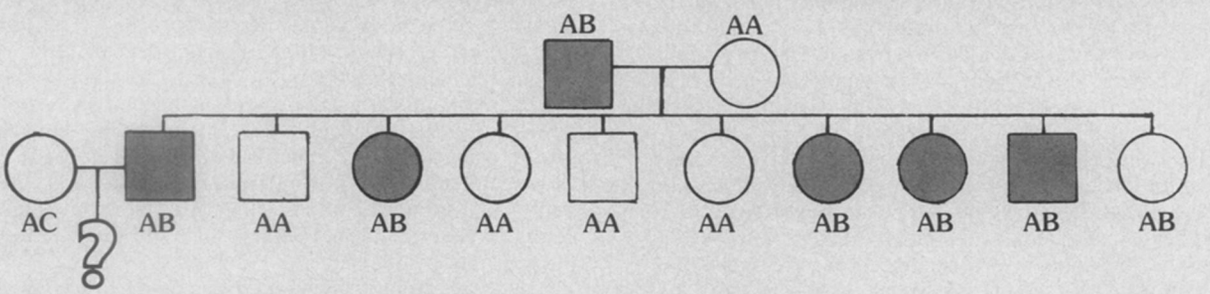
A person inherits from each parent a particular pattern of sites where the enzymes cut, and thus a pattern of fragments. A pattern can be used as a marker for a genetic disease if in a particular family one pattern is produced by the members with the disease, but not by the other members. Scientists must use large families of several generations to establish such an association. In the case of the Huntington's disease gene, James Gusella of Massachusetts General Hospital in Boston examined the genes of 25 living members of an American family and 570 members of a family in Venezuela. In the two families different specific fragment patterns were linked to the disease, but in both cases the linkage was established between the disease gene and a marker from a certain region, a few million nucleotides long, of chromosome 4.

Scientists can now predict from the DNA fragment pattern whether a member of one of these families will develop Huntingtons' disease. Prenatal diagnosis is also possible. The researchers are currently searching for a marker on the other side of the Huntington's disease gene, because flanking markers will increase the accuracy of diagnosis. They are also looking for enzymes that will cut the DNA in ways that give more variability between individuals, and thus a greater possibility that any given family will be suitable for this DNA analysis.

Among the other diseases being investigated with this technique are cystic fibrosis, dystonia, neurofibromatosis, Wolf's syndrome, the dominantly inherited thyroid cancer called multiple endocrine neoplasm II and a sex-linked form of muscular dystrophy. If the fragments represent all parts of the chromosomes, the presence or absence of some fragment can be expected to be associated with any disease-causing gene. Botstein estimates that scientists will need a minimum of 150 evenly spaced markers in order to find the location of all the genetic disease genes. He says it will take examination of about 1,000 markers to get that number of well spaced, useful probes. "We want to get a set of flags to mark every street," Botstein says.

Botstein outlines some limitations of this powerful method. Because family studies are necessary, it won't be useful in population screening, he says. And he expects application of the technique to continue to require experts. "It won't be a 'dipstick' test; it will be a reference lab business," he says. "It will always be complicated, like HLA typing."

—J.A. Miller



In this hypothetical family, the DNA fragment pattern B, rather than A, is associated with the dominant genetic disease indicated in gray. All family members with the disease have inherited from their father the chromosome giving the B pattern. The couple on the left could determine by the presence of the B pattern in DNA of amniotic cells or chorionic biopsy whether their fetus is likely to carry the disease. In the case of the daughter on the far right, it is likely there was breakage and recombination of the chromosome between the marker and the disease gene. Use of markers on either side of the disease gene would avoid misdiagnosis in such cases.

longer to perform than do monoclonal antibody assays, the development of a probe is generally a predictable, straightforward business. Creating a monoclonal antibody, on the other hand, is more "hit-or-miss," he says.

"The big advantage of the DNA probe is that you are dealing with a defined situation," Yolken says. But he warns that the technique is still young, "We will have to go through clinical studies to look at its usefulness for disease diagnosis."

The DNA probe technique, however, is already attracting interest both from the health products industry and the stock market. The Health Industry Manufacturers Association says DNA probes are well on their way to carving out a fair share of the medical diagnostic market. Stock analysts say they expect DNA probes to supplement current methods, including monoclonal antibody tests, and to further enlarge the size of the diagnostic test market. □