Computational analysis of mutation spectra

Igor B. Rogozin, Vladimir N. Babenko, Luciano Milanesi and Youri I. Pavlov

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Abstract
Mutation frequencies vary along a nucleotide sequence, and nucleotide positions with an exceptionally high mutation frequency are called hotspots. Mutation hotspots in DNA often reflect intrinsic properties of the mutation process, such as the specificity with which mutagens interact with nucleic acids and the sequence-specificity of DNA repair/replication enzymes. They might also reflect structural and functional features of target protein or RNA sequences in which they occur. The determinants of mutation frequency and specificity are complex and there are many analytical methods for their study. This paper discusses computational approaches to analysing mutation spectra (distribution of mutations along the target genes) that include many detectable (mutable) positions. The following methods are reviewed: mutation hotspot prediction; pairwise and multiple comparisons of mutation spectra; derivation of a consensus sequence; and analysis of correlation between nucleotide sequence features and mutation spectra. Spectra of spontaneous and induced mutations are used for illustration of the complexities and pitfalls of such analyses. In general, the DNA sequence context of mutation hotspots is a fingerprint of interactions between DNA and DNA repair/replication/modification enzymes, and the analysis of hotspot context provides evidence of such interactions.

MUTATION SPECTRA AND MUTATION HOTSPOTS
The process of mutation is fundamental in biology, being an essential evolutionary factor that creates genetic variation. Understanding complex mechanisms by which mutations occur spontaneously or are induced by mutagens is an important goal of molecular biology. A mutation spectrum is a distribution of frequencies of every type of mutation along nucleotide sequences of a target gene. Mutations in target sequences are usually revealed by either phenotypic selection in experimental test systems or, in the case of disease-causing genes in humans, by clinical studies in which certain genes are sequenced in groups of patients and in control groups. Both the experimental test systems and the clinical studies rely on detectable (mutable) positions, which are sites where DNA sequence changes cause phenotypic changes. A standard representation of a mutation spectrum is a nucleotide sequence of a target gene with all changes detected put above this sequence. The base substitution mutation spectrum in Figure 1(A) includes three principal elements: (1) the target sequence (Figure 1A; lower line of continuous DNA sequence); (2) the mutations in the target sequence (Figure 1A); and (3) a representation of all positions in the target sequence which can be detected using phenotypic selection (shown by lower case letters in Figure 1A). Mutations in DNA/RNA molecules are classified as point mutations, deletions/insertions, duplications, inversions and chromosomal rearrangements. Point mutations are subclassified as base pair substitutions, including transitions (purine (R) mutates to R or pyrimidine (Y) mutates to Y) and transversions (R mutates to Y or Y mutates to R), and +1 and −1 frameshifts (insertions and deletions of a single base pair). Complex mutations include combinations of several point mutations and are relatively rare.

Mutability varies significantly along
nucleotide sequences: mutations, whether induced or spontaneous, occur at higher frequencies at certain positions of a nucleotide sequence (mutation ‘hotspots’). Mutation hotspots often reflect a specific mechanism for generating mutations at a particular site and/or unusual properties of a phenotypic selection protocol. Thus, study of mutation hotspots can help reveal mutagenic mechanisms, or can reveal information about the functional domains of a target protein. Some mutation hotspots are thought to depend on the nucleotide sequence and the mechanism of mutagenesis per se; these hotspots are called intrinsic mutation hotspots, however. In contrast, some hotspots may be due to expression and selection of a protein (RNA) molecule encoded by the target sequence, for example hotspots in human p53 might reflect both intrinsic mutability and selection for tumorgenesis. This paper primarily discusses methods that are useful for analysis of intrinsic mutation hotspots.

**Figure 1:** The sequence of the lacI gene with spontaneous A:T → C:G mutations revealed in the mutT strain of E. coli, lower case letters denote the detectable positions, numbers above the sequence stand for the number of mutations at the position, detectable positions are asterisked, detectable sites are underlined (positions –2 to +2 are shown) (A); a distribution of observed mutation frequencies (B)
NUCLEOTIDE CONTEXT OF MUTATION HOTSPOTS

The examination of mutation hotspots provides evidence that they arise due to some structural features of hotspot subsequences (the local DNA sequence context of hotspots). There are several general DNA context features that can influence mutagenesis: homonucleotide runs, sites of potential Z-DNA formation, direct and inverted repeats, microsatellites, etc.6,10–14

Mutable motifs

In many cases, mutation hotspots emerge due to the influence of neighbouring nucleotides.6,10,11,13,14 Examples of mutable DNA contexts are shown in Table 1. Sequence context effects can act over a significant distance: in one example, a single base pair change altered the mutation rate 12 bases away (8-fold effect on 2-aminopurine induced mutagenesis).26 It was suggested that sequence context effects act as far as 80 bases away from a site of mutations.27

Local DNA sequence environment has been shown to be an important determinant of rates of base substitutions in human germinal cells. CpG is the usual context of mutation hotspots in human genes (Table 1), in which C•G → T•A mutations are thought to be the result of deamination of methylated cytosine.13,15 A more complex pattern of hotspots was found in the human dystrophin gene, however: the frequency of CpG mutations was found to be lower than reported for other human genes, while the motif of TGRRGA (sometimes referred as DNA polymerase α reaction termination site) was found to be associated with >50 per cent of single-base mutations.28 It was found that dipyrimidines that contain 5-methylcytosine are preferential targets for sunlight-induced (but not UVC-induced) mutagenesis in the methylated lacI transgene in cultured mammalian cells, which might be relevant to the observation of the large proportion of mutations in these sites among p53 mutations found in skin tumours in vivo.29

Observations of this kind provide clues for understanding of molecular mechanisms of mutations and require further experimental and computational analysis.14,30

Site-specific illegitimate recombination is associated with specific motifs recognised by specialised enzymes. A site-specific joining of immunoglobulin V-J and V-D-J segments is mediated by CACAGTG and ACAAAAACC sequences with a short spacer between them.31 Interestingly, RAG1 and RAG2 proteins interacting with these sites might

Table 1: Examples of mutable motifs

<table>
<thead>
<tr>
<th>Test system/mutagen</th>
<th>Mutable motif</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sn1-type alkylating agents, the lacI gene</td>
<td>RG</td>
<td>G is more mutable than AG11</td>
</tr>
<tr>
<td>Spontaneous G•C → A•T mutations in mammalian genomes</td>
<td>CG</td>
<td>May result from the spontaneous deamination of 5-methylcytosine15</td>
</tr>
<tr>
<td>Somatic mutations in immunoglobulin V genes</td>
<td>RGYW</td>
<td>AGYW is more mutable than GGYW16</td>
</tr>
<tr>
<td>Hotspots of errors produced by DNA polymerase I η</td>
<td>WA</td>
<td>TA is more mutable than AA17,18</td>
</tr>
<tr>
<td>B-oxoG induced hotspots in vitro and in vivo</td>
<td>RG</td>
<td>This motif was found to be mutable in some human genes20</td>
</tr>
<tr>
<td>UV-induced mutations in the phage lambda cI gene</td>
<td>YT</td>
<td>In vitro experiments19</td>
</tr>
<tr>
<td>Pyrimidine (6–4) pyrimidone photoproducts</td>
<td>YTTCA</td>
<td>In vitro DNA damages induced by UV21</td>
</tr>
<tr>
<td>Cyclobutane dimers photoproducts</td>
<td>YTT</td>
<td>In vitro DNA damages induced by UV21</td>
</tr>
<tr>
<td>Single-base deletions</td>
<td>YTTG</td>
<td>Spontaneous mutations23</td>
</tr>
<tr>
<td>Context of complex mutations in human disease genes</td>
<td>GTAAGT</td>
<td>In vitro experiments22</td>
</tr>
<tr>
<td>Target signal of retroposable elements</td>
<td>TTTAAA</td>
<td>LINEs and SINEs24</td>
</tr>
<tr>
<td>Signal of recombination in the B.subtilis mal gene</td>
<td>CATCGCTTRRT</td>
<td>Similar to gyrase binding sites25</td>
</tr>
</tbody>
</table>

Hotspot positions are underlined. R = A or G; Y = T or C; S = G or C; W = A or T; K = G or T; M = A or C; B = T, C, or G; H = A, T or C; V = A, C or G; D = A, T or G.
be evolutionary descendants of proteins encoded by a mobile element, and the whole joining recombination system may be derived from a selfish repetitive element.\textsuperscript{32} Incomplete collections of motifs which mediate site-specific recombination can be found in Badge et al.\textsuperscript{33} and in a compilation of recombination signals and mutable motifs (Table 2).

### Repetitive sequences

#### Homonucleotide runs and microsatellites

In 1966 Streisinger et al.\textsuperscript{34} proposed that short deletions and insertions within runs of a same base (homonucleotide runs, homopolymeric tracts) arise by misalignment of DNA strands during replication. This type of misalignment can lead to length heterogeneity within homopolymeric tracts and more complex tandemly repeated structures called microsatellites (for example, runs of a di- and trinucleotides).\textsuperscript{10,12,34–36} It was demonstrated that one base pair insertions and deletions are frequent in homonucleotide runs: the longer the run is, the higher is the mutation rate, which can be precisely explained by misalignment.\textsuperscript{37} Variation of this mechanism, called dislocation mutagenesis, involves transient misalignment in homonucleotide runs (Figure 2) and may be responsible for some substitution hotspots. This mechanism was first found in the spectrum errors produced by DNA polymerase-\(\beta\) during \textit{in vitro} DNA synthesis.\textsuperscript{38} Dislocation mutagenesis causing substitution hotspots was suggested to play an important role \textit{in vivo} at the control region of human mitochondrial DNA.\textsuperscript{39}

#### Direct repeats

Short direct repeats have been long known to mediate deletions and duplications.\textsuperscript{40,41} The mechanism could involve illegitimate recombination within short regions of similarity (<20 bases) or DNA polymerase slippage between repeated sequences. Recombination frequency increases with the length and GC content of repeated sequences and decreases with the length of the spacer between repeated sequences.\textsuperscript{42} When a DNA sequence flanked by short direct repeats could form a palindrome, recombination between them occurs at a higher frequency.\textsuperscript{43} In addition it was suggested that the repair of heteroduplexes formed by direct repeats may result in base substitutions and frameshifts. This mechanism was suggested for some classes of spontaneous mutations in bacterial and eukaryotic genes.\textsuperscript{21,44}

#### Inverted repeats

Long inverted repeats (40–150 bases) are particularly unstable in bacterial DNA.\textsuperscript{45} The proposed mechanism of deletions simulated by inverted repeats includes formation of hairpin structures in single

### Table 2: List of available databases and integrated software

<table>
<thead>
<tr>
<th>Databases</th>
<th>Web Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Genome Mutation Database</td>
<td><a href="http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html">http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html</a></td>
</tr>
<tr>
<td>List of databases</td>
<td><a href="http://arcel.ucs.unimelb.edu.au:80/~cotton/mdt.htm">http://arcel.ucs.unimelb.edu.au:80/~cotton/mdt.htm</a></td>
</tr>
<tr>
<td>List of databases</td>
<td><a href="http://info.med.yale.edu/mutbase/">http://info.med.yale.edu/mutbase/</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Integrated software</th>
<th>Web Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrated software for the analysis of mutations</td>
<td><a href="http://sunsite.unc.edu/dnam/mainpage.html">http://sunsite.unc.edu/dnam/mainpage.html</a></td>
</tr>
<tr>
<td>UMD (Universal mutation database)</td>
<td><a href="http://www.umd.necker.fr/">http://www.umd.necker.fr/</a></td>
</tr>
<tr>
<td>MutPlus software</td>
<td><a href="http://www.cs.brown.edu/people/gq/mutplus_home.html">http://www.cs.brown.edu/people/gq/mutplus_home.html</a></td>
</tr>
</tbody>
</table>
strand DNA. Inverted repeats elevate intra- and interchromosomal recombination (reviewed by Gordenin and Resnick). It was proposed that correction of a quasipalindrome to a perfect inverted repeat occurred by either inter- or intramolecular strand switch. Many mutations of this type had been observed in bacteria, yeast and human cells. This mechanism was suggested, in addition to direct repeat mechanism, to explain some classes of somatic mutations in immunoglobulin genes. An example of hypothetical correction of the imperfect hairpin formed by the quasipalindrome is shown in Figure 3.

Although a significant correlation between substitutions and direct and inverted repeats was detected in immunoglobulin genes, any impact of this mechanism and relevance to base substitution hotspots is not clear.

Z-DNA
Several chemicals were found to react preferably with Z-DNA formed by regions of alternating purine–pyrimidine sequences. For example, hydroxylamine reacts very strongly with cytosines at junctions between B- and Z-DNA and in Z–Z-DNA junctions. It was also suggested that repair is less effective in Z-DNA regions.

Local mononucleotide composition
There are indications that GC-rich segments of DNA might be subject to more errors than AT-rich segments during replication. A higher local AT content might increase the frequency of ultraviolet (UV) induced mutations in bacteria.

DNA conformation and oligonucleotide content
Curved DNA is a sequence-directed curvature of the helix axis of double-stranded DNA, which is determined by the oligonucleotide content of a target sequence. It was suggested that curved DNA has an important influence on various genomic rearrangements including deletions mediated by short direct repeats. For bending and curvature calculation, the BEND_TRI program can be used; it was shown that this program produces a good approximation of bending and curvature values. Another example of possible involvement of oligonucleotide content in recombination was suggested by Konopka; some trinucleotides are preferentially cleaved by topoisomerase I and thus may facilitate illegitimate recombination nearby.

Global factors
Many factors influence mutation frequency in a particular nucleotide sequence. In most cases, however, only local nucleotide sequence context was
studied. It is likely that other higher-level features of gene or chromatin structure also have significant influence on mutation frequency of a mutable motif at a specific site. For example, AGTA is more mutable in complementarity-determined regions than in framework regions of immunoglobulin genes. A factor could be the rate of DNA repair. DNA repair rates vary for transcribed and non-transcribed strands of the same gene and for more and less highly-expressed genes. Inherent asymmetry between the two DNA strands at the replication fork could also influence mutation frequency and specificity. Other potential factors include asymmetric base composition or higher-order chromatin structure (reviewed by Boulikas).

### METHODS FOR MUTATION SPECTRA ANALYSIS

#### Comparison of mutation spectra

Comparison of mutation spectra is the most common approach when studying two or more spectra induced differently (e.g., by different mutagens) in the same gene. Piezorsch and Bailer described statistical methods to compare two spectra based on an exact or pseudo-probability test (a Monte Carlo modification of the exact test). HG-PUBL and COMP12 programs for such comparisons were developed (Table 3).

In a case of a multiple spectrum, two types of analysis can be done: a test of overall homogeneity, and pairwise comparisons between mutation spectra. There are a number of programs available:

### Table 3: List of available methods and programs

<table>
<thead>
<tr>
<th>Analysed features</th>
<th>Program</th>
<th>Method description</th>
<th>URL/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation hotspot prediction</td>
<td>–</td>
<td>Several approaches including heuristic and binomial test-based reconstructions of consensus sequences</td>
<td>Methods that rely on arbitrary discrimination between informative and non-informative positions may lead to unreliable results</td>
</tr>
<tr>
<td>Context of mutation hotspots</td>
<td>–</td>
<td>The exact test can be used to test the null hypothesis that mutations are equally probable in mutable motifs and all other positions in the target sequence</td>
<td>The input numbers are MM, MA-MM, NP, NA-NP, where MM is the number of mutations in mutable motifs, NP is the number of such motifs, MA is the number of mutations and NA is the number of positions in the target sequence</td>
</tr>
<tr>
<td>Correlation between nucleotide sequence features and mutation spectra</td>
<td>–</td>
<td>Correlation between the oligonucleotide content and mutation frequencies</td>
<td>This approach requires a large number of detectable sites</td>
</tr>
<tr>
<td>Oligonucleotide composition</td>
<td>–</td>
<td>Substitutions frequency, mutations and hotspots clustering, periodicity</td>
<td>This kind of information is important for the understanding of molecular mechanisms of mutagenesis</td>
</tr>
<tr>
<td>Context-free mutations features analysis</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple spectra</td>
<td>HG-PUBL</td>
<td>Test of heterogeneity based on $2 \times N$ contingency table</td>
<td>ftp://sunsite.unc.edu/pub/academic/biology/dna-mutations/hyperg</td>
</tr>
<tr>
<td>Pairwise correlation analysis</td>
<td>CORR12</td>
<td>Correlation test based on the $2 \times N$ contingency table</td>
<td>ftp://ftp.bionet.nsc.ru/pub/biology/dbms/CORR12.ZIP</td>
</tr>
<tr>
<td>Multiple spectra comparison</td>
<td>COLLAPSE</td>
<td>Test of heterogeneity based on $R \times M$ contingency table</td>
<td><a href="http://anthropologie.unige.ch/arlequin.htm">http://anthropologie.unige.ch/arlequin.htm</a></td>
</tr>
<tr>
<td>Multiple spectra comparison</td>
<td>ARLEQUIN</td>
<td>Test of heterogeneity based on $R \times M$ contingency table</td>
<td>ftp://ftp.cefe.cnrs-mop.fr/pub/pk/msdos/genePOP</td>
</tr>
<tr>
<td>Multiple spectra comparison</td>
<td>GENEPOP</td>
<td>Test of heterogeneity based on $R \times M$ contingency table</td>
<td></td>
</tr>
</tbody>
</table>
ARLEQUIN and GENEPOP (Table 3) provide the exact test of the overall homogeneity in $n \times T$ matrices and the exact tests for the pairwise comparison between spectra as well. The COLLAPSE program\(^65\) (Table 3) allows collapsing (grouping, combining) of rows and columns of $n \times T$ matrices. It calculates expected frequencies, squared standardised residuals as well as Zehterman and Cressie-Read statistics, which were recommended for the analysis of mutation spectra when data are sparse.\(^64\) A new analytical strategy for mutation spectra comparisons was suggested recently, this approach is also useful for hotspot prediction and analysis.\(^66\) This strategy is based on comparison of mutation frequencies in each site of two studied spectra.\(^67\) The problem of pairwise and multiple spectra comparisons was discussed by Piegorsch and Baier,\(^64\) Khromov-Borisov et al.,\(^65\) Rogozin et al.\(^6\) and Lewis and Parry.\(^67\)

The Kendall’s tau correlation coefficient can be used as a complementary approach.\(^6,68\) If two mutation spectra are not significantly different, they may be assumed to be significantly similar only if a significant correlation is found between these two spectra, as shown by analysis with the CORR12 program (Table 3).\(^6,68\) The simultaneous application of exact and correlation tests is complementary, underlining different factors affecting the structures of mutation spectra. For example, Babenko and Rogozin\(^68\) identified a spectrum for which both tests produced statistically significant results. Based on this observation, two context factors affecting the spectrum in different ways have been determined.

Multiple linear regression analysis was successfully applied for comparison of three mutation spectra in the same mouse immunoglobulin target sequence.\(^69\)

## Tests of homogeneity

## Hotspot prediction

## Correlation analysis

**Revealing hotspots**

A mutation spectrum can be transformed into a distribution of observed mutation frequencies along a gene sequence (Figure 1B) which should be regarded as a sample of multinomial distribution (discussed by Piegorsch and Baier\(^64\)). Analysis of such distribution can be performed using a simulation, expectation, maximisation (SEM) classification approach.\(^70\) A general principle of mutation hotspot prediction in this approach is based on a threshold (Sh) value for the number of mutations in a mutable site. All sites with the number of mutations greater than or equal to Sh are defined as hotspots (Figure 1B). The threshold and resulting hotspot sites are defined for each mutation spectrum separately based on results of classification analysis.\(^70\) The CLUSTERM program (Table 3) is used for this purpose. This program decomposes a mutation spectrum into several homogeneous classes of sites. Each class is approximated by a binomial (or Poisson) distribution. Variations in mutation frequencies among sites of the same class are due to random reasons, since mutation probability is the same for all sites in one class. Differences between mutation frequencies among sites from different classes are statistically significant, however. A class (or classes) with the highest mutation frequency is called a hotspot class(es). Problems of hotspot prediction were discussed by Rogozin et al.\(^6\) and Fijal et al.\(^71\).

## Analysis of neighbouring bases

Nucleotide sequence context influences mutation probability.\(^2,5,11,13,15,16\) Several methods have been developed to analyse this phenomenon. A commonly used approach for analysis of neighbouring bases is to calculate the number of times a given base occurs next to a mutated base, immediately in the 5’ or 3’ direction (positions –1 and +1). A significant deviation from the expected numbers can be estimated by using various statistical tests.\(^5,19,72,73\) Krawczak et al.\(^13\) analysed nearest-neighbour effects with correction for codon usage and for different probability of detecting different amino acid substitutions in a clinical study, which may be useful in studying human disease susceptibility genes. Maximum
likelihood estimates of nearest-neighbour effects were developed by Zavolan and Kepler.5

A set of aligned hotspot sites (Figure 4) can be analysed separately to derive a consensus sequence74 using one of several available approaches.75 Methods that rely on arbitrary discrimination between informative and non-informative positions may lead to controversial and/or unreliable results. Simple consensus sequences can be misleading especially when the data set is small; however, they can be reconstructed using any mutation spectrum and any subset of positions. The binomial test can also be used to study consensus sequences at or near mutation hotspots.16,39 In this method, a number (N_{ij}) of a nucleotide I is calculated in each position J in a set of M aligned mutation hotspot sequences (Table 3). The probability P(N_{ij}, M, F) of finding N_{ij} or more nucleotides I in a position J is calculated taking a frequency F_i of a nucleotide I in a target sequence as an expected number of the nucleotide I in the position J. A nucleotide with the lowest probability P(N_{ij}, M, F) among all possible nucleotides in a position J is accepted as a consensus nucleotide for this position if P(N_{ij}, M, F) for this nucleotide is below a threshold value P^*. It is important to note that the estimate of P(N_{ij}, M, F) cannot be used for rejecting or accepting a statistical hypothesis owing to a multiplicity of binomial tests; moreover these tests are strongly interdependent for each position. To estimate a significance level for P(N_{ij}, M, F), Malyarchuk et al.39 developed a resampling procedure. In this procedure, M sites were randomly chosen from a target sequence. Thus, each ‘random’ sample was a mixture of hotspots and non-hotspots. The statistical analysis described above was repeated for each sample, and the minimal value P_{mr}(N_{ij}, M, F) was calculated for all positions. This procedure was repeated 10,000 times to calculate a significance level P^* that separates the right critical region of the distribution P_{mr}(N_{ij}, M, F) at 5 per cent level of significance, P^* may be significantly less than 0.05 (for example, P = 0.005 for the HVS1 spectrum).39

**Correlation between nucleotide sequence features and mutation spectra**

In cases when a context factor influencing the frequency of mutations is known a priori, a correlation between distributions of this factor (for example, a mutable motif) and mutations along a target sequence may be measured. This approach is discussed here with regard to analysis of multiple somatic mutations in the 5' flanking region of BCL-6 (Figure 5), a proto-oncogene encoding for a transcriptional repressor from B-cell lymphomas.76

![Figure 4](https://example.com/f4.png)

**Figure 4:** An alignment of putative hotspot and non-hotspot sites for the mutation spectrum described in Figure 1. The detectable position is displayed as a purine, using the appropriate DNA strand.
motif (the hotspot base is underlined) is a signature of somatic hypermutation (Table 1), and may play a role in somatic mutations observed in BCL-6. The Fisher exact test for analyses of 2x2 tables can be used to test the null hypothesis that mutations are equally probable in mutable motifs and all other positions of the target sequence. When one of the numbers is large (>300), the χ² test with one degree of freedom can be applied instead of the exact test. In the case of mutations in BCL-6 (Fig. 5), the correlation is statistically significant (P(χ²) < 0.05).

Statistical significance can be also estimated using a modified Monte Carlo procedure. This approach takes into account frequency of mutations in A, T, G and C bases, the presence of several mutations in a site and context properties of the target sequence. Weight W_j of site j was defined as the number of mutations in a mutable motif; however, more complex definitions were also used (discussed below). A distribution of statistical weights W_random was calculated for 10,000 computationally generated groups of random sites. Each group contained the observed number of mutations distributed similarly in all sites. The distribution in W_random was used to calculate probability P(W ≤ W_random). This probability is equal to the number of groups of random mutations in which W_random is the same or higher than W. Small probability values (P(W ≤ W_random) ≤ 0.05) indicate a significant correlation between mutable motif and mutation frequency. Modified versions of this approach were used to analyse a dislocation model in human mitochondrial DNA, mutability of direct and inverted repeats in immunoglobulin genes and gene conversion in immunoglobulin genes. A similar approach was used to analyse illegitimate recombination events. Theoretically, this method can be applied to any data where a reliable correlation measure (the weight W_j) between mutation/recombination events and the nucleotide sequence context can be derived.

Other approaches

Various multiple regression models can be used for simultaneous analysis of how several neighbouring positions influence mutation frequency. Nucleotide sequence context of mutation hotspots can also be analysed by focusing on oligonucleotides. For example, Smith et al. analysed the relative frequency of somatic mutations in 16 dinucleotide and 64 trinucleotide motifs. This approach revealed that the mutation frequencies in different di- or trinucleotides were significantly different. A meta-analysis of complex mutations causing human genetic diseases revealed a hotspot motif GTAAGT over-represented in the vicinity of these mutations. A local oligonucleotide composition is also the focus of studies on frameshift mutations in microsatellites. These mutation hotspots are affected by length and base composition of the microsatellite repeat. In general, both approaches require a large number of detectable sites (eg hundreds of sites for trinucleotide motifs) and mutations in a target sequence.

Several aspects of a mutation spectrum, including frequency of substitutions, clustering of mutations and hotspots, and periodicity of mutation can be considered as ‘context-free’ characteristics of the spectrum. These approaches were discussed by Rogozin and Pavlov. In general, various computational approaches can be used to analyse aligned
sequences of mutation hotspots. Many techniques have been developed for analysis of functional signals including information content, weight matrices, perceptron, k-tuple frequencies, discriminant analysis, hidden Markov models, linguistic approaches and neural network models.84–86 These methods are well established and have been tested on different types of data, but all of these methods require large data sets.

Mutation databases and integrated software

Databases

There are many mutation databases freely available via the internet.87,88 It is relatively easy to find various databases of mutation spectra on the internet, starting from the web servers listed in Table 2. Various information about mutation databases are regularly published in the first January issue of the journal Nucleic Acids Research; another valuable source of such information is the journal Human Mutation. Database formats vary significantly among databases, and a lot of work is required to provide and perform their analysis. Designing standard formats of mutation databases can help a lot in development and application of computational tools. An example of such an initiative is the Human Gene Mutation Database (HGMD). HGMD constitutes a comprehensive core collection of data on germ-line mutations in nuclear genes associated with human inherited diseases (Table 2). Each mutation is entered into HGMD only once in order to avoid confusion between recurrent and identical-by-descent lesions (discussed in the ‘Unsolved problems, perspectives and conclusions’ section).

UV-induced mutations

Databases and software applications for the analysis of DNA mutations at the human p53 gene, the human hprt gene and both the rodent transgenic lacI and lacZ loci have been developed.73 The databases are stand-alone dBASE files, and the software for analysis of these databases runs on PCs (Table 2). A piece of genetic software called the Universal Mutation Database (UMD, Table 2) allows development of locus-specific databases. The software includes an optimised structure to assist data entry and allows the input of various clinical data.89 A MutPlus program (Table 2) is another attempt to integrate different analytical tools and mutation data sets.

EXAMPLES OF MUTATION SPECTRA ANALYSIS

Mutation spectra induced by ultraviolet light

Ultraviolet (UV) light generates a complex spectrum of mutations including base substitutions, frameshifts, complex mutations, large deletions and duplications.10,90–94 Mutation spectra of UV light were studied in numerous experimental systems. These studies were conducted most frequently with short-wave UV in the range 180–280 nm (UVC), however, and may have somewhat limited relevance to the effects of sunlight itself, which is a mixture of wavelengths.30 Principal damage to DNA leading to UV-induced mutations are pyrimidine–pyrimidine cyclobutane dimers and pyrimidine (6–4) pyrimidone photoproducts.95,96 These lesions block replicative DNA polymerases and should be either repaired or bypassed by specialised mechanisms to escape cell death, mutagenesis and cancer.

Examining the context specificity of UV mutagenesis helps to determine which photoproducts are important premutational lesions, since neighbouring DNA sequence and the resulting local DNA conformation play a critical role in formation of these photoproducts.10,30 In bacteria, TC, TCC, CC and CCC are hotspots of UV-induced mutations; however, both cyclobutane dimers and pyrimidine (6–4) pyrimidone photoproducts are frequent in these mutable motifs.10,30 Early analysis of UVC-induced mutations did not reveal transitions in CT dinucleotides, suggesting that pyrimidine (6–4) pyrimidone photoproducts rather than cyclobutane dimers are primarily responsible for targeting mutations in...
bacteria, since pyrimidine (6–4) pyrimidone photoproducts are rare at CT sequences, whereas cyclobutane dimers are quite abundant.\textsuperscript{10,95,96}

Later it was shown that pyrimidine (6–4) pyrimidone photoproducts are more mutagenic than cyclobutane dimers in yeast and mammalian cells.\textsuperscript{97} It was not clear, however, why TT dinucleotides appear as frequent sites of transitions in \textit{E. coli}, since pyrimidine (6–4) pyrimidone photoproduct formation is sharply reduced at TT dinucleotides relative to TC and CC sequences.\textsuperscript{10} Since then it has been discovered that the same UV photolesions have different mutagenic potential in different organisms, for example bacteria and yeast.\textsuperscript{97} The complexity of UV-induced spectra may be illustrated using results of a correlation analysis between photoproduct hotspot consensus sequences and UV-induced mutation spectra (Table 4).\textsuperscript{21}

Apparent discrepancies between different experiments (Table 4) are not surprising taking into account that various repair/replication enzymes involved in UV mutagenesis and final UV-induced mutations in different species\textsuperscript{90,97–99} and the precise mechanisms of interspecies differences were not fully understood until recently, when damage bypass DNA polymerases were discovered. It was found that the superfamily Y of DNA polymerases with relaxed active centre exists to deal with UV- and chemically-induced DNA adducts. Translesion synthesis is a two-step process of insertion of bases opposite damaged site and further extension, that may require several DNA polymerases — sometimes belonging to different superfamilies.\textsuperscript{98–100} Mutagenesis depends on a lesion bypass event, including incorporation opposite the damage and extension from the thus formed DNA 3’ end. The family Y DNA polymerases are much less accurate than replicative DNA polymerases;\textsuperscript{11} however, even low fidelity of incorporation would substantially reduce the probability of mutation generation when replicating opposite the damage. The difference in mutagenic potential of photoproducts in different experiments (Table 4) may be easily explained by the involvement of polymerases with different properties (DNA polymerase V and DNA polymerase \eta, respectively) in this initial bypass event.\textsuperscript{98–100}

### Mutations induced by alkylating agents

SN1 alkylating agents preferentially generate G\textbullet C \rightarrow A\textbullet T transitions at RG sites, while SN2 agents do not.\textsuperscript{11} A comparative analysis of mutation spectra induced by SN2 alkylating agents using regression analysis, revealed various mutable motifs specific for SN2 alkylating agents.\textsuperscript{101} For the mutation spectra induced by N-methyl-N-nitrosourea (MNU) in the \textit{lacI} and \textit{gpt} genes\textsuperscript{102,103} different mutable motifs were revealed:

### Table 4: A non-random correlation ($P(W \!<\! W\text{random}) < 0.05$) between hotspots of UV-induced substitutions and consensus sequences of two major UV-induced photoproducts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Comments</th>
<th>cyclobutane dimers</th>
<th>Py(6–4)Pyo photoproduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacI</td>
<td>E. coli</td>
<td>Wild type\textsuperscript{31}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>lacI</td>
<td>E. coli</td>
<td>UVB-strain\textsuperscript{31}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SUP4-o</td>
<td>Yeast</td>
<td>UVB-induced\textsuperscript{31}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SUP4-o</td>
<td>Yeast</td>
<td>UVC-induced\textsuperscript{31}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>supF</td>
<td>Hamster</td>
<td>Cell line\textsuperscript{92}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>gpt</td>
<td>Human</td>
<td>XPV cell line\textsuperscript{94}</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Consensus sequences for pyrimidine (6–4) pyrimidone [Py(6–4)Pyo] and cyclobutane dimers photoproducts are YTCA and YTT, respectively.\textsuperscript{21} Consensus sequences were derived using regression analysis of pyrimidine (6–4) pyrimidone\textsuperscript{93} and cyclobutane dimers photoproduct spectra.\textsuperscript{95,96} The significance of correlations between the distribution of mutable motifs and mutations along a target sequence was estimated using a Monte Carlo approach.\textsuperscript{16}
three mutable motifs GGC, AGNG and GGKM for the lacI spectrum and one mutable motif GG for the gpt spectrum.\textsuperscript{101} The correlation between the former set of motifs and distribution of MNU-induced mutations in the gpt gene, however, was statistically insignificant\textsuperscript{101} — a Monte Carlo test\textsuperscript{16} (described above) was used. Sets of mutable motifs thus revealed were ambiguous and cannot be recommended as a signature of MNU-induced mutagenesis. In general, the analysis suffered from the small number of detectable positions (22 G•C \rightarrow A•T detectable sites) and the small number of mutations.

Somatic mutations in immunoglobulin genes

The wide variety of immunoglobulins in vertebrates results from the combinatorial joining of different variable (V), diversity (D) and joining (J) gene segments to create the primary antigen–receptor repertoire, followed by somatic hypermutation of variable (V) regions. These mutations are introduced at a rate estimated to be about six orders of magnitude greater than the normal rate of spontaneous mutations in the genome.\textsuperscript{31} A number of different models of somatic hypermutation have been proposed.\textsuperscript{104} Most models postulate involvement of mutator polymerases to account for the high frequency of mutagenesis in V regions. One important feature of somatic hypermutation in V regions is the non-random distribution of mutations.

Somatic mutation hotspots in V regions occur primarily within two DNA sequence motifs. RGYW hotspots\textsuperscript{16,17} are found in both strands and WA hotspots preferentially are found in only one strand.\textsuperscript{16–18,82} A candidate for a principal RGYW mutator is activation-induced cytidine deaminase (AID), which converts cytosine in DNA into uracil.\textsuperscript{105–107} Analysis of mutation spectra of errors made by various DNA polymerases during \textit{in vitro} DNA synthesis provided clues on which polymerase could operate during somatic hypermutation. A correlation between the WA motif and the error specificity of human DNA polymerase \(\eta\)\textsuperscript{17,108} and lack of A–T mutations in XP-V patients deficient in DNA polymerase \(\eta\)\textsuperscript{109} suggested that this polymerase may contribute to the WA hotspots. Additional analysis of this correlation using the same mouse immunoglobulin target sequence for \textit{in vivo} and \textit{in vitro} spectrum generation, combined with studies of mutable motifs and frequencies of substitutions, greatly improved the power of comparisons, allowing use of different statistical methods.\textsuperscript{69} It was found that two DNA polymerase \(\eta\) error spectra, determined while this polymerase synthesises the transcribed or non-transcribed strands, correlate in a mosaic fashion with a spectrum of somatic mutations \textit{in vivo}. This suggested that this polymerase contributes to somatic hypermutation in mice during short patch DNA synthesis on alternating DNA strands.\textsuperscript{69}

In general, analysis of mutable motifs became a standard procedure in studies of somatic hypermutation of immunoglobulin genes.

UNSOLVED PROBLEMS, PERSPECTIVES AND CONCLUSIONS

Many factors influence mutation frequency in a particular nucleotide sequence. In most cases, however, analytical methods only attempt to characterise factors related to local nucleotide sequence context. It is likely that other higher-level features of gene or chromatin structure also have significant influence on mutation frequency of a mutable motif at a specific site. Analyses of these features require large data sets obtained under different experimental conditions; small sample size is a major problem in analysis of mutation spectra. Even if the number of mutations is large, the number of mutation hotspots is likely to be small (eg Figure 4). A few approaches can be robust with small data sets (ie hotspot prediction, comparing mutation spectra, correlation between nucleotide sequence features and
Mutation assays

Hotspot context and molecular mechanisms of mutagenesis

Mutation spectra). Other methods may not be reliable when applied to small data sets.

It is very important to identify the detectable positions in a target sequence before analysing its mutation spectrum. This can be achieved by analysing large numbers of mutants or by systematic site-directed mutagenesis of all amino acids in the target gene. Non-phenotypic assays are rarely used since they are usually restricted to a few specific positions (e.g. using sites of restriction enzymes to detect mutations) or they require a very high frequency of mutation. In some tests a list of detectable positions is known a priori. This is the case for mutation spectra in amber (resulting in UAG stop-codons) and ochre (resulting in UAA stop-codons) nonsense sites. All such nonsense sites can be easily found in protein coding sequences. Unfortunately, for many other spectra the list of detectable positions is the most problematic component of a mutation spectrum. Population polymorphism becomes another important issue when the mutated sequences from one individual are compared with non-mutated sequences from another individual (such an approach is used sometimes for studies of somatic mutations in immunoglobulin genes). In such cases, each polymorphic position will be counted as a mutation, which biases mutation spectra. It is possible to misassign a functional mutation at a specific site even if a data set is carefully collected. This can occur in cases of multiple mutations when an unidentified distal mutation alters gene function, and the mutation in the assigned site does not have a functional effect. Thus, only well-characterised detectable sites, in which several independent mutations have been observed, should be used when a mutation spectrum is analysed.

Another problem of mutation spectra analysis, which is restricted only to the case of locus specific mutation databases, is the problem of repetitions (i.e. mutations that are identical by descent). Such repetitions should be counted as a single mutation. It is not always possible to detect them, however. In general, mutation spectra revealed by clinical studies do not represent random samples of all arising mutations. Rather, the vast majority of them are middling to highly deleterious mutations, whereas advantageous, neutral or slightly deleterious mutations are hardly or not at all represented. This is because for a mutation to be represented in a database, it was to come to clinical attention, otherwise it remains undetectable. The same complication applies to phenotypic selection systems. The non-random sampling of mutations may systematically bias context properties of detected mutation hotspots.

It should be emphasised that the context of hotspots may be very helpful in deep understanding of underlying molecular mechanisms of mutagenesis, however, the determinants of mutation frequency and specificity are complex and there are many analytical methods for their study. The most reliable results can be obtained if several methods are combined or used sequentially and if many different sources of information are considered. Simple, and thus robust, approaches should be used with small mutation samples, while combinations of simple and complex approaches can be used for large samples. Complex approaches are needed because mutation spectra reflect the simultaneous influence of multiple diverse local and global factors. It is a challenging task to analyse mutation spectra and, in some cases, the effort will be primarily descriptive in nature. In several well-documented studies, however, the analysis of mutation spectra has contributed substantially to understanding molecular mechanisms of mutagenesis. As analytical methods continue to be developed, more theoretical and experimental studies will contribute insights into the complex process of mutagenesis.
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