Articles

Disease Diagnosis by Recombinant DNA Methods

C. THOMAS CASKEY

Recombinant DNA procedures have now been applied to the problem of the identification of molecular defects in man that account for heritable diseases, somatic mutations associated with neoplasia, and acquired infectious disease. Thus recombinant DNA technology has rapidly expanded our ability to diagnose disease. Substantial advances in the simplification of procedures for diagnostic purposes have been made, and the informed physician has gained in diagnostic accuracy as a consequence of these developments. The wide application of recombinant DNA diagnostics will depend on simplicity, speed of results, and cost containment.

RECOMBINANT DNA METHODS ARE HIGHLY SENSITIVE and discriminating in detecting DNA mutations and differences between species. These properties have promulgated a rapid emergence of DNA applications for detection of inherited and acquired disease. This review will provide a selected survey of the diseases and recombinant DNA diagnostic techniques presently in use in clinical medicine. As will be illustrated, medical diagnosis via recombinant DNA methods has an expanding role in clinical medicine.

Common Disease Alleles

Sickle cell anemia, the most common heritable disease of blacks (*1*), has stimulated development of recombinant DNA diagnostic techniques now applicable to many heritable disorders. The β-globin complementary DNA (cDNA) and nuclear gene were the first disease-related mammalian DNA sequences to be cloned (*2*), thus facilitating study of globin mutants (*3*). The β-globin gene probes were then used to identify a restriction fragment length polymorphism (RFLP) (*4*) (Fig. 1A). By means of the Hpa I restriction endonuclease, Kan and Dozy found a 13-kb RFLP that occurred ~5 kb 3′ to the β-globin locus in a San Francisco black population and had a high frequency of association with the β-globin sickle cell (β^S), but not the normal (β^A) alleles. A nonrandom association of an RFLP with the β-globin alleles (linkage disequilibrium) emerged, since 87% of the U.S. population's β^S genes were associated with the 13-kb RFLP, while only a 3% association was found for β^A. Thus, two important principles were established in a single observation: (i) natural variation in DNA structure (polymorphisms) could be readily detected by RFLPs; and (ii) the presence of RFLP alleles could be correlated with disease alleles, which would facilitate disease diagnosis (*5*).

The author is associated with the Howard Hughes Medical Institute and Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030.

Recently a second RFLP linkage disequilibrium for a common recessively inherited disease gene has been identified (*6*). The Z allele, which is associated with the recessively inherited α-1-antitrypsin (AAT) deficiency, has shown strong linkage disequilibrium with an RFLP detected by the restriction endonuclease Ava II. Prenatal diagnosis of both sickle cell disease and AAT deficiency has been made possible by means of RFLP associations with the disease alleles (*7, 8*).

Improved, more precise diagnostic methods emerged that made use of DNA sequence information at the β^S mutation. Initially the restriction endonuclease Dde I (which recognizes CTNAG) (*9*) and subsequently Mst II (which recognizes CCTNAGG) (*10*) were used to detect the β^S mutant sequence CCTG*T*GGAG (in which A → T). This permitted a precise diagnosis by DNA hybridization analysis, since β^A and β^S Mst II fragments were associated with 1.15-kb and 1.35-kb fragments, respectively (Fig. 1B). A second common mutant allele β^C (CCT*A*AGGAG) (in which G → A) occurring in codon 6 of β^6 did not alter the endonuclease recognition site of either Dde I or Mst II.

Synthetic oligodeoxynucleotides provided the next technologic refinement for diagnostic accuracy—oligodeoxynucleotide complementarity (*11*). By means of separate allele-specific oligodeoxynucleotides (ASO) that perfectly matched the 19 nucleotides flanking and including the point mutation of sickle cell anemia, appropriate hybridization conditions were identified that permitted stability of only perfectly matched oligonucleotide DNA duplexes. Thus a detection method was available for β^A, β^S, and β^C (Fig. 1C). The detection of single copy genes by ASOs has, however, been limited by the high ratio of nonspecific to specific hybridization targets, leading to high gel backgrounds and requirements for 5 to 10 μg of purified genomic DNA. Recently Saiki *et al.* (*12*) have improved the sensitivity of the ASO method by selective amplification of the nuclear β-globin gene sequences in vitro with the polymerase chain reaction (PCR) (*13*). Two priming oligodeoxynucleotides complementary to opposite DNA strands that flank the β^S and β^C mutations were used to generate a 110-bp sequence that contained β^A, β^S, and β^C alleles and that could be amplified in vitro by successive cycles of DNA synthesis. This method permitted a 220,000-fold amplification of the target sequence with as few as 100 cells as starting material.

The identification of point mutations is feasible utilizing oligonucleotides derived by ASO alone or in combination with PCR amplification (Fig. 1D). These methods are sensitive and specific, with potential for wide application of other common disease alleles. A summary of common inherited diseases that have a high frequency of base-change alleles is given in Fig. 2. In each case, the DNA sequence immediately flanking the mutation is known, thus providing the opportunity for PCR amplification. The frequency of heterozygosity for β^S is 7.3% and for β^C is 2.8% in the U.S. black population. Although any of 23 different mutations (*3*) can cause β-thalassemia, the frequency of a specific mutant allele can be quite high in certain regions. The β^{39} (*14*) point mutation occurs at high

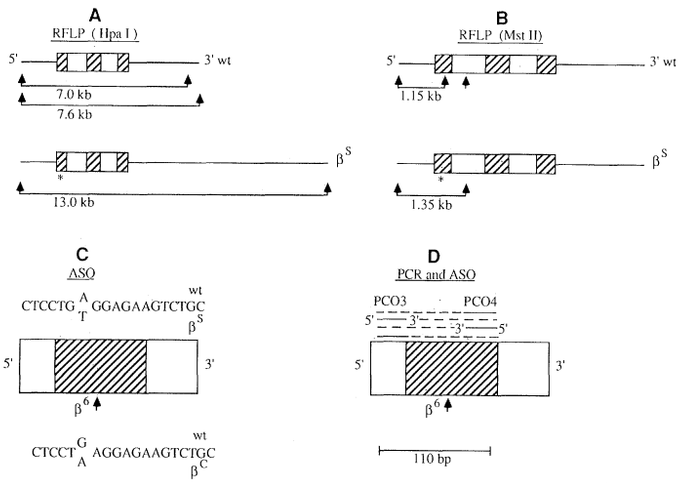

Fig. 1. Progression of β^S DNA diagnosis. (**A**) Hpa I identification of linkage association of the 13.0-kb RFLP with β^S. (**B**) Mst II cleavage at codon 6 of β^A, but not β^S, leading to mutation-dependent RFLPs. (**C**) Allele-specific oligodeoxynucleotide (ASO) recognition of β^A, β^S, and β^C alleles at codon 6 (β^6). (**D**) Polymerase chain reaction (PCR) primed by PCO3 and PCO4 including the β^6 within the amplified 110 bp (– – – –). Methods of Fig. 1C are used for the PCR product. Exons are indicated by hatched boxes; introns are indicated by open boxes. The asterisk indicates the site of β^S mutation in exon 1.

frequency in Sardinia and is common among Mediterraneans. The heterozygote frequency of β^{E26} is high (*15*) in Southeast Asians and the β^+ splicing defect is common in blacks (*16*). The recessively inherited α-1-antitrypsin deficiency, a disease manifested by liver disease and adult onset emphysema, occurs on the basis of two common mutant alleles (Z and S), each a single base mutation (*17, 18*). Recently, a point mutation in the 5′ splice donor site of intron 12 (IVS12) of the phenylalanine hydroxylase gene has been identified that accounts for 30% of the cases of phenylketonuria (PKU) which occurs at the rate of 1 in 10,000 in the Northern European population (*19, 20*). Thus, some common inherited disorders occur on the basis of a few point mutation allele(s), making ASO diagnosis of adult carriers and affected newborns possible. Both sickle cell anemia and PKU are included in state-directed newborn screening programs (*21, 22*), but present newborn screening tests lack the diagnostic precision provided by recombinant methods. Furthermore, testing for PKU by recombinant DNA procedures provides an opportunity to identify carriers by genetic screening. The practicality of wide diagnostic application depends on a resolution of medical, ethical, and economic issues. We are entering a new era in disease prevention and early therapeutic intervention.

Alleles Arising from New Mutations

The detection of a new mutational event at a gene locus is a formidable diagnostic challenge. The clinical importance is emphasized by the frequent occurrence of new mutational events in X-linked recessive diseases, such as Lesch-Nyhan syndrome [hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency (*23*)], the urea cycle defect resulting from a deficiency in ornithine transcarbamylase (*24*), and Duchenne muscular dystrophy (DMD) (*25*). The precise diagnosis of female carriers would be facilitated by detection of the mutations accounting for disease within a specific family. The ribonuclease A (RNase A) cleavage method developed by Myers *et al.* (*26*) and Winter *et al.* (*27*) has the ability to detect single base mismatches between the radioactive RNA probe (normal) and the patient's genomic or transcribed sequences (Fig. 3A). The method has been adapted for detection of mutations accounting for Lesch-Nyhan disease (*28*). Since HPRT is expressed in all cells, HPRT mutations can be detected in patients by probing messenger RNA (mRNA), thereby circumventing the problems of studying the large HPRT gene (44 kbp). Genomic deletion mutations are also identified by this method since they lack HPRT mRNA, resulting in total degradation of the single-stranded probe. The spectrum of identified Lesch-Nyhan mutations is summarized in Fig. 4. We estimate that the RNase cleavage procedure will detect 50% of Lesch-Nyhan mutations. There is a high degree of heterogeneity among mutations accounting for Lesch-Nyhan, since all 18 mutants characterized thus far differ.

New mutational events in genes expressed in specialized tissues such as hemophilia A (liver), ornithine transcarbamylase (OTC) (liver), or DMD (muscle) represent a considerable challenge. Each of these genes (*29–32*) is larger than the HPRT gene, thus further complicating analysis of mutants. The base change mutation in the mouse OTC mutant *spf* (*33*) was recently determined by a sequence of simple recombinant methods. The RNase A method was initially used to detect the mutation site with liver mRNA. Oligodeoxynucleotide primers that flanked the mutation site were then used to amplify the mutation site from liver mRNA, thus facilitating the molecular cloning and sequencing of this point mutation. This approach identified a single base change associated with the disease in a ~64-kb gene. It remains to be determined if adequate mRNA can be isolated from a percutaneous needle biopsy of liver or muscle, thus permitting a similar strategy for detection of mutations for OTC deficiency or DMD in humans.

Searching for mutations within the nuclear DNA will be difficult since each gene is quite large and contains multiple exons. Myers *et al.* (*34*) have developed an alternative method for detection of mutations (Fig. 3B) that may facilitate the study of large genomic

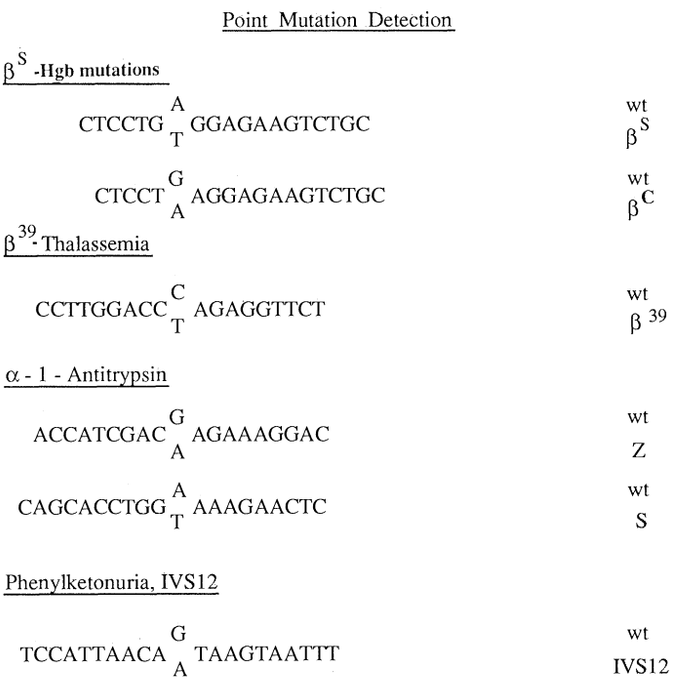

Fig. 2. Allele-specific oligodeoxynucleotides identifying disease mutations. The negative strand sequence for normal and mutant alleles are corresponding to β^S, β^C, β^{39}, α-1-antitrypsin, and phenylketonuria are shown. The normal sequence is shown above and the single-base substitution associated with the common disease alleles is shown below. The site of mutation in each is indicated by the alternate base.

sequences. Single base mismatches within probe DNA:sample DNA duplexes will initiate denaturation of the duplex that can be detected as a lack of mobility in a denaturation gradient gel. Mismatched duplexes denature more readily than the perfectly matched duplexes and thus become immobile earlier in the denaturation gradient. Knowledge of the DNA sequence is useful in predicting the denaturation characteristics of the sequences under study. Furthermore, addition of sequences with high duplex stability (clamps) makes genomic sequences amenable to denaturation analysis. Both the RNase A cleavage and the duplex melting methods have the advantage of searching for mutations or natural DNA variation in the absence of detailed knowledge of the cloned target sequence. The two methods (Fig. 3) would appear particularly useful for analysis of previously unknown mutations and perhaps detection of new classes of polymorphisms for gene mapping.

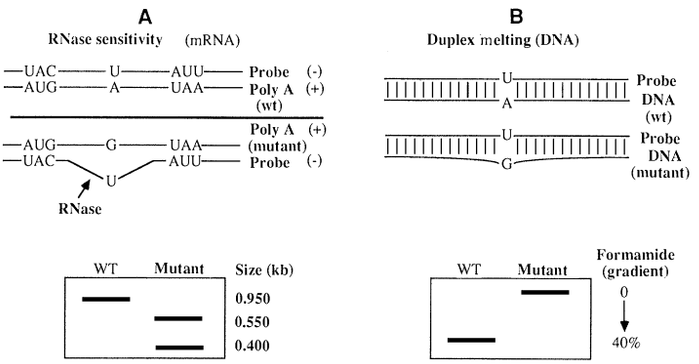

Fig. 3. Methods for detecting new mutations. (**A**) The detection of a single base change A → U reflected in the mRNA. Detection of the mismatch with normal negative-strand RNA probe. The RNase cleaved fragments are detected by gel electrophoresis and radioautography. (**B**) The detection of a single base change A → G reflected in nuclear DNA. This probe is a single-stranded normal sequence. The initiation of denaturation is illustrated. RNase A would not have detected this mismatch if the probe was RNA.

Genetic Linkage Analysis

The recognition of RFLPs (*4*), publication of the theoretical mapping paper of Botstein and White (*35*), and successful application of the methods to map Huntington's chorea to chromosome 4p (*36*) have set in motion a new approach for disease diagnosis—DNA linkage analysis in which the inheritance of a particular disorder in a family is associated with the presence of an RFLP. The strategy was particularly attractive for diseases whose gene defects were unknown such as Huntington's chorea (*36*), adult polycystic kidney disease (*37*), cystic fibrosis (CF) (*38*), and DMD (*25*). The usefulness of probes for linkage analysis is affected by the frequency of their RFLPs as well as their recombinational distance from the disease gene. Two types of DNA variation represent the molecular bases of RFLPs: single base alterations and repeated sequences. In some cases the RFLP has components of both (*39*). Single base RFLPs are recognized by restriction endonuclease cleavage and detected by DNA hybridization analysis as illustrated in Fig. 5A. The restriction endonuclease Taq I (which recognizes TCGA) has proven useful for RFLP identification, suggesting that CpG is a highly mutable site. This is supported by the alteration of Taq I sites in new mutational events giving rise to defects in the OTC (*24*) and hemophilia A genes (*40*). Jeffreys *et al.* (*41*) were the first to isolate probes that had a highly polymorphic character because of a variable number of tandem nucleic acid repeat sequences (VNTRs) (Fig. 5B). By means of oligodeoxynucleotides complementary to sequences of the highly variable region of myoglobin, they have isolated additional chromosomal probes with highly informative RFLPs due to VNTRs. Nakamura *et al.* (*42*) utilized selected synthetic oligodeoxynucleotides on the basis of the sequence of highly variable regions of the zeta-globin pseudogene, insulin, and the X-gene region of hepatitis B virus for isolation of cosmid clones. These synthetic oligodeoxynucleotides have in common a sequence of TGGGA. Genomic cosmid clones were found to frequently (20 to 25%) be associated with VNTR RFLPs, a frequency five- to tenfold higher than found in randomly selected cosmid clones. VNTRs have been proven clinically useful in the diagnosis of hemophilia A (*43*) and in recent studies in which adult polycystic kidney disease (*37*) has been linked to the α-globin locus of chromosome 16. Willard *et al.* have used α-satellite DNA for development of chromosome-specific probes that have associated RFLPs (*39*). VNTRs tend to be more informative than single base change RFLPs since frequently more than two alleles occur at a VNTR locus.

Linkage diagnosis is currently being applied to the X-linked recessive disease DMD, among a number of other X-linked recessive disorders (*44*). The mapping of the DMD gene to Xp21 was first determined by identification of females with X:autosome translocations involving Xp21 (*45*). Linkage of DMD to the short arm was achieved by means of X-chromosome specific probes constructed with DNA that had been isolated by fluorescence-activated sorting (*46*). Probes closer to the DMD locus were isolated by cloning strategies that led to the isolation of the X:21 junction (XJ1.1) of a female with DMD (*47*). Kunkel and his associates (*48*) developed a subtractive hybridization method [phenol-enhanced reassociation technique (pERT)] involving DNA from a normal male and from a patient with a chromosomal deletion encompassing DMD, retinitis pigmentosa, chronic granulomatous disease, and McCleod blood group phenotype regions (*49*). These collective efforts provided RFLP-associated Xp21 probes that are within and flank the DMD locus as illustrated in Fig. 6. A long-range restriction map covering 3 million base pairs around the DMD gene has been constructed by means of pulsed field gradient electrophoresis (*50*), a method capable of resolving DNA fragments of very high molecular weight (*51, 52*).

The pERT and XJ1.1 probes identified deletions in ~5% of DMD males. Since RFLPs are associated with the probes, a regional RFLP haplotype can be determined in families, which may be helpful in female carrier diagnosis and prenatal diagnosis of DMD males. As many as nine RFLPs are needed for the average family study of six members. Carrier detection is highly accurate in families with multiple affected males. It is less accurate for females in those families with a single affected male since the origin of the mutation is often difficult to identify (*53*). Similar features are true for application of linkage studies in prenatal diagnosis of DMD. Linkage analysis of DMD families for diagnosis is difficult due to the occurrence of new DMD mutations as well as the apparent high recombination rate between probes and DMD (pERT–DMD, 2 to 5%) (*54*). Since sequences within the pERT probes have recently been reported to be part of the putative DMD mRNA (*32*), intragenic recombination is likely. It is anticipated that knowledge of the DMD cDNA and genomic organization will improve DMD diagnosis in families with a single affected member and avoid the errors inherent in recombination between probe and mutation. Despite these difficulties, prenatal diagnoses by linkage analysis are being successfully carried out in the United States. Of 74 females requesting prenatal diagnosis, 29% were identified as carrier and 48% non-carrier for DMD, at >98% accuracy. The remainder showed carrier risk ranging between 2% and 41%. Of 33 carriers and probable carriers undergoing prenatal diagnosis, 10 were

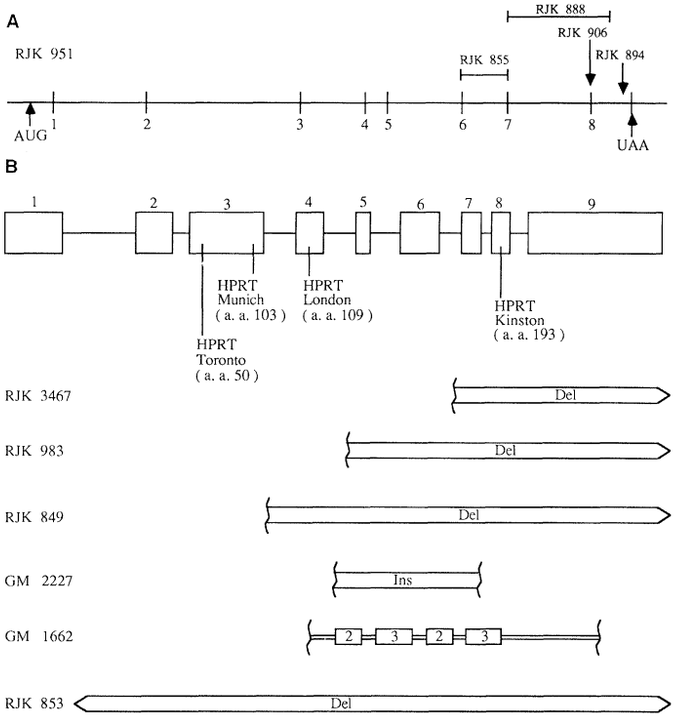

Fig. 4. Mutation detection—HPRT gene. (**A**) Detection of mutations by RNase cleavage of duplexes between patient mRNA and the RNA probe are indicated by the ⊢⊣ or ↓. The 3′ end of each exon is indicated. (**B**) Deletion of mutations with gene rearrangements are indicated by Del. The mutants GM2227 and GM1662 are insertion (Ins) and endoduplications, respectively. The mutations labeled Toronto, Munich, London, and Kinston are point mutations that have been detected by amino acid sequence analyses (*83*). The 35-kb HPRT gene is divided into nine exons ⊢□⊣ as illustrated.

predicted to bear unaffected males, 3 were affected males, 6 were possible affected males (2 to 41% risk of DMD), and the remainder were females (*53*). Pregnancy outcome data is pending. Prior to availability of DNA linkage diagnosis, families had to base pregnancy decisions solely on the identification of a fetus as male by prenatal diagnosis.

The prenatal diagnosis and family carrier detection of CF have also been made more accurate by DNA linkage analysis. Before the DNA diagnostic method was available, Brock (*55*) found intestinal alkaline phosphatase to be useful in CF diagnosis since its level is decreased in the amniotic fluid of CF fetuses. The genetic defect accounting for this most common recessive disease of Caucasians (1 in 2000) is unknown. After the mapping of CF to chromosome 7 by linkage of the disease to the anonymous (unknown gene) probe DOCRI-917 (*38*), the tightly linked pJ3.11 (*56*) and D7S8 anonymous probes (*57*) provided a linkage approach to prenatal diagnosis for families with an affected child. The order of the *met* oncogene and anonymous D7S8 probes relative to CF as well as their linkage (recombination between these probes and CF is rare—0.01%) has been the subject of collaborative study. These probes are predicted to be highly accurate for prenatal diagnosis of CF. Spence *et al.* (*58*) found recombinant DNA probes fully informative for RFLP linkage in 74.4% of 90 couples who had a one in four risk of bearing a diseased child. At the present time, both DNA and quantitation of the alkaline phosphatase in the amniotic fluid are being used for prenatal diagnosis. The outcome of seven pregnancies (five normal and two affected) is in agreement with the predictions by DNA analysis. The available probes are inadequate for CF carrier detection in the general population since they have only slight RFLP linkage disequilibrium with CF. They can, however, be used for carrier detection among siblings in a family with an affected and available patient.

Linkage diagnosis of Huntington's chorea, which is autosomal dominant, has recently been initiated. This adult-onset neurodegenerative disorder varies in clincial severity and has no effective therapy. Until now, no means of presymptomatic Huntington's chorea diagnosis or prenatal diagnosis were available. As the clinical symptoms frequently occur after childbearing age, families made reproductive decisions with low degrees of confidence. Gusella *et al.* (*36*) successfully mapped the Huntington's chorea gene to chromosome 4p and identified a tightly linked probe, G8, that is associated with RFLPs. Current research efforts are directed toward isolation of additional RFLP-associated probes that map to the distal side of 4p and flank the Huntington's chorea gene. The controversy over diagnostic application now appears resolved, since probes are in use (*59*). It remains to be determined how receptive Huntington's chorea families will be to linkage analysis and how effective presymptomatic diagnosis will be in reducing disease incidence.

A second adult-onset and variably expressive disease that shows an autosomal dominant pattern of inheritance, adult polycystic kidney disease (APCKD), is also amenable to DNA linkage diagnosis. APCKD has an estimated frequency of 1 in 1000 and accounts for a substantial fraction of patients undergoing chronic dialysis and renal transplantation. A VNTR polymorphic probe (3′ HVR) localized to chromosome 16 is tightly linked to the α-globin locus and APCKD (*37*). The medical benefits of APCKD heterozygote identification exceed those of Huntington's (where no treatment is currently available)—a factor which may be important in patient and family acceptance. The issues related to assured insurance coverage by private companies for asymptomatic heterozygotes identified by linkage diagnosis deserves study and resolution.

Forensic Applications of Allele Markers

Identification methods for individuals presently use phenotypic traits such as height, weight, facial, dental, and fingerprint features. The methods are difficult to apply in many medical forensic circumstances. Use of genetic markers has become routine in rape

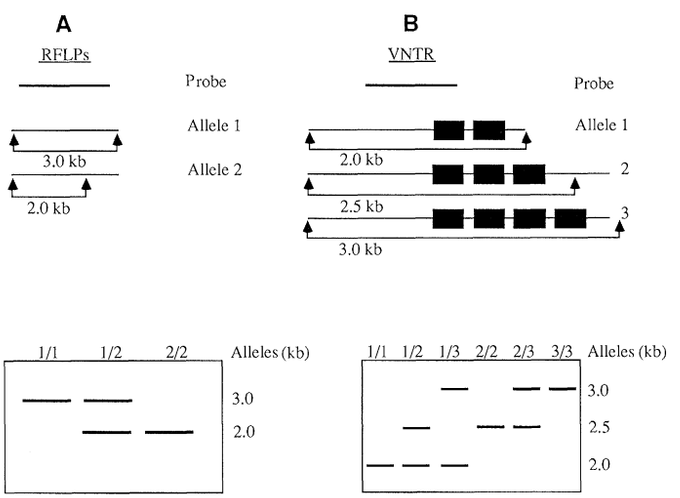

Fig. 5. RFLP mechanisms. (**A**) RFLPs associated with base changes are indicated by the difference in restriction endonuclease site (↑) cleavage, which yield different DNA molecular weight fragments. (**B**) RFLPs associated with variable tandem repeats (■) are indicated by the difference in size of the DNA fragments created by restriction endonuclease sites flanking the repeats. The size variations are illustrated below each diagram as they would appear in DNA hybridization (Southern) analysis.

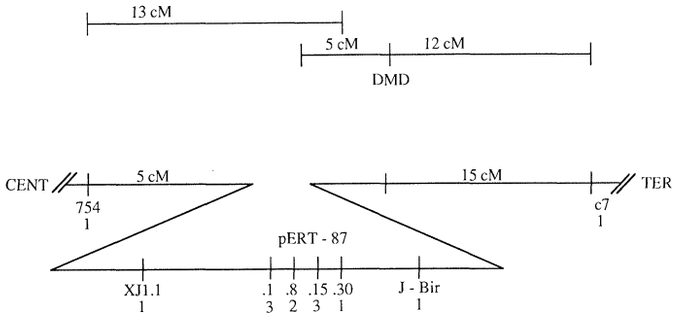

Fig. 6. Duchenne muscular dystropy (DMD) region Xp21. The recombinational distances between markers are given in centimorgans (cM) on the basis of family studies. The probes 754 and c7 flank the DMD locus and were isolated from X-chromosome sequence-enriched λ libraries. The XJ1.1 probe was isolated as an X–autosome junction DNA fragment from a female patient with DMD. The pERT-87 probe, isolated by subtractive hybridization, led to isolation of derivative probes from the region. The J-Bir probe was identified as a junction sequence in a deletion case whose deletion initiated in the pERT region. The recently isolated cDNA sequences were derived from cross-reactive sequences found in the pERT region. The number of RFLPs associated with each probe is given below the probe. The locus for DMD has been placed 5 cM from the pERT probes on the basis of recombinational studies of families with the DMD mutation. TER, terminus; CENT, centromere.

and paternity testing but requires a variety of test methods. The recent isolation of highly informative RFLPs at multiple loci with common sequence probes offers an opportunity for genetically identifying indiviuals by DNA variation. The VNTRs associated with the recently isolated probes (*39, 41, 42*) are highly discriminating and thus may provide a single method for identification. This approach would have the advantages of requiring small tissue or blood samples, a single analytic method for a stable compound, and having potential for automation.

Neoplasia—Acquired Genetic Alteration

Activation of oncogenes can occur by point mutation (*60*) and chromosomal rearrangements (*61, 62*). For example, the finding that c-*myc* (chromosome 8) transcription is enhanced in association with B-cell chromosomal translocations in Burkitt lymphoma first illustrated oncogene activation by chromosomal rearrangement. The c-*myc* was activated by translocations involving immunoglobulin sequences on chromosomes 2, 14, and 22 and c-*myc* coding sequences. The chromosomal breakpoints of the translocations are readily detected by cytogenetic techniques (*63*) and many DNA junctional sequences are detected with c-*myc* or immunoglobulin probes by DNA hybridization analysis (*64*). Recently T-cell leukemias have been found to be associated with chromosomal rearrangements (11:14) and (8:14) that involve c-*myc* and the α chain of the T-cell receptor (*65*). The precise site of joining for the (8:14)(q24:q11) has been identified and sequenced. The breakpoint for chromosome 8 is 3 kb 3′ to c-*myc* and for chromosome 14 is 36 kb 5′ to the gene for the constant region of the α chain of the T-cell receptor. The sequence data suggests that the translocation is the consequence of errors in V-J joining. Each junction was initially identified by DNA hybridization analysis with c-*myc* and T-cell receptor probes. If such joining occurs reproducibly via an aberrant homologous recombination involving specific nucleotide sequences such as GAAAGT, precise and simple methods for translocation detection should be possible. Other leukemias associated with chromosomal translocations include chronic myelogenous leukemia (*66*) and acute promyelocytic leukemia (*67*). The following techniques all offer methods for breakpoint detection over variable DNA distances: ASO (18 to 60 bp), PCR (250 to 500 bp), RFLP (500 to 30,000 bp), and PFGE (30,000 to 10^6 bp). The leukemia and lymphomas in which documented associated chromosomal rearrangements and probes flanking the translocation are available are well suited for development of cancer diagnostics.

The somatic cell loss of genetic sequences is associated with the autosomal dominantly inherited form of retinoblastoma, maps to chromosome 13q21–13q13. It is speculated that loss of repressor sequences is involved in the somatic transition to neoplasia. Cavenee *et al.* (*68*) found that retinoblastoma tumors had undergone somatic alteration leading to hemizygosity of DNA sequences in the tumor. RFLPs associated with these chromosome 13 probes have been used for linkage in families with autosomally dominant inherited retinoblastoma for prediction of tumor risk (*69*). The recent isolation (*70*) and DNA sequencing (*71*) of the putative retinoblastoma gene cDNA is an exciting development. This probe may increase accuracy for the prediction of retinoblastoma risk in families with clear autosomally dominant disease and should also improve the accuracy of genetic predictions in families with isolated retinoblastoma.

Correlation of severity of neoplasia and oncogene expression may be useful in the care of children with neuroblastoma (*72*). It has been proposed that neuroblastoma staging for therapy should be augmented by the quantitation of N-*myc* expression in the tumor. It is unknown at this time if quantitation of oncogene expression will be clincially useful for other neoplasias. Such analysis has the potential to augment or replace systemic or pathologic strategies of clinical staging of disease for therapy selection.

Infectious Disease

The application of recombinant DNA methods for diagnosis of infectious diseases has been most extensively explored for viral infections (*73*) where current methods are cumbersome and results are delayed. In situ hybridization of tissues or cultured cells has made diagnosis of acute and chronic herpes infection possible (*74*).

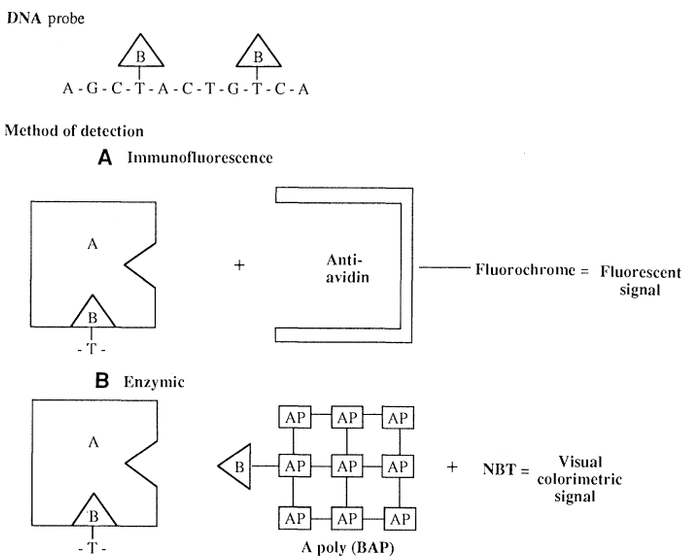

Fig. 7. Nonradioactive biotin probes. The incorporation of a biotinylated thymidine (16-residue side arm) into a probe is illustrated. Detection of the probe is achieved by either fluorescence (**A**) or enzymic (**B**) procedures. B, biotin; A, avidin; AP, alkaline phosphatase polymers; A poly (BAP), polymer containing bovine alkaline phosphatase; NBT, AP substrate.

Fresh and Formalin-fixed tissues have been reported to be suitable for detection of papillomavirus in invasive cervical carcinoma (*75*) and in the detection of HIV (*76*), while cultured cells have been used for the detection of cytomegalovirus (*77*) and Epstein-Barr virus (*78*). Recently several improvements in the methods for in situ hybridization of tissues with nonradioactive probes promise a general strategy for detection of acquired infectious agents (*79*). The application of recombinant DNA methods to the diagnosis of microbial diseases (*80*) has the potential to replace current microbial growth methods if cost-effectiveness, speed, and precision requirements can be met. Clinical situations where recombinant DNA procedures have begun to be applied include the identification of penicillin-resistant *Neisseria gonorrhoeae* by the presence of a transposon (*80*), the fastidiously growing chlamydia (*80*), microbes in foods; and simple means of following the spread of an infection through a population (*81*). The worldwide epidemiologic challenge of diseases involving such parasites as leishmania and plasmodia is already being met by recombinant methods (*82*). There is little doubt that the applications will expand.

Detection of Sequences

A simpler diagnostic method is needed. The labor-intensive, multistep, capricious character of DNA hybridization analysis, as well as the slow turn-around time (5 to 10 days) are severe limitations for widespread diagnostic application. The use of radioisotopes has a negative effect on widespread application because of laboratory safety and waste disposal requirements. Presently a limited number of academic and private laboratories provide diagnostic services by methods developed for research laboratories. Regional referral laboratories dedicated to the new technology and/or simplification of methods for hospital use are needed. The combined method of PCR and ASO is a significant advance toward streamlining of procedures as is the use of chemical analogs of nucleotides with alternative means for detections (*83*). The use of biotinylated nucleotides within a probe has permitted the development of labeling procedures that avoid the need for isotopes. Biotin-labeled analogs of TTP and UTP can be enzymatically incorporated into DNA and RNA, respectively (Fig. 7). The biotin-labeled probe can be coupled to avidin and antibody complexes that can then be detected by immunofluorescence (Fig. 7A), immunoperoxidase, or immunocolloidal gold techniques. However, each of these methods has a reduced capability for detection of single-copy sequences in a mammalian cell as compared to procedures in which radioisotopes are used. The use of polymers containing calf intestinal alkaline phosphatase (Fig. 7B) (*84*) and biotin now promises reliable single-copy detection capability. This highly amplified single complex binds to the biotinylated probe through avidin, giving a colorimetic signal. Undoubtedly the detection methods will continue to improve and perhaps advance to a simple protocol within the technical capability of many certified diagnostic laboratories.

Conclusion

There can be no doubt that DNA diagnosis has already made substantial contributions to the diagnosis of disorders such as sickle cell anemia, DMD, and CF. We predict that more genetic disorders will become amenable to analysis by recombinant DNA techniques and these techniques will be exploited in clinical studies of infectious diseases and cancer. Technical advances made in the research laboratory will be transferred rapidly to the clinical environment, and will lead to simple, speedier, and more accurate diagnoses. The ethical problems associated with some tests, and the economics of testing are issues that still require discussion, but we can be quite certain that recombinant DNA techniques will play an ever-increasing role in disease diagnosis.

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The Human Hematopoietic Colony-Stimulating Factors

STEVEN C. CLARK AND ROBERT KAMEN

The complementary DNAs and genes encoding the four major human myeloid growth factors—granulocyte colony-stimulating factor, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3—have all been molecularly cloned. These DNA clones have proved valuable for studying the molecular biology of these important regulatory molecules as well as for the large-scale production of the recombinant growth factor proteins. These advances have led to a much better understanding of the role of the myeloid growth factors in regulating hematopoiesis in vivo that should soon find practical application in clinical medicine.

THE REGULATED PRODUCTION OF BLOOD CELLS IS ONE OF the most complex examples of multilineage differentiation and one of those most amenable to scientific investigation. A common set of pluripotent stem cells, residing mostly in the bone marrow, gives rise to the large numbers of red cells, neutrophils, basophils, eosinophils, monocytes, platelets, and lymphocytes circulating in the blood. As many of these blood elements are short-lived, they must be continually replenished. Moreover, the levels of mature cells can respond dramatically to environmental stress. For example, granulocyte counts can vary from approximately 5,000 per microliter in normal humans to more than 50,000 in cases of severe infection; this variation, which corresponds to the production of approximately 2×10^{11} cells, can occur within a matter of only a few days (*1*).

A key advantage in studying the hematopoietic differentiation system has been that the blood is a liquid tissue composed of unicellular components. The development, more than 20 years ago, of cell culture systems for the clonal growth of hematopoietic progenitor cells in semisolid media was the key to the establishment of the hematopoietic lineages and the discovery that cell division and differentiation are dependent on the continuous supply of highly specific protein factors which act as regulators of hematopoiesis (*2*) (Fig. 1). Because these proteins were initially identified through cell culture colony formation assays, they were named the hematopoietic colony-stimulating factors, or CSFs.

CSF research has recently been intensified by cellular and molecular biologists for reasons beyond a purely scientific fascination with the hematopoietic system. Disease states caused by hematopoietic dysfunction or hyperplasia (leukemias) are significant medical problems. Furthermore, the possibility that CSFs could function as regulators of blood cell production in vivo suggested broad therapeutic utility and thus attracted the attention of the biotechnology industry. As a result of highly synergistic interactions among academic investigators, clinical researchers, and the scientists and technologists within industry, the field has advanced remarkably since the last review of the subject in *Science* by D. Metcalf (*3*). Most significant to those of us whose involvement stems from biotechnology, CSFs have now progressed from the laboratory to the clinic.

Several different colony-stimulating factors were distinguished through careful analysis of the cell types found in hematopoietic colonies grown with various sources of growth factor activity (*2*). In the murine system, four major types were identified. Two of these proved to be relatively lineage specific; that is, colonies grown in the presence of granulocyte-CSF (G-CSF) were found to consist largely of neutrophilic granulocytes and their precursor cells (*4*), whereas those grown in the presence of macrophage-CSF (M-CSF, also known as CSF-1) consisted largely of macrophages (phagocytic cells derived from circulating monocytes) (*5*). In contrast, the colonies grown in the presence of multi-CSF (also known as interleukin-3 or IL-3) were occasionally found to contain many different cell lineages (*6*), whereas those found in cultures grown in the presence of granulocyte-macrophage-CSF (GM-CSF) were found to contain neutrophilic granulocytes, macrophages, eosinophils, and other cell types (*7*). These results are believed to define a hierarchy of progenitor cells along the various cell lineages as summarized in Fig. 1. In this model, G-CSF and M-CSF are postulated to support the growth and proliferation of only relatively late progenitors already

S. C. Clark is director of hematopoiesis research and R. Kamen is senior vice president of scientific affairs at Genetics Institute, Inc., 87 Cambridge Park Drive, Cambridge, MA 02140.