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Molecular Pathology in Clinical Practice

Second Edition

Springer

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To Greg
With love and thanks

Preface

Today, molecular and genomic information is informing the patient care decisions in many, if not most, areas of healthcare. Clearly, cancer diagnosis, prognosis, and treatment are driven largely by the molecular variants that drive the cancer and are the targets for new therapies. Medical genetics is moving beyond the classic single gene genetic disorders as we understand the genetic risk factors that drive the common chronic diseases that are costly to our healthcare system. While the clinical relevance of all areas of the human genome is not yet understood, our knowledge is growing rapidly and expanding well beyond the protein-coding genes to include many regulatory-coding regions, such as microRNAs and long noncoding RNAs (lncRNAs), in regions of the genome which used to be considered "junk." For infectious diseases, we are beginning to understand not only the well-known and emerging infectious agents, but that health and disease also relates to the symbiotic relationship of each patient with their microbiomes. Finally, the technologies available to the clinical molecular laboratory have advanced so the genome of individual patients can be analyzed for clinical care, even resulting in the definition of genomic critical values, which are recommended to be reported any time an exome or genome is sequenced for clinical purposes.

Molecular Pathology in Clinical Practice addresses all areas of clinical molecular pathology practice in a single textbook. This second edition has 12 new chapters, in addition to updates on the chapters from the first edition. The new chapters cover diseases not included in the first edition, plus two chapters on next-generation sequencing applications in genetics and cancer, and a proteomics chapter. The purpose of this textbook remains to provide a comprehensive reference for the practicing molecular pathologist as well as a resource for pathologists in any area of practice. The book also will continue to be used by training programs, both for Anatomic and Clinical Pathology and for Molecular Genetic Pathology trainees. This book is not meant to be a recipe book for clinical molecular tests, simply because the specifics of testing change quite rapidly in molecular pathology as new technologies emerge and are integrated into clinical molecular practice. Instead, the emphasis remains the molecular variants being detected for clinical purposes, the clinical usefulness of molecular test results, and the clinical and laboratory issues that require special attention. While this textbook focuses on molecular and genomic testing, with only a single chapter covering proteomics, the reader must understand that the genome does not drive all disease and health, but works in concert with the environment, the metabolome, the methylome, and other determinants of disease and health.

As we move toward genomic medicine, the molecular pathologist and all pathologists will play a significant role in the proper utilization of molecular and genomic tests to improve patient outcomes and the cost-effectiveness of the care we deliver. In the era of US healthcare reform, the promise of genomic medicine aligns almost perfectly with the healthcare reform goals of improving individual patient outcomes, improving the health of populations, and reducing the cost of healthcare. While much of genomic research focuses on the clinical

significance of pathogen and patient genomic variants for diagnosis and therapy, evidence of the value of genomics in clinical care also is needed, especially as we move toward population health management and global payment models.

My hope is that you apply the information in *Molecular Pathology in Clinical Practice* to the care you provide for your patients.

Burlington, VT, USA Debra G.B. Leonard

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Basics of Molecular Biology

1

Deborah Ann Payne

Abstract

Molecular biology entails the analysis and study of the chemical organization of the cell. Molecules comprise the smallest chemical component capable of performing all the activities (structural or catalytic) of a substance. One or more atoms constitute each molecule. Many molecules comprise the various cellular and subcellular components of an organism. Molecules form not only the physical structure of the organism but communicate information between the various compartments of the cell. This communication can be the transfer of information from DNA to RNA and finally to protein or the subtle regulation of the cell's internal homeostatic processes. This communication relies on the interaction of various molecules to insure the fidelity of the message or cellular regulation. This chapter describes the physical organization of cells, cellular organelles, and molecules important in cell division, inheritance, and protein synthesis and describes how genetic information is communicated within the cell.

Keywords

Molecular biology • Genetic • Gene • Nucleic acids • DNA • RNA • Protein • Nucleotides • Amino acids • Codon • Transcription • Translation • Replication • Chromatin • Chromosomes • Complementary • Cell cycle • Hybridization • Denaturation • Mitochondria • Mutation • Ribosome • Polymerase • Exon • Intron

Introduction

Molecular biology entails the analysis and study of the chemical organization of the cell. Molecules comprise the smallest chemical component capable of performing all the activities (structural or catalytic) of a substance. One or more atoms constitute each molecule. Many molecules comprise the various cellular and subcellular components of an organism. Molecules form not only the physical structure of the organism but communicate information between the various compartments of the cell. This communication can be the transfer of information from DNA to RNA and finally to protein or the subtle regulation of the cell's internal homeostatic processes. This communication relies on the interaction of various molecules to insure the fidelity of the message or cellular regulation. This chapter describes the physical organization of cells, cellular organelles, and molecules important in cell division, inheritance, and protein synthesis and describes how genetic information is communicated within the cell.

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Organization of the Cell

The cell is a mass of protoplasm surrounded by a semipermeable membrane [1]. Cells constitute the smallest element of living matter capable of functioning independently; however, within complex organisms, cells may require interaction with other cells. To function independently, cells must produce nucleic acids, proteins, lipids, and energy. In complex

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organisms, these organic processes form and maintain tissues and the organism as a whole.

Genes consist of discrete regions of nucleic acids that encode proteins, and control the function of the cell. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) comprise the two types of nucleic acids found in all cells. Chromosomes, made up of double-stranded DNA complexed with proteins, contain all the genes required for the cell to live and function.

Prokaryotic Cells

Prokaryotic cells are simple organisms lacking subcellular compartments, such as bacteria. The majority of prokaryotic nucleic acids form circular strands comprising approximately 1×10^6 base pairs (bp) (Table 1.1) [2]. Additional extrachromosomal genetic elements consist of circular plasmids also known as episomes and linear mobile genetic elements called transposable elements or transposons. Plasmids range in size from 2,686 to 500,000 bp and first gained notoriety in the 1950s by being associated with antibiotic resistance in bacteria [3, 4]. Transposons also may confer antibiotic resistance on the host bacteria. All these genetic elements exist in direct contact with the bacteria's cytoplasm.

Table 1.1 Comparison of sizes (in base pairs) of various genetic elements [2–5]

Genetic element	Size in base pairs
Human chromosome	3.3×10^9
Bacterial chromosome	$1–4 \times 10^6$
Mitochondrial chromosome	16,569
Bacteriophage	39,000
CAM plasmid	500,000
pUC19 plasmid (engineered plasmid)	2,686
Retrotransposon (i.e., SINE to LINE 1)	75–7,000
Long intergenic noncoding RNA (lincRNA)	>200
Transcribed ultraconserved regions (T-UCR)	>200
Telomeric repeat containing RNAs (TERRA)	>200
Small nucleolar RNA (snoRNA)	60–300
Promoter upstream transcripts (PROMPTs)	<200
Promoter-associated small RNAs (PASR)	22–200
Transcription start site-associated RNA (TSSa-RNA)	20–90
PIWI-interacting RNA (piRNA)	26–31
microRNA (miRNA)	22
Transcription initiation RNA (tiRNA)	17–18

Eukaryotic Cells

Cytoplasm

In contrast to prokaryotic cells, eukaryotic cells are complex, highly compartmentalized structures. The cytoplasm contains multiple membrane-bound compartments known as organelles. The cellular membrane separates the cellular cytoplasm from the external environment. The membranes consist of hydrophobic lipid bilayers. The lipid bilayer contains proteins that serve as receptors and channels.

Nucleus and Nucleolus

The nucleus of the cell contains the cell's linear chromosomes and serves as the primary locus of inherited genetic material. Inner- and outer-pore-containing membranes define the nucleus and separate the chromosomes from the surrounding cytoplasm. Further partitioning occurs within the nucleus to generate the nucleolus, which functions as the ribosome-generating factory of the cell. Instead of additional membranes, fibrous protein complexes separate the nucleolus from the rest of the nucleus. In this structure, the nucleolus organizer (a specific part of a chromosome containing the genes that encode ribosomal RNAs) interacts with other molecules to form immature large and small ribosomal subunits. Following processing, immature subunits depart the nucleolus and enter the nucleus. Eventually, mature ribosomal subunits and other molecules exit the nucleolus through the nuclear pores and enter the cytoplasm.

Mitochondria

Mitochondria are membrane-bound organelles within the cytoplasm of cells that have several cellular functions. Inheritable genetic material, independent from the nuclear chromosomes, resides in mitochondria. These maternally derived organelles contain their own circular chromosome (16,569 bp) and replicate independently from the cell and one another. As a result, not all mitochondria in a given cell have the same mitochondrial DNA (mtDNA) sequence. The genetic diversity of these organelles within and between different cells of the same organism is known as heteroplasmy. A range (approximately 39–1,283) of mitochondrial genomes are present per cell, and this number may vary with different disease states [6, 7]. Mitochondrial genes encode mitochondria-specific transfer RNA molecules (tRNA). In addition, the mtDNA contains genes that encode proteins used in oxidative phosphorylation, including subunits of the cytochrome c oxidase, cytochrome b complex, some of the ATPase complex, and various subunits of NAD dehydrogenase. Other components of the oxidative phosphorylation pathway are encoded by nuclear genes. For this reason, not all mitochondrial genetic diseases demonstrate maternal transmission. Mutations associated with mitochondrial diseases can be found at MITOMAP

(http://www.mitomap.org/MITOMAP). The higher copy number per cell of mtDNA compared with genomic DNA (i.e., approximately 100 to 1) enables the detection and characterization of mtDNA from severely degraded samples and scant samples. For this reason, mtDNA is suitable for paleontological, medical, and forensic genetic investigations. Analysis of mtDNA has applications for diagnosis of mitochondrial-inherited genetic diseases, disease prognosis, as well as forensic identification of severely decomposed bodies [6–9].

Other Cellular Organelles

Membranes not only segregate heritable genetic molecules into the nucleus and mitochondria, but also separate various cellular functions into distinct areas of the cell. The compartmentalization of cellular functions (such as molecular synthesis, modification, and catabolism) increases the local concentration of reactive molecules and improves the biochemical efficiency of the cell. This partitioning also protects inappropriate molecules from becoming substrates for these processes. One example of this segregation is the endoplasmic reticulum (ER), which consists of a complex of membranous compartments where proteins are synthesized. Glycoproteins are synthesized by ribosome-ER complexes known as rough ER (RER), while lipids are produced in the smooth ER. The Golgi apparatus possesses numerous membrane-bound sacs where molecules generated in the ER become modified for transportation out of the cell. In addition, peroxisomes and lysosomes segregate digestive and reactive molecules from the remainder of the cellular contents to prevent damage to the cell's internal molecules and infrastructure. The pathologic accumulation of large molecules within lysosomes occurs when enzymes cannot chemically cleave or modify the large molecules. Lysosomal storage and mucopolysaccharide storage diseases are associated with a variety of genetic variants and mutations. Similarly, peroxisomal diseases are associated with genetic defects in the peroxisomal enzyme pathway [1].

Biological Molecules

Carbon can covalently bond to several biologically important atoms (i.e., oxygen, hydrogen, and nitrogen) and forms the scaffold for all biomolecules. Basic subunit biomolecules can combine to form more complex molecules such as carbohydrates, nucleic acids, and amino acids.

Carbohydrates

Carbohydrates serve as energy reservoirs and are a component of nucleic acids. In addition, carbohydrates also attach to lipids and proteins. The basic unit of a carbohydrate consists of the simple sugars or monosaccharides. These molecules have carbon, oxygen, and hydroxyl groups that most commonly form ringed structures. The oxygen can react with the hydroxyl group of another simple sugar to form a chain. As a result, the formula for a simple sugar is $(CH_2O)^n$, where n represents various numbers of these linked building block units.

Two pentose sugars, deoxyribose and ribose, comprise the sugar element of DNA and RNA molecules, respectively. As the name indicates, deoxyribose ("de-," a prefix meaning "off" and "oxy," meaning "oxygen") lacks one hydroxyl (OH) group compared with ribose.

Nucleic Acids

Nucleic acids are composed of chains of nucleotides. Each nucleotide is composed of a sugar (either ribose or deoxyribose), a phosphate ($–PO_4$) group, and a purine or pyrimidine base. The nucleotides are joined into a DNA or RNA strand by a sugar-phosphate-linked backbone with the bases attached to and extending from the first carbon of the sugar group. The purine and pyrimidine bases are weakly basic ring molecules, which form N-glycosidic bonds with ribose or deoxyribose sugar. Purines are comprised of two rings, a six-member ring and a five-member ring ($C_5H_4N_4$), while pyrimidines consist of a single six-member ring ($C_4H_2N_2$). Purines (guanine, G, and adenine, A) pair with pyrimidines (cytosine, C, and thymine, T) via hydrogen bonds between two DNA molecules (Fig. 1.1). The additional hydrogen bond that forms between G and C base pairing (i.e., three hydrogen bonds) dramatically enhances the strength of this interaction compared to the two hydrogen bonds present between A and T nucleotides. This hydrogen-bonding capacity between G:C and A:T forms a pivotal molecular interaction for all nucleic acids and assures the passage of genetic information during DNA replication, RNA synthesis from DNA (transcription), and the transfer of genetic information from nucleic acids to the amino acids of proteins.

Numerous types of base modifications increase the number of nucleotides beyond the classic four types (i.e., A, T, G, and C). Although these modifications do not alter the base's hydrogen bonding characteristics, modified nucleotides serve various functions in the cell including (1) regulating gene function, (2) suppressing endoparasitic sequence reactivation, (3) identifying DNA damage, and (4) facilitating translation. Modifications such as 5-methylcytosine, 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine influence gene expression. Most endoparasitic sequences such as retrotransposons (e.g., long interspersed nucleotide elements [LINE 1]) are hypermethylated in normal tissue but hypomethylated in cancer tissue [10].

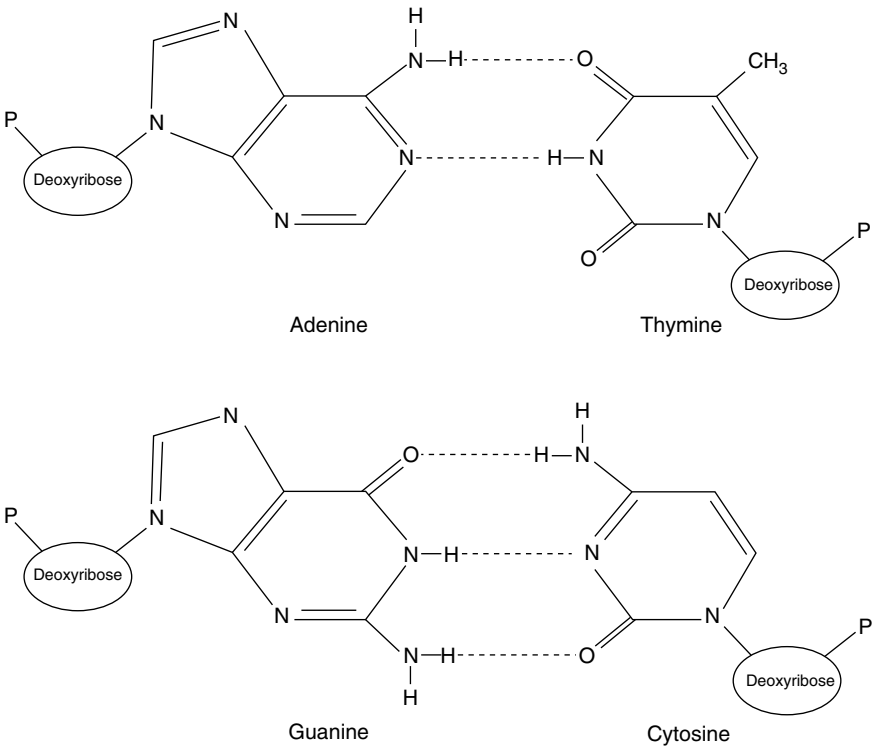

Figure 1.1 DNA base pairing. DNA nucleotides are composed of three moieties (e.g., sugar, base, and phosphate groups). The bases are either purine (adenine and guanine) or pyrimidine (thymine and cytosine). Note the difference in hydrogen bonds between adenine and thymine base pairs, with two hydrogen bonds, compared to cytosine and guanine base pairs, with three hydrogen bonds. Reprinted with permission from Leonard D. Diagnostic Molecular Pathology. 2003:1–13. Copyright Elsevier (2003)

Presumably the hypermethylation of the LINE 1 sequences prevents various insults to the host genome by inactivating the ability of these elements to transpose themselves. Methylation also regulates the phenomenon of imprinting. Methylation mechanisms include P-element-induced wimpy testis (in *Drosophila*, PIWI) proteins and PIWI-interacting noncoding RNAs (specifically, piRNA) [11]. Additionally, certain modifications such as 8-oxoguanine and 8-oxoadenine are associated with DNA damage. Base pair modifications are not limited to DNA but also influence the function of tRNAs [12]. Some of these modifications include 5-formylcytidine, queuosine, 5-taurinomethyluridine, and 5-taurinomethyl-2-thiouridine. Certain tRNA modification defects result in mitochondrial disease [13]. Modifications of rRNA include 2′-O-methylation and pseudouridylation and enable rRNA folding and stability. Such modifications result from interactions of the bases with small nucleolar ribonucleoproteins and noncoding small nucleolar RNAs [5]. With the advent of methodologies that simplify the detection of modified bases, the role of modified bases in human disease may become better understood [14].

Amino Acids

Amino acids are the building blocks of proteins. Amino acids linked together via peptide bonds form large, complex molecules. Amino acids consist of an amino group (NH_3), a carboxyl group (COO–), an R group, and a central carbon atom. The R group can be a simple hydrogen, as found in glycine, or as complex as an imidazole ring, as found in histidine. Twenty different R groups exist (Table 1.2), and determine whether an amino acid has a neutral, basic, or acidic charge. The amino group of a polypeptide is considered the beginning of the protein (N-terminus), while the carboxyl group is at the opposite end, providing directionality to the protein.

Genetic Molecules

Nucleic acids encode genetic information but also participate in additional physiological processes ranging from metabolism to energy transfer. Nucleotides constitute the monomeric units of nucleic acids (Fig. 1.1). Nucleosides consist of two components (ribose or deoxyribose in RNA and DNA, respectively, and either a purine or pyrimidine base). A nucleotide is produced from a nucleoside by the addition of one to three phosphate groups through a covalent bond with the hydroxyl group of the 5′ carbon of the nucleoside's sugar ring.

Nucleic acids consist of chains of nucleotides linked by phosphodiester bonds between the 3′ carbon of the first nucleotide's sugar ring and the 5′ carbon of the adjacent nucleotide's sugar ring. The phosphodiester linkages cause nucleic acids to have a 5′ to 3′ directionality. The alternating

Table 1.2 Amino acids

Amino acid	Amino acid symbols		Linear structure
	Three letter	Single letter	
Alanine	ala	A	CH_3–$CH(NH_2)$–COOH
Arginine	arg	R	HN=$C(NH_2)$–NH–$(CH_2)_3$–$CH(NH_2)$–COOH
Asparagine	asn	N	H_2N–CO–CH_2–$CH(NH_2)$–COOH
Aspartic acid	asp	D	HOOC–CH_2–$CH(NH_2)$–COOH
Cysteine	cys	C	HS–CH_2–$CH(NH_2)$–COOH
Glutamic acid	glu	E	HOOC–$(CH_2)_2$–$CH(NH_2)$–COOH
Glutamine	gln	Q	H_2N–CO–$(CH_2)_2$–$CH(NH_2)$–COOH
Glycine	gly	G	NH_2–CH_2–COOH
Histidine	his	H	**N**H–CH=N–CH=**C**–CH_2–$CH(NH_2)$–COOH
Isoleucine	ile	I	CH_3–CH_2–$CH(CH_3)$–$CH(NH_2)$–COOH
Leucine	leu	L	$(CH_3)_2$–CH–CH_2–$CH(NH_2)$–COOH
Lysine	lys	K	H_2N–$(CH_2)_4$–$CH(NH_2)$–COOH
Methionine	met	M	CH_3–S–$(CH_2)_2$–$CH(NH_2)$–COOH
Phenylalanine	phe	F	Ph–CH_2–$CH(NH_2)$–COOH
Proline	pro	P	**N**H–$(CH_2)_3$–**C**H–COOH
Serine	ser	S	HO–CH_2–$CH(NH_2)$–COOH
Threonine	thr	T	CH_3–$CH(OH)$–$CH(NH_2)$–COOH
Tryptophan	trp	W	**Ph**–NH–CH=**C**–CH_2–$CH(NH_2)$–COOH
Tyrosine	tyr	Y	HO–Ph–CH_2–$CH(NH_2)$–COOH
Valine	val	V	$(CH_3)_2$–CH–$CH(NH_2)$–COOH

The two bolded atoms in each of histidine (N–C), proline (N–C), and tryptophan (Ph–C) are covalently bonded to each other. Ph is a phenyl ring.

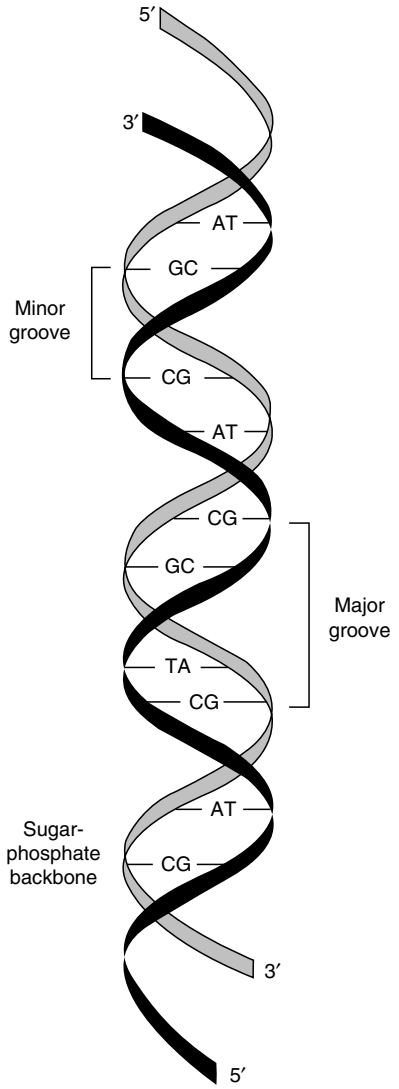

Figure 1.2 Double-stranded DNA. The two DNA strands are oriented in an antiparallel relationship, with asymmetric base pairing of two DNA strands that generates the minor and major grooves of the DNA double helix. Reprinted with permission from Leonard D. Diagnostic Molecular Pathology. 2003:1–13. Copyright Elsevier (2003)

sugar-phosphate chain forms a continuous molecule with bases extending from the 1′ carbon of each sugar. For this reason, the sugar-phosphate chain is referred to as the backbone of nucleic acids (Fig. 1.2). The phosphate groups give nucleic acids a negative charge that imparts important physiochemical properties to nucleic acids. The negative charge of DNA facilitates the binding of mammalian DNA to various proteins and allows separation of nucleic acid molecules by charge and size during gel or capillary electrophoresis.

Structure

In double-stranded DNA, the two DNA strands are held together by exact A:T and G:C hydrogen bonding between the bases of the two strands, in which case the two strands are said to be complementary. The two strands are oriented in opposite 5′ to 3′ directions, such that one strand is oriented 5′ to 3′ and the complementary strand is oriented 3′ to 5′ in an antiparallel fashion (see Fig. 1.2). In this case, "anti-" refers to the head (or 5′ end) of one DNA strand being adjacent to the tail (or 3′ end) of the opposite strand.

The molecular curves of the two DNA strands form antiparallel helices known as the DNA double helix. This double-helix form (the B form) has ten nucleotide base pairs per turn, occupying 3.4 nm. Because the bonds between the sugar and the base are not perfectly symmetrical, the strands curve slightly. The slight curve of the offset glycosidic bonds results in major and minor grooves characteristic of the B form of the double helix [15]. Many clinical molecular tests target the minor groove of DNA with sequence-specific probes known as minor groove-binding (MGB) probes. Two other forms of DNA exist as the Z and A forms. The Z form