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## Current methods of mutation detection

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### Summary

Mutation detection is important in all areas of biology. Detection of unknown mutations can involve sequencing of kilobases of DNA, often in many patients. This has led to the development of methods to screen DNA for mutations as well as methods to detect previously described mutations. This review discusses current methods used for such purposes with special emphasis on genetic diseases of humans. However, savings can be made by similar means in other areas of biology where repetitive or extensive sequencing for comparative purposes needs to be done. This review covers the methods used for detection of unknown mutations, namely the ribonuclease, denaturing gradient-gel electrophoresis, carbodiimide, chemical cleavage, single-strand conformation polymorphism, heteroduplex and sequencing methods. Once mutations have been defined they can be searched for repeatedly by methods referred to as diagnostic methods. Such methods include allele-specific oligonucleotide hybridization, allele-specific amplification, ligation, primer extension and the artificial introduction of restriction sites. We can now choose from a range of excellent methods, but the choice will usually depend on the background of the laboratory and/or the application in hand. Screening methods are evolving to more satisfactory forms, and the diagnostic methods can be automated to screen whole populations inexpensively.

The rate of identification and characterisation of genes which cause specific inherited diseases in humans is rapidly increasing. Also the numbers of mutations identified in a particular gene as causative in disease are increasing rapidly. This is likely to gain further impetus from the Human

Genome Project. Thus methods to detect unknown mutations and previously described mutations are assuming increasing prominence and use. The importance of such studies is enhanced as knowledge of the molecular basis of cancer has increased, given that mutations in oncogenes and tumour suppressor genes are now well-documented as causes of cancer. Changes of such magnitude have not occurred in genetics other than the area of human disease, but nevertheless the methods to be discussed are potentially advantageous in these areas.

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There are 3 main areas where such methods are used in human disease, (a) the research laboratory attempting to find mutations in a gene which causes a specific disease, (b) the clinical diagnostic laboratory which needs to look for known and unknown mutations causing a specific disease for prenatal or other diagnostic purposes, as well as polymorphic harmless mutations for linkage studies, and (c) the screening of populations for specific mutations (such as has occurred with Tay Sachs disease and as is beginning to occur in cystic fibrosis).

For this review, methods will be divided into screening methods and diagnostic methods. The former are usually used to detect unknown mutations, but there is an increasing tendency to use screening methods to screen for a number of known mutations together with any unknown mutations in the diagnostic setting (see below) (Fig. 1).

Methods of detecting mutations have been reviewed several times in recent years (Caskey, 1987; Grompe et al., 1989; Rossiter and Caskey, 1990; Cotton, 1989, 1991, 1992). One of these was particularly detailed (Cotton, 1991) and reviewed the area up to the end of 1990 and a subsequent review (Cotton, 1992) is essentially an update of this review to near the end of 1991. The field is evolving so rapidly that frequent reviews are necessary to monitor important new methods and modifications of older methods; it is also important to assess the effectiveness of methods after a time in operation.

This review aims to provide a brief description of the principles and practice of methods available at this time together with their variants, and a discussion of their advantages and disadvantages. Key illustrative applications will be given. For more detail (and further examples) the reader is referred to an earlier review (Cotton, 1991). Only those methods used actively at present or those described in the last few years will be covered. Methods to detect the more obvious deletion/insertion mutations have been covered earlier (Rossiter and Caskey, 1990) and will not be covered here, where detection of point mutations will be emphasized. It should be noted that some of the methods mentioned below will be reviewed in more detail in another issue of *Mutation Research*.

## Screening methods

The screening methods can be divided into two types: (a) those simple methods which rely on differences in electrophoretic properties being generated between mutant and wild-type nucleic acid by point mutations (these methods cannot, as currently used, detect all mutations, do not localize them within the fragment, and can only be applied to DNA fragments hundreds of bases long), (b) and another group which includes cleavage methods and the carbodiimide method (which can screen kilobase lengths and localise the mutations to within 10 bases in the fragment examined). The subcategory of chemical methods have the potential to detect *all* mutations. Sequencing is more frequently used to detect unknown mutations than it is for diagnostic purposes.

### *Ribonuclease cleavage (RNAase)*

Many ribonucleases cleave single-stranded RNA after pyrimidine residues. This finding was exploited when it was found that single base-pair mismatches in RNA:RNA heteroduplexes were cleaved by ribonuclease (Freeman and Huang, 1981; Winter et al., 1985) as well as in RNA:DNA heteroduplexes (Myers et al., 1985a).

The method was given considerable impetus when uniformly labelled probes could be conveniently produced as described in 1984 using the SP6 system (Melton et al., 1984). Application of the method directly to unamplified genomic DNA has been reported (Myers et al., 1985a; Kaufman et al., 1990). Cleavage of the DNA to which the cleaved RNA was hybridized is possible via S1 nuclease (Atweh et al., 1988). Cleaved RNA has been detected after transfer to a membrane and hybridization with probe (Genovese et al., 1989).

The main advantage of the method is that it is a simple single-step reaction which locates the mutations within the fragment. This is, however, offset by the fact that special RNA probe has to be prepared and that only about 70% of all mutations are detected (Myers et al., 1985a). This is because when purines appear in the probe at the mismatch most mismatches are not cleaved.

Despite the aforementioned disadvantages the method has been used until the present day. For

example, variation in HIV isolates have been studied (Lopez-Galindez et al., 1991), the pattern generated after digestion by RNAase being indicative of geographical distribution and temporal appearance of resistance to AZT. In addition, a number of mutations in the *apc* gene were identified with ribonuclease (Nishisho et al., 1991). The method has also been applied to the intensively studied p53 gene in tumours and cell lines (Kim et al., 1991).

The fact that around 30% of mutations are missed with this method is a considerable shortcoming, if a simple single-step screening method capable of detecting 100% of mutations becomes available the RNAase method is bound to decrease in both use and value.

#### *Denaturing gradient-gel electrophoresis (DGGE) and related techniques*

When double-stranded DNA is electrophoresed into a gradient of increasing denaturant a portion of a given strand separates but the strands are anchored together by the portion (higher melting domain) which has not melted at this point. This split in the duplex suddenly arrests the movement of the molecule in the gel. If a single-base change is present in a similar duplex in the split portion, the denaturant concentration for strand separation is usually different, thus the arrest of movement occurs at a different position in the gel and a mutation can be detected by the differential positions of arrest (Myers et al., 1985b). The difference between the positions of arrest is greater if heteroduplex molecules (between mutant and wild-type) are used. The gel is poured with an increasing gradient of denaturant (formamide) and run at 60°C in a special apparatus needed to keep the temperature constant. The length screened is 50–500 bp and it is possible to use unlabelled DNA.

There has been considerable evolution of the method since it was first described, and also there are a number of variants. Changes have been directed either to increasing the percentage of mutations detected or to simplifying the methodology. The most important modification has been the placing of a high melting point 40-base GC rich sequence (the GC clamp) at one end of the fragment to be screened. Most recently this has

been achieved using PCR technology, with special primers being synthesized with a clamp attached (Sheffield et al., 1989, 1992a). This means the whole area to be screened is in a low melting point domain and that "almost all" mutations, instead of about 50%, can be detected. In the practical situation it was found that mutant samples had to be mixed with normal DNA to ensure heteroduplexes were formed in order to ensure detection of a maximal number of mutations (Cai and Kan, 1990; Higuchi et al., 1990). Kilobase lengths of genomic DNA can be screened for polymorphisms (60% of any base changes) by digestion with restriction enzymes, separation by DGGE, blotting onto a membrane and then probing with relevant genes (Gray, 1992).

Further modifications have attempted to avoid the use of the special apparatus altogether. Smith et al. (1988) melted the duplexes in solution containing stepwise increases in denaturant and analysed them by standard polyacrylamide gel electrophoresis. Another variation has been to use a temperature rather than a liquid-denaturant gradient (Rosenbaum and Reissner, 1987). The most recent modification leading to greater simplicity has been the constant denaturant gel electrophoresis (CDGE) method (Hovig et al., 1991). Here separation is undertaken at that concentration of denaturant which corresponds to that of the melting domain of the fragment being analysed. The authors reported detection of 6 of 7 mutations at a particular locus whereas 3 of 7 were found with conventional DGGE. This low detection rate with conventional DGGE was despite the use of a GC clamp which is rather surprising.

One of the special and important advantages of the above method (and other methods separating intact mutant and wild-type molecules during analysis (see below)) is the fact that mutant molecules can be isolated from gels for further analysis such as sequencing. This feature was exploited in the study of errors during PCR amplification (Keohavong and Thilley, 1989). Other advantages are the fact that it can be used in unlabelled mode, it can be used directly on unamplified genomic DNA, and a result can be obtained in 24 h. A particular disadvantage of almost all variants is that either preliminary ex-



periments or computer analysis has to be performed before screening different DNA fragments. Also for the original DGGE method special apparatus has to be used. The method cannot guarantee detection of all mutations.

The method has been frequently used since its first description until the present day. Most applications have been in two major categories (a) screening a newly characterized gene for mutations and (b) screening a well-studied disease gene to detect known, and possible unknown, mutations in the clinical setting. Examples of the former include application to Factor VIII deficiency (Higuchi et al., 1991), Rhodopsin mutations (Sung et al., 1991) and mitochondrial DNA (Yoon et al., 1991) and the latter include  $\beta$ -thalassaemia (Loosekoot et al., 1990),  $\alpha$ -1-antitrypsin deficiency (Johnston et al., 1991) and porphyria carriers (Bourgeois et al., 1992). Application of the CDGE method to p53 has been described (Borresen et al., 1991).

Because of its ability to detect "almost all" mutations this method and its variants are likely to be used for some time yet. However, its relative complexity (apparatus and the preliminary studies which are required) is likely to see it lose ground to SSCP (see below) due to the latter's all round simplicity, even although SSCP is likely to detect fewer mutations than DGGE and its variants.

#### *Carbodiimide modification (CDI)*

Carbodiimide is a bulky reagent that reacts with the imino sites of T and G bases and reacts more rapidly with unpaired bases than with paired bases (e.g. Kelly and Maden, 1980). It was recently found that mismatched T and G bases in DNA heteroduplexes react more rapidly than matched T and G bases (Novak et al., 1986). Thus when reacted with heteroduplexes between mutant and wild-type DNA, any base change (and therefore any mutation) should be potentially detectable. For example, a C  $\rightarrow$  G change will give rise to heteroduplexes with C  $\cdot$  C and G  $\cdot$  G mismatches and both and bases should be detectable. With a T  $\rightarrow$  G change the mismatches generated, T  $\cdot$  C and G  $\cdot$  A, should both be detectable.

The original method (Novak et al., 1986), described detection of the reaction of CDI with the mismatches by slowing of the electrophoretic mobility of the DNA fragment during electrophoresis. Later, detection by cleavage of the heteroduplex at the derivatized mismatch point by ABC excinuclease was used (Thomas et al., 1988). Neither of these methods were applied to a practical situation. Detection of the derivatized mismatch by electron microscopy was made possible by the production of antibodies to the carbodiimide (Ganguly et al., 1989). A more practical protocol was established when it was shown that the derivatized T and G bases would block primer extension (Ganguly and Prockop, 1990) and this is the protocol that has mainly been applied to the practical situation. Thus detecting electrophoresis of products shorter than the full segment length, signals the presence of a mismatch.

The main advantage of the most recent protocol is that no probes have to be produced, since the label is incorporated during primer extension; also, the *one* chemical which is used is relatively non-toxic. Some specific minor problems are mentioned in the description (Ganguly and Prockop, 1990) but it seems that two sets of incubation conditions are needed to guarantee complete detection. Because two detectable mismatched bases are generated from any mutational change any mutation has two chances of detection. A theoretical objection is that when a T  $\cdot$  G mismatch is generated, as it is the most stable mismatch (Cotton, 1989), both chances of detection may be negative. While examples of all possible T and G mismatches have been shown to be detectable, the method has not seen wide enough application for potential problems to be discovered.

All practical applications so far have been from the laboratory where the method originated. Point mutations in the collagen genes COL1A1 (Zhuang et al., 1991) and COL1A2 (Ganguly et al., 1991; Spotilla et al., 1991) were detected and positioned by the carbodiimide method. It should be noted that in one of these cases (Ganguly et al., 1991) the position of a splice site mutation was ascertained in plasmid inserts 3–4.5 kb long by immuno-electron-microscopy.

This illustrates a special feature of the carbodiimide method, which is the ability to screen for mismatches in very large heteroduplexes (7.2 and 4.9 kb) by immuno-electron-microscopy (Ganguly et al., 1989). This capability, for those with ready access to immuno-electron-microscopy, is unique to the carbodiimide method and could be of enormous value in future. The limited application so far has not allowed specific problems of the CDI method to be identified and possibly eliminated. Other than this, it remains a viable alternative to the chemical cleavage method but it is not yet available in an unlabelled form.

#### *Chemical cleavage of mismatch (CCM)*

This method is based on the fact that mismatched C bases and mismatched T bases in heteroduplexes were found to be more reactive with hydroxylamine and osmium tetroxide respectively than equivalent matched base-pairs (Cotton et al., 1988). The point of reaction can be readily ascertained by further reaction with piperidine, which cleaves the strand containing the mismatched base (Cotton et al., 1988). Mismatched G and A bases are ascertained by use of the same probe but of opposite sense which transposes them to mismatched C and T bases respectively. Use of mutant probe as well as wild-type probe ensures each mutation has two chances of being detected making it unlikely that any mutation is missed (see below).

There have been many variations and improvements described since the initial publication. The method has been shown to be readily applicable to mRNA (Dahl et al., 1989) and viral RNA (Cotton and Wright, 1989). In this latter application, end-labelled probe and incomplete reaction allowed a pattern of difference or fingerprint to be generated between viral strains. Detection of mutations can be indirect, since mismatches can have a destabilizing influence on nearby matched T and C bases such that they become reactive with the two chemicals (Cotton and Campbell, 1989). To determine if a mutation is present in the homozygous or heterozygous state after it has been detected, mutant probe can be made for hybridization with mutant sample. Thus homozygous samples will give no signal whereas heterozygous samples will give a detectable mis-

match (Dianzani et al., 1991a). A class of T·G mismatches which were unreactive with osmium tetroxide (Theophilus et al., 1989; Forrest et al., 1991; Anderson et al., 1992) led to a modification in the strategy recommended for mutation detection using CCM, when it was suggested that mutant probe be made for hybridization with wild-type DNA (Forrest et al., 1991). This would ensure detection of the complementary C·A mismatch with hydroxylamine. It was first expected that this would generate twice as much work but it was found that by hybridizing mutant and wild-type probe together in equimolar quantities and reacting them in the one tube mutations could be readily detected (J. Saleeba, unpublished). Earlier protocols recommend 10 × excess unlabelled target. This protocol was independently published by others (Han and Sternberg, 1990). Labelling the probe with <sup>35</sup>S has been shown to give sharper bands (Saleeba and Cotton, 1991). Most recently an unlabelled variant has been developed (Saleeba et al., 1992) where mutant and wild-type DNA are mixed in equimolar quantities, reacted with the chemicals and the gels are stained with silver after electrophoretic separation. This procedure is thought best applied to heteroduplexes less than 600 bp long. Permanganate has been suggested as an alternative chemical to osmium tetroxide for mismatched T bases (Gogos et al., 1990), but confirmatory studies have not been published and preliminary experiments do not support this suggestion (R. Cotton, unpublished).

The main advantage of this method is its ability to detect 100% of mutations and it must therefore be compared with using direct sequencing as a screening method. Its ability to simultaneously screen 1–2 kb lengths of DNA is also a significant advantage over direct sequencing with an 11 kb screen taking only a person week (Roberts et al., 1992). Its major drawback is the need for many manipulations to be performed in a fumehood due to the toxic chemicals, and also the need for the two-step reaction and treatment with 3 chemicals.

Applications have been steadily increasing since the first description. Most of these applications have been searches for mutations in genes in order to define those causing disease. These

have recently included p53 mutations in colorectal cancer (Rodrigues et al., 1990), simultaneous screening for mutations in  $\beta$ -thalassaemia (Danzani et al., 1991b), ornithine transcarbamylase (Grompe et al., 1991a), collagen (Pro  $\alpha$  1(1)) (Valli et al., 1991), BCL2 oncogene (Tanaka et al., 1992),  $\beta$ -hexosaminidase (Akli et al., 1991), topoisomerase II (Bugg et al., 1991) and cystic fibrosis (Strong et al., 1991; Jones et al., 1992). Mutations in a bacterial gene have also been detected (Grompe et al., 1991b). Large lengths of DNA have been screened for mutations in individual patients. These include factor VIII in a screen of about 8 kb (Naylor et al., 1991) and dystrophin cDNA in a screen of about 11 kb (Roberts et al., 1992). The fact that large tracts of DNA can be screened is underlined by the 80 kb screened around gene targeted sites (Zheng et al., 1991). A novel application was that of mutation profiling of selectable elements. Here a selectable element containing randomly distributed point mutations was subjected to selection. Mutations were then assayed by CCM and the cleavage fragments were compared with the unselected controls (Wurst and Pohl, 1991).

The future may see increased use of the unlabelled form of the method as well as its improvement. Where tens of kilobases need to be screened it may well be the method of choice even over sequencing as currently performed. Despite numerous trials, alternative chemicals have not been encountered (R. Cotton, unpublished).

#### *Single-strand conformation polymorphism (SSCP)*

The SSCP method relies on the fact that single-strand DNA in solution under certain conditions has a defined secondary structure. This secondary structure can be altered when one of the bases is changed and the alteration in secondary structure is detected by electrophoresis in non-denaturing gels. Thus normal and mutant strands will have a different mobility (Orita et al., 1989a,b). The method is analogous to DGGE in analytical terms, but is far simpler.

Quite a few improvements and adaptations have been described in the last two years. The adaptation of precast gels and the application of silver staining has eliminated the use of radioactivity, and has improved resolution (Dockhorn-Dwor-

nickzak et al., 1991; Mohabeer et al., 1991) but ethidium bromide staining is a more rapid procedure to perform (Yap and McGee, 1992). Because mutations can be most effectively screened for in fragments several hundred base-pairs long, two strategies have been introduced to increase the length of the screen. In one case DNA (900 bp) is cut with several frequent-cutting restriction enzymes and subject to SSCP analysis (Iwahawa et al., 1992). This both increases the length screened and improves the chances of mutations being detected due to different fragment contexts for the mutation. In the other case, 2.7-kb DNA was cut with frequent-cutting restriction enzymes, separated according to size in a denaturing gel in a first dimension, and then analysed on a non denaturing gel in the second dimension by SSCP (Kovar et al., 1991). As in DGGE, alleles and minor components can be separated for direct sequencing (Suzuki et al., 1991; Hata et al., 1990). More recently the ARMS technique (see below) has been combined with SSCP for HLA genotyping (Lo et al., 1992). This involves selection of specific groupings of polymorphisms by allele specific amplification (ARMS) followed by analysis of variation within these groups by SSCP with silver staining or fluorescent primers. This should allow rapid and complete analysis for matching purposes and for analysis of other complex systems. Recently the most marked modification has been to transfer the analysis to RNA copies of DNA (Danenberg et al., 1992; Sarkar et al., 1992). The rationale is that RNA can assume more elaborate and greater numbers of conformational forms, and these conformational forms appear to be sensitive to single-base substitutions. Both of the groups demonstrated the increased sensitivity of RNA SSCP over SSCP, but the latter group performed a detailed comparison (Table 1).

It is thus clear that RNA SSCP leads to detection of an increased percentage of mutations.

The most clear cut advantage of SSCP as used on DNA is the *apparent* simplicity of the method. Use without label is also an important advantage. However, this is offset by the most serious drawback which is the lack of 100% detection. In one of the so far rare analyses of the proportion of mutations detected, the number of mutations missed in the 183- and 307-bp pieces is cause for



TABLE 1  
MUTATIONS DETECTED BY DNA SSCP AND RNA  
SSCP (%)

|              | Number of mutations studied | DNA SSCP | RNA SSCP |
|--------------|-----------------------------|----------|----------|
| 183 bp       | 12                          | 83       | 93       |
| 307 bp       | 22                          | 58       | 77       |
| 520 bp       |                             | Minority | Minority |
| IX mutations | 20                          | 35       | 70       |

Data from Sarkar et al. (1992).

concern, particularly in the blinded study of the factor IX mutations (Sarkar et al., 1992) (Table 1). It was stated that if shorter pieces were analysed a better percentage of these mutations would be detected but it would take “substantially more effort”. 1% false positives were reported in this study. When compared with DGGE, SSCP detected 5 of 7 polymorphisms found with the former (Sheffield et al., 1992b).

A definite improvement in the proportion of mutations detected was shown with the use of RNA SSCP (Table 1), but this is at a cost of an

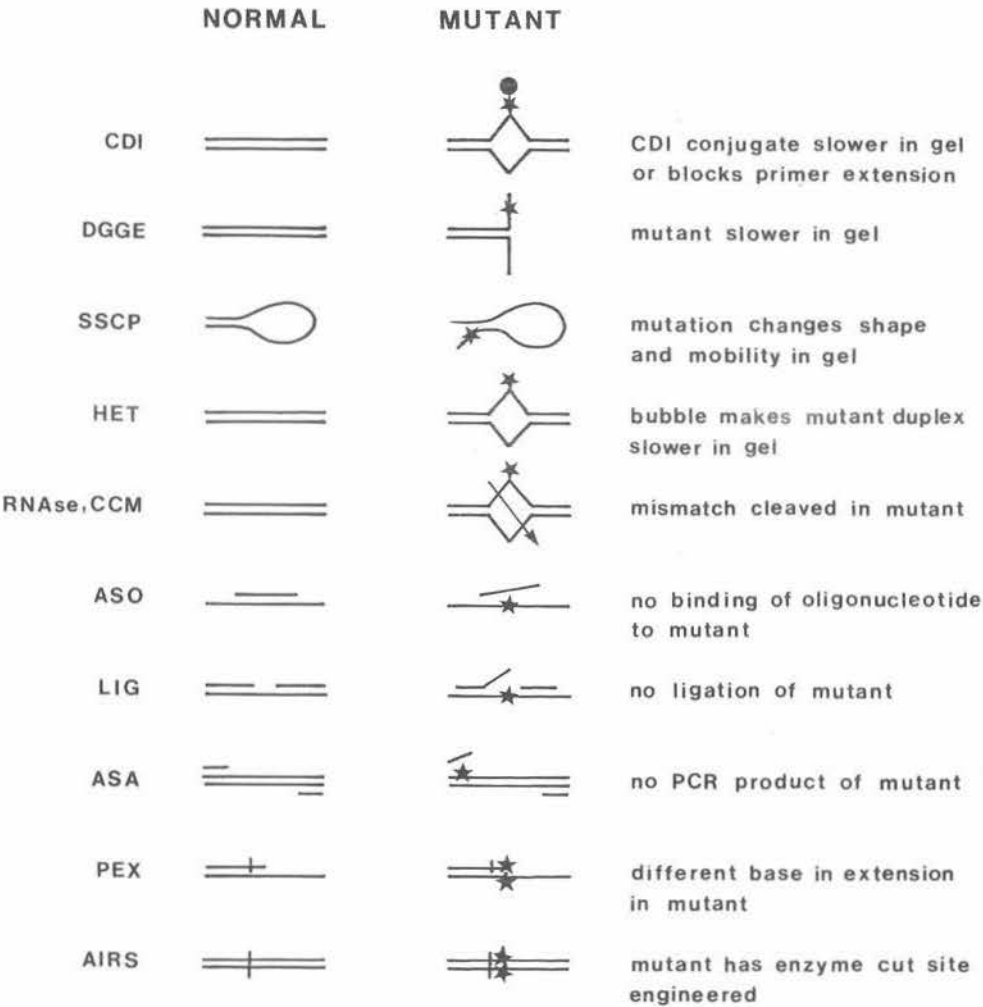


Fig. 1. Principles of the methods covered in this review. The abbreviations are given in the text. The asterisk represents the position of the mutation.

extra transcription step and longer primers to incorporate the promoter sequences, still without the attainment of 100% mutation detection. Combination of SSCP with the HET method (see below) has been suggested to lift mutation detection to closer to 100% as it has been shown to detect mutations not detectable by SSCP (White et al., 1992). In a recent review of the method (Hayashi, 1991) estimates that 99% and 89% of mutations are detected in 100–300- and 300–450-bp fragments respectively which differs from the above estimates.

The simplicity of the method has seen frequent use of the SSCP method over the last 1–2 years. There have been many examples of simple detection of unknown mutations in genes. For example in p53 in astrocytoma (Fults et al., 1992), in the Hup2 paired domain gene in Waardenburg's syndrome (Baldwin et al., 1992), p53 in gastric cancer (Yamada et al., 1991), aspartylglucosamidase in aspartylglucosaminemia (Ikonen et al., 1991) and CFTR in cystic fibrosis (Dean et al., 1990). SSCP has been used to match donor and recipient HLA antigens by analysis of patterns produced after amplification and separation of the second exon of an HLA class II gene (Summers et al., 1992). Polymorphism has been detected in *ALU* repeats by SSCP (Iizuka et al., 1992). RNA SSCP has recently been used to detect a mutation in DT-diaphorase (Traver et al., 1992).

The simplicity of this method has made it very popular recently despite its failure to detect 100% of mutations, and will ensure its future use. If an application can tolerate a maximum of around 80% detection, SSCP is the method of choice. Such applications might include detection of polymorphisms in a family for use in disease gene linkage studies, or finding mutations in a particular gene to incriminate it in a particular disease. Some improvement has been made via RNA SSCP in improving the proportion of mutations detected, but it is difficult to see modification bringing it up to a certain 100%.

#### *Heteroduplex analysis (HET)*

Heteroduplexes containing single base-pair mismatches can be accurately separated from related heteroduplexes on nondenaturing gels

(Keen et al., 1991a; Perry and Carrell, 1992; White et al., 1992). Others (Triggs and Rhine, 1991; Bidwell and Hui, 1990) performed separation of heteroduplexes on normal gels which detected deletions, but their method probably would not detect point mutations and can thus be considered a *different method* of lesser sensitivity. Thus far there have been few modifications.

The main advantage of the HET method is simplicity (as for SSCP) but its application has not been so widespread, partly because of its later description and partly because of the need for "Hydrolink" gels in the initial description (Keen et al., 1991a). The main disadvantage is the lack of 100% detection, in one study on 8 characterized antithrombin variants, 2 of the alleles were undetected and another 2 were detectable only after a much longer run (Perry and Carrell, 1992). In another model study, 1 of 9 single-base substitutions was not detected (White et al., 1992). This makes the proportion of mutations detected about 80%, which is similar to SSCP. In this latter study, mutations were deliberately placed at the end of a hairpin loop to test the efficiency of detection of SSCP. The authors found that only 2 out of 9 mutations were detectable by SSCP. This led to the suggestion that both methods could be used on samples in order to bring detection closer to 100%. In fact a new product (MDE) has been developed with this combination of methods in mind (R.J. Molinari, personal communication). Like SSCP, the HET method can only be applied to fragments hundreds of base pairs long (for example 200–300 bp; see Keen et al., 1991).

Applications include detection of a mutation in the human homologue of the PAX-3 paired box gene in Waardenburg's syndrome (Tassebehji et al., 1992) and rhodopsin mutations in retinitis pigmentosa (Keen et al., 1991b). PCR fingerprinting (Bidwell and Hui, 1990; Clay et al., 1991) relies on multiple mismatches between alleles and pseudogenes to generate specific patterns for individuals after PCR and non-denaturing electrophoresis. Thus when one amplifies HLA-DR/DW genes from an individual, heteroduplexes form between the two different alleles possessed by the individual as well as between those alleles and nearby related but different

pseudogenes. This generates a number of heteroduplexes characteristic of the genes an individual carries and the pattern after electrophoresis displays this. These patterns have been used as an aid in matching marrow donors (Clay et al., 1991).

It is possible that future modifications may move the detection closer to 100%, but perhaps this method is best used in combination with SSCP to allow possible detection of *nearly* all mutations.

### *Direct sequencing (DS)*

Sequencing is so common it requires only passing comment in this review. Sequencing can be used either as a "screening" or a "diagnostic" method. Two methods are used. One uses end-labelled probe and partial chemical cleavage is achieved at all the bases with particular chemicals. Size of fragments and the chemicals which produced them can then be used to define the sequence (Maxam and Gilbert, 1980). The other method uses enough nucleotide analogue (di-deoxy) to allow some chain termination at each base, and again the base used and the length of the fragments defines the sequence (Sanger et al., 1977). The latter method is most frequently used.

The Sanger method is used with little modification, except that recently PCR products are now being used instead of fragments cloned into M13 sequencing vector (Wong et al., 1987). Vent DNA polymerase is reported to give enhanced genomic sequencing (Garrity and Wold, 1992). Coupling of amplification and sequencing has been reported (Ruano and Kidd, 1991). Capillary gel electrophoresis has been used for analysis of products as an alternative to gel electrophoresis (Swerdlaw and Gestel, 1990). Different bases have a different reaction rate with formamide, so that fragments have a different intensity depending on the base cleaved to produce that end-labelled fragment (Negri et al., 1991).

The main advantage of direct sequencing is as a reference method, in that it detects and defines the mutation. However, many manipulations are necessary, stimulating the design of robotic systems for use in sample reactions. Gels have to be poured and analysed, although automated "se-

quencers" are now available for the analysis. In some sequences (such as GC rich areas), compression can occur and some PCR products can not be sequenced making M13 cloning necessary (see for example Kerem et al., 1990).

Laboratories are found in which sequencing of multiple samples is still used to find mutations. For example, the p53 gene cDNA (about 1.5 kb) was sequenced in 30 tumors and mutations were found in 14 of them (Sayaki et al., 1991). Earlier Koeberl et al. (1990) used genome amplification with transcript sequence (GAWTS) sequencing to screen 8 regions (2.46 kb) in each of 60 successive haemophilia B patients.

Whilst progress can be made with existing sequencing methods, faster methods are needed. This is particularly so for the Human Genome Project, where vast tracts of DNA need to be sequenced with a minimum of time and expense. Rapid, simple inexpensive methods would also be appreciated by those in the clinical situation. Such methods need a completely new approach. One approach which is receiving thought is the hybridization method thereby eliminating gels. In this method, identifiable, mixed and defined oligomers are fixed on a membrane and mixed with target. Their pattern of hybridization defines the sequence (Bain, 1991; Streyoska et al., 1991; Baringa, 1991).

### **Diagnostic methods**

Once mutations have been described they can be searched for again with a completely different set of methods (although screening methods can be used; both DGGE and CCM have been used this way in B thalassaemia, for example). 3 of these rely on mismatch (or its lack) to obtain discrimination between mutant and wild-type. The others either have a particular base inserted depending on the allele, or else rely on PCR to create a restriction site. Some aspects of this area have been reviewed recently (Landegren, 1992).

### *Allele-specific oligonucleotide (ASO)*

This is the oldest general targeted method for mutation detection, and relies on mismatches between mutant target genomic (or PCR amplified)

DNA and synthetic wild-type oligonucleotides showing decreased binding of the oligonucleotide relative to a mutant oligonucleotide (Wallace et al., 1981; Connor et al., 1983). This is sometimes referred to as the sequence-specific oligonucleotide method (SSO). One of these two components is usually attached to a membrane or other solid support.

The original method description involved probing separated bands which had been transferred to a membrane, but more recently the sample has been directly transferred to the membrane (the dot blot). With recent application of the PCR technique colorimetric detection is possible since probes can be labelled with horse radish peroxidase (HRP). As more alleles were assayed per sample, instead of increasing the number of dots each to be probed with a different oligonucleotide, different probes were spotted onto the membrane and the biotin-labelled target sample applied. The probes forming duplexes without mismatches and retaining the target became stained with HRP avidin. This has been referred to as the reverse blot (Saiki et al., 1989).

The main advantage of the current method is that it does not require electrophoresis and the method is non-radioactive. Of particular importance is the fact that normal and mutant probe (or target) is used and thus built in controls are available. Some have suggested that the method is too sensitive to hybridization conditions, but use of trimethylammonium chloride avoids this problem (Wood et al., 1985). Another disadvantage is that 2 primers have to be synthesized for amplification and 2 more, mutant and wild-type, for mutation detection.

Applications are almost too numerous to require specific examples to be given. However, some recent topical examples include Rhodopsin mutations (Sung et al., 1991), Pro alpha 1(1) collagen mutations (Valli et al., 1991) and topoisomerase II variants (Bugg et al., 1991).

It is likely that this method will continue to be used as a low technology method where only a few mutations need to be studied in a particular disease. When automated using 96-well plates it will compete with the methods below for any population screens which become practicable.

### *Allele-specific amplification (ASA)*

By placing one of the primers of a PCR amplification pair at the point of a known mutation and making this primer in either the mutant or wild-type form one can amplify only the mutant or wild-type respectively. When the PCR products are identified it is possible to determine if mutant or wild-type genes are present in the target sample.

In 1989 at least 6 papers were published describing major variants of this approach. The methods are known as Competitive Oligonucleotide Priming (COP) (Gibbs et al., 1989), Colour Complementation Assay (CCA) (Chehab et al., 1989), Amplification Refractory Mutation System (ARMS) (Newton et al., 1989), Allele-Specific Polymerase Chain Reaction (ASPCR) (Wu et al., 1989) PCR Amplification of Specific Alleles (PASA) (Sommer et al., 1989) and Allele-Specific Amplification (ASA) (Okayama et al., 1989). All varied in the length of the specific oligonucleotide (12–30 bp) and mode of detection (autoradiography, fluorescence or ethidium bromide) and extent of testing. However, the main groupings depend on whether the mismatch of the specific primer is in the middle (COP, CCA) or at the 3' end (ARMS, ASPCR, PASA, ASA). The former variant relies on differential competition of primers and thus on hybridization conditions for amplification/non amplification. The latter relies on a mismatch at the 3' end allowing, or not allowing, extension and hence amplification. Detection of the allele amplified originally involved electrophoresis of two target samples, one amplified with mutant primer and the other with wild-type primer. Presence of a band defines the presence of that allele. The CCA method used different fluorescent dyes for mutant and wild-type probes thus avoiding electrophoresis, since colour defined the allele amplified. With the use of allele-specific primers with mismatches in the centre and two annealing temperatures, the assay can be carried out in a single tube (Ye et al., 1992). This is similar to a strategy described earlier for detection of Haemoglobin Constant Spring except that the primer with the mismatch has it at the 3' terminus and fluorescent labels are used (Kropp et al., 1991). More recent developments have been the placing of



each of the PCR primers at a site where mutations are found. This is called Double ARMS (Lo et al., 1991) or PCR Amplification of Multiple Specific Alleles (PAMSA) (Dutton and Sommer, 1991). Thus if two alleles are present, which allow both primers to match on a single-strand, amplification occurs. With the use of 4 different primer pairs in different tubes, assay of which alleles are on specific strands is possible. One group has also shown that double ARMS can be used with inverse PCR (Lo et al., 1991). Multiple PCR amplification of specific alleles (PAMSA) has been shown to be possible by use of primers of different length (Dutton and Sommer, 1991). The general nature of the PASA assay has been demonstrated by Sommer et al. (1992) despite earlier reservations on this point (Kwok et al., 1990). In fact, Sommer et al. (1992) showed that the PASA assay could be made to discriminate 69 pairs of alleles.

The main advantage of the method is that it requires only a single PCR reaction and a result is obtained in a matter of hours. Other important advantages include avoiding electrophoresis, radioactivity, and even label. It can be automated for large numbers of samples. There seems to be few requirements, except the need for multiple primers and a cycling machine.

Sommer et al. (1992) have optimized conditions in such a way that alleles can be detected around 5 disease genes. Old et al. (1990) have used it for the rapid detection and prenatal diagnosis of B thalassaemia in Indian and Cypriot populations. It has also been used to screen for 6 Gaucher's alleles in 12 patients (Mistry et al., 1992).

It is clear that the simplicity and rapidity of this method will see it used increasingly both in research/clinical situations and in screening of populations.

### *Ligation (LIG)*

This assay relies on differential ligation of two abutting oligonucleotides when hybridized to target DNA. The primer on the 5' side has its 3' base placed at the position of the variation (mutation or polymorphism). If hybridization to DNA involves no mismatch at the 3' base the ligase

enzyme joins the oligonucleotides, whereas when there is a mismatch there is no ligation. Ligation status can be readily assayed (Landegren et al., 1988; Alves and Carr, 1988; Wu and Wallace, 1989).

The original method (oligonucleotide ligation assay-OLA; Landegren et al., 1988) used biotin labelling of the 5' end of the 5'-oligonucleotide and  $^{32}\text{P}$  for the 3' end of the 3'-oligonucleotide. A wild-type set is added to one portion of the sample and mutant set to the other. If ligation occurs avidin beads will bind radioactivity, if not there will be no binding. Fluorophores were also described as label in this study so that an automated sequencer could be used. A later development has made the whole assay compatible with robotics with 96-well plates (Nicherson et al., 1990). The wells are coated with streptavidin, and one oligonucleotide is bound to this via biotin. The other oligonucleotide is labelled with digoxigenin and its ligation to the other oligonucleotide is detected by alkaline phosphatase conjugated anti-digoxigenin antibodies. This should be suitable for large-scale screening of populations.

A completely different use has been made of ligation for amplification of DNA, but obviously only of those portions covered by the oligonucleotides. This procedure can be used to detect specific sequences in a sample (ligation amplification assay, LAR, or ligase-chain reaction, LCR). Thus excess of two pairs of abutting oligonucleotides, one pair for each sense, are added to a sample. If there is no mismatch, and/or if the sequence is present, the oligonucleotides are joined by ligase after melting and annealing. When this melting and annealing cycle is repeated 20–30 times readily detectable product is formed. If no target is present or a mismatch is present at the 3' end of the 5' side primer no product is formed. This might be used to assay for mutant or wild-type sequences (or viral DNA for example Wu and Wallace, 1989). Amplification can be either linear or exponential and is an alternative to straight PCR amplification of the sample, since the LAR performs the amplification and detection steps simultaneously. A discussion of patent aspects has appeared recently (Weiss, 1991). The discovery of a thermostable ligase (Barany, 1991a) has obviated the need for

addition of ligase at each cycle. A review of the LCR has recently appeared (Barany, 1991b).

The main advantage of the method, either in the PCR-LIG form or in LAR (or LCR), is that it is amenable to automation, which is of course useful for a large number of samples. The former system is said to be able to process 1200 samples per day for example (Nicherson et al., 1990).

No published applications have been noted to date. It is highly likely that one of the above forms will be developed in a robotics format with a custom designed instrument for processing large numbers of samples.

#### *Primer extension (PEX)*

This method relies on differential extension of the 3' end of an oligonucleotide by a single-base, the identity of the coupled base being dependent on the allele present one base away from the end of the oligonucleotide paired with the target (Sokolov, 1989; Kornher and Livak, 1989).

In one of the original forms (Sokolov, 1989), primer was synthesized up to the base before the position where mutant and wild-type differ. The primer was then annealed to genomic DNA in two different tubes. Radiolabelled ( $^{32}\text{P}$ ) base complementary to wild-type allele base was added to one and radiolabelled base complementary to mutant allele base was added to the other. Electrophoresis of samples indicated incorporation or non-incorporation after extension. Variants of the technique involve the biotinylated probe being captured in an avidin coated well, and the base which extends the probe was either  $^3\text{H}$  or digoxigenin labelled. The latter leads to a non-radioactive mode (Syvanen et al., 1990). Use of the original form (but on PCR amplified DNA) was also described by Kuppaswamy et al. (1991). A variant is based on the dideoxy sequencing method. This involves use of a labelled sequencing primer with its 3' close to (but not at) the mutation site, and use of the dideoxynucleotide, which permits addition of a different number of bases in mutant and wild-type (Lee and Anvret, 1991). The products are run on the usual sequencing gels to define the sizes of labelled extended fragments.

The main advantage of the PEX method is its simplicity, together with the facts that it can be

used in a non-radioactive form and can be automated for application to large numbers of samples.

There have been several applications apart from the model studies. A simplified protocol of the "mini sequencing" PEX technique was used to determine the prevalence of 2 mutations in the aspartyl glucosaminidase gene in 115 Finnish patients (Syvanen et al., 1992).

The quantitation available with the technique allowed a pooled sample from 1350 individuals to be analysed, leading to an estimate of 1 in 36 as the frequency of carriers of the dominant disease allele. Others applied it to mutations in the Factor IX and CFTR gene (Kuppaswamy et al., 1991). This method is likely to be useful in research and clinical situations as well as in large-scale population screening.

#### *Artificial introduction of restriction sites (AIRS)*

When a mutation changes a restriction site, it can readily be sought in a genome. Most mutations do not change a restriction site, however, introduction of a restriction site by mismatched primers in the region of a mutation for either the wild-type or mutant sequence has been a useful strategy (Cohen and Levinson, 1988). Apart from increasing use, the only major modification has been its adaption to the detection of minority alleles present in as few as 1 in 1000. In this method an artificial restriction site is introduced at the point of the mutation, at a site near to (but inside) a natural invariant sequence used in a first round of amplifications by PCR. The DNA is digested and in this case wild-type is digested and is unavailable for a second round of amplification using the same primer in the region of the mutation and a different primer inside the other initial primers. This then prevents amplification of the DNA of the artificially cut allele after the first cycle thereby allowing differential enrichment of the mutant sequence (Chen and Viola, 1991; Levi et al., 1991).

The special advantage of the simple version of this method is its absolute simplicity and low technology demand. However, it is not all embracing and it is probable that not all mutations can be found in this way. The technique has however already been applied to PK (Elken et al.,

1991), cystic fibrosis and retinitis pigmentosa (Sorscher and Hwang, 1991), B thalassemia (Lindeman et al., 1991; Ward et al., 1990),  $\alpha$ -1-antitrypsin deficiency (Dry, 1991) and cystic fibrosis (Ng et al., 1991).

The simplicity of this method will ensure its continued use in the research and clinical laboratory.

#### General strategies

The PCR method (Saiki et al., 1986) has had an enormous impact on the efficiency of mutation detection and almost all methods currently incorporate it. Alternative isothermal methods of DNA amplification have appeared but have not yet become widely used (Guatelli et al., 1990; Walker et al., 1992). A review of one of these methods has recently appeared (Fahy et al., 1991).

For human applications, one of the more important discoveries in recent years has been the finding of small amounts of message in tissues which are not the prime tissue(s) of expression (Chelly et al., 1989; Sarkar and Sommer, 1989). For example, messenger RNA of the liver specific phenylalanine hydroxylase was found in white blood cells (Sarkar and Sommer, 1989). This was only possible because of the extreme sensitivity of the PCR technique. Amplification of cDNA followed by screening with a method such as RNAase CCM or CDI then becomes possible. An alternative would be amplification of individual short exons in genomic DNA for screening with DGGE, SSCP or HET. For example, in the case of phenylalanine hydroxylase instead of having to synthesize 13 pairs of primers for 13 different reactions only a minimum of two pairs would be needed plus another pair for nesting. An idea of the saving of work by a screening method is shown in Table 2.

Practical examples include amplification of the illegitimate transcript of the CF gene of 27 exons in 6 segments ranging in size from 699 to 1033 bp (Chalkley and Harris, 1991) and amplification of the illegitimate transcripts of dystrophin (Roberts et al., 1992).

In DNA heterozygous for a particular mutation the heteroduplexes form without the need for an additional annealing step during PCR amplification of DNA. This is most useful saving of

TABLE 2

#### SAVINGS BY USING ILLEGITIMATE TRANSCRIPTS FOR MUTATION DETECTION

Minimum reactions and/or lanes needed.

|      | 13 Exon gene | 1.4 kb cDNA | % detection |
|------|--------------|-------------|-------------|
| SEQ  | 104 (13) *   | 48 (7)      | 100         |
| SSCP | 13 (13)      | 6 (7)       | 80          |
| CCM  | 26 (13)      | 2 (2)       | 100         |

\* The numbers of primer pairs required are in parentheses.

a step for methods dependent on heteroduplexes (DGGE, CDI, CCM, HET). Normal DNA can be added before amplification to ensure heteroduplex formation (Higuchi et al., 1990; Cai and Kan, 1990).

For linkage studies (in inherited disease), in the past restriction enzymes were used to define polymorphisms present in families so they could be used to track genes for diagnostic purposes. SSCP (or HET) with approximately 80% detection is an excellent alternative due to the much greater coverage and extreme simplicity. For very large pieces of DNA in the order of 10 kb the CDI and CCM methods are really the only practical methods, the former possibly being also used in the EM mode (see above).

Some workers have used directed searches at sites where cleavage of pre-proteins occur, or else CpG at sites, both of which might be sites for mutations causing disease. Example include important sites in Factor VIII cleavage (Gitschier et al., 1988) using ASO, and changes in the *taq*I sites in the Factor VIII gene since CpG included in the *taq*I site is a hot spot for mutations (Millar et al., 1991).

Patterns of difference between alleles/viral segments have been defined by several of the methods. Examples include using end labelled probe with the CCM method for Dengue virus (Cotton and Wright, 1989), uniformly labelled RNA probe for HIV with the RNAase method (Lopez-Galindez et al., 1991) and SSCP for matching for transplants (Summers et al., 1992).

#### Comparison and conclusions

It is difficult to compare screening methods since many factors must be taken into account

TABLE 3  
COMPARISON OF SCREENING METHODS

|        | Nominal<br>detection (%) | Steps <sup>a</sup> | Advantage                    | Disadvantage                |
|--------|--------------------------|--------------------|------------------------------|-----------------------------|
| RNAase | ~ 70                     | 2                  | simple, locates              | 70% detection, probe prep.  |
| DGGE   | "Almost all"             | 1                  | almost all, separation       | complex theory, GC clamp    |
| CDI    | 100?                     | 3                  | 100% detection, long lengths | little used, many steps     |
| CCM    | 100                      | 3                  | 100% detection, long lengths | toxic chemicals, many steps |
| SSCP   | ~ 80                     | 1                  | very simple, separation      | 80%, short lengths          |
| HET    | ~ 80                     | 1                  | very simple, separation      | 80%, short lengths          |

<sup>a</sup> Steps for processing when DNA obtained and including analysis.

when choosing a method. The most important of these factors include the numbers of alleles/people to be screened/diagnosed, the experience of operators, the technical sophistication of the laboratory, the availability of technical staff, the length of region to be screened, whether or not the laboratory is dedicated to a particular disease, and the percentage of mutations which has to be detected. Comparisons of the screening methods are shown in Table 3.

The simplicity of HET and SSCP will ensure their continued use, but for some purposes the lack of complete detection is a disadvantage as is the short length which can be screened in one strand. Increased detection is made more likely by combining HET and SSCP (White et al., 1992). The CCM method (and possibly CDI) is more of a precision method when used in its equimolar mutant and wild-type mode, with the attendant 3-step process since large lengths can be effectively and confidently screened for mutations. DGGE is popular (despite the attendant theory) because it detects most mutations, but it certainly cannot guarantee detection of all mutations. Once set up for a particular disease it can be a satisfactory routine diagnostic process.

The RNAase method, apart from having a low detection rate also needs an RNA probe, but despite this it is still used. DGGE and SSCP and possibly HET are the only methods capable of separating small amounts of mutant species for sequence or other analysis. The DGGE, SSCP and HET methods are more suited to smaller fragments and CDI, CCM and RNAase methods to larger fragments.

There is still much room for improvement of screening methods. For example, a single-step method which cleaves at a mismatch, is able to operate on kilobase lengths of DNA, and can detect 100% of the mutations, would be ideal.

All of the diagnostic methods excepts AIRS have evolved to automated forms suitable for screening large populations. Equally, all methods (and especially perhaps AIRS) are well suited to diagnostic and small research laboratories.

In conclusion, we now have available a set of excellent methods of several different basic for mutation detection. The precise method of choice will depend as much on the background of the laboratory as on the task at hand.

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