1

INTRODUCTION

The genetic blueprint of all organisms consists of nucleic acids. In nature this choice is based on the stability of nucleic acid molecules due to the presence of phosphodiester linkages, which are the strongest among all chemical bonds (Westheimer, 1987). The stability of nucleic acid is a very important feature in maintaining the integrity of the genetic material. However, during the life of an organism the stability of phosphodiester linkages is compromised at times to facilitate certain other important processes such as the removal of damaged DNA and its repair in order to restore the accuracy of the genetic blueprint. Such breakage of phospodiester bonds in DNA chains is also allowed to provide the recombination of genes in a chromosome or the salvage of genetic material in a cell for reutilization of nucleotides or their components or for their final disposal in a cell destined for apoptosis during normal development of a multicellular organism as complex as human. Thus, it is not surprising that all living systems contain a group of enzymes capable of hydrolyzing the phosphodiester linkages present in nucleic acids; these enzymes are called *nucleases*.

The facts that these phosphodiester linkages are the most stable among all chemical bonds found in biological molecules and that nucleases can hydrolyze such phosphodiester linkages make them the most unique of all enzymes. No other groups of enzymes influence the physiology of an organism in such diverse ways as the nucleases. The fact that nucleases catalyze an array of biochemical reactions, all involving just one chemical bond, namely, the phosphodiester linkage, has no parallel in the biochemistry of enzymes. A number of nucleases possess other enzymatic functions in addition to their principal property of catalyzing the hydrolysis of phophodiester linkages. Many nucleases possess other associated catalytic activities such as DNA polymerase, ligase, helicase, or kinase and other functions such as repressor.

Initially, nucleases were considered to play only a degradative role in the salvage pathway of nucleic acid metabolism because of their association with the pancreas (Kunitz, 1940). However, they are now shown to play a multitude of important roles in different aspects of basic genetic mechanisms. These include their participation in (a) the processes of DNA replication, repair, recombination, and mutagenesis (Clark, 1971; Hanawalt et al., 1979; Kornberg and Baker, 1992), (b) the control of gene expression by determining the nature of transcript (Sharp, 1981) and its turnover and (c) transposition and other programmed gene rearrangements (Borst and Greaves, 1987). They are also a part of the host defense mechanism against alien nucleic acid molecules (Luria and Human, 1952). Nucleases play a great role in the mechanisms of immune systems in mammals by controlling the assembling of immunoglobulin genes, their allelic exclusion, and class switching and in the determination of the membrane-bound or secreted forms of the immunoglobulins, leading to immunoglobulin diversity (Sakano et al., 1980; Tonegawa 1983, 1985; Yancopoulos and Alt, 1985, 1988; Yancopoulos et al., 1986) and antigenic variation (Myler et al., 1984; Borst and Cross, 1982). Nucleases also play an important role during programmed cell death in the development of multicellular organisms, including human.

Thus the study of nucleases has been very useful from both the conceptual and technical points of view. The pancreatic ribonuclease was the first enzyme whose entire amino acid sequence was determined (Moore and Stein, 1973; Anfinsen, 1963). The amino acid sequence of the bovine pancreatic ribonuclease is presented in Figure 1.1. Knowledge of the amino acid sequence of the pancreatic ribonuclease was crucial in the confirmation of the idea that the secondary and tertiary structures of a protein are controlled by its primary structure (Anfinsen, 1964). Determination of the structure of the ribonuclease led to the development of an entirely new technology of protein synthesis and protein engineering (Gutte and Merrifield, 1971; Merrifield, 1986; Gutte, 1992). Nucleases have been studied extensively in order to seek answers to the question of the mechanism of enzyme catalysis and polypeptide folding (Anfinsen, 1964; 1973; Tucker et al., 1979; Cotton et al., 1979; Shortle, 1983; Botstein and Shortle, 1985; Kippen et al., 1994). Ribonucleases were used in the complete sequencing of the first tRNA molecule which led to the understanding of its role in the mechanism of genetic coding (Holley, 1965). Use of these nucleases led to the elucidation of the nucleosomal organization of eukaryotic chromosomes (Hewish and Burgoyne, 1973). Above all, the discovery of a new class of nucleases called *restriction endonucleases* (Smith and Wilcox, 1970) resulted in the development of recombinant DNA technology (Jackson et al., 1972), DNA sequencing methodology (Maxam and Gilbert, 1977; Sanger et al., 1977), and new methods for genetic mapping (Southern, 1975, 1982; Kan and Dozy, 1978; Botstein et al., 1980; White and Lalouel, 1988) and the extensive mapping of human chromosomes (Donis-Keller et al., 1987). Furthermore, the use of restriction endonucleases has been crucial in the development of a new branch of genetics called *reverse genetics* (Ruddle 1984; Orkin, 1986). The methods of reverse genetics have been very useful in the understanding of the molecular basis of several human diseases—for example, Huntington's chorea, cystic

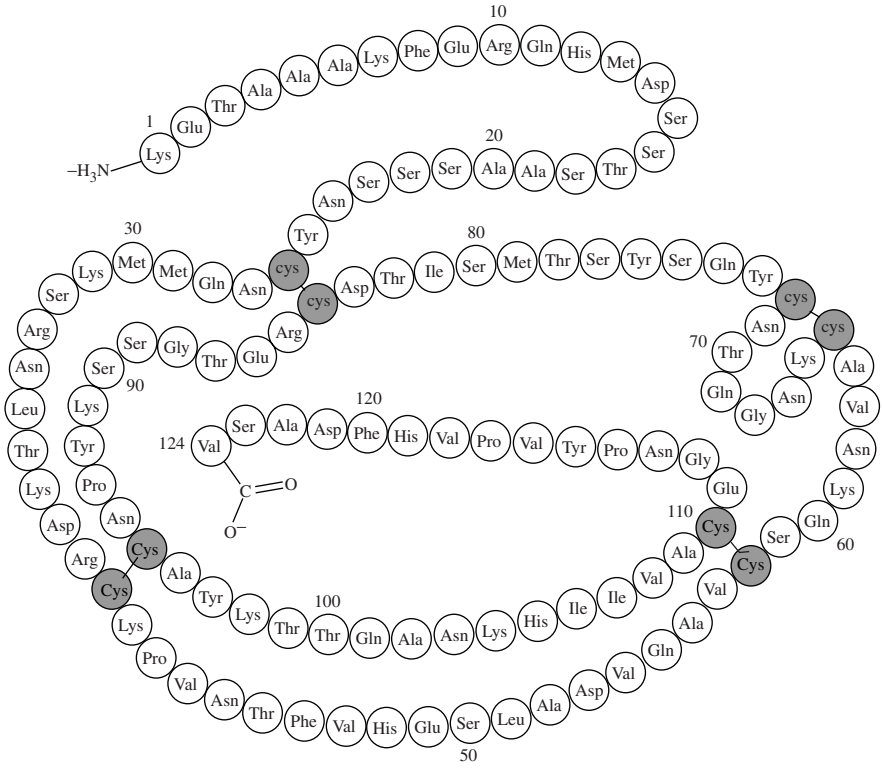


Figure 1.1. Amino acid sequence of the bovine pancreatic ribonuclease. Black circles represent cysteine residues forming disulfide bonds. (From *Biochemistry* by L. Stryer, copyright W. H. Freeman and Company, 1982, used with permission.)

fibrosis, retinoblastoma, arthritis, and many others (White, 1986; Gusella, 1986; Caskey, 1987; Davies, 1991; Mark, 1987; Monaco and Kunkel, 1987; Woron and Thompson, 1988; Martin, 1987; Buxton, 1992)—which may pave the way for the possible alleviation of certain inherited human diseases by gene therapy (Weatherall, 1982; Anderson, 1992; Ailliton, 1985; Nichols, 1988; Mulligan, 1993). All these new methodologies of genetics in which nucleases play critical roles hold great promise for the future of mankind through the development of science, technology, and commerce (Mishra, 1985).

I. HISTORICAL PERSPECTIVES

The enzymatic breakdown of nucleic acid was first observed in the early twentieth century (Araki, 1903). The enzymes involved in this process were named *nucleases* (Iwanoff, 1903). The fact that nucleases are indeed phosphodiesterases was, however, established much later (Brown and Todd, 1955; Hilmoe et al., 1961;

Laskowski, 1967; Westheimer, 1987; Gerlt 1992, 1993). Soon two groups of nucleases designated as ribonuclease (RNase) and deoxyribonuclease (DNase), capable of hydrolyzing ribonucleic acids and deoxyribonucleic acids, respectively, were described by Kunitz (1940, 1950). This specificity of nucleases toward the sugar moiety (i.e., deoxyribose or ribose) of nucleic acid was considered the most important criterion for the classification of nucleases (Kunitz, 1940, 1950). Studies of the properties of crystallized nucleases (Kunitz, 1940, 1950) laid down the foundation for the understanding of the biochemical nature, genetic control, and physiological role of these enzymes. Early studies of nucleases and their chemical modifications provide an excellent model for understanding the nature of the biochemical reaction mechanisms and the role of catalytic sites that facilitate substrate binding. These conclusions were later confirmed by x-ray crystallography. Analysis of the crystal structures of a number of nucleases such as *Escherichia coli* DNA polymerase I, RNaseH, endonuclease III, topoisomerase I, Hin invertase, T_4 endonuclease DNaseI, a number of recombinases and structure-selective nucleases pancreatic RNases, and several other RNases has provided insight into the structure and function of enzyme proteins and their specificity of action (Joyce and Steitz, 1987; Suck et al., 1984; Nakamura et al., 1991; Morikawa et al., 1992; Sevcik et al., 1990; Lima et al., 1994).

The study of nucleases, particularly the mechanistic view of the interaction of their active sites with their substrates, has provided a better understanding of the mechanisms of enzyme catalysis (Gerlt, 1992, 1993). Nucleases must possess the ability not only to recognize and bind with a DNA sequence, but also to hydrolyze it. Nucleases differ from other DNA recognizing and binding proteins such as transcription factors; the latter usually possess leucine zipper or zinc finger or helix-turn-helix motifs. Nucleases lack such motifs and therefore must recognize and bind with DNA by other methods. Certain restriction endonucleases (see Chapter 4) may undergo mutation to produce the mutant forms of the enzyme which can recognize a DNA sequence and bind with it but are unable to hydrolyze it.

II. PROTEIN, RNA, DNA, AND OTHER MOLECULES AS NUCLEASES

Enzymes are biocatalysts that facilitate chemical reactions in biological systems. Enzymes are traditionally known to be proteins. However, proteins are no longer the only molecules that have a catalytic function. In recent years a number of RNA and DNA molecules have been shown to possess such functions. Thus, it is no surprise that a number of nucleases are RNA or DNA even though a majority of nucleases are made up of proteins. In addition to proteins and RNAs or DNAs, a number of other smaller molecules (such as bleomycin and phenanthroline and others) have been shown to possess nucleolytic property. It is proposed here that the term *enzyme* or *proteinzyme* should be used to indicate all biocatalysts that are proteins. This nomenclature would be consistent with the fact that a majority of molecules with biocatalytic function are proteins. The RNA and DNA molecules

with catalytic activities have been designated as *ribozyme* or *RNAzyme* (Kruger et al., 1982) and *deoxyribozyme* or *DNAzyme* (Breaker and Joyce, 1994; Breaker, 2000; Wilson and Szostak, 1999) respectively. Certain small molecules and chemical reagents with enzyme-like catalytic functions are called *chemzymes* (Corey and Reichard, 1989; Waldrop, 1989). The occurrence and the role of DNAzymes and chemzymes in biological systems remain to be established. An understanding of the divergent nature of the biocatalysts has made it possible to design synthetic or semisynthetic nucleases that can be targeted to a specific nucleic acid sequence. These nucleases are called *artificial* or *designer* or *chemical nucleases* (Sigman and Chen, 1990). The ability to design such nucleases may revolutionize the field of genome mapping (Ebright et al., 1990; Oakley and Dervan, 1990). Nucleases as protein enzymes are described in the different chapters of this book. The nature and role of ribozymes, deoxyribozymes, and certain chemzymes as nucleases are discussed in Chapter 9. All protein enzymes and ribozymes are encoded by genes or DNA molecules; therefore the DNA molecules are their genotypes and they represent phenotypes, whereas in the case of DNAzyme, both the genotypes and phenotypes are represented by the same molecule.

III. NATURE OF ENZYMATIC REACTIONS CATALYZED BY NUCLEASES

Nucleases are, in essence, phosphoesterases that hydrolyze the internucleotide linkage in a nucleic acid molecule. There are different kinds of phosphoesterases. Phosphomonoesterases do not act on internucleotide linkage but cleave the terminal phosphate from a nucleotide chain. Among the three classes of phosphodiesterases, the first group of enzymes acts on phosphodiester bonds not involving internucleotide linkage. The enzymes responsible for the breakdown of cAMP and cGMP are examples of such phosphodiesterases. The second group of enzymes acts on different types of phosphodiester bonds, including internucleotide linkages; the snake venom phosphodiesterases belong to this group of enzymes. The third group specifically acts on internucleotide phosphodiester linkage(s); protein and ribozyme nucleases belong to this group of phosphodiesterases. Both protein and ribozyme nucleases act by hydrolysis of phosphodiester linkages in the nucleic acid chain. This, however, is not true of the chemzyme nucleases; they cause cleavage by disruption of the sugar moiety via oxidation or by alkylation of bases (Sigman and Chen, 1990).

Most enzymatic reactions are reversible in nature. Both the synthesis and degradation of micromolecules are usually carried out by the same enzymes. However, the degradation and biosynthesis of macromolecules in all biological systems are carried out by distinct sets of enzymes (Kornberg, 1974). Nucleases that cleave internucleotide linkages during the degradation of nucleotides cannot reform the internucleotide linkage leading to the biosynthesis of nucleic acid molecules. Instead, the internucleotide linkages are either (a) formed by DNA or RNA polymerases during the synthesis of specific nucleic acid molecules or (b) joined

by the enzyme ligase. However, there are exceptions to this rule. A number of DNA polymerases carry nuclease activity in different parts of the same polypeptide. Also, a class of nucleases called *topoisomerases* has been shown to combine both properties; that is, they can hydrolyze the internucleotide linkages transiently and then rejoin them as discussed in Chapter 6. The action of different enzymes that act on nucleic acids are illustrated in Figure 1.2. Nucleases and phosphodiesterases

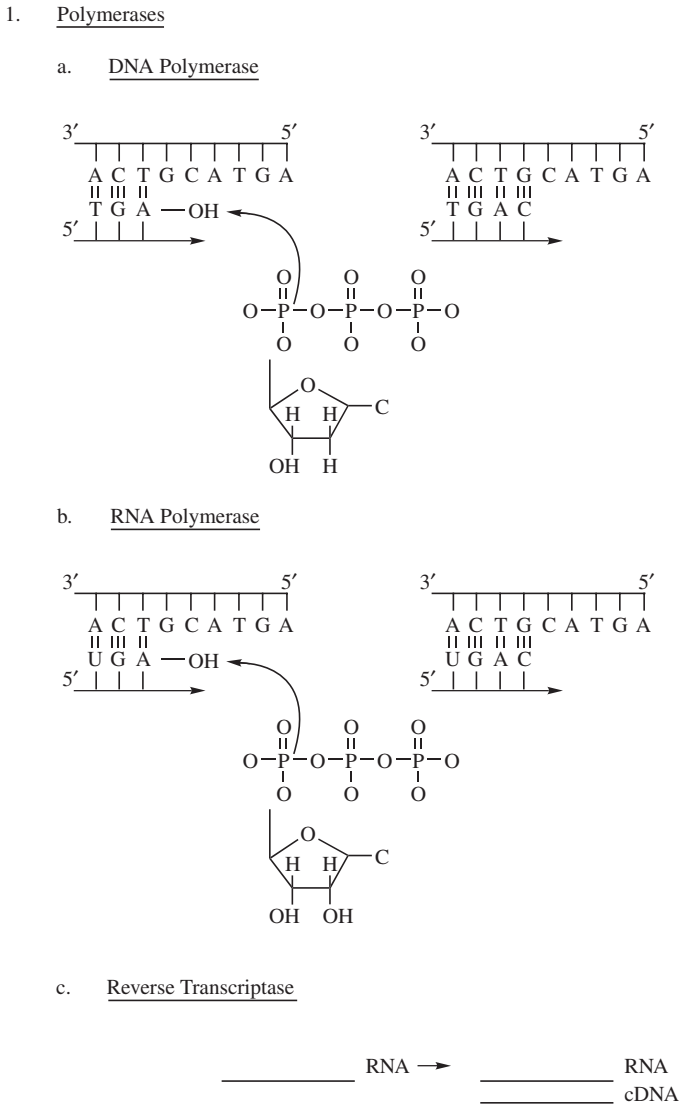
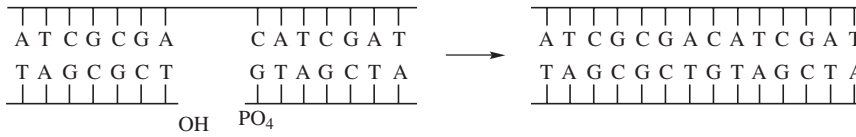


Figure 1.2 Action of different enzymes on nucleic acids.

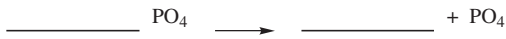
2. Ligase



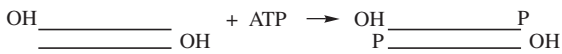
3. Terminal Transferase

4. Phosphatase and Polynucleotide Kinase

a. Phosphatase

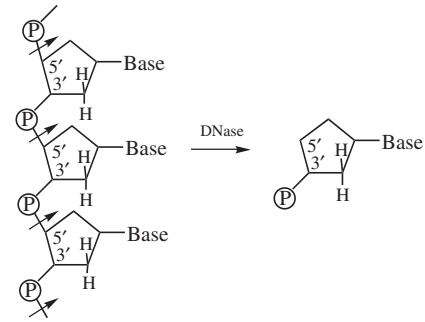


b. Polynucleotide Kinase



5. Nucleases

a. DNase



b. Restriction Endonuclease

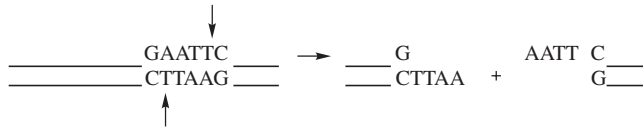
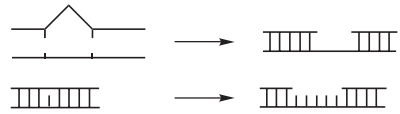


Figure 1.2 (*continued*)

c. Damage-Specific Nuclease

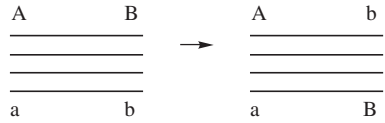


d. Topoisomerase

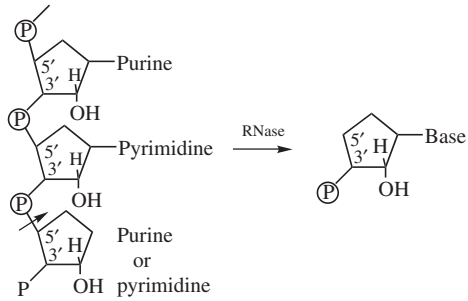
(i) Relaxed DNA —Gyrase→ Supercoiled DNA

(ii) Supercoiled DNA —Topoisomerase I→ Relaxed DNA

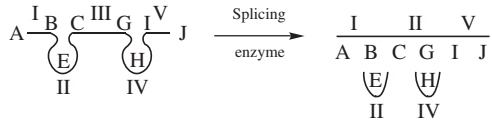
e. Recombinase



f. RNase



g. RNA Splicing Enzyme



h. RNaseH

DNA RNA hybrids

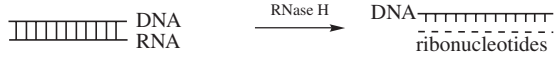
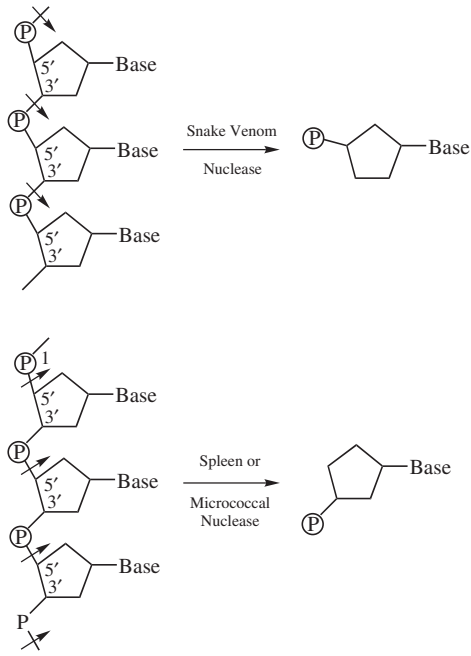


Figure 1.2 (*continued*)

i. Nonspecific Nucleases or Phosphodiesterases



6. DNA Methylase

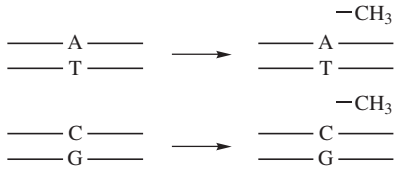


Figure 1.2 (*continued*)

cleave the bond between phosphorus and oxygen in the internucleotide linkage of nucleic acid (Hilmoe et al., 1961) as shown in Figure 1.3.

Protein nucleases are characterized by their processive or distributive mode of action. A processive enzyme remains engaged with the substrate molecules, resulting in the hydrolysis of successive internucleotide linkages before it leaves to act on another nucleic acid molecule. In contrast, a distributive nuclease will usually hydrolyze only a few internucleotide linkages before it falls off the substrate molecule and will then be free to engage with the same or another nucleic acid molecule.

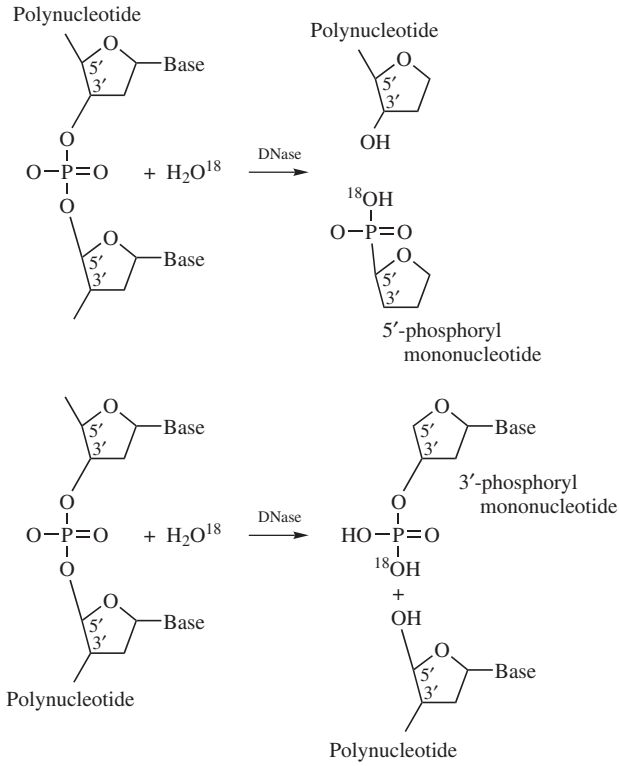


Figure 1.3. Nuclease acts on bond in P–O.

IV. CLASSIFICATION

Kunitz (1940) classified nucleases into two major groups, ribonucleases and deoxyribonucleases, based on the specificity of their nucleolytic attack toward the sugar moiety of the different nucleic acid molecules. Even though snake venom phosphodiesterase hydrolyzed both ribonucleotides and deoxyribonucleotides (Schmidt, 1955), the classification of nucleases into DNase and RNase by Kunitz was considered valid at that time. This was partly because of the nonspecific nature of snake venom phosphodiesterase that could attack phosphodiester linkages other than those found in nucleic acid. The classification of nucleases into DNase and RNase, however, appeared somewhat inappropriate with the discovery of micrococcal nuclease and snake venom phosphodiesterases that attacked both DNA and RNA (Laskowski, 1982). Soon a new class of sugar-nonspecific nucleases was added to the list of nucleases. This method of classification led to a trend in which the terms DNase and RNase were used for the sake of convenience. Later, several new groups were added as dictated by new evidence.

In view of these difficulties with the classification of nucleases, Bernard (1969) and Laskowski (1959, 1982) introduced the idea of consensus criteria (instead of

absolute criteria) for the classification of nucleases. These include (a) the nature of substrate hydrolyzed (DNA, RNA), (b) the type of nucleolytic attack (exonuclease and endonuclease), (c) the nature of the nucleolytic products (i.e., mono- or oligonucleotides terminated by a 3′- and 5′-phosphate group), and (d) the nature of the bonds hydrolyzed. Additional criteria, such as the nature of the substrate DNA (mismatch, damaged or topological isomers), site-specificity, structure-selectivity, and functional ability to restructure DNA molecules (i.e., to facilitate genetic recombination), were added to this list at a later stage (Laskowski, 1982; Linn, 1982a; Lieber, 1997 and Suck, 1998; Mishra, 1995). This set of consensus criteria for the classification of nucleases seems compatible with the recent advances made in the understanding of nucleases as a whole. This eliminates many of the problems in the classification of nucleases, and it also provides an easy method for the accommodation of an enzyme with exceptional properties into a particular group.

However, this system is still riddled with difficulties, by the fact that the pancreatic DNase can attack a polynucleotide chain containing a mixture of both ribo- and deoxyribonucleotides (Pruch and Laskowski, 1980). Furthermore, exonucleases were initially considered to be enzymes that sequentially removed mononucleotides from a free terminus. This group was later expanded to include enzymes that attacked internal phosphodiester linkages but required a free terminus for the identification of polynucleotide chain to be hydrolyzed (Frankel and Richardson, 1971). This view was based on the observation that a covalently closed circular DNA could be broken by an endonuclease, but not by an exonuclease, since the latter required a free terminus for the recognition of the substrate. However, recently it has been shown that some exonucleases are capable of opening a supercoiled DNA 10,000 times faster than a relaxed DNA (Pritchard et al., 1977), suggesting that an exonuclease is, in essence, an ‘‘exophilic’’ nuclease that prefers an open terminus (Laskowski, 1982). Furthermore, certain nucleases such as micrococcal nuclease can attack a nucleic acid molecule either endo- or exonucleolitically. The properties of these nucleases and their possible roles in the basic genetic mechanisms that occur during the life of an organism are discussed in the different chapters of this book. The major classification groups of nucleases are based on a set of consensus criteria. Some of the other major criteria considered during the classification of nuclease as discussed by Laskowski (1982) are described below.

A. Nature of Substrates

Nucleases differ significantly in the nature of the substrates that they can hydrolyze. This is implicit in the idea of the consensus criteria discussed above. Nucleases hydrolyze the phophodiester linkages in DNA and RNA. Both of these molecules can exist in different helical structures. DNA can exist as a right-handed or a left-handed helical structure (Figure 1.4). The biologically active form of DNA found in the living systems is the right-handed form of DNA called B-DNA. The DNA whose structure was first elucidated by the Watson–Crick model of DNA is the B-DNA. This DNA has 10 base pairs per turn. The B-DNA depending on hydration or ionic environment may transform into several isomeric forms such as the

B′, C, C′, C″, D, E, and T forms. The phage T2 DNA occurs in the B form under conditions of relative high humidity but transform directly into T-DNA. The other right-handed DNA helix is the A-DNA, which possesses 11 base pairs per turn—in contrast to the naturally occurring B-DNA, which passesses 10 base pairs per turn. In addition, DNA can exist as a left-handed helix with 12 base pairs per turn; this left-handed DNA is called a Z-DNA. There is no evidence for the existence of Z-DNA in nature, but alternating purine–pyrimidine occurring as tandem repeats of dinucleotide pair CA/AG found in intergenic DNA and in introns and rarely in the coding DNA may assume Z-DNA form. Most nucleases act on B-DNA. Only one naturally occurring nuclease such as Mung bean nuclease has been found to make cuts in the intergenic DNA, suggesting its ability to use Z-DNA as substrate. A number of chemical nucleases can utilize Z-DNA as substrate. The structure of biologically active forms of DNA is presented in Figure 1.4.

Most RNAs occur as single-stranded structures, but these may include a certain double-helical domain that is very characteristic of tRNA ribosomal RNA and many mRNA. Double-stranded RNAs assume a helical structure in two isomeric

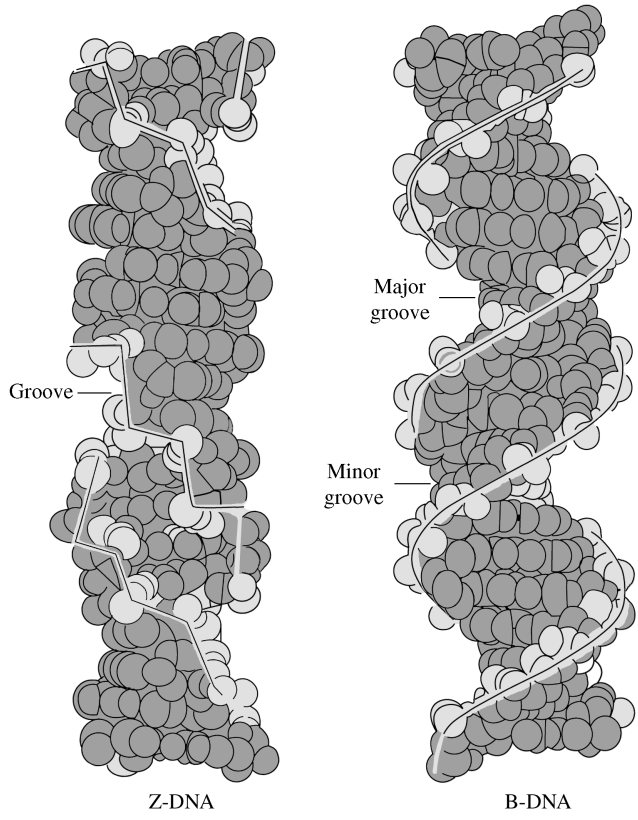


Figure 1.4. Z- (*left*) and B- (*right*) DNA. The dark lines connect phosphate groups. (Reproduced with permission from the *Annual Review of Biochemistry*, Volume 53, Copyright 1984 by Annual Reviews, Inc.)

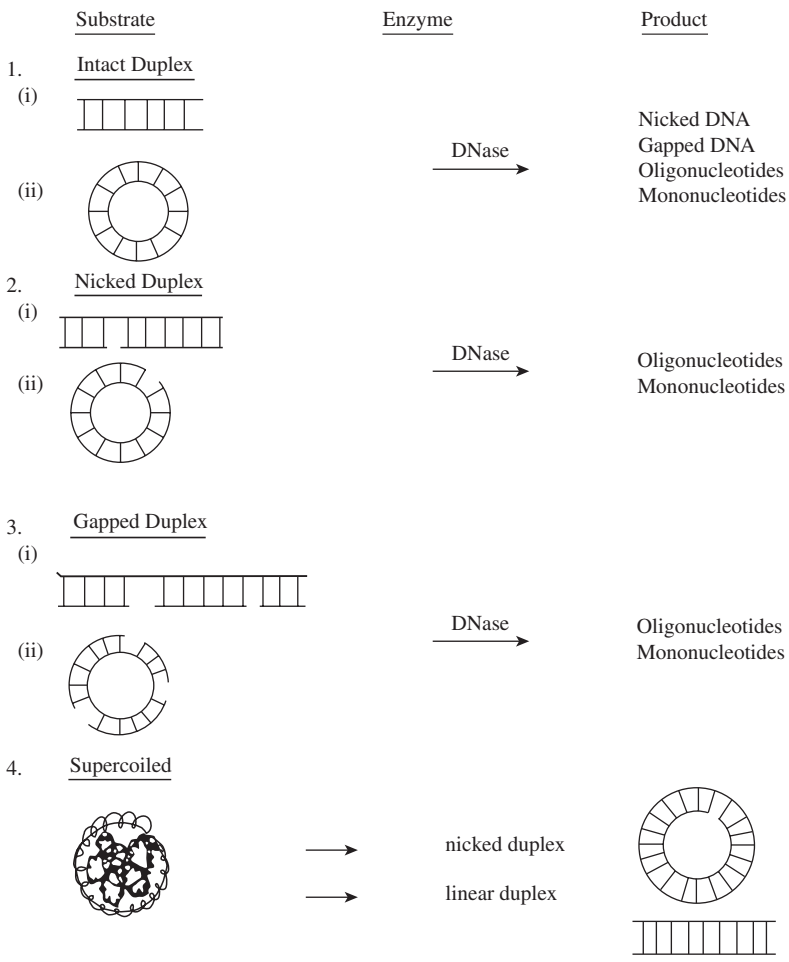


Figure 1.5. Nature of substrate for protein nucleases.

forms called A-RNA and A′-RNA depending on the ionic concentration of the media. At low salt concentration, the RNA helix exists as A-RNA with 11 base pairs per turn; however, at high salt concentration the A-RNA may assume the A′-RNA form with 12 base pairs per turn. Both forms of the double-stranded RNA show features typical of Watson–Crick base pairs; however, certain homopolymers may yield triple-stranded RNA structure simultaneously showing Watson–Crick and Hoogsteen base pairing. Different forms of DNA and RNA substrates are shown in Figure 1.5.

In addition to DNA and RNA, small DNA pieces with a hairpin structure containing a stem with a fluorophore-quencher pair and a loop with sites for cleavage by nucleases are used as the substrates to probe the nuclease activity of unknown molecules. The nuclease activity of the unknown molecules can be measured by the amount of fluorescence released due to the separation of quencher from the fluorophore after the digestion of the DNA substrate (Figure 1.6).

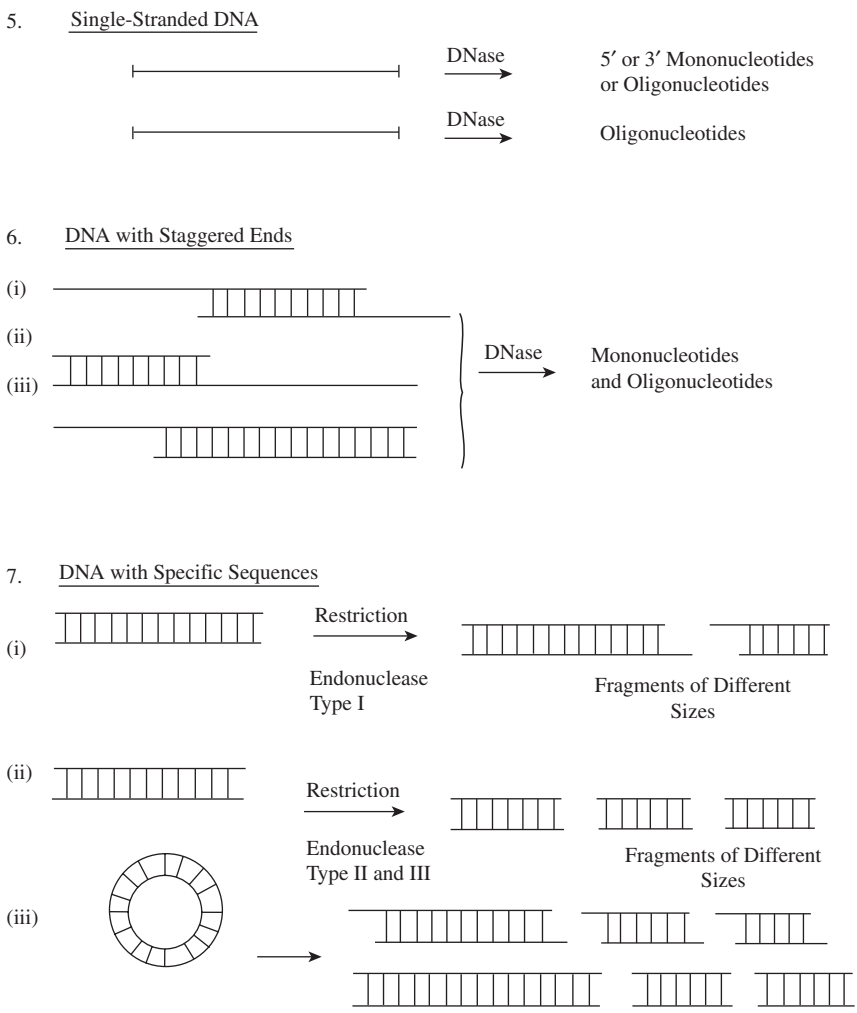


Figure 1.5 (*continued*)

Peptide nucleic acids (PNAs), a class of artificially synthesized oligomers containing a neutral peptide-like backbone instead of ribose phosphate backbone, mimic nucleic acids. PNAs can hybridize to complementary DNA/RNA with higher affinity or specificity than the corresponding oligonucleotides. PNAs can be recognized by different DNA polymerases as substrate (Lutz et al., 1999). However, PNAs are never recognized as substrates by nucleases even though binding of a complementary PNA oligomer to DNA may target a single-strand-specific nuclease such as S1 nuclease in a sequence-specific manner to DNA, leading to a double-strand DNA cleavage (Demidov et al., 1993).

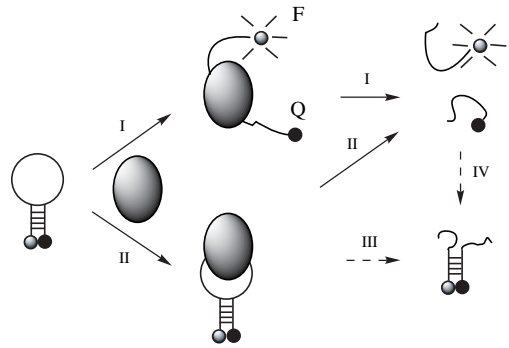


Figure 1.6. Schematic representation of the fluorescence mechanism of the molecular beacon during cleavage by single-stand-specific DNA nuclease (indicated by solid arrows). The solid arrows indicate two paths (I and II) leading to fluorescence enhancement during digestion. The dashed arrows represent two possible processes (III and IV) in which no fluorescence enhancement is produced. Only the first cut is shown here. Even though the nuclease may keep on cutting one single strand many times, only the first cut contributes to the fluorescence signal increase. The ball represent the nuclease. MB, E, and Q represent molecular beacon, fluorophore, and quencher, respectively. Here the fluorophore and quencher are tetramethylrhodamine (TAMRA) AND 4-(4′-dimethylaminophenylazo) benzoic acid (DABCYL), respectively. (From Nucleic Acid Research, reproduced with permission.)

B. Mode of Attack

Nucleases differ in their modes of attack. Enzymes may attack either from the 3′ end or from the 5′ end of the nucleic acid molecule; some nucleases can attack from either end. The mode of nucleolytic attack is presented in Figure 1.7.

Nucleases hydrolyze a wide spectrum of substrates with different modes of attack and produce either mono- or oligonucleotides as products. However, the nucleolytic products are always either 3′- or 5′-phosphorylated. The same nuclease is not known to produce both 3′- and 5′-phosphorylated products, regardless of which direction the enzyme may traverse the substrate. Thus, this is the only criterion which can be strictly applied to the classification of all nucleases without exception. Recently, this criterion has been a point of major consideration in the classification of a large number of protein nucleases (Linn, 1982a). However, this criterion is not applicable to topoisomerases, recombinases and damage-specific nucleases. The system of nuclease classification devised by Linn (1982a) represents the most suitable system of classification. The classification of protein nucleases is presented in Table 1.1.

Presumably the active site of the nuclease may use either the 3′ or 5′ carbon in the internucleotide linkage as a guide to make the incision; it can hydrolyze the P–O bond adjacent to 3′ carbon or 5′ carbon of the sugar moieties involved in the internucleotide linkage. The fact that the topography of the region encompassing the 3′ or 5′ carbon of the sugar molecule attached to a particular base is significantly different may explain why a particular nuclease produces either a 5′ or 3′ mononucleotide but never both. The enzyme must have evolved to recognize distinctly

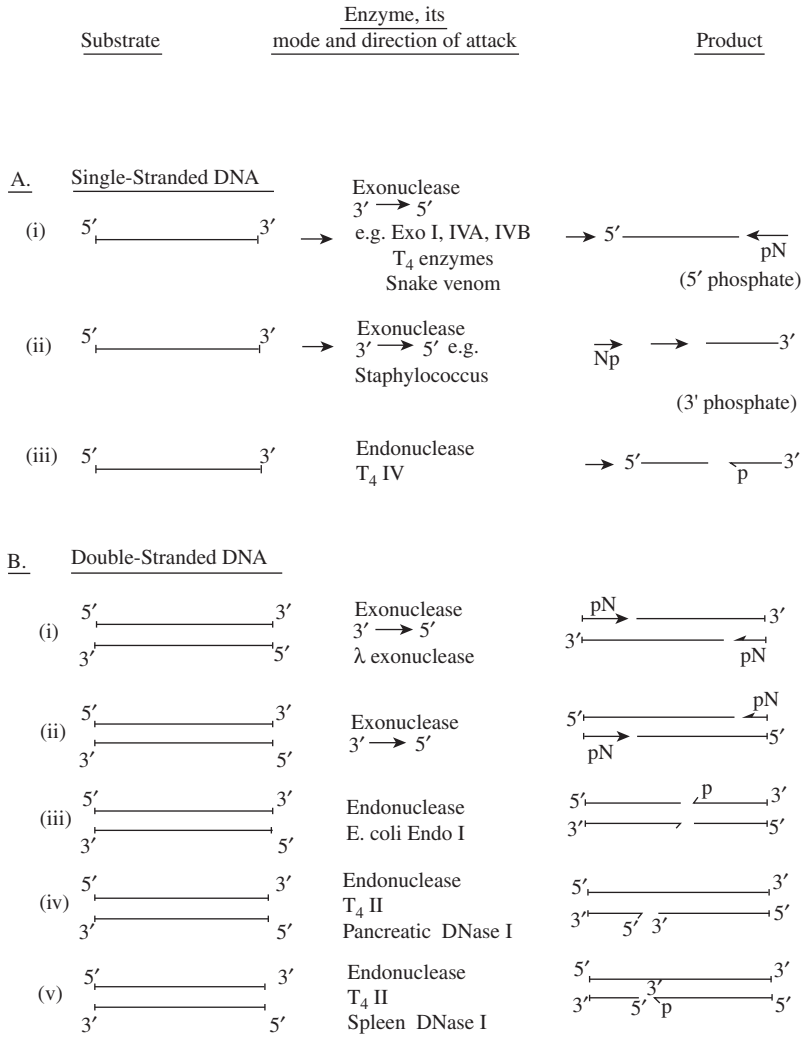


Figure 1.7. Mode of nucleolytic attack.

these two regions of the sugar moiety encompassing the internucleotide phosphodiester linkage of a nucleic acid chain. There is some evidence that recognition of oxygen atoms adjacent to the phosphorus atom may be used as a guide by a nuclease before it cleaves the phosphodiester bond. This view is based on the fact that nucleotides containing sulfur in place of oxygen in the α phosphate are good substrates for polymerization (by DNA polymerase I) or for ligation (by DNA ligase) but not for removal from a polynucleotide chain by an exonuclease (Putney et al., 1981; Kunkel et al., 1981a,b).

Table 1.1 Classification of protein nucleases[a]

Sugar-Specific Nucleases		Sugar-Non-Specific Nucleases
Ribonucleases	**Deoxyribonucleases**	
I. *Exoribonuclease*	I. *General deoxyribonucleases*	I. *Exonucleases*
a. 3′-Phosphomonoester producer	A. Exodeoxyribonucleases	a. 3′-Phosphomonoester producer
b. 5′-Phosphomonoester producer	a. 3′-Phosphomonoester producer	b. 5′-Phosphomonoester producer
II. *Endoribonuclease*	b. 5′-Phosphomonoester producer	II. *Endonuclease*
a. 3′-Phosphomonoester producer	B. Endodeoxyribonuclease	a. 3′-Phosphomonoester producer
b. 5′-Phosphomonoester producer	a. 3′-Phosphomonoester producer	b. 5′-Phosphomonoester producer
	b. 5′-Phosphomonoester producer	
	II. *Restriction endonucleases*	
	a. Type I	
	b. Type II	
	c. Type III	
	III. *Damage-specific deoxyribonucleases*	
	IV. *Topoisomerases*	
	Topoisomerases I	
	Topoisomerases II	
	V. *Recombinase*	

[a] This classification is based on a consensus of criteria as reflected in earlier classification (Linn, 1982).

C. Site-Specificity and Structure-Selectivity

A variety of nucleases such as restriction endonucleases, certain recombinases, and a number of ribonucleases are site-specific. They recognize a sequence of (two or more) nucleotides and make a cut either within these sequences or outside of the recognition sequence. Certain other nucleases such as micrococcal DNase and the topoisomerases also make site-specific cleavages (Horz and Altenburger, 1981). Site-specific cleavage is a characteristic property of several RNases which were used in the sequence analysis of RNA (Holley, 1965). However, the apparent site-specificity of certain RNase was found to be dependent on the size, stereospecificity, or secondary structure of the substrate RNA molecules (Penswick and Holley, 1965). Certain nucleases, although not specific to nucleotide sequences, are specific to distortions in the DNA structure caused by a damage in the DNA structure or by mismatches in complementary base pairing. Certain nucleases are specific to cruciform structures or to bends in DNA molecules. However, none are known to make distinctions among the different forms of DNA such as B-DNA and Z-DNA and thus are unable to make cleavages in a specific DNA form. There seems to be an exception to this rule because mung bean nuclease can make a cleavage at the beginning or end of a gene by recognizing a short stretch of Z-DNA, presumably present at sites preceding and following a gene (McCutchen et al., 1984).

In contrast to those nucleases that make cleavage at a particular site with a specific nucleotide sequence, a number of nucleases can instead recognize particular structural characteristics of their corresponding DNA substrates (Suck, 1998). Such structural features for recognition and subsequent action by these structure-specific nucleases may include (a) the strandedness of the substrate nucleic acid molecules such as nucleases P1 and S1, (b) helical parameters (i.e., width and/or flexibility) of DNA grooves such as DNase I, (c) distortion in DNA helical structure due to thymine dimer or abasic sites such as exonuclease III and HAP1, and (d) certain specialized DNA structures including a flap DNA recognized by viral, bacterial, and eukaryotic FEN-1 or a four-way junction of Holliday structure by T4 endonuclease VII or RuvC or yeast enzyme.

Both site-specific or structure-selective nucleases show little sequence homology but contain certain structural motifs that allow such sequence or structural selectivity.

V. METHODS FOR THE STUDY OF NUCLEASES

A. Methods for the Assay of the Enzymatic Activity

Several methods for the assay of nucleases are available. These include:

1. Viscocity measurements: This method (Laskowski and Siedel, 1945) is based on the decrease in the viscosity of a nucleic acid polymer in a solution upon its digestion with nucleases.
2. Spectrophotometric method: This method measures changes in hyperchromacity of nucleic acid after enzyme digestion (Kunitz, 1950; Privat de

Garilhe and Laskowski, 1956). The increase in the optical density of DNA molecules in solution increase upon nuclease digestion because of the release of the nucleotides that absorb more UV light. Such chromic shift is also seen during the process of denaturation and denaturation of DNA molecules in solution.

3. Increase in the amount of inorganic phosphate: Phosphates are released due to phosphatase activity of certain nucleases.
4. Increase in the amount of acid soluble (or decrease in the amount of acid insoluble) radioactivity due to mono- or oligonucleotides released after enzyme digestion of radioactively labeled nucleic acid molecules as substrate (Roth and Milstein, 1952). In the case of damage-specific nuclease, radioactivity can be released only when a radioactively labeled damaged DNA is used as substrate.
5. The release of radioactivity from ^{3}H-labeled DNA substrate, bound (either directly or through anti-DNA antibodies) to plastic depression plates after incubation with nuclease preparation, has been used to measure nuclease activity. This method has proven to be very accurate for nucleases that attack native DNA, single-stranded DNA, and damaged DNA.
6. A rapid and sensitive assay for endonucleolytic activity of DNase is based on the fact that nitrocellulose filters can retain only large fragments of denatured DNA. In this method, radioactively labeled denatured DNA is treated with enzyme and then passed through nitrocellulose filters. The decrease in retention of radioactivity by the filter is correlated to enzyme activity (Eron and McAuslan, 1966; Geiduschek and Daniels, 1965).
7. A change in the size or conformation of the nuclease-treated DNA as determined by their mobility in agarose gels after electrophoresis (Wang, 1971). This method is useful for the assay of the activity of restriction endonucleases, topoisomerases, and recombinase. Histochemical and immunological methods are also available for the *in situ* demonstration of the localization of nucleases (Sierakowska and Shugar, 1977).
8. Several quantitative assays of nucleases are based on the digestion of nucleic acids incorporated into the medium on which an organism secreting nuclease has been grown or when nucleases are added as a disc overlay. Nuclease activity is then visualized as a ''halo'' around the growing colony or around the disc overlay containing the enzyme either after the precipitation of undigested nucleic acid with HCl or due to the change in color of metachromatic dyes bound to the DNA. The use of several dyes such as Toluidine blue and green has been described for the detection of nuclease activity (Lacks et al., 1974). Changes in color due to the release of dye as a result of the digestion of nucleic acid can be visualized as a halo.
9. The activity of nuclease can also be detected on an electrophoretogram. The DNA polyacrylamide gel is appropriately incubated to facilitate the digestion of DNAs by nucleases, and then the gel is stained to visualize DNA. In

such a gel, the nuclease activity is visualized as a band without a stain. These methods of visualization of nuclease activity can be very useful in screening mutants deficient in nuclease activity or in identification of different protein components with nuclease activity, in a cell-free extract resolved by electrophoretic analysis. This aspect is discussed further in the section on genetics of nucleases.

10. Enhancement of a fluorescence signal due to separation of a fluorophore and a quencher (see Figure 1.6) in a DNA probe used as a substrate for digestion by different nucleases such as restriction endonucleases and other DNases and chemzymes has been developed to measure the activity of nucleases (Li et al., 2000; Biggins et al., 2000).
11. Flow cytometry has been developed to measure DNA cleavage by the structure-specific nuclease FEN-1. This technique is very fast: It can measure the enzymatic cleavage in approximately 300 millisecond (Nolan et al., 1996).

The use of a specific method is also dictated by the nature of the enzyme being assayed and the nature of information one wishes to obtain (Schein, 2001). Viscosity and hyperchromacity measurements can readily provide some insight into the mode of the nucleolytic attack by a specific nuclease. Snake venom phosphodiesterases can cause a significant change in the hyperchromacity of calf thymus DNA without making any significant change in the viscosity of the DNA. This suggests that the snake venom enzyme is an exonuclease; in contrast, pancreatic DNaseI can cause significant drop in viscosity of the calf thymus DNA without producing significant changes in the hyperchromacity. This suggests that pancreatic DNaseI is essentially an endonuclease. The micrococcal nuclease, which can produce significant changes in the hyperchromacity and viscosity of DNA, is now known to possess both endo- and exonucleolytic modes of attack. The nature of the products of nuclease digestion has been usually determined by sucrose gradient analysis or by electrophoresis. However, at times, these methods do not provide the complete information regarding the nature of the nuclease digestion. For example, when examined by these methods, a sample of DNA of various lengths usually appears as a smear without providing any information about the nature of the smaller molecule. A better picture of the nature of the nuclease digestion products—such as their sizes, nicked or relaxed, and/or strandedness—can be determined by the application of the atomic force microscopy because of the unique ability of this methodology to visualize individual molecules present in the pool of the nuclease digests (Umemura et al., 2000).

B. Methods for the Study and Characterization of Nucleases

A large number of nucleases have been characterized with respect to their biochemical and molecular properties, both as enzymes and as the genes encoding them, using methods of classical biochemistry and genetics and that of molecular biology.

Traditionally, the study of nucleases includes their biochemical purification and characterization of their enzymatic properties such as substrate specificity, K_m and K_i, and other such parameters. Purification may involve several steps such as precipitation with ammonium sulfate and column chromatography, leading to the separation of proteins based on their net electrical (cation/anion) charge, molecular size, and affinity to a particular matrix. Nucleases purified to homogeneity, as ordinarily revealed to contain a single band of protein upon polyacrylamide gel electrophoresis, may further be subjected to crystallization and the determination of three-dimensional (3-D) structure via x-ray and/or NMR analyses leading to establish the active site and the role of the different N- and C-termini peptide segments in determining the enzymatic and other properties of the nuclease. Pancreatic RNase was the first nuclease crystallized (Kunitz, 1940) and its 3-D structure determined. Nucleases are relatively thermostable and have been the favorite of biochemist for the analysis of the structure and function of the proteins. Essentially, purification and crystallization and 3-D structure analysis have been the usual methods to understand the structuture and function of ribozyme nucleases as well. The Hammerhead ribozyme is a typical example of such nucleases other than protein nucleases.

The methods of classical biochemical genetics (Beadle and Tatum, 1941) involving comparision of the properties of the wild-type and mutant enzymes have been routinely used for understanding the structure and function of nucleases as well. Such analysis involves the identification of mutants in the natural population of an organism and/or creation of mutants in the laboratory for the comparision of their biochemical and structural properties. Such analysis has been further aided by the application of *in vitro* mutagenesis to create mutation at a specific site in the gene encoding the nucleases resulting in an enzyme with a site-specific change which is then used to evaluate the role of specific amino acid (or nucleotide in the case of ribozyme) in the enzymatic and other role of the nucleases. Study of nucleases has been further facilitated by the application of the modern methodologies of genomics, proteomics, and bioinformatics, leading to the comparision of their nucleotide and inferred amino acid sequences and their 3-D structure and the identification of different enzymatic and regulatory motifs helpful in the understanding of the multifunctional nature of several nucleases and finally the process of evolution of these nuclease and the role of nuclease in the evolution of the organisms possessing them.

VI. GENETICS OF NUCLEASES AND BIOLOGICAL ROLES

The fact that nucleases were initially obtained from animal pancreas led to the conclusion regarding their possible degradative role. This view was further supported by their occurrence as a lysosomal component in different cells. However, the fact that the cell possesses a variety of nucleases in different cellular components, besides those present in the lysosome, suggests biological roles of nucleases in

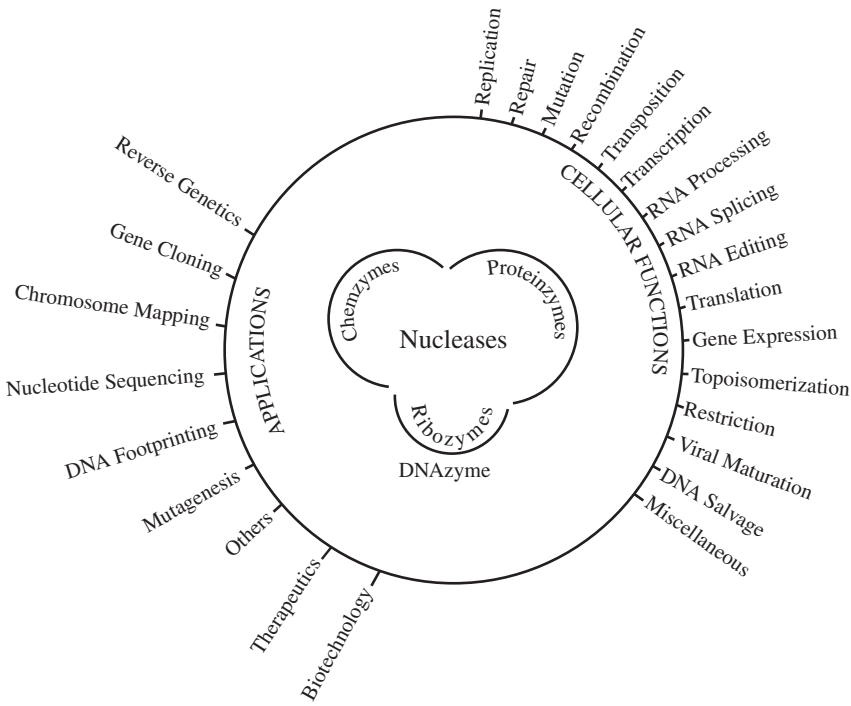


Figure 1.8. Biological role of nucleases and their application.

addition to the degradative role. Nucleases have been shown to participate in a variety of basic cellular and genetic processes.

Within the last few years, the role of nucleases in basic genetic mechanisms (as implied in Figure 1.8) has been elucidated to a great extent. This has been made possible through the genetic analysis of nucleases. A comparison of wild-type and mutant enzymes has been very useful in dissecting the role that a protein may play in controlling a specific biological function. Such an approach, first initiated by Beadle and Tatum (1941), has become crucial for investigating the biochemical, or molecular, basis of any biological component and its function. An alternative method, involving characterization of the gene and its product following the cloning of a specific gene, has also become available due to the development of recombinant DNA technology and *in vitro* site-directed mutagenesis (Jackson et al., 1972; Barany, 1985; Smith, 1985; Clarke et al., 1988; Botstein and Shortle, 1985; Shortle et al., 1981). Such methods of molecular genetics have been utilized in the characterization of micrococcal nuclease (Shortle, 1983). Both methods have been utilized in the study of nucleases.

An assessment of the role that nucleases play in several genetic processes has been made possible by the isolation and characterization of specific genetic mutants. A majority of these mutants have been obtained by the variety of both

general and novel methods. Some of these are summarized here. First, mutants with a possible defect in specific biological processes have been obtained and then characterized to uncover the specific role of different nucleases. Mutants defective in DNA replication, repair, and recombination belong to this group of mutants (see Clark, 1971, 1973; Hanawalt et al., 1979; Kornberg, 1980; Thompson et al., 1980). Second, mutants defective for particular nucleases were characterized in depth to assess their roles in biological processes (Weiss, 1981). Such nuclease defective mutants have been obtained by ''brute force'' methods (Milcarek and Weiss, 1972; Yajko and Weiss, 1975). Weiss and his colleagues (1972, 1975) developed a method to screen large numbers of *Escherichia coli* in order to detect a mutant colony deficient in nuclease. Likewise, yeast nuclease mutants that are defective in splicing of tRNA were identified by the brute force screening of a large number of putative mutants (Winey et al., 1989). Third, nuclease mutants have been isolated using their resistance to particular inhibitors; several topoisomerase mutants have been obtained by this method (Cozzarelli, 1980; Andoh et al., 1987). Fourth, a number of nuclease mutants have been obtained by certain novel methods such as (a) the isolation of *Neurospora* and yeast nuclease mutants by their inability to utilize nucleic acid as the sole phosphorus source in a defined medium (Ishikawa et al., 1969; Hasunuma and Ishikawa, 1972; Forsthoefel and Mishra, 1983; Mishra and Forsthoefel, 1983); (b) the identification of nuclease mutants by the lack of ''halo'' around the growing colony on a DNA–or RNA–agar medium after treatment with trichloroacetic acid or perchloric acid (Badman, 1972; Käfer and Fraser, 1979; Fraser et al., 1980); (c) the isolation of certain nuclease mutants of *E. coli* by their ability to survive in the presence of toluene as bactericidal agent; mutants unable to degrade DNA and RNA from the bacterial cell were able to survive the bactericidal effects of toluene and were identified after staining with basic dyes; (d) isolation of nuclease mutant by the change in the color of the medium containing metachromatic dye as previously mentioned; a number of mutants of *Diplococcus* and *Staphylococcus* have been obtained by this method (Shortle, 1983); (e) isolation of certain nuclease mutants by their altered repressor function; certain recombinases such as yeast Flp and δ resolvase have been known to possess repressor function in addition to their nuclease activity (Lebreton et al., 1988); and (f) the isolation of nuclease mutants following *in vitro* mutagenesis (Shortle, 1983; Botstein and Shortle, 1985). A number of staphylococcal nuclease mutants have been isolated after *in vitro* mutagenesis of the cloned nuclease gene. The mutant colonies were identified by the color change in the medium containing Toluidine blue (Shortle, 1983). Finally, a large number of nuclease mutants in phage, bacteria, fungi, and animal cells have been isolated by their sensitivity to UV light, x-ray, chemical mutagens, and carcinogens.

As depicted in Figure 1.8, there is hardly any basic biological process involving nucleic acids which is independent of participation by nucleases. As a matter of fact, one could learn a good deal about the molecular biology of nucleic acid structure, organization, transactions, and expression just by knowing the various properties of the different nucleases. Some of the properties of nucleases and their possible biological roles have been described earlier (Privat de Garilhe, 1967;

Davidson, 1972; Boyer, 1981, 1982; Linn and Roberts, 1982; Linn et al., 1993). Most of these descriptions are specialized, and at least some of them are now out of date in view of the developments of the last few years. In addition, there has been a change in the emphasis on the nature of approach to study the properties of the enzymes in general, due to the arrival of the techniques of molecular biology and reverse genetics. The new methodology has a tendency to ignore the details of the hard-core biochemistry of protein (Kornberg, 1987). Instead, the methodology of molecular biology relies on deducing the biochemical properties of an enzyme from its cloned DNA sequences. The molecular biological approach has certain advantages over the methods of hard-core biochemistry. First, it is much faster. Second, it can provide a better insight into the functional aspects of the enzyme that cannot be obtained readily, via classical approach of the hard-core biochemistry. The identification and characterization of a number of recombinases and other nucleases would not have been possible either without the molecular cloning and the amplified expression of the genes encoding these enzymes, at times via *in vitro* transcription and translation or without characterization of gene and enzyme after genetic complementation of a defective function in the mutant by the cloned wild-type gene.

An attempt is made in this book to provide the reader with an introduction to the properties and biological roles of nucleases, discuss their contribution to the emergence of modern biology, and, above all, elucidate the role of nucleases in evolution via their participation in various aspects of DNA transaction such as DNA replication, repair, recombination (including transposition), and transcription. Nucleases are the only common group of enzymes that participate in all these genetic processes by acting directly or in conjunction to other proteins, or in response to environmental (or SOS) factors. In addition, nucleases are triggered during the apotosis of cells and are therefore crucial for controlling the life and death of cells and in causing cancer. In addition, nucleases are the basis for a number of devastating human diseases such as blindness or Kalazar in tropical countries caused by parasites like trypanosomes and leschmania. Nucleases also control the unfolded protein response (UPR) which is the cause of several other devastating human diseases (such as cystic fibrosis, amphysema, and osteoporesis imperfecta). On the other side of the world of ribonucleases, DNase has been used to improve the lung function of a large number of cystic fibrosis patients, and certain RNases such as onconase is under stage III of trial for treatment of cancer. Above all, nucleases have been instrumental in the development of recombinant DNA-based biotechnology.

VII. APPLICATIONS OF NUCLEASES

There are several major considerations underlying the application of nucleases. The first is their utility in (a) the construction of recombinant DNA molecules crucial for gene-cloning, (b) the generation of RFLP useful for identification of genes controlling diseases, and (c) the mapping of genes on chromosomes and utilization in

forensic science. The second is the fact that certain nucleases are the target of anti-exitcancer drugs such as camptothecin, which interferes with resolution of the DNA-topoisomerase intermediate in cancer cells, leading to cell death; thus the understanding of nucleases structure and function can become the basis for drug development; likewise the manipulation of certain recombinases such as cre-lox could be utilized for temporal control of the expression of certain genes, as already seen to occur in nature by various microorganisms. The third is their ability to control the expression of certain genes by destroying certain mRNA; ribozymes have been exclusive tools for this approach called *antisense strategy*, which is being developed to treat certain diseases via control of the expression of certain genes. The fourth nucleases are known for their notoriety in digesting nucleic acid molecules during the purification of the latter for scientific or commercial and other investigative purposes; therefore, much of the application strategy is directed in protecting the digestion of the nucleic acids. The fifth is about the strategy of developing nucleases that completely removes nucleic acids in the cell extract in order to facilitate the preparation of proteins by reducing the viscosity of the cell extracts or to remove the viscous material in the lungs of patients suffering from the genetic disorder cystic fibrosis due to the abundance of nucleic acid in the bacterial cellular debris. The sixth basis for the application of nucleases includes breakdown of oligonucleotides by nucleases for the making of certain additives used as food flavoring. Finally, nucleases are used for diagnostic purposes as an indicator of environmental pollutants or infectants.

The fact that nucleases can be obtained in the purified form in a large amount has led to their utilization in science, medicine, and biotechnology. In 1970, the discovery of restriction enzyme opened the avenue for the cloning of genes and led to the coming of biotechnology based on recombinant DNA technology. Nucleases have been utilized as the major tool in developing the methodologies for recombinant DNA technology and molecular cloning of genes. Genentech, one of the first biotechnology companies founded in 1976 based on recombinant DNA technology, developed the procedure for producing human insulin; this was the first drug produced by recombinant DNA technology that was approved by the US Food and Drug Administration (FDA) and later marketed by Eli Lilly Company. In 1985 Genentech became the first company that developed human growth hormone via gene cloning and marketed this hormone for the treatment of children with genetic growth disorders. In 1995 the complete genome sequence of the first organism, *Hemophilus influenzae*, was obtained. This was followed by deciphering a number of other organisms, including yeast and fruit flies. Finally, in the spring of 2000 the announcement of the completion of the entire sequence of human genome was made possible; none of these were feasible without the application of recombinant DNA technology and gene cloning utilizing nucleases. Nucleases have been used for the diagnostic purposes in identifying the genes controlling human and other diseases and for the treatment of certain diseases. The gene controlling Huntington's disease was the first human disease gene that was cloned by positional cloning utilizing nucleases. The genetic disorder of the severe combined immunodeficiency disease (SCID) was the first human genetic disease that was treated by gene therapy

using the cloned human adenosine deaminase (ADA) gene; the SCID patients are defective for the ADA gene. Nucleases find their application in forensic science by their utilization in DNA fingerprinting/DNA profiling. Industrial use of nucleases involves making of certain sweetener and flavoring materials. Nucleases have great potential to be used in medicine and agriculture via the application of recombinant DNA technology and of course in tackling many environmental problems.