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TOWARDS DEVELOPMENT OF TECHNIQUES TO OBSERVE MUTATIONAL SPECTRA IN HUMAN TISSUE SAMPLES

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Summary. - *We have devised a protocol with the resolving power to uncover mutational spectra within DNA sequences from human genomic DNA. The protocol is a combination of: (a)* in vitro *DNA amplification of the desired DNA sequence; (b) denaturing gradient gel electrophoresis of the amplified DNA to separate the wild type and mutant sequences; (c) isolation of the mutant sequence(s); (d) determination of the nature of the base-pair change(s). Data based on reconstruction experiments using exon 3 of the human hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene suggest that the protocol can detect mutant sequences present at mutant fractions equal to or greater than* 10^{-4}.

Riassunto (Sviluppo di tecniche per il rilevamento di spettri di mutazione in campioni di tessuti umani). - *E' stato messo a punto un protocollo sperimentale che permette di rilevare gli spettri di mutazione di sequenze di DNA di genoma umano. Il protocollo è una combinazione di: (a) amplificazione del DNA* in vitro *della sequenza di DNA di interesse; (b) elettroforesi su gel con un gradiente di sostanze denaturanti del DNA amplificato per separare le sequenze mutanti dalle sequenze inalterate; (c) isolamento delle sequenze mutanti; (d) determinazione della natura dell'alterazione genetica. Dati basati su esperimenti di ricostruzione usando l'esone 3 del gene umano hypoxanthine-guanine phosphoribosyl-transferase (HPRT) suggeriscono che il protocollo è in grado di rilevare sequenze mutanti presenti a livelli di frazioni di mutanti uguali o maggiori di* 10^{-4}.

Introduction

Human beings suffer from a wide variety of diseases which begin with genetic changes in germ line or somatic cells. These include point mutations, chromosome rearrangements, large deletions and aneuploidy. Chemicals found in the environment can cause these types of genetic changes in human cells in tissue culture. It is a primary goal of genetic toxicology to discover if there is a causal link between environmental mutagens and human mutations. If such a link is established, it will then become the responsibility of genetic toxicologists to discover which environmental factors are significant contributors to human genetic change in order that appropriate measures to protect public health may be adopted.

We have previously proposed that the phenomenon of mutational specificity discovered by Benzer [1] can be used to diagnose the actual causes of human genetic changes. Mutagens often show remarkable specificity with regard to the nature and locations of the mutations, thus the pattern of mutation produced, called mutational spectra, may be diagnostic for exposure to a particular mutagen. This chapter is a progress report of attempts to obtain human mutational spectra by adopting, improving and combining several of the techniques of molecolar analysis of DNA.

In 1961, Benzer [1] showed that mutagens used to treat T4 bacteriophage gave a unique and reproducible distribution of mutations within the rII region of the bacteriophage genome. In particular, these sets of induced mutations were different from the set of spontaneous mutations occurring in the same region. Similar observations of specific mutagen-induced mutational spectra were reported for the *lac*I gene of *E. coli* [2-5]. Based on this concept, Thilly *et al.* [6] have suggested an approach to determine whether genetic change caused by chemicals is prevalent in human population. In this laboratory, Dr. Phaik Mooi Leong (Ph.D thesis, MIT, Cambridge, 1985) examined the ability of different mutagens to induce resistance to the drugs 6-thioguanine (6-TG), ouabain (OUA), podophyllotoxin (PPT) and 5,6 dichlororibofuranosyl benzymidazole (DRB). For OUA, DRB and PPT, only restricted kinds of missense mutations were expected because these toxins binds to essential proteins, thus the functionality of these proteins is necessary for cell survival. However, in the case of 6-TG resistance, since the hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene product is not essential

for cell survival, a wide variety of mutations were expected at this locus. From data of the frequencies of spontaneous mutations and mutations induced by ethyl methane sulfonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and nitroquinoline oxide (NQO), she showed the existence of reproducible mutational spectra in human cells. The ratio of $DRUG^r/TG^r$ was found to vary with different mutagens, and it was possible to discriminate among the mutagens based on the OUA^r/TG^r, DRB^r/TG^r and PPT^r/TG^r ratios.

An important limitation of this technology was that the low frequency of specific missense mutations obligated one to use large number of human cells. About 3 x 10^8 cells were necessary for this approach. These are easily obtained in human lymphoblastoid tissue culture but would represent about 300 ml of human blood were one to seek the same information from human $6TG^r$ T-cells. However, Leong made it clear that mutational spectra of marked specificity did exist in human cell populations. Seeing in mutational spectra a sufficient and perhaps unique means toward discovering the cause(s) of human mutations, we set out to divise a strategy and develop necessary technology to obtain mutational spectra from human blood samples and other tissues.

At this time, two techniques have been significantly improved and used in tandem to create what is believed to be a major advance in the study of point mutations: denaturing gradient gel electrophoresis (DGGE) and high fidelity DNA amplification (HFDA).

Denaturing gradient gel electrophoresis

Fischer and Lerman [7] used DGGE to separate homoduplex DNA sequences differing by only a single basepair. DNA consists of cooperative melting regions (domains). When a DNA sequence migrates into a polyacrylamide gel containing an increasing concentration of denaturant, the low temperature melting domain will melt (become single-stranded) at a spcecific concentration of denaturant. At this point, the DNA molecule has both helical and random coil segments. This partially denatured molecule has a reduced mobility and will focus in the gel at a position characteristic of the stability of the low-temperature melting domain. Mutations occurring in the low-temperature melting domain will often change the stability of the domain and the corresponding partially melted form will focus at another denaturant concentration which is specific for the nature and position of the mutation.

Thilly [8] suggested that DGGE could be applied to separating mixed sets of mutant/wild type heteroduplexes and thus yield mutational spectra as a series of bands on a DGGE gel.

Myers *et al.* [9] demonstrated the fact that heteroduplex DNA sequences did yield excellent separation of mutant from wild type sequences. We have since demonstrated that the technique separates mutants in exon 3 of human HPRT gene [10] which contains a natural high-temperature melting domain. However, a major problem with this approach is the limit of detection of a minor mutant species in an essentially wild type population. A solution to this is to use a polymerase chain reaction [11] to amplify the sequence directly from genomic DNA.

DNA amplification

Saiki *et al.* [11] and Mullis and Faloona [12] have demonstrated the ability to obtain DNA sequences directly from genomic DNA by DNA amplification *in vitro*. Basically, one uses a pair of primers to hybridize to both ends of the desired segment of the genomic DNA. DNA chain synthesis is then carried out simultaneously from each primer by a DNA polymerase. This procedure is repeated until a sufficient quantity of the desired segment is accumulated. This ability permits one to begin with a few copies of a DNA sequence in genomic DNA and to amplify them to a sufficient quantity for analysis by DGGE.

Two parameters are particularly important for this method: the efficiency or yield per cycle of the sequence amplified, and the fidelity of the amplification.

Efficiency of DNA amplification *in vitro*

The DNA amplification can be carried out using DNA polymerases from different sources: a) Klenow fragment used in the original method [11, 12]; b) T4 DNA polymerase [13] which has been reported to carry out DNA synthesis *in vitro* with higher fidelity than does Klenow fragment [14]; c) a thermostable DNA polymerase, *Taq* polymerase [15] which greatly simplifies the amplification process by eliminating repeated addition of the enzyme, and d) modified T7 DNA polymerase (Keohavong *et al.*, manuscript in preparation). The efficiencies, expressed in terms of overall net DNA synthesized per cycle of amplification, vary from about 60% for Klenow fragment and for T4 DNA polymerase [11-13], to 83% for *Taq* polymerase [15] and over 90% for modified T7 DNA polymerase (Keohavong *et al.*, manuscript in preparation).

Fidelity of DNA amplification *in vitro*

DNA polymerases produce errors during the DNA synthesis *in vitro*, and error rate varies according to the DNA polymerase used [14, 16]. DNA sequences containing polymerase-induced mutations produced during the amplification process can be separated from the wild type by DGGE. Consequently, the frequencies of these

polymerase-induced mutant sequences are a limiting factor masking the pre-existing mutant sequences, especially those present at very low mutant fractions. Comparative analysis by DGGE of the pattern of mutant exon 3 sequences generated by each of the above DNA polymerases showed significant differences in the fidelity of DNA amplification. T4 DNA polymerase produced the fewest errors while *Taq* polymerase induced the most errors (Keohavong *et al.*, manuscript in preparation). These data demonstrate that two DNA polymerases have the ability to carry out amplification with sufficiently high fidelity and efficiency to permit the detection of mutant sequences by DGGE from a set of mutants isolated by standard selective systems.

Combination of high fidelity DNA amplification and denaturing gradient gel electrophoresis to obtain mutational spectra in human cells

The basic idea is to amplify the mutant sequences directly from a mixture containing mutant and wild type sequences and then separate them by DGGE. The resolving power of DGGE is such that mutant sequences often separate from one another and mutant sequences can be isolated, and the nature of the mutations determined by direct sequencing. Using this procedure, one expects to eliminate many steps required to obtain the same information using a clone-by-clone approach.

For the technique to be useful in obtaining mutational spectra from human cells, a number of obvious criteria must be considered: a) the original cell sample must contain a sufficient number of mutants to constitute a spectrum; b) the mutants themselves must be recoverable and analyzable at the DNA sequence level in order to test the nature and quality of the information provided by the means developed.

Reconstruction experiments to probe the precision and accuracy of the protocol

It is important to estimate the minimum mutant fraction capable of being identified in a mixed-cell population. We have shown that a B-cell line obtained from a patient with gout ($HPRT_{Munich}$) contains a base-pair substitution in the low-temperature melting domain of HPRT exon 3 [10]. This mutant sequence was easily separated from the wild type by DGGE. Wild type cells (TK6) and $HPRT_{Munich}$ cells were mixed at ratios of 10^{-2} to 10^{-4}, the DNA was amplified using modified T7 DNA polymerase and analyzed by DGGE. This permitted the detection of 100 copies in a wild type background of 10^6 copies. The limit of detection was a mutant fraction of 10^{-4} (Keohavong *et al.*, manuscript in preparation). In the case of HPRT mutants, this means that in a large mixed population of 6-TG resistant cells, "hot spots" comprising greater than 10^{-4} of the entire population should be directly observable.

Application of the techniques for direct observation of mutational spectra in exon 3 of the human HPRT gene

In applying this technology to human cells, one would expect the availability of cells to be the limiting factor in blood and tissue samples. A blood sample, which can be easily isolated directly from an individual, gives approximately 10^6 T-lymphocytes per ml [17]. For the sake of argument, we will consider a sample of 10^8 T-lymphocytes (100 ml of blood). We know that the fraction of HPRT mutants (6-thioguanine resistant) in human blood T-lymphocyte-cell populations is about 5-10 x 10^{-6} [17]. Fewer than 20% of them represent large deletions or rearrangements visible by Southern blot analysis [17-22]. The remaining 80% are assumed to be point mutations. One of the consequences of using the stringent selection conditions of 6-TG resistance is that we expect only frameshift mutations, mutations that generate a stop codon and a small subset of missense mutations to be detected. However, by using DNA amplification and DGGE on unselected cell populations, we might expect to detect all the base-pair substitutions. What might their frequency be?

A variety of base-pair substitutions occurs in human DNA but 6-TG resistance identifies only a fraction of them. If we assume that half of the 6-TG resistant mutations generate a stop-codon and that the ratio of the nonsense on the missense mutations is 24/1 for random base-pair substitutions for the remaining half, we arrive at a frequency of: $5 \times 10^{-6} \times 0.5 \times 24 = 6 \times 10^{-5}$. This frequency represents base-pair substitution mutations distributed over the 654-bp coding region, assuming a random sequence. Within exon 3, we expect an overall mutant fraction of $184/654 \times 6 \times 10^{-5} \sim 2 \times 10^{-5}$ for point mutations that we wish to study directly from human blood samples.

With 10^8 T-lymphocytes (100 ml of a blood sample) as a starting point, we should be able to calculate the number of mutant copies and consider what needs to be done to observe these copies through amplification and DGGE resolution. For a male, the number of initial mutants will be $10^8 \times 2 \times 10^{-5} = 2 \times 10^3$. Such a mutant fraction is expected to be detected using our protocol by choosing an appropriate DNA polymerase to carry out the amplification step.

The detection of mutations can also be achieved by examining a multi-copy gene, such as the 45s ribosome gene, which is present in the human genome at an estimated 40 to 400 copies. Using multi-copy genes, one would need a maximum of 2.5×10^6 cells (or 2.5 ml of blood), to obtain 10^8 DNA copies, instead of 10^8 cells needed when using a single copy gene.

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