Topic Introduction

Methods for In Vitro Mutagenesis

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Several different methodologies for mutagenesis have been developed to introduce mutations at predetermined sites or regions within mammalian genes. These methods of in vitro mutagenesis have had a transforming effect on the understanding of functions of protein, transcription regulatory elements, and noncoding RNAs and are now integral to molecular biology investigations. In this introduction, we summarize the most commonly used experimental approaches for mutagenesis and their major applications.

MUTAGENESIS APPROACHES

Prokaryotic and eukaryotic cells are under constant exposure to extrinsic and intrinsic stimuli that cause DNA damage, which can lead to mutations if unrepaired (Lombard et al. 2005; Boesch et al. 2011). To survive under these constant genotoxic conditions and still faithfully replicate the genome, prokaryotic and eukaryotic cells have developed specific dedicated DNA repair machineries that repair the DNA damage and slow the accumulation of genetic mutations (Lombard et al. 2005; Boesch et al. 2011). However, not all mutations have an effect on the biological function of a protein. Once a mutation is identified in an organism, functional characterization is still required to understand the effect of the mutation on the function of a specific protein or a noncoding RNA.

Several different methodologies for mutagenesis have been developed to introduce mutations at predetermined sites or regions within mammalian genes (see Box 1). Below, we describe the three major types of mutagenesis approaches and provide a list of detailed protocols for commonly used methods (see Table 1). These nine different methods for performing mutagenesis can be used for introduction of mutations based on the requirements of the researcher. Mutagenesis terminology is defined in Box 2.

Oligonucleotide-Directed Mutagenesis

Oligonucleotide-directed mutagenesis is used to test the role of particular residues in the structure, catalytic activity, and ligand-binding capacity of a protein. In the absence of a three-dimensional (3D) structure, this type of protein engineering relies on informed guesses concerning the structure of the protein and the contribution of individual residues to protein stability and function. A major problem is distinguishing mutations that affect local structures from those that have profound and deleterious effects on the folding or stability of the entire protein. Consider a typical experiment in which several point mutations have been generated at various sites in a gene coding for an enzyme. When the activities of these mutants are assayed, some of them show a reduction in catalytic

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BOX 1. HISTORICAL BACKGROUND

The road to developing modern in vitro mutagenesis methods has been paved with Nobel Prizes as progress moved from the level of treating whole organisms to that of targeting specific sites in the DNA molecule. Hermann J. Muller (1890–1967), then at the University of Texas, electrified the scientific world in 1926 when he described evidence of gene mutations and chromosomal changes in *Drosophila* produced by X-irradiation. He announced his results at a scientific meeting in 1926 and then published them in the paper “Artificial Transmutation of the Gene” in *Science* (Muller 1927). The production of mutations by X-irradiation played a major role in the experiments on *Neurospora* published in 1941 by George Beadle and Edward Tatum in which they proposed their one gene–one protein hypothesis (Beadle and Tatum 1941).

H.J. Muller spent some time at Edinburgh University from 1938 to 1940, and while there he worked with Charlotte Auerbach (1899–1994) on substances that could cause mutations. With J.M. Robson in the early 1940s, Auerbach used mustard gas to induce mutations in *Drosophila* (Auerbach and Robson 1947; Stevens et al. 1950). In a later publication, Auerbach related that:

It seems to me that there may be many ways of affecting the chemical specificity of the gene or the physical integrity of the chromosomes: direct chemical reaction with proteins or nucleic acids; release of energy close to a chromosome; inactivation of enzymes concerned with chromosome metabolism; interference with gene reduplication by competitive analogs, and so on (Auerbach 1951).

The issues raised by Auerbach became approachable after the determination of the structure of DNA in 1953 by James D. Watson and Francis H.C. Crick—a discovery that changed the nature of mutagenesis studies. Mutagenesis techniques became very specific as a result of Michael Smith’s (1932–2000) studies of oligonucleotide synthesis and his 1975 sabbatical in Fred Sanger’s laboratory sequencing *Escherichia coli* phage ϕ X174. Smith realized that a mutagenic method was needed to target specific bases. His studies showed that small oligonucleotides could form stable duplexes at low temperatures, while work by Clyde A. Hutchison III and Marshall H. Edgell showed that “point mutations could be reverted by annealing mutant phage ϕ X174 with fragments from the complementary strand of wild-type DNA before transfection.” Using ϕ X174 DNA as a template, a 120-nt oligomer with a single-nucleotide mismatch as primer, and *E. coli* DNA polymerase, they produced a closed-circular double-stranded DNA with the oligonucleotide incorporated into one strand (Hutchison et al. 1978). At about the same time, the first specific restriction endonucleases were being discovered, making it possible to isolate specific DNA fragments (Smith and Wilcox 1970).

A final key element fell into place in 1983 when Kary Mullis invented the idea of the polymerase chain reaction (Saiki et al. 1985; Mullis et al. 1986). Polymerase chain reaction (PCR) soon became an integral part of in vitro site-directed mutagenesis techniques.

As for the Nobel Prize count, Muller’s (Physiology or Medicine) was awarded in 1946, and Smith’s in 1993 (Chemistry) was shared with Mullis. Beadle and Tatum were cited in 1958 (Physiology or Medicine), and Watson and Crick in 1962 (Physiology or Medicine).

function and others do not. In the absence of any other data, it is not possible to draw firm conclusions about the structure of the enzyme from this result. There is no way to know whether the substitution of one amino acid for another has affected only the function of the active site or whether it has had more global effects. The problem would remain even if the 3D structure of the wild-type enzyme were known because no algorithms have yet been devised that accurately predict the perturbations in protein structure caused by the substitution, addition, or deletion of amino acid residues. However, these difficulties can be alleviated by developing independent assays for the folding of the protein of interest. Such assays commonly include the ability of the protein to react with monoclonal or polyclonal antibodies that are specific for native or unfolded epitopes, the proper movement and post-translational modification of the protein within a cell, the retention of catalytic or ligand-binding functions, and the sensitivity or resistance of the mutant protein to digestion with proteases.

If reliable assays are available to confirm that the mutagenized protein is correctly folded, oligonucleotide-directed mutagenesis becomes an analytical technique with both exquisite specificity and extraordinary breadth. Mutations that could never be found in nature can now be placed precisely in the target gene, functions of proteins can be mapped to specific structural domains, undesirable activities of enzymes can be eliminated, and their desirable catalytic and physical

TABLE 1. Comparison of mutagenesis protocols

Protocol ^a	Template	Method used to enrich for mutant	Characteristics/advantages	Drawbacks
1	Double-stranded DNA	Selective PCR amplification of mutated DNA	A wide spectrum of mutations can be introduced.	Mutations cannot be targeted at specific positions.
2	Double-stranded DNA	Overlap extension	i. Used for the generation of a specific point mutation, insertion, or deletion within a particular DNA sequence of interest ii. Does not require the use of restriction enzymes i. Mutations can be placed precisely in the target gene. ii. 80% of the transformed colonies will contain plasmids with the desired mutation.	The researcher needs to have a specific mutation in mind to implement.
3	Double-stranded DNA	Selection of mutants with DpnI	Like other site-directed mutagenesis methods, targets a predetermined region within a DNA sequence.	Low efficacy with plasmids longer than 7 kb
4	Double-stranded DNA	Altered β -lactamase activity of mutant vector	Mutations can be placed precisely in the target gene.	Requires the presence of antibiotic selection marker in the plasmid.
5	Double-stranded DNA	Elimination of a restriction site (USE mutagenesis)	Used to introduce a library of site-specific changes into a specific DNA sequence within a target gene	The rate of mutant recovery is low.
6	Double-stranded DNA	Based on SapI digestion	A broad spectrum of amino acid changes can be placed at targeted positions.	Requires the presence of SapI digestion site.
7	Single-stranded DNA	Selection against uracil-substituted DNA	Multiple independent mutations can be introduced by following a single cloning step procedure.	The mutagenic process takes place during in vitro DNA synthesis and generates plasmid libraries with skewed distributions of variant amino acids at the target region.
8	Double-stranded DNA	Selection of mutants with DpnI	i. Mutations can be placed precisely in the target DNA sequences. ii. The mutagenesis process can be accomplished by only two rounds of PCR that are performed sequentially in the same tube.	i. This method requires as many rounds of PCR as the target mutation sites to be introduced. ii. When more than three mutations are to be generated, it is quicker to use a commercial kit. The yield of mutants has an efficiency of only ~82%.
9	Double-stranded DNA	Megaprimer		

^a1, Protocol: Random Mutagenesis Using Error-Prone DNA Polymerases (Forloni et al. 2018a); 2, Protocol: Creating Insertions or Deletions Using Overlap Extension Polymerase Chain Reaction (PCR) Mutagenesis (Forloni et al. 2018b); 3, Protocol: In Vitro Mutagenesis Using Double-Stranded DNA Templates: Selection of Mutants with DpnI (Forloni et al. 2018c); 4, Protocol: Altered β -Lactamase Selection Approach for Site-Directed Mutagenesis (Forloni et al. 2018d); 5, Protocol: Oligonucleotide-Directed Mutagenesis by Elimination of a Unique Restriction Site (USE Mutagenesis) (Forloni et al. 2019c); 6, Protocol: Saturation Mutagenesis by Codon Cassette Insertion (Forloni et al. 2019b); 7, Protocol: Random Scanning Mutagenesis (Forloni et al. 2019a); 8, Protocol: Multisite-Directed Mutagenesis (Forloni et al. 2019d); 9, Protocol: Megaprimer Polymerase Chain Reaction (PCR)-Based Mutagenesis (Forloni et al. 2019e).

BOX 2. MUTAGENESIS TERMINOLOGY

There is a plethora of mutagenesis terminology, some of it confusing, with different names for the same approach. The following list clarifies the terminology for the multitude of mutagenesis approaches.

5' Add-On Mutagenesis

A PCR-based method that is useful for adding a new sequence or chemical group to the 5' end of a PCR product.

Alanine Scanning Mutagenesis

Method used to determine the structure–function relationship of a given protein. Alanine is the substitution residue of choice because it eliminates the side chain beyond the β -carbon and yet does not alter the main-chain conformation, nor does it impose extreme electrostatic or steric effects. (See also Scanning Mutagenesis.)

Cassette Mutagenesis

Used for efficient insertion of mutagenic oligodeoxynucleotide cassettes, which allow saturation of a target amino acid codon with multiple mutations.

Chemical Mutagenesis

Exploits the nature of chemical mutagens, like hydroxylamine and *N*-ethyl-*N*-nitrosourea, to introduce random mutations into the target DNA sequence.

Circular Mutagenesis

Introduces site-directed mutations into circular DNA molecules of interest by means of mutagenic primer pairs.

Codon Cassette Mutagenesis

Relies on the use of universal mutagenic cassettes to deposit single codons at specific sites in double-stranded DNA.

Deletion Mutagenesis

Used to produce both randomly positioned and targeted deletions over large regions of DNA for constructing deletion mutants.

Directed Evolution

Used in protein engineering to harness the power of natural selection to evolve proteins or RNA with desirable properties not found in nature.

Directed Mutagenesis

See Site-Directed Mutagenesis.

Domain Mutagenesis

Used to introduce multiple mutations into a defined region of cloned DNA.

Insertional Mutagenesis

Used to mutagenize a DNA template by the insertion of one or more bases. These insertions can occur naturally, such as through transposons, or artificially in a laboratory setting.

Linker Scanning Mutagenesis

In linker scanning mutagenesis, a collection of 5' and 3' deletants is first created, and the termini are ligated to a linker oligonucleotide. Depending on the DNA sequence of individual deletants, paired combinations are chosen and used to create a new DNA fragment in which the linker sequence precisely replaces a part of the original sequence without altering the spacing of surrounding nucleotides.

Megaprimer PCR-Based Mutagenesis

Two external oligonucleotide primers and one internal mutagenic primer are used in two rounds of PCR to mutagenize a DNA sequence. The first round of PCR is performed using one of the external primers and the mutagenic primer containing the desired mutation. This generates an intermediate PCR product that is then

BOX 2. *Continued*

used as a “megaprimer” for the second round of PCR, along with the other external primer. The final PCR product is cloned into appropriate vectors and used in downstream applications.

Misincorporation Mutagenesis

Uses reverse-transcriptases as error-prone polymerases to create mutations.

Mismatch Mutagenesis

Creates specific DNA-based pair mismatches.

Multisite Directed Mutagenesis

Permits mutagenesis at multiple sites simultaneously with only a single oligonucleotide primer per site.

Oligonucleotide-Directed Mutagenesis

Uses a mutagenic oligonucleotide primer to introduce a mutation into a DNA strand. It may or may not involve PCR.

PCR Mutagenesis

Any technique that uses the PCR to generate a mutation in a specific DNA sequence.

PCR Site-Directed Mutagenesis

Uses mutagenic oligonucleotide primers to introduce the desired mutations.

Random Mutagenesis

Random mutations are produced in a specific DNA sequence by, for example, UV irradiation.

Random Scanning Mutagenesis

An oligonucleotide-based method for generating all 19 possible replacements at individual amino acid sites within a protein.

Retroviral Insertional Mutagenesis

Retrovirus particles are used to create insertional mutagenesis.

Saturation Mutagenesis

A form of site-directed mutagenesis, in which one tries to generate all possible (or as close to as possible) mutations at a specific site or narrow region of a gene.

Scanning Mutagenesis

Used to understand structure–function relationships by creating a library of mutants with an alanine or cysteine residue at each position of protein. (See also Alanine Scanning Mutagenesis.)

Sequence Saturation Mutagenesis

A method to create mutations at every single nucleotide position in a given target sequence.

Signature-Tagged Mutagenesis (STM)

A genetic technique used to study gene function. STM can be used to infer the function a particular gene has by observing the effects of mutations on the phenotype. The original and most common use of STM is to discover which genes in a pathogen are involved in virulence in its host, so that better medical treatments can be designed.

Site-Directed Mutagenesis

Also called “site-specific mutagenesis” or “oligonucleotide-directed mutagenesis,” this is a technique in which a desired mutation is created at a defined site in a DNA molecule.

Site-Specific Mutagenesis

See Site-Directed Mutagenesis.

Transposon Mutagenesis

Also called “transposition mutagenesis,” this is a biological process that allows genes to be transferred into a host organism’s chromosome, interrupting or modifying the function of an extant gene.

properties can be enhanced. In short, oligonucleotide-directed mutagenesis has become the genetic engineer's alchemy.

Saturation Mutagenesis

Saturation mutagenesis is used to generate mutations at many sites in a particular coding sequence. Every effort is made to introduce mutations in an unbiased fashion; preconceptions and knowledge about the functions of individual amino acids in the wild-type sequence are disregarded. The aim is to gather information about the entire “sequence space,” that is, about the relationship between the amino acid sequence and the 3D structure of the protein. Saturation mutagenesis is usually performed on small segments of DNA that encode an individual structural domain. At its best, the method can provide catalogs of amino acids or combinations of amino acids that are tolerated within a domain without deleterious effect on structure and function. Studies of the bacteriophage λ repressor, for example, have shown that a large number of combinations of amino acids can satisfy the structural and functional requirements of the hydrophobic core and α -helices of the molecule (Reidhaar-Olson and Sauer 1988; Lim and Sauer 1989).

Scanning Mutagenesis

Alanine-scanning mutagenesis is used to analyze the function(s) of particular amino acid residues on the surface of a protein. The charged residues that normally dapple the surface of proteins are not usually required for structural integrity, but they are generally involved in ligand binding, oligomerization, or catalysis. Systematic replacement of charged amino acids with alanine residues eliminates side chains beyond the β -carbon and disrupts the functional interactions of the amino acids without changing the conformation of the main chain of the protein. Alanine scanning is therefore a powerful method for assigning functions to particular regions of the protein surface (Cunningham and Wells 1989). In an extension of this approach—cysteine-scanning mutagenesis—unpaired cysteine residues are used to replace individual amino acid residues at particular sites in the protein. Unpaired cysteine residues are of average size, uncharged, and hydrophobic. Because they react efficiently with modifying reagents such as *N*-ethylmaleimide, cysteine residues introduced by scanning mutagenesis can be used as biochemical tags to verify the topology of transmembrane proteins and to measure the accessibility of residues to modifying reagents in the aqueous or lipid phases (e.g., see Akabas et al. 1992; Dunten et al. 1993; Kurz et al. 1995; Frillingos and Kaback 1996; He et al. 1996; Frillingos et al. 1997a,b, 1998).

RESEARCH GOALS

In vitro mutagenesis is used specifically to change the base sequence of a segment of DNA. The changes may be localized or general, random, or targeted. More general and less specific methods of mutagenesis, such as random mutagenesis using error-prone DNA polymerase (see Protocol: **Random Mutagenesis Using Error-Prone DNA Polymerases** [Forloni et al. 2018a]), are better suited to analysis of regulatory regions of genes, whereas more precise types of mutagenesis, such as overlap extension PCR, site-directed mutagenesis, or, alternatively, megaprimer PCR-based mutagenesis (see Protocol: **Creating Insertions or Deletions Using Overlap Extension Polymerase Chain Reaction (PCR) Mutagenesis** [Forloni et al. 2018b], Protocol: **In Vitro Mutagenesis Using Double-Stranded DNA Templates: Selection of Mutants with DpnI** [Forloni et al. 2018c] and Protocol: **Megaprimer Polymerase Chain Reaction (PCR)-Based Mutagenesis** [Forloni et al. 2019e], respectively) are used to understand the contributions of individual amino acids, or groups of amino acids, to the structure and function of a target protein. Both methods—random and targeted—share the virtue of generating mutants in vitro, without phenotypic selection.

During the past decades, several different types of mutagenesis methodologies have been developed that respond to different needs. Although these methods sometimes overlap each other, it is still possible to group the techniques based on the final goal of the research. For example, if the aim of

researchers is to analyze the function(s) of particular amino acid residues on the surface of a protein, scanning mutagenesis is well suited for this need. An extension of this approach referred to as “random-scanning mutagenesis” allows researchers to test a broader spectrum of amino acid changes at the targeted positions. For a more comprehensive description of this technique, see Protocol: **Random Scanning Mutagenesis** (Forloni et al. 2019a).

Alternatively, if the aim is to gather information about the entire “sequence space” (i.e., about the relationship between the amino acid sequence and the 3D structure of the protein), researchers should make use of “saturation mutagenesis.” For further details on this technique, see Protocol: **Saturation Mutagenesis by Codon Cassette Insertion** (Forloni et al. 2019b).

If the aim of the project is to test the role of particular residues in the structure, catalytic activity, or ligand-binding capacity of a protein, the methodology of choice should be “oligonucleotide-directed mutagenesis.” For a complete description of this methodology, see Protocol: **Altered β -Lactamase Selection Approach for Site-Directed Mutagenesis** (Forloni et al. 2018d) and Protocol: **Oligonucleotide-Directed Mutagenesis by Elimination of a Unique Restriction Site (USE Mutagenesis)** (Forloni et al. 2019c). Multisite-directed mutagenesis and megaprimer PCR-based mutagenesis are presented in Protocol: **Multisite-Directed Mutagenesis** (Forloni et al. 2019d) and Protocol: **Megaprimer Polymerase Chain Reaction (PCR)-Based Mutagenesis** (Forloni et al. 2019e).

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