# PHYSIOLOGY OF THE GASTROINTESTINAL TRACT

FOURTH EDITION

Volume 1

Editor-in-Chief • Leonard R. Johnson

Associate Editors

Kim E. Barrett • Fayez K. Ghishan
Juanita L. Merchant • Hamid M. Said
Jackie D. Wood

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**FOURTH EDITION**

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# Physiology

## of the

# Gastrointestinal Tract

**FOURTH EDITION**

*Volume 1*


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# Contents

## Section II. Neural Gastroenterology and Motility

## Section V. Digestion and Absorption

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# Preface to the First Edition

As with any publishing venture and especially one of this magnitude, one must first ask, "Why?" The Associate Editors and I were motivated primarily to collect in one set of volumes the most up-to-date and comprehensive knowledge in our field. Nothing comparable has been attempted in the area of gastrointestinal physiology during the past fourteen years. During this time, there has been a rapid expansion of knowledge and many new areas of investigation have been initiated.

More than fifty leading scientists—physiologists, clinical specialists, morphologists, pharmacologists, immunologists, and biochemists—have contributed chapters on their various areas of expertise for these volumes. Our original goal was to review the entire field of gastrointestinal physiology in one work. After examining all of the chapters, however, it was apparent that the final product encompassed more than physiology. The chapters reflect the backgrounds of the authors and the approaches of their different disciplines. As such, these volumes contain information for not only the investigator working in these fields but for the clinician or graduate student interested in the function of the gastrointestinal tract. Anyone involved in teaching gastrointestinal physiology of pathophysiology can readily find the latest and most pertinent information on any area in the discipline.

This work is divided into five sections. The first consists of topics such as growth, the enteric nervous system, and gastrointestinal peptides, each of which relates to all areas of the gastrointestinal tract. The second section contains material describing smooth muscle physiology and gastrointestinal motility. The third section presents treatment of the functions of the stomach and pancreas. The fourth series of chapters treats the entire field of digestion and absorption. These chapters vary from basic electrophysiology and membrane transport to reviews of mechanisms leading to clinical conditions of malabsorption. The final section contains chapters on areas peripheral to physiology (such as immunology, parasitology, and prostaglandins) yet necessary for a comprehensive understanding of the subject.

No one person can presume to organize and edit a scientific work of this scope. I was fortunate to enlist the aid of four preeminent scientists whose expertises cover the entire field. James Christensen was primarily responsible for the chapters on smooth muscle and motility. Eugene D. Jacobson solicited and edited most of the chapters dealing with secretory mechanisms as well as those covering many of the general topics. Chapters relating to regulation were primarily handled by Morton I. Grossman, and those covering aspects of digestion and absorption were organized and reviewed by Stanley G. Schultz. I am exceedingly grateful to these four men without whom this work would not have been possible.

L.R.J.

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# Preface

This fourth edition of *Physiology of the Gastrointestinal Tract* follows 12 years after the third edition. The delay was mainly due to buyouts and mergers of the involved publishing houses, certainly not to a lack of new information. On the contrary, the explosion of information at the cellular level, made possible, in part, by the continued emergence of powerful molecular and cellular techniques, has resulted in a greater degree of revision than that of any other edition. Section I, now titled "Basic Cell Physiology and Growth of the Gastrointestinal Tract," contains numerous new chapters on topics such as transcriptional regulation, signaling networks in development, apoptosis, and mechanisms in malignancies. Most of the chapters in the first section have been edited by Juanita L. Merchant. Section II has been renamed "Neural Gastroenterology and Motility" and has been expanded from 7 chapters with rather classic titles to more than 20 chapters encompassing not only the movement of the various parts of the digestive tract but also cell physiology, neural regulation, stress, and the regulation of food intake. Almost all of the chapters in the second section have been recruited and edited by Jackie D. Wood. Section III is entirely new and contains chapters on "Gastrointestinal Immunology and Inflammation," which were edited by Kim E. Barrett. Section IV, "Physiology of Secretion," consists of chapters with familiar titles but with completely updated information to reflect the advances in our understanding of the cellular processes involved in secretion. Section V, "Digestion and Absorption," contains new chapters on the intestinal barrier, protein sorting, and ion channels, together with those focusing on the uptake of specific nutrients. These chapters have been recruited and edited by Hamid M. Said and Fayez K. Ghishan.

The original purpose of the first edition of this textbook—to collect in one set of volumes the most current and comprehensive knowledge in our field—was also the driving force for this edition. As mentioned earlier, this edition includes completely new chapters that cover many new areas. Although the number of chapters has increased by 15, some chapters from the previous edition have been eliminated, some with identical titles have been written by different authors, and a few have been updated by the original authors. The final product again encompasses more than physiology. The information provided is relevant not only to the researcher in the various specialized areas but also to the clinical gastroenterologist, the teacher, and the student. The authors have done an excellent job of presenting their knowledge in a style that is readable and understandable.

Much of the effort in organizing and editing these volumes has come from five preeminent scientists whose interest and expertise cover the entire field. Drs. Barrett, Ghishan, Merchant, Said, and Wood met with me to decide on chapter topics, authors, and the overall organization of the material. They were responsible for recruiting authors and for the scientific editing of most chapters. The enthusiasm and abilities of these individuals simplified my task as editor, and without them this work would not have been possible. I also am especially grateful to Philip Carpenter of Elsevier, who contacted authors, tracked submissions, and assisted me in many ways.

My Associate Editors and I are all grateful to the contributing authors who were generous enough to make their expert knowledge available. Their efforts have made this work more than a mere review of past contributions to a field. The various chapters synthesize and criticize this accumulated knowledge and identify voids in it, pointing out future directions for research; many of them are superb presentations of information in fields that have been reviewed nowhere else.

L.R.J.

# Acknowledgments

From the organization stage to actual production the following people provided invaluable assistance, helpful suggestions, and a great deal of support. Their role and efforts have been much more than what is normally provided by a publisher, and I express my thanks to them.
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CHAPTER 1

# Transcriptional and Epigenetic Regulation

Juanita L. Merchant and Longchuan Bai



With the completion of the human genome sequencing project at the dawn of the third modern millennium, we have come to appreciate that we are only at the start of a new era of genomic enlightenment. Perhaps the most important piece of information that we have learned is that the clues to our genetic destiny are contained in more than just the primary sequence of DNA. Apparently, what distinguishes humans from other life-forms, and most interestingly, other mammals, lies in the complex modifications and function of the 20,000 to 30,000 genes. Not only are these 25,000 or so genes alternatively spliced, but these products are chemically modified to change their function. Therefore, as opposed to our genetic template being composed of a mere 25,000 genetic units, we are actually controlled by 25,000 to the $n^{th}$ power. The latter value has yet to be determined, but likely results in an enormous combination of genetic events. This chapter reviews what has led us to reformulate our notions of gene expression in the postgenomic era.

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## OVERVIEW OF GENE ORGANIZATION

### Gene Composition

The molecular definition of a eukaryotic gene is complex, but in the simplest terms, it is a nucleic acid sequence that encodes one polypeptide or messenger ribonucleic acid (mRNA) molecule (1). Genes are composed of two intertwining polymers of DNA that are noncovalently attached to a variety of proteins, including histones and specialized proteins (e.g., polymerases and various accessory proteins). The association of DNA, histones, and specialized nuclear proteins collectively is called chromatin. Chromosomes are composed of continuous strands of chromatin that have been compacted by supercoiling and looping to fit into the nucleus. Most importantly, they are the basic heritable unit in the mammalian cell. In humans, there are 46 chromosomes, or 23 pairs. The smallest unit of the DNA polymer is a nucleotide, a base attached to the first carbon of a five-carbon sugar phosphorylated at its fifth carbon (Fig. 1-1). Nucleosides do not contain phosphates; thus, they differ from nucleotides, which contain one, two, or three

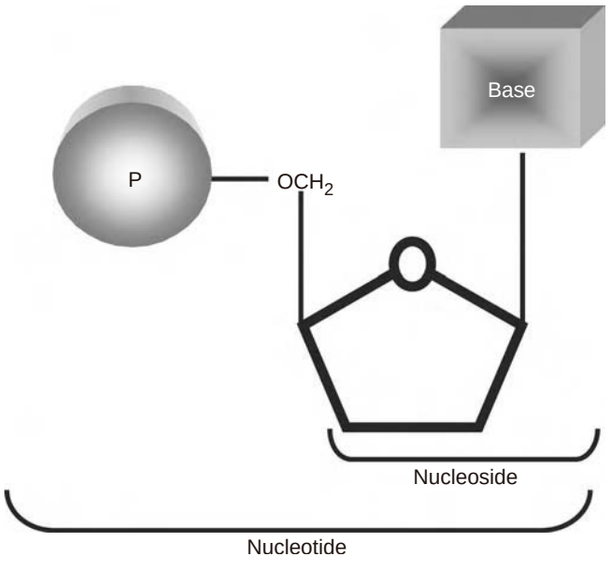


**FIG. 1-1.** Nucleic acid structure. A nucleoside consists of a purine or pyrimidine base covalently linked to the first carbon of the pentose ring. The addition of one, two, or three phosphate groups yields a nucleotide monophosphate, diphosphate, or triphosphate, respectively. The type of sugar determines the type of nucleic acid: ribose in ribonucleic acid (RNA) and deoxyribose in DNA.

phosphate groups. The four nucleotides are distinguished by the type of base that they contain: adenine (A), thymine (T), cytosine (C), or guanine (G). DNA contains the sugar *deoxy*ribose, whereas RNA contains the sugar ribose and the base uracil (U) instead of thymine.

Polymers of nucleotides or nucleic acids (also called nucleoside monophosphates, diphosphates, or triphosphates) are formed when the free phosphate group attached to the fifth carbon of an adjacent nucleotide of the pentose sugar condenses with the hydroxyl group on the third pentose carbon to produce two ester bonds and water (phosphodiester bond). Accordingly, the proximal end of each DNA strand (5′ end) contains a phosphate group in the 5 position of the deoxyribose sugar residue. The terminal nucleic acid at the 3′ end of each DNA strand contains a free hydroxyl group in the 3 position of the deoxyribose ring. By convention, nucleotide sequences are written from 5′ to 3′, reading from left to right, with the sense strand presented as the upper strand. The antisense strand, written on the bottom, is antiparallel and complementary to the sense strand so that the 5′ to 3′ direction proceeds from right to left. Each nucleotide within the polymer is base paired with a particular nucleotide on the opposing strand by hydrogen bonds; adenine pairs with thymine, and guanine pairs with cytosine. The DNA strand containing the same sequence as the mRNA is designated the *sense strand,* and the strand that it pairs with is designated the *antisense strand.* The antisense strand becomes the template sequence that will be transcribed by RNA polymerase II (Pol II) into mRNA and subsequently translated into amino acids.

Most studies on transcriptional control focus on genes transcribed by the seven-subunit enzyme Pol II, and thus are designated as class II genes (2). It is Pol II that is responsible for transcribing gene sequences into protein-encoding mRNA. Only 4% of the total RNA in the cell is mRNA. Many of these initial primary transcripts (heterogeneous nuclear RNA [hnRNA]) are further processed as discussed later. Nine percent of cellular RNA is hnRNA, the bulk of which are small nuclear RNA (snRNA; e.g., U2 involved in RNA splicing, 4%) and small nucleolar RNA (e.g., U22 snoRNA comprising 1%). The other 4% of hnRNA is mRNA. An additional 1% of total cell RNA is called guide RNA, which edits mature mRNA transcripts (3). RNA polymerase I (Pol I) transcribes all of the ribosomal genes except for the *5S* gene. Ribosomal RNA represents about 75% of the RNA in the cell. RNA polymerase III (Pol III) transcribes the *5S* ribosomal gene and the genes encoding transfer RNA. Transfer RNA represents about 15% of the total RNA in the cell. Pol I and III transcribe genes that will not be further translated into peptides, although their primary transcripts are also processed before reaching the cytoplasm. Because Pol II transcribes genes encoding proteins and peptides, Pol II–regulated genes are the primary focus of this chapter.

One may conceive of a gene as being analogous to a long sentence read from left to right and composed of letters organized into words separated by spaces and marks of punctuation. Specific DNA sequences "punctuate" the gene with important start and stop signals for transcription and translation. One gene may comprise several hundred to several thousand DNA base pairs. These base pairs (the alphabet) are organized into functional groups (phrases) based on whether a particular sequence is untranscribed, only transcribed, or both transcribed and translated (Fig. 1-2). Exons are DNA sequences that are transcribed into mRNA by Pol II and *ex*it the nucleus. Within the cytoplasm, exons may or may not be translated into peptides. Those exons that are transcribed *and* translated form the coding sequences (coding exon). In general, the term *intron* is used to describe the *in*tervening DNA sequence that is transcribed but is removed from the primary transcript by RNA splicing (RNA processing) before it exits the nucleus as a mature transcript (see Posttranscriptional Processing later in this chapter and also Chapter 2). DNA sequences or elements that regulate transcription and are not transcribed into mRNA usually reside in the 5′ portion of a gene upstream (to the left of) of the promoter. The promoter is a group of DNA sequences that binds Pol II in concert with accessory proteins to initiate the synthesis of mRNA. Accessory proteins control the accuracy and rate of polymerase binding. The first nucleotide transcribed into mRNA is assigned the number *1* with subsequent nucleotides (downstream or to the right of the promoter) assigned positive numbers as transcription proceeds toward the 3′ end. Nucleotides preceding the promoter (upstream or 5′) are assigned negative numbers. DNA sequences that encode a polypeptide (open reading frame) begin with the translational start site codon ATG (encoding methionine) and end with one of the three stop codons: TAA, TAG, or TGA.

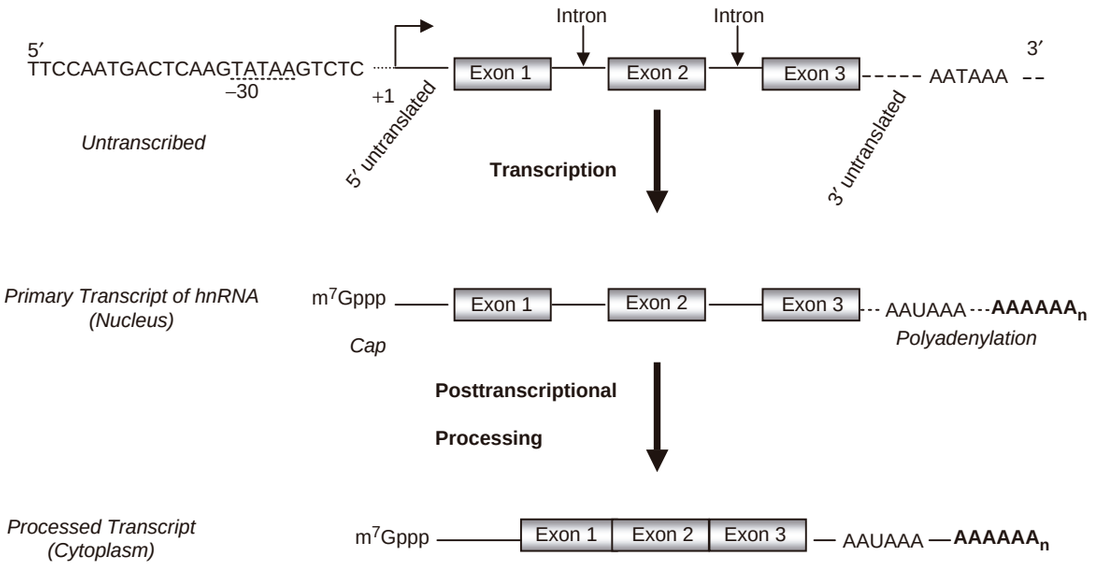


**FIG. 1-2.** Gene structure, transcription, and posttranscriptional processing. A gene is composed of several hundred to several thousand base pairs, subdivided into functional elements. The location of 5′ and 3′ untranslated sequences, exons, and introns are shown. The 5′ flanking sequences contain specific DNA elements (e.g., TATA box). Ribonucleic acid (RNA) polymerase II transcribes DNA into heterogeneous nuclear RNA (hnRNA) during *transcription.* Twenty base pairs after the sequence AATAAA is transcribed to AAUAAA, messenger RNA (mRNA) are cleaved and the polyadenylate tail is added to the 3′ end. A methylated guanylate residue is added to the 5′ end of the mRNA through a triphosphate linkage. Before exiting the nucleus, intron segments are removed by splicing factors during *posttranscriptional processing.*

(The translational start and stop codons, respectively, are transcribed into mRNA as AUG, UAA, UAG, and UGA.) Because one amino acid is encoded by three nucleotides or a triplet (codon), two or three peptides may be encoded by overlapping codons simply by shifting the reading frame by one or two nucleotides. Regulatory sequences that are transcribed but not translated reside at both the 5′ and 3′ ends of the mature RNA transcript. Both 5′ and 3′ untranslated regulatory sequences, which range from 10 to several thousand nucleotides, are thought to participate in the fidelity of translation and mRNA stabilization or destabilization.

RNA molecules that encode proteins (except most histone proteins) are distinguished from ribosomal and transfer RNA by the series of adenosines added to the 3′ end of the molecule (poly(A) RNA; see Fig. 1-2). This feature is a useful means to isolate mRNA from other, more abundant RNA species (transfer and ribosomal RNA) and also designates the functional termination of the protein-encoding portion of the gene. During transcription, the primary RNA transcript is cleaved 20 bp downstream of the AAUAAA site at the 3′ end, and ~150 to 200 adenine nucleotides are added to form the poly(A) tail (4–6). The 5′ end of the mRNA transcript receives a protective "cap" after synthesis of the first 30 nucleotides, which consists of a guanylate residue methylated at the 7 position and linked to the first nucleotide of RNA by three phosphates. The RNA cap is a high-affinity binding site for ribosomes (7,8). Notably, the element AATAA that signals the site of the poly(A) tail is not necessarily the functional end of the gene. Rather, the 3′ untranslated region (3′UTR) and 3′ untranscribed regions may still contain regulatory elements that can modulate gene expression. Therefore, just as the 5′ end of a gene must be determined empirically, so must the 3′ end of the gene.

The 5′ border of a gene is identified by the promoter region (functionally determined) and structurally by the first nucleotide transcribed into mRNA (cap site) as determined by various reverse transcriptase methods—for example, primer extension analysis or anchored polymerase chain reaction (PCR) (9). These techniques use reverse transcriptase to synthesize complementary or copy DNA (cDNA; Fig. 1-3). Radiolabeled primers complementary to the 5′ end of the DNA sequence to be copied are allowed to anneal to mRNA. Reverse transcriptase then adds deoxynucleotides to the primer in the 3′ to 5′ direction. Synthesis of the cDNA will terminate when the 5′ end of the mRNA is reached. Template mRNA molecules are removed by ribonucleases (RNases), and the synthesis of a double-stranded cDNA is completed through the action of DNA polymerase. Because the newly synthesized cDNA is radiolabeled at the 5′ end, the length of the cDNA (and hence the transcriptional start site) is determined by resolving the fragments on a denaturing polyacrylamide gel and comparing the length observed in base pairs to the known cDNA sequence. cDNA is also a useful tool for making probes to detect complementary nucleotide

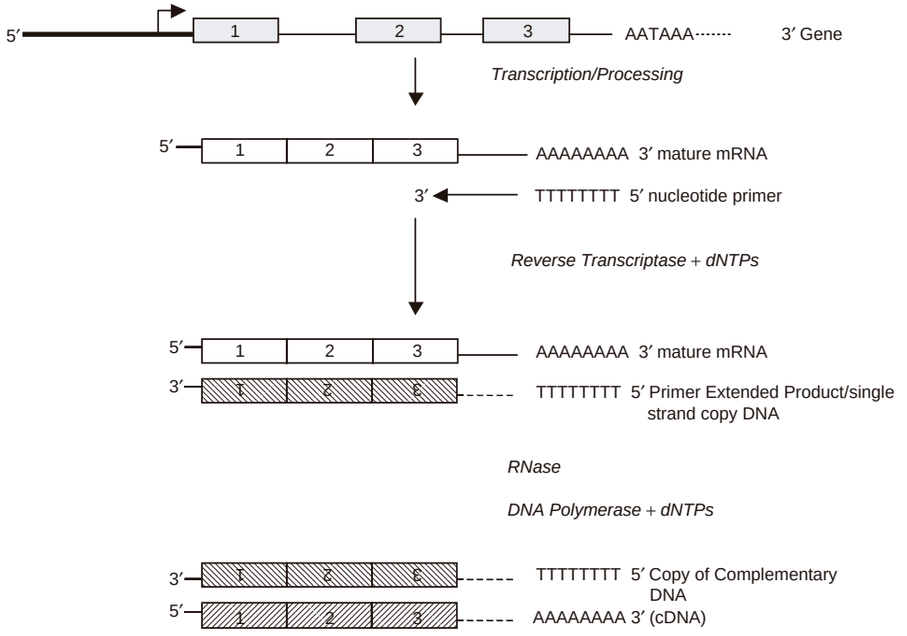


**FIG. 1-3.** Complementary DNA (cDNA). Primers complementary to a portion of the messenger ribonucleic acid (mRNA) are allowed to anneal. For unknown sequences, as in the synthesis of cDNA libraries, a primer complementary to the poly(A) tail is used, that is, poly (dT). Reverse transcriptase added together with all four deoxynucleotides (dNTPs) will transcribe mRNA in the 3′ to 5′ direction to make cDNA. The mRNA template is removed by RNases, and double-stranded cDNA is made using DNA polymerase. In primer extension analysis, the 5′ end of mRNA (the cap site) is identified by annealing primers of a known sequence near the 5′ end of mRNA.

sequences and for making cDNA libraries that reflect the spectrum and relative abundance of specific mRNA within a given cell. These cDNA libraries must be contrasted with genomic phage libraries in which the DNA sequences in the phage heads reflect the number of times that a particular gene sequence is represented in the host genome, which is usually once.

The 5′ sequences flanking the gene are defined functionally by various methods other than simple structural information. These sequences direct the developmental, tissue-specific, and inducible expression of the gene and can range from a few hundred to several thousand base pairs (10). It is possible to identify the sequences conferring these regulated gene activities by using methods such as DNA transfer into cell lines (11,12) and transgenic mouse models (13,14). For example, the expression of gastrin in the adult occurs in the antrum of the stomach and in the first portion of the duodenum (15–17). However, gastrin is never expressed in skin or kidney. Thus, if 1000 bp of 5′ flanking sequence permits the expression of gastrin in a fibroblast or kidney cell line, but 20,000 bp do not, it may be concluded that the untranscribed sequences between −1000 and −20,000 bp from the promoter are important in shutting off expression of gastrin in skin and kidney, sites where gastrin is never expressed *in vivo.* Thus, the 5′ regulatory sequences important in normal expression of the gastrin gene may extend as far upstream as −20,000 bp from the start site of transcription. Alternatively, the 5′ or even 3′ borders may extend even further if functional data indicate that a larger sequence is required for the appropriate tissue and temporal expression to be observed with the native gene. Recently, it has been found that there are specific DNA elements called Insulator elements that mark the boundary of genes (18). These elements, originally identified on the globin gene, bind a transcription factor called CTCF and are capable of preventing the spread of histone acetylation between adjacent genes (19). Specific examples of tissue-specific elements have been reported within the promoters of several gastrointestinal (GI) peptides (e.g., gastrin and secretin), as well as for specific intestinal proteins (e.g., sucrase-isomaltase) (20–26).

Similar experiments may also be performed in transgenic mice with constructs containing various lengths of 5′ flanking sequences regulating reporter gene expression. Instead of transferring these reporter constructs into cell lines, they are injected into fertilized eggs and reimplanted into ovulating female mice to be expressed in the mouse germ line (13,14). The expression of these constructs in the offspring is analyzed by cytochemical detection of reporter gene products in various organs or in response to physiologic inducers (27). The transgenic approach to gene expression, like the experiments described earlier, permits anatomic, environmental, and developmental analysis in the whole animal (28,29). This approach is particularly valuable in understanding the

regulatory sequences important for the tissue-specific expression of the genes in different cell types of the same organ such as the small intestine (30,31). Given the requirement for larger and larger pieces of DNA to recapitulate native expression in transgenic mouse models, techniques have been developed to clone and manipulate large pieces of DNA (more than 50 kilobases; e.g., yeast artificial chromosomes [YACs] and bacterial artificial chromosomes [BACs]) (32,33). Recombineering is a powerful technique performed in bacteria that permits introduction of foreign DNA or point mutations into these large plasmids that are eventually introduced into transgenic mice (34–37).

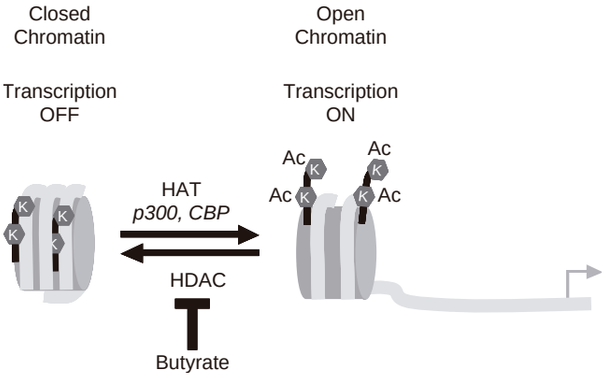


**FIG. 1-4.** Nucleosome structure. The double-strand DNA helix winds twice around a complex of the four core histones assembled as dimers. Unacetylated histones are positively charged and adhere tightly to the negatively charged DNA, preventing access by transcription regulatory proteins. Histones that are acetylated are less positively charged and do not adhere as tightly to chromatin, allowing access of regulatory proteins to the DNA. The addition or removal of acetyl groups to the ends of histones is regulated by histone acetyltransferase (HATs) and histone deacetylase enzyme complexes (HDACs). The short-chain fatty acid butyrate inhibits the activity of HDACs.

## EPIGENETIC INFLUENCES

*Epigenetics* literally means "outside of or beyond genetics," and it refers to the "study of genetic modifications that are mitotically and/or meiotically heritable yet do not change the DNA sequence" (38). Thus, mutations or deletions alter the character or length of the sequence that, in turn, alters the primary sequence of the protein. By contrast, epigenetic influences chemically modify the nucleotide or amino acid structure that, in turn, changes how that particular residue is recognized by nuclear proteins, without changing the sequence itself. Although it is now clear from the completed sequence of the human genome that there are only about 20,000 to 30,000 gene loci, the complexity of the genetic information encoded in human chromosomes must enlist other features of chromatin (39). The epigenetic influences on chromatin appear to be one of the critical features that enhance genomic complexity. Major targets of epigenetic changes are histones, basic proteins coating the naked DNA double helix. The N-terminal tails of histones (H1, H2A, H2B, H3, H4) are positively charged because of the basic amino acid lysine. The positively charged histones attach to DNA because of the negatively charged phosphate backbone of DNA. The ionic interaction is reduced if the positive charge on the lysines is removed. Specific enzymes called histone acetyltransferases (HATs) acetylate the lysine side group, effectively eliminating the positive charge (Fig. 1-4). The loss of the ionic interaction between the histones and phosphate groups on DNA permit greater access to the DNA helix by accessory proteins such as polymerases, transcription factors, and coactivators or repressors. DNA in the form of chromatin becomes open, accessible, and readily transcribed. By contrast, there are enzymes that will "close" chromatin by removing the acetyl groups from the lysines at the N-terminal tails of histone proteins. These enzymes are called histone deacetylases (HDACs). Removal of the acetyl group restores the positive charge to the histones allowing the ionic interaction between histones and DNA to be restored. The nonhistone proteins such as polymerases and transcription factors become excluded from DNA, transcription is silenced, and chromatin is inactive.

Collectively, the histones and accessory proteins associated noncovalently with DNA are what forms chromatin. Chromatin exists in two forms: euchromatin and heterochromatin (40). Euchromatin contains the actively transcribed genes and becomes decondensed during DNA replication. Euchromatin is also centrally located in the nucleus. By contrast, heterochromatin contains transcriptionally silent genes that remain condensed at the periphery of the nucleus. The DNA sequences within heterochromatin are repetitive, and only 15% of nuclear chromatin is heterochromatin. The major forms of epigenetic modifications in mammalian cells occur on DNA and histones and include such covalent modifications as acetylation and methylation, but also via the addition of other organic residues. These epigenetic changes affect such events as chromatin folding, gene expression, X-chromosome inactivation, and genomic imprinting (41). Epigenetic events are essential for development and differentiation, during which clusters of genes must be activated or silenced at precisely timed intervals to allow for the organism's growth and maturation.

### Histone Modifications

The basic repeating unit of chromatin is the nucleosome. Each nucleosome is composed of 147 bp of DNA wrapped twice around a histone protein octamer consisting of 2 molecules of each of the 4 core histones (H2A, H2B, H3, and H4). The linker histone H1 sits alone between each core nucleosome, facilitating further compaction (42). Each histone contains a structured globular domain with a histone-fold motif important for nucleosome assembly and a highly charged unstructured amino-terminal tail of 25 to 40 residues,

which protrudes from the body of the nucleosome to latch onto the phosphate backbone. The amino termini are the major sites for histone modifications (43). Histones can be modified by acetylation, methylation, phosphorylation, adenosine diphosphate (ADP)-ribosylation, ubiquitination, and sumoylation (44). The mixture of these covalent modifications creates a "code" on the surface of the histone molecule that is subsequently recognized by bromo and chromo domain–containing proteins mediating chromatin compaction, transcription, and DNA repair (45). Acetylation, methylation, ubiquitination, and sumoylation occur on the lysine residues, whereas methylation also occurs on arginine residues. Phosphorylation occurs on serines and threonines, and ADP-ribosylation occurs on glutamic acids. Most of these modifications, particularly acetylation, alter the charge distribution on the amino terminus and also alter nucleosome structure, which may, in turn, regulate chromatin structure (46,47). Some covalent modifications act as molecular switches, enabling or disabling subsequent covalent modifications, which explains the functional complexity of epigenetic modifications (48). Therefore, each modification correlates with a specific physical status of chromatin.

### *Histone Acetylation*

Acetylation of histones occurs at the ε-amino side group of specific lysines within the N termini of histones. HATs transfer an acetyl group from acetyl-coenzyme A as a donor to the histone terminal lysines (49). In hypoacetylated chromatin, the positive charges on unacetylated lysines are attracted to the negatively charged DNA, producing compact, closed chromatin thereby repressing transcription (50). In contrast, acetylation of the lysines removes their positive charges, resulting in a less compact, open chromatin structure, which facilitates gene transcription. Therefore, HAT activity, and subsequently histone acetylation, is linked mainly to transcriptional activation (51) (see Fig. 1-4). Removal of the acetyl group (deacetylation) by HDACs restores the positive charge on lysines, and chromatin becomes compacted and less accessible to regulatory proteins required for transcription. Thus, HDACs and deacetylation are primarily associated with transcriptional repression (see Fig. 1-4).

HATs are divided into five families. These include the p300/CBP (cyclic 3′,5′-adenosine monophosphate [cAMP] response element binding [CREB] protein) HATs (p300 and CBP), Gcn5-related acetyltransferases (GNATs; including Gcn5, p300/CBP-associated factor [PCAF], etc.), MOZ, Ybf2, Sas2, and Tip60 (MYST) (monocytic leukemia zinc finger protein [MOZ], Ybf2/Sas3, Sas2, and Tip60)-related HATs, the general transcription factor (GTF) HATs (TFIID subunit TAF250 and TFIIIC), and the nuclear hormone-related HATs (SRC1 and ACTR) (52). The most consistent functional characteristic of the HATs is that they are transcriptional coactivators. These proteins are components of large multisubunit complexes that do not bind DNA directly, but instead form protein–protein interactions with DNA-binding transcription factors (53).

The more numerous mammalian HDACs have been grouped into three protein classes (54). Class I includes HDACs 1, 2, 3, and 8. Class II includes HDACs 4, 5, 6, 7, 9, and 10. The class III HDAC family consists of the conserved nicotinamide adenine dinucleotide (NAD)–dependent Sir2 family of deacetylases. Like HATs, HDACs do not bind directly to DNA but rather are recruited by large multisubunit complexes to function primarily as corepressors of transcription (55).

The function of HATs and HDACs are of particular relevance in the GI tract because of the effect of butyrate, a by-product of colonic bacterial fermentation, on histone acetylation (see Fig. 1-4). Epidemiologic studies uniformly concur that a diet high in fiber is protective against colon cancer (56). The short-chain fatty acid butyrate is one of several fiber-derived fermentation products capable of maintaining epithelial cell differentiation (57). The differentiation effects were initially demonstrated after treatment of erythroleukemic cells with butyrate (58). Subsequently, it was discovered that the induction of differentiation by butyrate correlated with histone hyperacetylation (59–61) due to suppression of HDACs (62–66). Thus, the HDAC effects of butyrate and resulting histone hyperacetylation may, in fact, be one mechanism by which dietary fiber exerts its anticancer effects (67).

Reviews support the viewpoint that butyrate is a potent anticancer agent (68–70). Collectively, early studies emphasized the global effects of butyrate on chromatin remodeling, but the molecular basis for the gene-specific effects of butyrate remains poorly defined. HDAC inhibitors regulate less than 10% of actively transcribed genes. Most of those are up-regulated through GC-rich sites (71,72). In addition to histone acetylation, it is now known that DNA-binding proteins can become acetylated (52). Thus, a possible mechanism by which hyperacetylation induced by butyrate might target specific genes is through acetylation of specific transcription factors. The proposed function of acetylated transcription factors varies and includes increased or decreased DNA binding, as well as protein stability (73). In many instances, the genetic targets of butyrate are GC-rich sequences that bind Sp1 and Sp3. Gamma glutamyl transferase (74), insulin-like growth factor (IGF) binding protein 3 (75), G α(i2) (76), galectin (77), Cox1 (78), and intestinal alkaline phosphatase (79) are all up-regulated by butyrate through Sp1 sites. Sp1 binding sites are also implicated in the butyrate induction of $p21^{WAF1}$ gene expression (80). HAT p300, recruited to the $p21^{WAF1}$ promoter, cooperates with Sp1 and Sp3 to mediate the effects of butyrate (81). However, Sp1 does not cooperate directly with p300, but instead binds HDAC1 (82,83). The Sp1-HDAC1 complex, in turn, forms complexes with other corepressors such as Sin3A (84). Thus, Sp1 appears to be the factor that confers $p21^{WAF1}$ promoter repression by recruiting HDACs and corepressor complexes.

HDACs can have opposing functions, especially in cancer. HDACs can prevent the activation of tumor suppressor genes and block the ability of a cancer cell to undergo

apoptosis (85). However, HDAC2 silencing can trigger apoptosis (86). Another important feature of HDACs is their interaction with DNA methylation. HDACs cooperate with DNA methyltransferases (DNMTs) by removing the acetyl groups that would otherwise block methylation targets on histones or DNA (87,88).

### *Histone Methylation*

There are two types of histone methylation, targeting either lysine or arginine residues. Histone methyltransferases perform these modifications using S-adenosyl-methionine as the methyl group donor. Lysine methylation is implicated in changes in chromatin structure and gene regulation, whereas arginine methylation correlates with the active state of transcription, such as acetylation (89).

#### *Histone Methylation at Lysines*

Methylation of lysines residues (K) occurs on histone H3 primarily at K4, K9, and K27 and on H4 at K20 (Fig. 1-5). The lysine residues can be monomethylated, dimethylated, or trimethylated at the ε-amino group. The methylation of H3 is associated with an open chromatin configuration and gene activation (90,91). In contrast, the methylation of H3 at K9 is associated with condensed, repressed chromatin (92).

In general, there are at least four families of lysine methyltransferases. All of the lysine methyltransferases are distinguished by the presence of Su(var)3-9, Enhancer of Zeste, and Trithorax (SET) domains. The fourth family of these methyltransferases contains other protein domains aside from the SET domain. SET protein domains are approximately 130 residues homologous to amino acid segments in SET, three *Drosophila* proteins with intrinsic methyltransferase activity (93,94). The mammalian form of Su(var)3-9 is SUV39H and is involved in stabilizing heterochromatin by trimethylation of histone H3 at lysine K9. The trimethyl group creates an atomic feature or imprint on H3 that, in turn, is recognized by HP1, a chromatin organization modifier (chromo domain proteins) (95). The methylated or acetylated imprints on DNA or histones are recognized by two classes of proteins: those with chromo domains that recognize methyl group imprints and those with bromo domains that recognize acetyl group imprints. Transcriptional coactivators such as CBP, p300, and PCAF are HATs that contain bromo domains. They acetylate histones and other nuclear proteins; thus, not surprisingly, they also recognize an acetyl group imprint. These proteins are discussed in greater detail later in this chapter in Chromatin-Binding Proteins.

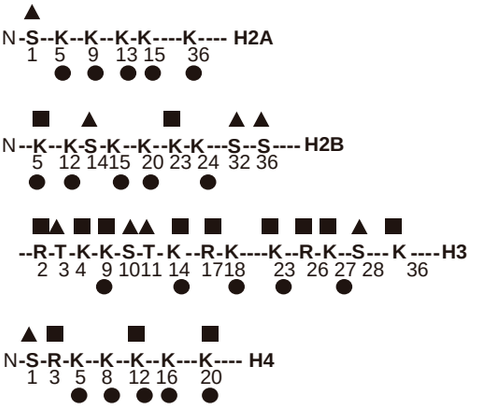


**FIG. 1-5.** Histone modifications on histone tails. Shown are the amino-terminal histone residues modified by acetylation (*filled circles*), methylation (*filled squares*), and phosphorylation (*filled triangles*).

#### *Histone Methylation at Arginines*

Methylation at arginines occurs within the tails of histones H3 (R2, R17, and R26) and H4 (R3) and is catalyzed by coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine *N*-methyltransferase 1 (PRMT1), respectively, in mammalian cells (see Fig. 1-5). Like lysines, arginines can be either monomethylated or dimethylated (asymmetric or symmetric) on the guanidino nitrogen, and this process is antagonized by human peptidylarginine deiminase 4 (PADI4), which converts methyl-Arg to citrulline (96,97). Less is known about the fate of histones methylated at arginines. However, initial studies indicate that the methylated arginines create an imprint recognized by coregulatory molecules, for example, p300 and switching/sucrose nonfermenting (SWI/SNF) (98,99).

### *Histone Phosphorylation*

Histone phosphorylation occurs on all four core histones: H2A (S1), H2B (S14), H3 (S10 and S28), and H4 (S1) (see Fig. 1-5). The phosphorylation of S10 in H3 is associated with transcriptional activation (100) and chromosome condensation during mitosis (101). In addition, phosphorylation of S10 in H3 is also associated with the transduction of external signals to chromatin, leading to the transient expression of immediate early genes (102,103). The phosphorylation of H3 is mediated by several specific kinases, activated by distinct pathways. For example, mammalian mitotic H3 phosphorylation is associated with Aurora B kinases (104,105), H3 phosphorylation by IKKα is important for the activation of nuclear factor (NF)-κB (106), and the immediate early gene response is mediated mainly by *m*itogen and *s*tress-activated *k*inases MSK1 and MSK2 (107). Histone H2B phosphorylation condenses the chromatin and is involved in apoptosis (108,109). The downstream effects of phosphorylation of H2A and H4 are unknown.

Of the histone modifications, acetylation and phosphorylation are reversible. Consequently, if the presence of a modification influences transcription in a particular way, its removal may have the opposing effect. In this way the cell could effectively respond to changes in environmental cues. Different histone modifications may be linked mechanistically. For example, phosphorylation of S10 on H3 enhances

histone acetylation by Gcn5 (110,111), whereas H3 K9 methylation inhibits phosphorylation at H3 S10 (93). Given the number of sites and the variety of possible modifications, the combinatorial possibilities are extremely large. The combinatorial pattern of N-terminal modifications results in a heterogeneous identity for each nucleosome that the cell interprets as a readable code from the genome to the cellular machinery directing various processes to occur. This concept is commonly referred to as the "histone code hypothesis" (45). The precise modification status of a given histone tail on a given gene can also change during the process of transcriptional regulation and each of these different constellations of histone modifications may elicit distinct downstream transcriptional signals (45).

### DNA Methylation

DNA methylation is a postsynthesis modification that normal DNA goes through after each replication. This modification is catalyzed by DNMTs and occurs on the C-5 position of cytosine residues within CpG dinucleotides located primarily in the promoter of a gene. There are three major DNMTs (DNMT1, DNMT3a, and DNMT3b). Each DNMT plays a distinct and critical role in cells. Murine knockouts of DNMT1 and DNMT3b exhibit embryonic lethality (112). The DNMT3a homozygous knockout mouse appeared normal at birth but died by aged 4 weeks (112, 113). In humans, mutations of DNMT3b are linked to ICF syndrome (*i*mmunodeficiency, *c*entromere instability, *f*acial anomalies) (112,114). Sixty percent of human genes contain a CpG island (115). Although methylation can also occur in other parts of the gene, CpG dinucleotides tend to be underrepresented in the genome, and when they are found, they appear in clusters ranging from 0.5 to several kilobases with a GC content greater than 55% (116). These clusters are known as CpG islands (117). Methylation of CpG islands is a late evolutionary development and functions to maintain genome stability by repressing transposons and repetitive DNA elements (118).

DNA methylation is an important player in many processes, including transcriptional repression, X-chromosome inactivation, and genomic imprinting. CpG islands located in the promoter region of genes are normally hypomethylated about 40% of the time (116). Their hypermethylation causes stable heritable transcriptional silencing. As observed with HDACs and deacetylation, the methylation status in cancers may seem contradictory. Aberrant *de novo* hypermethylation of CpG islands is a hallmark of some human cancers and is found early during carcinogenesis (119–121). Tumor suppressor genes are locally hypermethylated in some cancers to silence their expression, whereas oncogenes may be hypomethylated (116). Tumor cells globally demonstrate an overall hypomethylation of DNA, a process that has more recently been linked to nutrition (122). S-adenosylmethionine is the primary methyl donor in the cell and is reduced in conditions predisposed to cancer (123).

Genomic imprinting occurs in gametogenesis and is necessary for development. One of the X chromosomes in female individuals is not expressed because of the heavy methylation of the inactive X chromosome. The epigenetic phenomenon whereby expression of a gene depends on whether it is inherited from the mother or the father is called imprinting, and is caused by differential methylation of specific cytosine bases on the maternal versus the paternal genes.

### Chromatin-Binding Proteins

The remaining histone methyltransferases also recognize methyl groups on other regulatory proteins; therefore, they are discussed here. The second family of SET domain proteins is related to the *Drosophila* protein Enhancer of Zeste, with the prototypical mammalian protein named EZH2. EZH2 is part of a complex of proteins called the Polycomb group (PcG). Two variants of these complexes have been designated Polycomb repression complexes 1 (PRC1) and 2 (PRC2). EZH2 belongs to the PRC2 complex that also includes EED and SUZ12; whereas PRC1 includes the proteins RNF2, HPC, EDR, and BMI1. BMI1 has received increased attention because it is an important marker of normal and cancerous hematopoietic stem cells (124–126). The Polycomb group of proteins with their SET domains not only participates in histone lysine methylation, but the complexes that they form (PRC1, PRC2) are also important in recognizing the methylated protein imprint.

A human homolog of *Drosophila* Trithorax is the mixed leukemia gene 1 (MLL1). There are four human MLL homologs. MLL1 has been shown to be a specific methyltransferase for H3 at K4 (127). In turn, it forms protein–protein interactions with coactivators, for example, CBP and corepressors chromatin remodelers (e.g., SWI/SNF) (128,129). Other Trithorax homologs (e.g., Ash1, Trx) form complexes with different coregulatory complexes. Collectively, members of the Trithorax group (TrG) of proteins can either activate or repress transcription depending on the coregulator with which they associate.

Retinoblastoma protein-interacting zinc finger protein (RIZ), SMYD3, and MDS-EVI1 form a fourth family of SET domain proteins because they have two isoforms that exhibit opposing functions. The isoform containing the SET domain has tumor suppressor function, whereas the isoform missing the SET domain is cancer promoting. This "yin-yang" theory put forth by Huang (123) is especially true for RIZ and MDS-EVI1, in which by an unclear mechanism, the cancer disturbs the normal ratio between the two isoforms. The SMYD3 protein contains another DNA-binding domain called MYND, in addition to a SET domain, and is overexpressed in colorectal and hepatocellular carcinomas (130).

Cross talk between DNA methylation and the histone modifications exists. These interactions were shown by the observation that HDAC1 forms a complex with DNMT1 and 5-methyl-cytosine binding protein (MBP) on a methylated promoter to silence gene expression (131). Similar cross talk

occurs between the HDACs SUV39 and HP1, the HDACs PRC2 and PRC1, and the HATs MLL1 and BRM (47).

### Epigenetics and Development

The epigenetic control of gene expression is a fundamental feature of mammalian development, as indicated by the occurrence of developmental arrest or abnormalities in mutants deficient in methylation or acetylation. X-chromosome inactivation is an example of sequence-identical alleles being maintained stably in different functional states. In humans, X-linked inactivation serves to normalize the level of expression of X-linked genes in female (XX) and male (XY) individuals. Mutations in genes that affect global epigenetic profiles can cause human diseases. For example, the Fragile X syndrome results when a CGG repeat in the Fragile X Mental Retardation gene 1 *(FMR1)* 5′ regulatory region expands and becomes methylated *de novo,* causing the gene to be silenced and creating a visible "fragile" site on the X chromosome under certain conditions (132). On a more global level, mutations in the *DNMT3b* gene (which regulates the DNA methylation) lead to ICF syndrome (112,114), and CBP (with acetyltransferases activity) mutations cause Rubinstei–Taybi syndrome (133).

### Epigenetics and Cancer

Epigenetic changes play an important role in tumorigenesis. The major epigenetic changes that take place during the development of cancer are generally the aberrant DNA methylation of tumor suppressor genes and histones. Chapter 17 covers in greater detail the role of epigenetic influences in cancer, but a few highlights are mentioned here to conclude this section.

Genomic methylation patterns are frequently altered in tumor cells, with global hypomethylation accompanying region-specific hypermethylation events. When hypermethylation events occur within the promoter of a tumor suppressor gene, this can silence expression of the associated gene and provide the cell with a growth advantage in a manner similar to deletions or mutations. Although cancer cells are hypomethylated in the genome compared with normal tissues, many tumor-suppressor genes are silenced in tumor cells because of hypermethylation. This aberrant methylation occurs early in tumor development and increases progressively, eventually leading to the malignant phenotype. For example, a high percentage of patients with sporadic colorectal cancers with a microsatellite instability phenotype show methylation and silencing of the gene encoding MutL protein homolog 1 (MLH1) (134). Other methylated tumor suppressors include p16CDKN2A, p14ARF, Rb, E-cadherin, and breast cancer gene-1 *(BRCA1).* Deregulation of genomic imprinting can also play a role in cancer development, as exemplified by loss of imprinting of the *IGF2* gene in Wilms' tumor (135).

Chromatin remodeling also plays an important role during tumorigenesis. Loss or misdirection of HATs has been linked to embryonic aberrations in mice (136,137) and to human cancers (138,139). Misdirection of HAT activities as a result of chromosomal translocations is associated with multiple human leukemias (140–142). In acute promyelocytic leukemia, the oncogenic fusion protein promyelocytic leukemia-retinoic acid receptor-α (PML-RARα) recruits an HDAC to repress genes essential for the differentiation of hematopoietic cells (143). Similarly, in acute myeloid leukemia (AML), AML1-eight-twenty-one (ETO) fusions recruit the repressive N-CoR-Sin3-HDAC1 complex that, in turn, inhibits normal myeloid development (144).

That many human diseases, including cancer, have an epigenetic cause has encouraged the development of a new therapeutic option called "epigenetic therapy" (145). Many agents have been discovered that alter methylation patterns on DNA or the modification of histones, and several of these agents currently are being tested in clinical trials.

## ANATOMY OF THE PROMOTER

### DNA Elements

RNA Pol II and its accessory factors bind to a DNA sequence called the *promoter,* which is located upstream of protein-coding sequences to direct RNA transcription (146). Without the promoter, the genetic sequences that encode the information to make a functional peptide product will not be transcribed. Other 5′ flanking sequences or DNA elements that participate in transcription are sequence-specific binding sites for proteins that regulate the fidelity, rate, and timing of Pol II binding, formation of the preinitiation complex (PIC), and initiation of transcript elongation under basal and regulated conditions (147–149). These sequences are defined as *cis-acting* elements because they are a part of the same (cis) gene (150–153). DNA elements are categorized according to their ability to regulate transcription as a function of their distance and orientation from the promoter. Sequences that are contained within the first 30 to 100 bp of the promoter and operate in one orientation are considered promoter-dependent, cis-acting elements. If they are *positive-acting* elements and increase the rate of transcription, they are considered *activating DNA elements,* whereas if they are *negative-acting* DNA elements and decrease or repress the rate of transcription, they are *repressor elements* (154–156).

The structure of the promoter includes several critical elements that include the TATA element, which lies upstream of the transcription start site, the initiator sequence (Inr) that spans the start site, upstream regulatory elements that bind either transcriptional activators or repressors, and finally downstream poly(dA-dT) elements (157). The TATA element, or "TATA box," is an element with a DNA sequence that is TATA or variants thereof (151,158–161). This sequence resides at a fixed distance 25 to 30 bp upstream from the transcriptional start site in many Pol II promoters, and its

location relative to the start site is dependent on position and distance (162–164). However, it became apparent that many genes did not have TATA sequences. These "TATA-less promoters" still remain dependent on assembly of the TATA-binding protein (TBP) at the promoter to form the PIC, but the recruitment of TBP is not rate limiting (165).

Inr elements, although initially identified at the "TATA-less promoters" (166,167), have subsequently been found in both TATA-containing and TATA-less promoters. Their role appears to be in directing the accuracy of Pol II initiation (168). These Inr elements reside within the first 60 bp of the transcriptional start site and directly overlap the start site itself, but they do not have a clearly defined consensus sequence (169). Many of the genes encoding GI peptides (e.g., gastrin, somatostatin, cholecystokinin [CCK], glucagons, and secretin) contain TATA elements (170–174); however, the gene encoding the growth factor, transforming growth factor-alpha (TGF-α), does not (175).

Regulatory elements are generally sequence-specific DNA elements that bind transcription factors. In the case of transcriptional activators, there are two variations, upstream activating sequences (UASs) and enhancers. Both elements are orientation and distance independent. However, UAS elements do not function downstream of the TATA box. Thus, their function is restricted by their location relative to the TATA box (176,177). UAS elements, which bind transcription factors, facilitate assembly of the PIC directly by forming protein–protein interactions with GTFs, or indirectly by complexing with coactivators. Upstream repressor sequences (URSs) use several approaches to disrupt formation of the PIC. They can interfere with the activation domain of the activator complex, disrupt interaction with the core promoter factors, or recruit corepressors (e.g., Sin3-Rpd3, HDACs). Homopolymeric dA-dT sequences are required for normal levels of transcription. The repetitive dA-dT sequence has intrinsic structural ability to impair nucleosome assembly or stability (178,179).

Models describing the formation of the Pol II initiation complex are constantly evolving and essentially involve the convergence of information gathered from biochemistry, structural biology, and genetics, particularly yeast genetics (148,180,181). Elucidation of the three-dimensional crystal structure of the TBP has advanced our understanding of preinitiation assembly complexes (182,183). Protein folding of TBP into a β-sheet forms a "saddle-shaped" concave surface of sufficient size to contact helical DNA (Fig. 1-6A). On the opposing convex surface are potential binding sites for various regulatory proteins, for example, TBP-associated factors (TAFs), GTFs, and Pol II (see Fig. 1-6A). At least 10 to 14 different human TAFs have been identified from HeLa cells, with their molecular weights ranging from 18 to 250 kDa (167,184,185). TAFs are multiple subunit proteins that associate with TBP to form the essential transcription factor TFIID. The proteins are conserved from yeast to humans with the bulk of our understanding of these factors coming from experiments in yeast and *Drosophila.* An interesting finding is that TAFs are not universally required for transcription, but each one is required for only a subset of genes. Thus, for example, one TAF is required for transcription of 8% of genes, whereas three different TAFs are required for 60% of transcribed genes. In addition, TAFs are found in protein complexes other than with TBP. In fact, some TAFs have HAT activity, whereas others are similar to histones. Still other TAFs (e.g., TAFII250) have numerous enzymatic features including ubiquitin-conjugating activity (186). The conclusion from these studies is that TAFs are involved in promoter selection through yet to be defined mechanisms (185).

TBP is not specific to Pol II promoters, but also forms PICs at the start site of Pol I and III promoters, as well as Inr promoters that do not contain TATA elements (167,187,188) (see Fig. 1-6B). For example, in Pol I promoters, TBP does not bind DNA directly in a sequence-specific manner, but instead forms protein–protein interactions with the selectivity factor complex (SL1) and the upstream binding factor (UBF) (189). In Pol III promoters, TBP complexes with TFIIIB and TFIIIC (190). In TATA-dependent and -independent Pol II promoters, TBP forms protein–protein interactions with spatially constrained upstream activators that bind DNA; for example, CCAAT-enhancer binding protein (C/EBP) and Sp1. Thus, TBP forms the core of the PIC through both DNA–protein and protein–protein contacts in TATA-dependent promoters but primarily protein–protein interactions in non-TATA promoters (see Fig. 1-6). Apparently, the selection of a promoter by TBP preceding the assembly of the PIC is determined by the type of accessory factors recruited (TAFs, SL1, Sp1, TFIIIC) (188,190,191). Moreover, this recruitment may be regulated by temporal and tissue-specific influences. Inhibition of transcription (repression) may occur simply by preventing one of these general TAFs from participating in the assembly of the PIC (182,187). Like Pol II itself, many TAFs and GTFs are composed of multiple subunits. Thus, there is an enormously complex pattern of assembly of proteins (TBP + TAFs = TFIID, other GTFs, and upstream activators) on specific DNA elements at the promoter (e.g., TATA, INR, UAS) that results in the initiation and elongation of mRNA (182,188,192).

Other GTFs besides the TFIID complex include TFIIA, TFIIB, TFIIE, TFIIF, TFIIG, TFIIH, TFIII, TFIIJ, and TFIIK (160,167,193–195). There appears to be a strict requirement for these factors to assemble at the promoter in a specific order (181,182,192). TFIID binds to the TATA elements first, followed by protein–protein interactions of TFIID with TFIIA and TFIIB. The 12-subunit Pol II binds next. TFIIF is then recruited to the TFII-diaminobenzidine complex and facilitates binding of other general (basal) transcription factors E, J, H, and K. Many of these basal factors do not bind DNA directly (e.g., TFIIB, TFIIE, TFIIF), but instead form bridging complexes between the general Pol II transcriptional machinery and TAFs with specific upstream regulators. GTFs are required for the basal activity of the promoter, whereas UAS enhancers are dispensable.

Specific functions of some of the GTFs have been elucidated. For example, the larger subunit of TFIIF (Rap74)

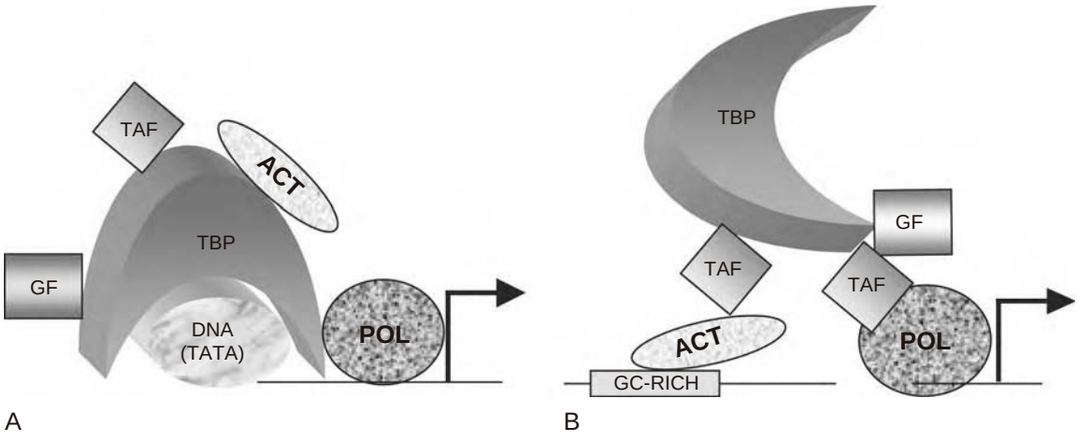


**FIG. 1-6.** Schematic diagram of a polymerase (POL) II initiation complex. The saddle-shaped TATA-binding protein (TBP) **(A)** binds DNA directly at the TATA sequence and **(B)** is tethered between TBP-associated factors (TAFs) in non-TATA promoters. Thus, TBP forms the core of a complex consisting of TAFs, general transcription factors (GF), upstream activators (ACT), and ribonucleic acid (RNA) POL I, II, and III. (Modified from Comai and colleagues [187], by permission.)

functions as an ATPase-dependent helicase to unwind DNA ahead of the transcription complex (196). TFIIF appears to play a role in promoter stability rather than selectivity. TFIII, a helix-loop-helix (HLH) protein, binds preferentially to Inr promoter with or without TATA elements and cooperates with upstream regulatory factors and the general transcription complex (197). TFIIH is one of several C-terminal domain (CTD) kinases that phosphorylates the CTD of Pol II to signal elongation of the nascent mRNA chain (192,194). Other kinases are now known to phosphorylate CTD (198).

Certainly, all genes are not transcribed concurrently; thus, the cell must have various mechanisms for silencing genes either permanently or in response to extracellular cues. The mechanisms for repressing genes may be general (e.g., DNA methylation [199,200]; see also #1375 in Bird [201]) or sequence-specific (202). Alternatively, loss of the ability to inhibit transcription of a gene (derepression) may permit certain cellular functions to proceed unchecked. Examples of the interaction between positive and negative regulators occur during cellular proliferation and differentiation (203). During fetal development, most cells are in the process of rapid proliferation. This period is followed by one of regulated differentiation during which the genes controlling proliferation are repressed. However, proliferative pathways may be derepressed (reactivated) during periods of organ repair or during neoplastic transformation (203,204). Examples include the reexpression of fetal proteins during liver regeneration and neoplasia (e.g., α-fetoprotein) or GI mucosal neoplasia (e.g., carcinoembryonic antigen) (205–207). Negative promoter elements or repressors may serve as the binding sites for proteins that sterically hinder the binding of GTFs (e.g., TFIID) or upstream activators (e.g., Sp1) critical in the formation of the Pol II transcription PICs (DNA–protein interactions). Alternatively, proteins responsible for gene repression may act by preventing the recruitment of required general or accessory factors (e.g., TFIIB or TAFs) to the bound PIC (protein–protein interactions) (202).

The DNA elements CCAAT and GGGCGG, which bind the nuclear proteins C/EBP and Sp1, respectively, are examples of promoter-activating elements that are distinct from the TATA box (151). These *upstream promoter elements* are distinguished from the TATA element in that mutation or removal of these UASs reduces basal promoter activity without completely eliminating it, whereas mutation or elimination of the TATA sequence completely abolishes transcription. DNA elements that function independently of their position on the gene or their orientation (3′ to 5′ or 5′ to 3′) are called *enhancers* if they bind nuclear proteins that activate transcription and *silencers* if they bind nuclear proteins that inhibit transcription (208–210). Many of these enhancer and silencer elements occur far upstream within the 5′ flank, but they may also occur within introns, exons, or 5′ or 3′ untranslated sequences.

To identify cis-acting enhancer elements, constructs are made by ligating the regulatory elements to be studied in front of a functional promoter expressing a gene encoding a protein or enzyme that is easily assayed. Typical reporter genes encode proteins that are not normally expressed by the transfected cell. By systematically deleting portions of 5′ flanking sequence, the transcriptional activity of the promoter under various conditions is altered and the regulatory elements of interest are identified. DNA elements responsible for tissue specificity can be identified by transfecting (transferring DNA into eukaryotic cell lines) cell lines derived from different tissues. Transcriptional initiation from a promoter that requires a particular cis-acting sequence for expression in a specific cell type is diminished or abolished if this sequence is eliminated or mutated.

Cis-acting sequences conferring inducible responses are also identified by this method. Alternatively, elements that are only active during development must be identified in eukaryotic systems in which differentiation of a cell line can be controlled, or in transgenic animal models.

### DNA-Binding Proteins

DNA-binding proteins are also referred to as *trans-acting factors* by virtue of their ability to bind to the 5′ flanking regions of genes in a sequence-specific manner and regulate transcription (211–213). The term *trans* was coined to acknowledge that the protein product of one gene regulates the transcription of a different gene. With the genes for several hundred trans-acting factors now cloned, the study of their primary and secondary amino acid structures has demonstrated characteristic protein domains (214,215). In general, these proteins contain specific DNA-binding, transactivation, and oligomerization domains (Fig. 1-7). The amount of a transcription factor binding to a particular sequence initially is considered to be the primary mechanism of control. However, it is now clear that the proteins themselves are regulated by a variety of mechanisms in addition to controlling their levels in the nucleus and include activation or inactivation by proteolysis (e.g., NF-κB), covalent modification (e.g., phosphorylation, acetylation), and ligand binding (e.g., steroid receptors), in addition to regulating translocation to and from the nucleus and transcriptional induction or repression of the trans-acting factor (216).

The DNA-binding domain is the portion of the protein that contacts DNA in a sequence-specific manner. However, flanking amino acids may also influence DNA-binding through noncovalent interactions. Examples of four major designs for DNA-binding domains are proteins with a helix-turn-helix domain, "zinc finger" domains, amphipathic helices (e.g., basic-zipper [bZip], HLH), and β-ribbon (prokaryotic proteins) (215) (Fig. 1-8). Most of the protein-DNA contacts occur in the major groove through noncovalent interactions (e.g., hydrogen bonds, hydrophobic interactions, and van der Waals interactions). An α-helical structure appears to be a common motif used in the formation of the DNA-binding domain.

The helix-turn-helix motif was initially identified in prokaryotic DNA-binding proteins, but similar motifs have now been identified in the homeodomains of eukaryotic transcription factors (217–219) (see Fig. 1-8). Homeobox factors are a class of DNA-binding proteins that predominantly play a role in the developmental expression of genes. Their discovery arose from the idea that developmental regulation involves control of gene expression by a few regulatory transcription factors called "master switch genes" (220). These DNA regulatory proteins initially were identified in simpler organisms such as the roundworm *Caenorhabditis elegans (C. elegans)* or *Drosophila,* in which the genetic development from the single-cell stage to maturity is well defined.

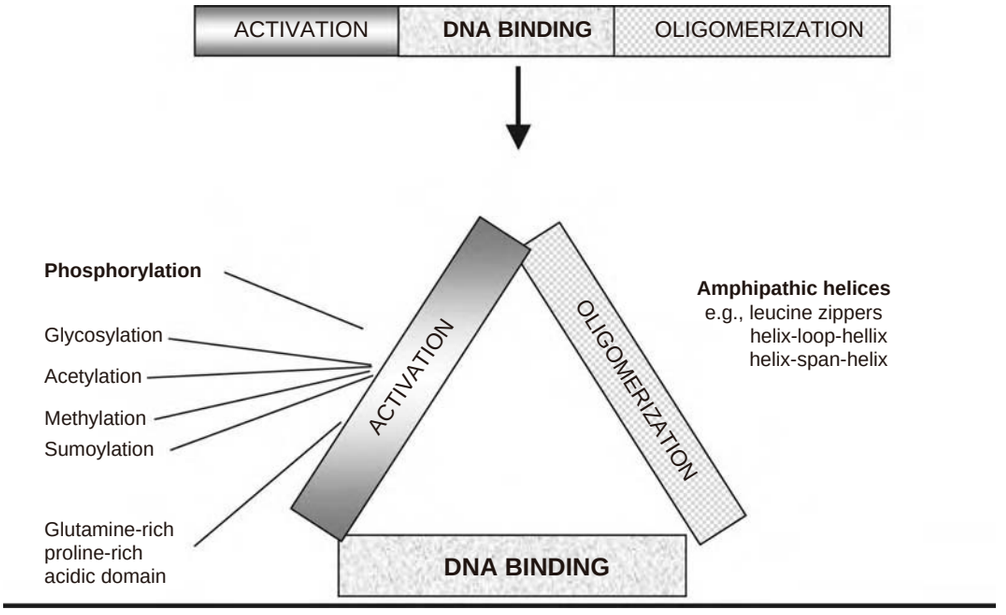


**FIG. 1-7.** DNA-binding protein domains. DNA-binding proteins have three functional domains: (1) a surface to bind DNA, (2) a surface to interact with other proteins containing similar oligomerization (dimerization) motifs, and (3) a surface to interact with the signal transduction pathways or the preinitiation complex. Examples of motifs associated with these domains are listed.

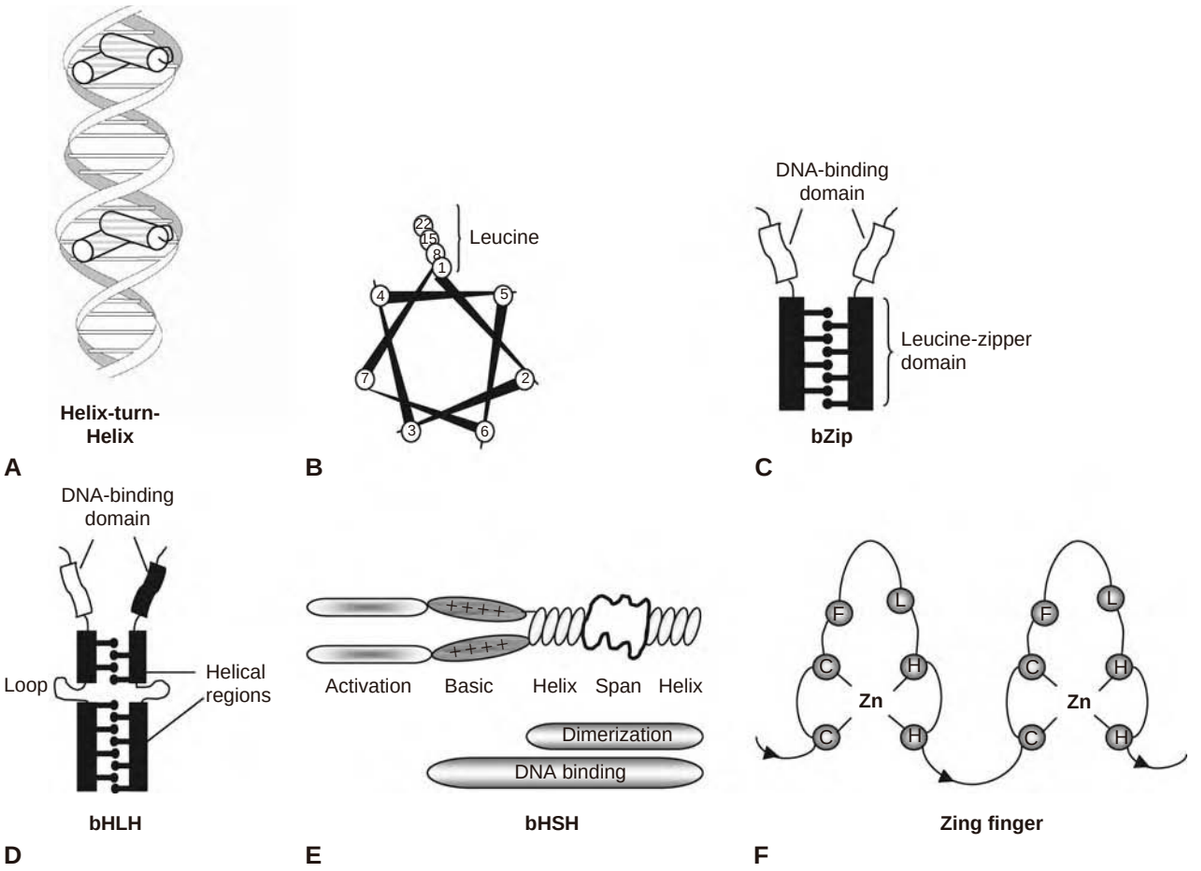


**FIG. 1-8.** Example of DNA-binding motifs. **(A)** Helix-turn-helix. **(B)** Amphipathic helix formation. An α-helical structure is formed so that all leucines or hydrophobic amino acids line up at one surface. Shown are **(C)** a leucine zipper adjacent to basic DNA-binding domain (bZip); **(D)** a helix-loop-helix adjacent to a basic DNA-binding domain (bHLH); **(E)** and a helix-span-helix adjacent to a basic domain (bHSH). **(F)** A zinc finger motif of the C2H2 type with tetrahedral coordination of a zinc ion between two histidines and two cysteines. (Modified from Berg [241], Falke and Juliano [255], Ellenberger and colleagues [266].)

Through site-directed mutagenesis studies, a specific protein domain required to effect developmental progression of these organisms was identified. This domain shared significant homology with a region within proteins controlling cell lineage in the pituitary (Pit-1) and immune system (B-cell octamer proteins, Oct-1 and -2) (221–225). These proteins also shared significant homology with the *C. elegans* "homeotic gene," *unc-86*. Thus, the ~60- to 75-amino-acid region of shared homology was renamed the "POU" domain after the three proteins Pit-1, Oct-1, and unc-86. Initially, the POU domain was named without knowledge of function or the ability to form specific secondary protein structure. Although some bind similar AT-rich consensus DNA-binding sites (octamer proteins bind an eight-nucleotide sequence ATTTGCAT; Pit-1 [also called GHF-1] binds a nine-nucleotide consensus site $^{T}_{A}{}^{T}_{A}$TATNCAT), others do not (*Drosophila eve* protein recognizes TCAGCACCG) (217). Mouse homeobox genes have nomenclature based on their similarity to *Drosophila* homeobox genes (e.g., *caudal*, *forkhead*) and have been associated with control of gut development (226–228).

In fact, homeobox genes have emerged as critical regulatory factors in the development of both the luminal GI tract and pancreas (229,230). Homeobox genes in the luminal GI tract are related to the 39-member *Hox* gene family of transcriptional regulators that control anterior-posterior patterning, and they are related structurally to the *Drosophila Antennapaedia* gene (229). *Hox* genes are so strongly conserved in evolution that this cluster of genes has been repeated four times in mammals on different chromosomes (231). Collectively, the replicated genes are called Hox clusters and are expressed primarily in either the mesoderm or ectoderm (e.g., skin, muscle, neural tissue), but not in endodermal tissues. Rather, an evolutionarily related cluster of homeotic genes call the *Para-Hox* genes appear to play the more important role in endodermal tissue, and therefore gut patterning (232). These genes include *Pdx1,* which is

essential to the correct development of the pancreas and duodenum (233,234), and the genes related to the *Drosophila caudal* gene, *Cdx1, Cdx2,* and *Cdx4* (229,235). *Cdx2* is not only relevant to development of the luminal gut, but it also is an indicator of neoplastic transformation, especially in the upper GI tract (236,237). The *forkhead* family of homeotic genes is another group of transcriptional regulators with important implications in the gut because of their role in GI cancers (238,239). There are at least 43 members of the *forkhead* family spread over three chromosomes. The "winged helix" motif of the forkhead DNA-binding proteins is a variant of the 60-amino-acid homeodomain helix-turn-helix because it has additional peptide domains that have been described as "wings" (240). The forkhead transcription factors are downstream targets of the hedgehog pathway, which is an important developmental signaling cascade originally described in *Drosophila* (see Chapter 9 for a more detailed discussion).

The zinc finger motif is distinguished by the occurrence of cysteine and histidine resides tetrahedrally coordinating a zinc ion (241–243) (see Fig. 1-8F). Two subcategories of zinc finger proteins have been identified: those regulatory proteins in which only cysteine contacts the zinc ion (e.g., the steroid receptor family, GAL4 [244,245]), and those in which both cysteine and histidine residues are involved (e.g., Sp1 and Zif 268 [245,246]). The X-ray crystallographic structures of several zinc finger and helix-turn-helix proteins have now been identified, with identification of more structures still to follow (247–253). Through crystallographic studies and computer modeling, investigators have been able to identify which amino acids within the DNA-binding domain contact particular nucleotides within the DNA element. It is anticipated that most of these interactions will be defined sufficiently well to predict protein–DNA contacts at the molecular level for other trans-acting factors. In the future, this will facilitate the targeting of specific transcription factors (natural or synthetic) to specific promoter sequences (248,254–257).

Landschulz and coworkers (258) originally described the bZip/coiled-coiled DNA-binding motif as a dimerization domain (see Fig. 1-8). However, this motif, which consists of 55 to 65 amino acid residues, actually forms two domains: one for dimerization and a second for DNA binding (259). Seven repeating leucine residues forming an α-helical coil compose the dimerization domain (Zip domain) (see Fig. 1-8). Immediately adjacent to the Zip domain, toward the amino terminus, lies the basic/hydrophobic domain (b domain) (215). Thus, the bZip family of proteins, the first of the amphipathic helices to be described, must dimerize to form a complete DNA-binding domain (260,261). Other transcriptional regulatory proteins containing the same heptad repeat are able to dimerize with each other to form a "coiled coil" (262). For stable binding to DNA to occur, some bZip proteins prefer that each dimerization partner be the same (e.g., CREB, C/EBP, or general control of amino acid synthesis 4 [GCN4] homodimers [see #564 in Pu (260); 263,264]), whereas other bZip proteins form more stable complexes as heterodimers (e.g., Fos/Jun), although lower affinity binding is also possible as homodimers (e.g., Jun/Jun) (265). The first report of a crystal structure for a bZip protein, the yeast transcription factor GCN4, confirmed the predicted model of two α-helical coils, which merge into diverging b domains that straddle and grip the major groove of DNA like "forceps" (266).

Other amphipathic helices, which combine a dimerization domain with a basic DNA-binding domain, have been described; however, less is known about their three-dimensional structure. The helical domains contain hydrophobic amino acid residues arrayed in an α helix so that they are clustered on one face of the helix, whereas hydrophilic residues reside on the opposing face (see Fig. 1-8). According to thermodynamic principles, the hydrophobic face is sequestered away from the aqueous environment by noncovalent interactions when they dimerize with similar domains on other proteins. In addition to the bZip model described earlier, the HLH and helix-span-helix (HSH) motifs were coined to describe other subclasses of amphipathic helices, albeit with longer linker sequences between the two α helices (267–269) (see Fig. 1-8). In the case of the leucine zipper, the hydrophobic face is formed by a series of leucine residues spaced seven amino acids apart (258). In contrast, the HLH and HSH proteins use a variety of different hydrophobic amino acids in addition to leucine to form two amphipathic α helices separated by a stretch of amino acids ("loop or span") that do not form a helix. Like the bZip family, HLH and HSH regulatory proteins bind DNA through an adjacent basic domain.

Thus, bZip proteins (e.g., CREB, activator protein 1 [AP1], activating transcription factor [ATF], Fos, Jun) are potentially interchangeable partners within homodimeric or heterodimeric complexes with the corresponding ability to recognize a greater repertoire of DNA-binding elements (270–272). For example, the Fos/Jun-binding site differs from the CREB/ATF-binding site by 1 bp: CREB/ATF binds TGACGTCA, whereas Fos/Jun binds TGAGTCA. Likewise, the bHLH proteins that recognize the CANNTG consensus binding site are also able to complex with each other (273). Currently, there are three family members of the transcription factor AP2, which are the only members of the bHSH family (269, 274,275). An HLH protein without the basic DNA-binding domain called Id was cloned (276). This protein has been shown to combine with three bHLH proteins (MyoD, E12, and E47) and to prevent the formation of normal homodimers or heterodimers, thereby functioning as a dominant negative mutant. Similar types of negative regulatory proteins have been identified for bZip proteins (277,278). Therefore, the combinatorial ability of transcription factors permits flexibility in responding to extracellular signals at the level of DNA–protein and protein–protein interactions.

The transactivation domains of regulatory proteins consist of predominantly acidic, basic (glutamine), or proline residues (152,279). These non-DNA-binding surfaces interface with signal transduction pathways and other proteins, but their specific function is not completely understood. Domains with a high degree of acidic charges are thought to represent important contact points for interaction with the Pol II PIC

(e.g., Gal 4, VP16). Ptashne (280) coined the phrase *acidic blobs* to describe such negatively charged trans-activating domains. Glutamine-rich (Sp1) and proline-rich (C/EBP, CTF) domains also presumably cooperate with the transcriptional machinery through protein–protein interactions (160,184,281–284). However, it has more recently been confirmed that transcription factors form protein–protein interactions with other transcription factors not within the same DNA-binding domain family. The most common transcription factor exhibiting this property is Sp1. Sp1 can interact directly with other transcription factors, for example, YY1, Smads, or Jun family members (285,286). A functional interaction between cJun and Sp1 has been shown to mediate epidermal growth factor activation of lipoxygenase gene expression (287). Presumably, the "acidic blob" in the transactivation domain of Sp1 creates a "sticky" surface on which new partnerships are formed at various promoters in response to a variety of extracellular signals. Likewise, Smad proteins, which mediate TGF-β signaling, are also promiscuous in their ability to partner with other transcription factor family members (288,289). Although at one time undetected, protein–protein interactions among transcription factors are now recognized as common occurrences, particularly because there are convenient means to identify the interactions genetically through two-hybrid cloning methods, or biochemically using affinity chromatography, immunoblot assays, and mass spectroscopy.

Many of the mechanisms involving transactivation of transcription factors involve protein phosphorylation and dephosphorylation (290). Phosphorylation by protein kinases occurs at serine, threonine, or tyrosine amino acid residues. Several classes of protein kinases exist within the cell; however, the best studied are the protein kinase A (PKA) and C (PKC) pathways. PKA is activated indirectly by the catalytic subunit of adenylate cyclase. Signals that increase intracellular cAMP will activate PKA (291,292). In contrast, PKC is activated by calcium released from intracellular stores and by the phospholipid diacylglycerol (293). Phospholipase Cγ catalyzes the hydrolysis of phosphatidylinositol to diacylglycerol. The tumor promoter 12-0-tetra-decanoyl phorbol-13-acetate (TPA) is a lipid-soluble compound that mimics diacylglycerol and directly activates PKC. Hundreds of additional protein kinases within both the cytoplasm and the nucleus exist that may be implicated in the specific phosphorylation of transcription factors (294). Ligand binding triggers a variety of different activation pathways that appear to result in the direct phosphorylation of transcription factors by protein kinases other than PKC and PKA; for example, casein kinase II (CKII), glycogen synthase kinase III, and several DNA-dependent protein kinases (295–297). Direct phosphorylation of the DNA-binding protein may result in a conformational change that enhances its ability to induce transcriptional activation (e.g., CREB, cJun, C/EBP-β) or inhibition (e.g., yeast protein A[298]DRI) (290). Alternatively, phosphorylation of an inhibitory subunit may release the transcription factor from an inactive state (e.g., NF–κB) (299–303). Phosphorylation can also regulate the ability of a protein to dimerize, thereby broadening or narrowing the repertoire of DNA sequences that are recognized (e.g., signal transducer and activator of transcription [STAT] and Fos/Jun family) (304,305).

The removal of phosphate groups by sequence-specific phosphatases is an additional mechanism by which the transcriptional activity of DNA-binding proteins may be altered (290,306,307). Interestingly, dephosphorylation appears to be a more common mechanism for regulating transacting factor binding than is kinase-mediated phosphorylation (295). Binding of the Jun family (bZip class), homeodomain proteins, and cMyb to DNA is regulated by dephosphorylation. Phosphorylation of sites within or adjacent to the DNA-binding domain of these proteins inhibits DNA binding, whereas removal of phosphates enhances binding. In contrast, activation of DNA binding by phosphorylation has fewer documented examples. One example is the serum-response factor (SRF) that binds to and activates the cFos promoter (308,309). SRF appears to be activated by phosphorylation at sites adjacent to the DNA-binding domain by CKII. This observation is supported by studies involving both mutational analysis of these phosphorylation sites and increasing cellular CKII kinase activity through microinjection of the enzyme into cells (310,311).

Although glycosylated proteins are usually observed on the plasma membrane of cells or in the lumen of intracellular organelles, nuclear proteins have been shown to contain O-linked glycosylated residues as well (312). Sp1 represents the prototypical glycosylated transcription factor, the activity of which is enhanced by the presence of carbohydrate residues (312–315). Other eukaryotic transcription factors such as CTF, AP1, and AP4 are also known to be glycosylated, but the effect of the carbohydrate residues on their transcriptional activity is unknown. Glycosylation may regulate the transcriptional activity of individual transcription factors, perhaps by increasing their resistance to proteolysis, by targeting them to the nucleus, by blocking potential phosphorylation sites, or by facilitating their interaction with coactivators (316).

### Coregulatory Proteins

By the mid 1990s, it became clear that DNA-binding factors were working in a combinatorial manner, not only with other DNA-binding factors, but with non-DNA-binding proteins that were closely linked to chromatin structure and the PIC. These large molecular weight proteins were initially identified as factors interacting with the steroid hormone receptors, which are DNA-binding proteins that translocate to the nucleus after binding hydrophobic ligands in the cytoplasm (317–319). At about the same time, it was discovered that phosphorylation of the cAMP-activated transcription factor CREB induced its interaction with a 300-kDa coactivator protein called CBP. Subsequent to the discovery of CREB, the homologous transcriptional coactivator designated p300 was also identified (320). Coactivators were

found to facilitate transcriptional activation through intrinsic HAT activity, resulting in an "open" chromatin state at the start site of transcription. There are now several of this class of proteins that include PCAF and GCN5 (321). Conversely, the protein complexes that inhibited transcription were multiprotein complexes that recruited histone deacetylators, which, in turn, deacetylate histones returning chromatin to its closed, inactive state (322). The prototype corepressors were identified because of their ability to suppress activation by the retinoid and thyroid hormones (SMRT/N-CoR) (216). It is now known that there are transcriptional corepressors of a variety of signal transduction pathways, including Sin3A, a corepressor of the cMyc bHLH transcription factor family, and PIAS/SUMO, a corepressor of the STAT signaling pathway (323–328).

Collectively, these proteins are considered to be coregulatory factors because they do not contact DNA directly as transcription factors do, but rather form protein bridges between the sequence-specific DNA-binding proteins and the Pol II assembly apparatus, bringing with them enzymatic activity, for example, acetylase and deacetylase activity involved in remodeling chromatin (329–331). Currently, there are three broad categories of coactivators (332). p300 and CBP are the prototypes of the HAT class of coactivators. The TRAP/DRIP/Mediator/ARC complex compose the second class and are proteins that bind transcription factors and recruit RNA Pol II without having intrinsic histone modification capabilities. The third class comprises the yeast SWI/SNF and their mammalian homologues BRG1/BRM. This third class of coactivators contains intrinsic ATP-dependent DNA-unwinding activity required for efficient *in vivo* transcription. Coactivators increase the transcriptional activation of a promoter through its interaction with a sequence-specific DNA-binding protein, but it is not yet clear how the coactivator selects one group of promoters over another. Two concepts have been considered (332). For example, a promoter might need a "threshold level" of positive signals to be activated. Alternatively, some promoters might have a greater requirement for the presence of one coactivator than another.

The precise mechanisms of transcriptional activation continue to evolve, and certain themes are emerging. In rare instances, positive or negative enhancer activity is dependent on a single DNA-binding protein that functions as a master switch to activate a family of related genes, for example, the myogenin MyoD family in muscle differentiation (333). However, further scrutiny of this model has indicated a large network of transcription factors that interact with non-DNA-binding complexes involved in chromatin remodeling, for example, histone acetyltransferase proteins p300 and CBP (334–337). Therefore, the more common mechanism implies that most cells respond to their environment by recruiting subsets of ubiquitous and promoter-specific transcription factors that combinatorially produce the desired cellular phenotype (204,338-341). Corepressors SMRT and N-CoR both recruit HDACs, yet they mediate activation downstream of different kinase cascades (342). In addition to the recruitment of classic HDAC-associated corepressors (e.g., mSin3A and Groucho) (343–345), the runt-related transcription factor (RUNX) proteins exert gene silencing by associating with histone methyltransferases (e.g., SUV39H1) (346). Bifunctional attributes of transcription factors have been attributed to their regulated association with either coactivators or corepressors.

## METHODOLOGY

This section summarizes some of the molecular techniques used to study transcriptional control of genes. These methods are used to study either genetic structure or function. Three systems have been used to study function: reconstituted cell-free transcription assays, cell culture models, and whole-animal studies. Methods that analyze structural interactions include those techniques that assess DNA–protein interactions and those that assess protein–protein interactions.

### Functional Methods

#### *Reconstituted Transcription Systems*

The most basic approach to the functional study of a gene is an *in vitro* transcription system in which the minimal components required for transcription are isolated and reconstituted to produce the gene product (347,348). mRNA is transcribed from cloned cDNA in the presence of radiolabeled nucleotides, RNA polymerase, and accessory factors isolated from nuclear extracts. The radiolabeled RNA synthesized *in vitro* is resolved by gel electrophoresis after extraction from the cell. Changes in basal levels of transcription are measured by quantifying the amount of newly synthesized RNA transcripts produced in the presence or absence of cloned or purified gene-specific DNA-binding proteins (349). In this way, differences in gene expression attributable to the activity of a purified transcription factor or enriched nuclear fraction may then be studied under tightly controlled assay conditions.

#### *Cell Culture Models*

The study of transcriptional regulation has been advanced greatly by the use of cell lines derived from the same tissues as the endogenous gene of interest. These cell lines have become the vehicles in which the study of gene expression is performed. Two major advantages of using cell lines are that they are homogeneous populations and they continue to divide in minimal culture conditions. However, in many situations, the cell lines are derived from neoplastic tissues, which may have lost the normal regulatory mechanisms that maintain the differentiated state. In a dedifferentiated state, cells tend to express a variety of genes outside of the repertoire expressed by their normal counterparts. Therefore, studies with cell lines always carry the caveat that they may not reflect activities of native cells.

The use of cell lines permits the direct study of regulators of endogenous gene expression, avoiding the confounding effects of contaminating cell types. However, this approach does not permit alteration of the regulatory domains of genes to assess their contribution to transcription. Therefore, techniques have been developed to insert altered genetic material into cells by chemical, electrical, or viral mechanisms. In this way, specific elements controlling transcription can be isolated and studied. To tag the inserted gene, the promoter from which transcription will be initiated is ligated upstream of the coding sequences for a reporter gene, for example, chloramphenicol acetyltransferase, β-galactosidase, growth hormone, green fluorescent protein, or luciferase (9,350–353). The products of the reporter gene are easily measured, and spurious detection of reporter gene activity is kept to a minimum because their products are not normally expressed by most mammalian cells. Regulatory sequences to be analyzed are ligated upstream of a promoter with basal transcriptional activity in the test cell line. Taking advantage of various restriction sites, sequentially shorter 5′ flanking sequences are created, and each resulting construct is then tested by assaying the reporter gene product as an indicator of gene expression.

### *Whole-Animal Models*

Whole-animal studies have been useful in assessing the contribution of transcriptional control to the regulation of several GI peptides, including gastrin, CCK, and somatostatin (354–356). Brand and Stone (357) showed that gastrin mRNA levels in the antrum increase under conditions of chemical or surgical achlorhydria and coincide with a reciprocal decrease in somatostatin mRNA. These observations are correlated with prior observations that gastrin plasma levels increase under conditions of achlorhydria (354, 358). Furthermore, infusion of the somatostatin analogue octreotide blocks the increase in gastrin mRNA (357). Walsh and coworkers (359,360) found that gastrin mRNA levels are predictably regulated by cycles of fasting and refeeding. Recently, infusion of the proinflammatory cytokine interferon-γ into mice has been used to recapitulate the effect of *Helicobacter pylori* infection on gastrin and somatostatin (361). Similarly, studies on the dietary control of CCK gene expression have been reported (355). Although such studies permit the linkage of transcriptional regulation to physiologic events, they do not allow dissection of the responsible regulatory elements.

### *Transgenic Animals*

Through transgenic animals it is possible to introduce genetic information into the mouse genome such that there is permanent alteration of the genetic makeup in both the founder line and successive generations (13,14). Transgenic studies afford the opportunity to study the importance of specific genetic sequences in cell, organ, and whole-animal function. By breeding mice with different transgenic lineages, the interaction between these artificially produced genotypes on the overall phenotype may be amplified or abolished. In many situations, these alterations reproduce clinically relevant pathologic states (362–366). Chapter 53 provides specific details on transgenic technology including the powerful technique of homologous recombination.

### *Cell-Based Knockout Strategies*

Once a genetic target is identified, whether DNA, RNA, or protein, the next step is to determine the significance of the molecule in a particular signaling, developmental, or neoplastic cascade. This usually is done by blocking, reducing, or removing the gene product at the cellular level before applying the extracellular signal. A change in the expected phenotype would confirm that the gene product makes a significant contribution. At the cellular level, the traditional approach has been to use small molecules, for example, pharmaceutical inhibitors. Once DNA vectors were developed in the early 1980s, antisense and dominant negative approaches to inhibit gene expression came into vogue (367). With the emergence of transgenic technology, it became apparent that one could remove the gene product through genetic manipulation specifically by homologous recombination to disrupt the gene in mice (362,368). With the discovery of snRNA molecules that interfere with either transcriptional initiation or translation, the commercial availability of synthetic "interfering" RNA molecules has emerged (369,370). High-throughput methods using RNA silencing are now being used to complement the gene discovery methods of DNA microarray technologies (371). Nevertheless, RNA interference technology, although relatively easy to use, does not eliminate the gene product as effectively as direct gene targeting. Therefore, genetic methods must be used to generate a complete null cell line. Cell lines are either created from a null mouse model (e.g., embryonic fibroblasts), or somatic cell gene targeting can be performed in the cell line of choice (372,373). The advantage of creating the null cell line from a mouse is that the cells will be from normal tissue and not a tumor cell line. However, unless molecules are introduced to immortalize the cells, the lines are not permanent. Gene targeting in a somatic cell line has not been as widely used because of the difficulty in performing the technique, but it is a powerful approach that permits the study of a null locus without incurring the expense of mice.

## Structural Methods

Once functional regulatory DNA elements have been identified, assays that assess DNA–protein interactions are performed (374). Indeed, in circumstances where a long sequence (>50 bp) must be analyzed, it is simpler to identify DNA–protein interactions first, and then determine whether these DNA elements are involved in transcriptional regulation. DNase I footprinting assays are used to identify DNA-binding elements that interact with crude or purified nuclear proteins by protecting them from chemical or enzymatic

cleavage (375,376). Such assays are particularly well suited for studying cooperative interactions among proteins bound to adjacent DNA elements. The technique can be performed *in vivo* or *in vitro* (9). However, *in vivo* footprinting has been superseded by chromatin immunoprecipitation (ChIP) assays (see the next section). Electrophoretic gel mobility shift assays (EMSAs; gel shift, gel delay, or band-shift assays) permit a more detailed analysis of the following: (1) the type of protein complexes that bind to individual DNA elements, and (2) the specificity of the protein interaction with a specific base pair (377–379) (Fig. 1-9). This assay system is also rapid and easier to use than footprinting assays. Methylation interference assays extend the power of the gel shift assay by identifying specific nucleotide contacts that are required for DNA binding (380). DNA affinity precipitation is a DNA–protein interaction assay that uses the biotinylated DNA binding site to identify the proteins that are recruited to the element (381). The assay uses the DNA element to isolate the protein factors, coupled with immunoblots to identify the proteins that form both the protein–DNA and protein–protein interactions. Southwestern blot analysis takes advantage of specific DNA elements that are used to detect nuclear proteins separated on a denaturing gel and transferred to nitrocellulose or produced by a phage expression library (382–384).

### *Chromatin Immunoprecipitation Assays*

ChIP analysis is now the most effective method to document an *in vivo* interaction at DNA (385–387). First, a fixative, usually formaldehyde, is used to cross-link proteins to DNA. Then antibodies are used to immunoprecipitate the DNA-binding proteins. After a series of extractions to remove the protein from DNA, specific primers are used to PCR amplify the DNA-binding element precipitated with the protein and antibody. Variations of this method are used to identify the *in vivo* preferred binding sites of known DNA-binding proteins. Alternatively, the immunoprecipitate is resolved on a sodium dodecyl sulfate gel, and mass spectroscopy can be used to identify the proteins that coprecipitate and are likely involved in protein–protein interactions with the DNA-binding proteins. The technique completely depends on the quality of the antibodies, the quantity and quality of genomic DNA precipitated, and primer specificity. ChIP assays complement *in vitro* DNA–protein interaction assays such as EMSAs or footprinting. Expression vectors or cell-based knockout strategies using dominant negative constructs, antisense technology, or RNA interference may be used to demonstrate functional significance (388). These approaches are rapid and useful to perform before using transgenic mouse approaches.

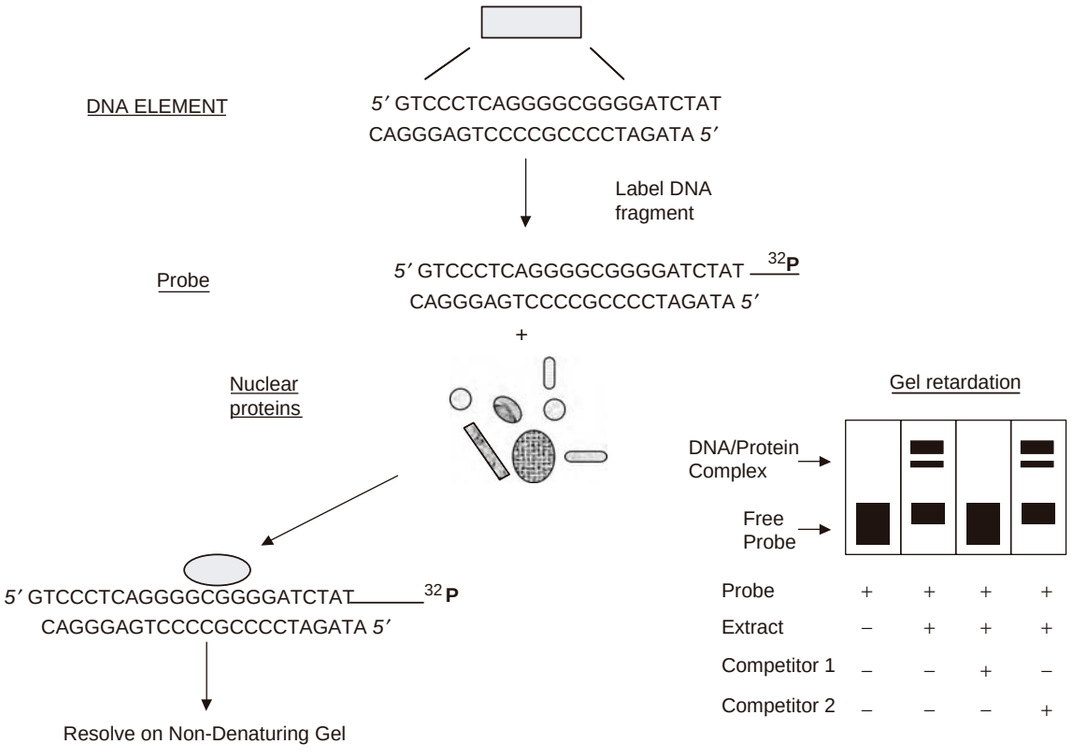


**FIG. 1-9.** Electrophoretic mobility shift assay (EMSA, gel shift). A DNA element ~30 to 100 bp in length is labeled, and then is incubated with crude nuclear extract or purified protein. A band on the autoradiogram is detected if the radiolabeled probe is retarded and does not migrate to the bottom of the gel. The specificity of binding is determined by competing with unlabeled DNA sequences. Competitor 1 is related to the probe sequence, whereas Competitor 2 is unrelated to the probe sequence.

### *Microarray Technology*

The latest method to comprehensively analyze gene expression is by microarray technology. At the transcription level, DNA array technology increases by several orders of magnitude the number of genes that can be examined simultaneously under different conditions (389–391). The number of genes that are either stimulated or inhibited under various conditions can be studied simultaneously with the limitations being the number of genomic sequences that are spotted on the glass slide. A glass slide is able to hold the genomic sequences of 25,000 to 30,000 genes, which is the current estimate of the total number of genes in the human genome. Two types of arrays are available: EST/cDNA and oligonucleotide (Affymetrix, [Santa Clara, CA]) based. The EST microarray chips use *e*xpressed *s*equence *t*ags that are fragments of DNA corresponding to segments of the genome that encode mRNA. The Affymetrix gene chips spot commercially designed oligonucleotide sequences. These DNA fragments are subsequently "arrayed" onto glass slides. In most instances, several regions of the genomic sequence unique to that gene are spotted in multiple copies to ensure reproducibility. Different genetic domains are plated because of differences in hybridization affinity. RNA is isolated from cells or tissue after treatment with an extracellular molecule or from cells at different stages of development or transformation. cDNA are then generated and tagged fluorescently, then hybridized under stringent conditions to the DNA arrayed on the glass slide followed by analysis by a special plate reader. Computer-generated algorithms are required to interpret the fluorescent signals and rank the degree of change from baseline fluorescence. The technology is being used to study the gene expression pattern found in various tissues at designated stages, for example, developmental or transformation stages (392–394). The significance of the findings must be confirmed by alternative methods including Northern blot analysis or quantitative PCR.

### *Proteomics*

Analogous high-throughput approaches have been developed to study protein modifications (395). However, the techniques used to detect protein posttranslational modifications are more complex and use more labor-intensive technology. Protein is extracted from the cell or organelle of interest and resolved by two-dimensional gel electrophoresis, in which proteins are separated by both size and ionic charge (along a pH gradient). The proteins are visualized with a dye either directly on the gel or after transfer to a paper substrate. Both substrates (gel or paper) can be used for further analysis. However, proteins transferred to a paper substrate permit several options for analysis. Resolved proteins that are transferred to paper can be submitted for analysis with an antibody (immunoblot) that might recognize phosphorylated or acetylated peptides. Differences in the size of the spot corresponding to the amount of a particular protein version (phosphorylated, acetylated) can be quantified by computer. Proteins that cannot be identified by antibody can be analyzed by mass spectroscopy. Therefore, proteomic studies allow the monitoring of regulatory changes that occur because of posttranslational modifications and quantification for large numbers of proteins simultaneously. Taking advantage of the technology used to develop DNA arrays, companies are now developing protein arrays that will be applied to new drug discovery (396).

## TRANSCRIPTIONAL CONTROL OF GASTROINTESTINAL PEPTIDES

Although knowledge in the transcriptional control of GI peptides has accelerated over the last several years, the field is still hampered by the paucity of gut-derived cell lines that express regulatory peptides. The problem has been circumvented somewhat through the use of neural and endocrine-derived hormone-producing cell lines, but application of data obtained with these models to the gut requires assumptions that may not be accurate. Future work in this field will be assisted greatly by the application of high-throughput and transgenic technologies and the development of immortalized and transformed cell lines using *in vitro* DNA transfer techniques. An overview of what has been accomplished with respect to specific GI peptides can be found primarily in Chapters 4 through 6. Nevertheless, a few peptides deserve brief mention. To date, most studies of the transcriptional control of peptide hormones have focused on somatostatin and vasoactive intestinal peptide because they are expressed in islet or neural-derived cell lines (397–400). The downside of this is that little is known about how somatostatin is regulated in gut-derived tissues; for this reason, the peptide should become a priority for future transcriptional control studies in the GI tract. Studies on the transcriptional control of gastrin have been slow for similar reasons and have been reviewed recently (401). Information on the transcriptional control of secretin and CCK has increased because of the use of transgenic mouse models (25,402–404).

## POSTTRANSCRIPTIONAL PROCESSING

### Polyadenylation

Three major events occur at the end of transcription: (1) The poly(A) tail is added, (2) adenine bases are methylated, and (3) hnRNA is processed by removing introns before exiting the nucleus (see Fig. 1-2) (405). All mRNA, except those encoding most histone proteins, have poly(A) tails. The length of the poly(A) tail that is added ranges from 200 to 250 bp and is quite uniform among eukaryotic organisms. Once the transcript reaches the cytoplasm, the length of the poly(A) sequence decreases with the age of the transcript (406). Thus, polyadenylation contributes to mRNA stability and translational activation, processes that also involve a synergistic interaction with the cap site (407–409).

Because there is no poly (dT) sequence within DNA, addition of the poly(A) tail represents a posttranscriptional modification of the newly synthesized mRNA. The AATAAA site in DNA is transcribed as AAUAAA and signals endonuclease cleavage of hnRNA ~20 bp after this RNA element (410). Several factors are required for specific recognition of the AAUAAA element before the addition of adenylate residues by poly(A) polymerase (411,412). Polyadenylation occurs in two phases: (1) an AAUAAA-dependent phase marked by addition of the first 10 residues, and (2) an AAUAAA-independent phase marked by rapid elongation and catalyzed by a poly(A)-binding protein (413). In addition, endonuclease cleavage of polyadenylated histone H1 transcripts have also been shown to require the presence of small nuclear ribonucleoproteins (U7 snRNP, pronounced "snurp"), which are trans-acting factors that participate in RNA splicing reactions (414). Transcription can proceed for up to 2 kb past the polyadenylation site and may terminate prematurely 30% of the time. Adenylate residues within exons are methylated at the sixth nitrogen and are thought to serve a protective role for those sequences that will eventually be translated (415). It is now known that formation of the PIC is linked to the assembly of factors involved in polyadenylation (416).

## RNA Splicing

### *The Spliceosome*

Soon after the termination of transcription, most vertebrate hnRNA (pre-mRNA) will be posttranscriptionally processed after exiting the nucleus into a form that can be translated (see Fig. 1-2). This involves removing intervening sequences that in some transcripts contain transcriptional regulatory signals (cis-acting elements). Splice sites are identified by comparing the genomic sequence with the cDNA prepared from an RNA template. The cis-acting elements within the intron that regulate RNA splicing are GU (GT in the genomic sequence) at the 5′ splice border, AG at the 3′ splice border, and a pyrimidine-rich element that defines the area of the branch point 20 bp upstream from the 3′ splice junction (Fig. 1-10). The branch point lies just upstream of the pyrimidine-rich region $(PyPy)_n$ and is a highly conserved sequence in yeast (UACUAAC) but much less so in vertebrates.

Five snRNA-U1, U2, U5, U4, and U6-combine with subsets of about 10 different proteins to form small nuclear ribonucleoproteins (snRNPs) (417,418). The snRNA, ranging in size from 56 to 217 nucleotides, are quite abundant in the nucleoplasm and contain a trimethylguanylate cap. Some proteins are components of all five major snRNPs, whereas others are unique to one snRNP. The U7 snRNP, which is present in low concentrations, participates in the 3′ posttranscriptional processing of hnRNA [poly(A)] (419). The five major snRNPs assemble into large multicomponent complexes called *spliceosomes* to perform the splicing reactions (420). There reactions occur in three steps: cleavage at the 5′ exon-intron border with formation of a branch point, excision of the branch point as a lariat, and joining of the exons. Splice site selection can be influenced by subtle changes in flanking exon sequences (421–423).

The basic steps in RNA processing illustrated in Figure 1-10 are as follows (419): U1 snRNP binds in a sequence-specific manner to the 5′ exon-intron junction of capped pre-mRNA (424). An U2 snRNP accessory factor (U2AF) then binds to the pyrimidine-rich element before sequence-specific recognition of the branch point element by U2 snRNP (425,426). The 5′ exon is released by cleavage of the 5′ exon junction. This allows the freed 5′ guanylate residue to form a phosphodiester bond at the 2′ site of an adenylate residue within the branch point. U4 and U6 snRNPs are paired together by complementary bases and function as a single snRNP complex (427). The recruitment of the U4/U6 snRNPs to the spliceosome is essential to the last excision step and final removal of the intron from the pre-mRNA. U4/U6 snRNP cooperates with the U2 branch point complex without direct contact with RNA (428). U5 snRNP binds just upstream of the 3′ splice junction to initiate cleavage of the 3′ intron border. Finally, the intron is removed as a lariat and the two exons are joined. More recent evidence indicates that small RNA catalyze the splicing reactions without the presence of specific enzymes (429,430). As observed for polyadenylation, the splicing events coincide with transcriptional events (431). It is therefore somewhat surprising that the events involved in splicing are not better understood. Nevertheless, with the understanding that the complexity of the human genome lies beyond the DNA sequence and at the level of epigenetics and alternative splice products, the next decade will likely witness heightened attention to this additional nuclear process (431,432).

### *Alternative Splicing*

Eukaryotic cells have applied the mechanics of RNA splicing to generate the protein diversity necessary to meet their multiple demands. Thus, in contrast with the original definition of a gene in which only one transcript is produced, complex genes can generate multiple protein isoforms from multiple RNA transcripts through alternative splicing (433). This can be achieved by altering which introns and exons are included in or excluded from the mature mRNA transcript that is used as the template for peptide chain elongation. Accordingly, the definition of introns and exons for each gene is actually a fluid concept because an intron for one gene product may become an exon within another transcript. Alternative splicing is a mechanism used by many protein classes, including muscle-related genes, hormones, and transcription factors (434–438).

### *Regulated Posttranscriptional Mechanisms*

In addition to cis-acting DNA elements, the cis and trans models of regulation also occur at the posttranscriptional level (439). Ferritin and the transferrin receptor (TfR),

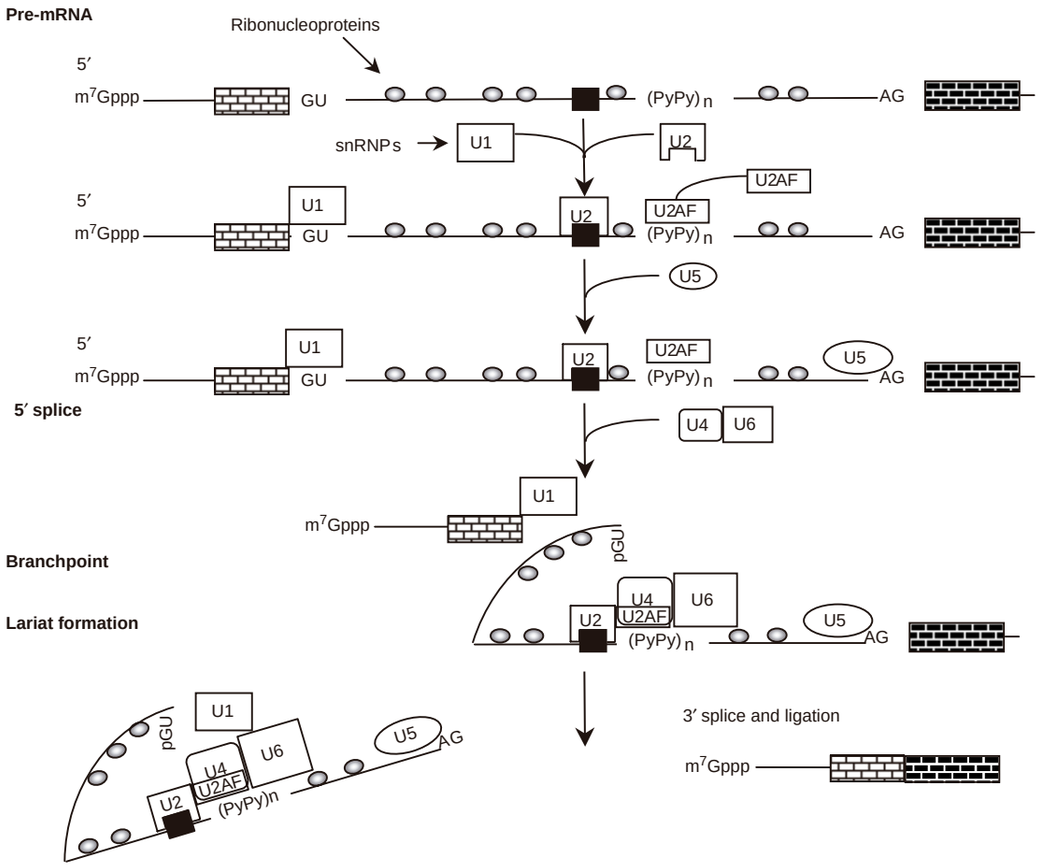


**FIG. 1-10.** Ribonucleic acid (RNA) splicing reactions. First, small ribonucleoproteins (snRNPs, pronounced "snurps") and accessory factors (U2 accessory factor [U2AF]) bind in a sequence-specific manner to the branch point and intron-exon borders. Second, the 5′ exon-intron border is cleaved, and a "lariat" is formed by the free end of the intron at the branch point. Third, the 3′ intron-exon border is cleaved, the exons are joined, and the excised intron is removed in the form of a lariat.

which regulate the storage and uptake of iron, are the best known examples of regulated posttranscriptional control (440). Cis-acting RNA elements, responsible for conferring iron regulation on both proteins (iron-response elements [IREs]), reside in the 5′ UTR and 3′ UTR of ferritin and TfR mRNA transcripts, respectively. The same iron-binding protein (IRE-BP) that binds to the IRE in the 5′ UTR of ferritin to block translation can also bind to the 3′ UTR of TfR to block mRNA degradation (439,441,442). Therefore, regulation of iron homeostasis ultimately depends on post-transcriptional mechanisms that either block translation or increase mRNA stability.

## TRANSPORT ACROSS THE NUCLEAR MEMBRANE

As noted earlier, RNA is synthesized initially as a much larger primary transcript molecule that in many instances undergoes posttranscriptional modification (e.g., splicing, degradation). However, for any mature RNA transcript to be translated, it must be transported from the nucleus to the cytoplasm. In contrast, nuclear regulatory proteins are translated in the cytoplasm and are eventually returned to the nucleus, either immediately after synthesis or after a dormant state from which they are activated in response to signals (443). This bidirectional shuttling of macromolecules between the cytoplasm and the nucleus occurs through the nuclear pore complex, a specialized compartment of the nuclear membrane regulated by a group of transport receptors called karyopherins. Both import and export processes through the nucleus require energy in the form of the Ras-related GTPase Ran and specific targeting signals on the cargo to be transported (nuclear localization and export signals) (444). The three-dimensional structure of the nuclear pore complex shows a doughnut-shaped structure comprising eight subunits (445). From the eight subunits emanate spoke-like structures that radiate inward to form a central plug (446, 447). The cytoplasmic surface of the nuclear pore complex (NPC) is closely associated with ribosomes. Its nuclear

surface is thought to participate in the organization of the genome by binding to specific DNA sequences within transcribed genes with products that may be destined for export from the nucleus (gene-gating hypothesis) (448).

## CONCLUSION

With the dawn of the postgenomic era on us, our next challenge is to apply the volumes of available genetic, molecular, and cell biological information to tackle questions of GI physiology and development. To accomplish this task and make optimal use of past, ongoing, and future discoveries, physiologists will need to acquire the basic vocabulary of several disciplines including bioinformatics. It is our hope that this chapter has laid the initial foundation necessary to understand those aspects of physiology that pertain to transcriptional control.

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CHAPTER 2

# Translation and Posttranslational Processing of Gastrointestinal Peptides

Cheryl E. Gariepy and Chris J. Dickinson



## TRANSLATION

Translation is the complex process by which a sequence of codons of messenger ribonucleic acid (mRNA) directs the synthesis of a polypeptide chain. Beyond the sequence of codons, the mRNA contains untranslated regions (UTRs) with structural and regulatory sequences that determine its translational fate. Translation involves hundreds of molecules including mRNA, transfer RNA (tRNA), ribosomal RNA, activation enzymes, and many RNA-binding proteins, as well as energy in the form of guanosine triphosphate (GTP) and adenosine triphosphate (ATP).

To be translated into protein, mRNA must contain, in addition to a string of codons, information that specifies nuclear export, translation, and stability. Much of this information is communicated by specific RNA-binding proteins. These proteins first associate with pre-mRNA (primary transcripts of genomic DNA-containing exons and introns) cotranscriptionally and undergo a dynamic series of rearrangements involving the binding and dissociation of numerous proteins throughout the life of mRNA. The mRNA nucleoprotein complex (mRNP) communicates information to the cytoplasm about the structure of the gene from which the mRNA was formed and the processing steps experienced by the mRNA. The mRNP therefore carries significantly greater information than the sequence of the mRNA itself (1).

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### Initiation

Translation requires the positioning of an elongation-competent 80S ribosome at the initiation codon (AUG). Binding of the small (40S) ribosomal subunit at the 5′ end of the mRNA is rate limiting and requires energy in the form of ATP. It then forms a 43S preinitiation complex with eukaryotic initiation factors (eIFs) 3, 1, 1A, and 5, and a ternary complex including the methionine-loaded initiator tRNA that will recognize the AUG codon and eIF2 that is coupled to GTP. The preinitiation complex recognizes the mRNA by the binding of eIF3 to the eIF4 protein complex associated with the 5′ cap structure (eIF4F) (2). The eIF4F protein complex contains an enzyme (eIF4A) that unwinds RNA duplexes, allowing the 43S complex to bind and scan the mRNA, and a scaffold protein (eIF4G) that serves as a platform for the assembly of other proteins and interacts with the poly(A)-binding protein. This interaction is thought to loop the mRNA and bring the 3′ UTR in close proximity to the 5′ end of the mRNA (3). This provides a means by which sequences in the 3′ UTR can regulate translation initiation. Most known translational regulatory sequences are found within the 3′ UTR.

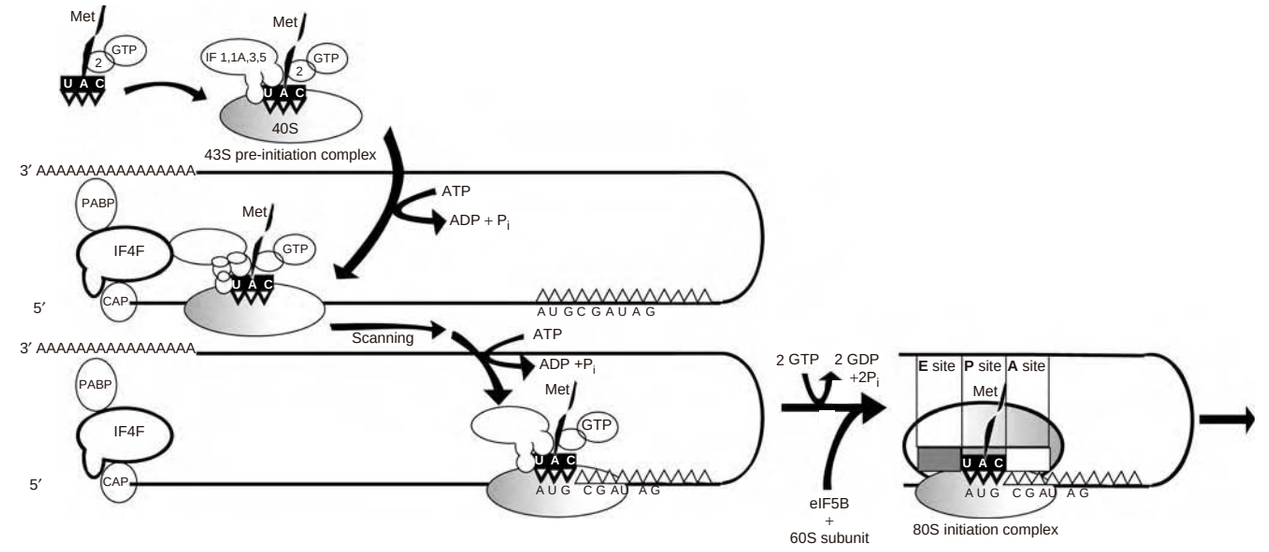
The 43S complex recognizes the initiation codon through the formation of base pairs (bp) between the initiator tRNA and the start codon. Subsequently, eIF2-bound GTP undergoes hydrolysis, a reaction that is necessary for the 60S ribosomal subunit to join the initiation complex. This appears to release most of the initiation factors from the small ribosomal subunit, leaving the initiator tRNA associated with the ribosome (in the P site). Formation of the 80S initiation complex capable of catalyzing the formation of a peptide bond occurs with the hydrolysis of a second molecule of GTP on eIF5B (Fig. 2-1A).

### Regulation of Initiation

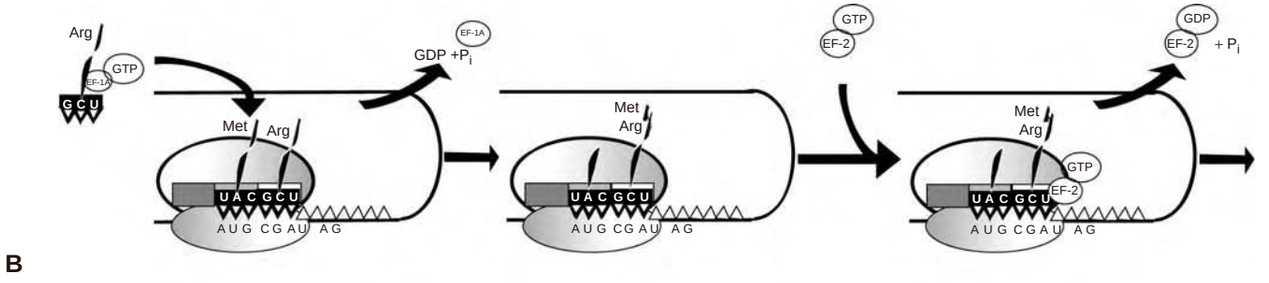
Although the specific translational regulatory mechanisms active in peptide hormone synthesis are not yet clear, translation is generally controlled at the initiation step where regulation may be global or mRNA specific. Global control of mRNA translation generally occurs through changes in the phosphorylation state of initiation factors or regulators that interact with them. Proteolytic cleavage of translation factors can also reduce translation of all mRNA species within the cell. mRNA-specific regulation of translation can be achieved by steric blockage, interference with the eIF4F complex, and cap-independent inhibition of the early initiation steps. Steric blockage refers to the binding of regulatory proteins to message-specific response elements that results in insufficient space for the binding of critical initiation complex proteins (4–7). Interference with the eIF4F complex is achieved by mRNA-specific binding proteins that block eIF4E recognition by eIF4G (8–11). Cap-independent inhibition of translation refers to proteins that bind to specific sites in both the 5′ and 3′ UTRs and recruit corepressors to the 3′ UTR. This affects stable association of the small ribosomal subunit with the mRNA (12–14).

Translation can also be controlled later in the initiation process. RNA-binding proteins have been described that prevent the binding of the 60S ribosomal subunit to the 40S subunit at the initiation codon, apparently through interference with initiation factors (15). The existence of more than one open reading frame on an mRNA and the sequence distance between the open reading frames can also play a significant role in determining the likelihood of translation. For example, amino acid deprivation reduces global protein

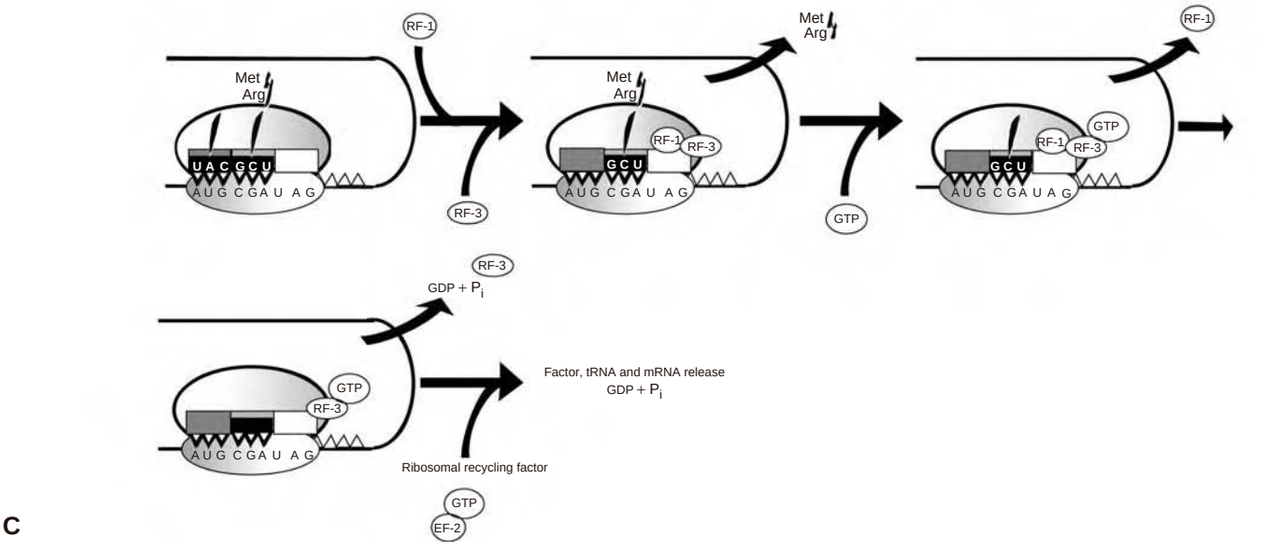
**FIG. 2-1.** Translation of messenger RNA (mRNA) into protein, highly simplified. Some translation-initiation factors are omitted (see Preiss and Hentze [526], Ramakrishnan [527], and Hershey [528] for more complete descriptions). **(A)** Cap-mediated initiation: The methionine-containing ternary complex (methionine-loaded transfer RNA [tRNA], eIF2, and GTP) binds to the 40S ribosomal subunit and other initiation factors (eukaryotic initiation factor-1 [eIF1], 1A, 3, and 5) to form the 43S preinitiation complex. The preinitiation complex recognizes the mRNA through the binding of eIF3 to eIF4 in the cap-binding complex. The cap-binding complex contains eIF4A, an RNA helicase that unwinds the secondary structure of the mRNA during the subsequent scanning step. The cap-binding complex also contains eIF4G, which contacts the poly(A)-binding protein (PABP). This contact is thought to bring the 3′ region of the mRNA in close proximity to the 5′ cap. The 43S preinitiation complex scans the mRNA from 5′ to 3′ until the initiation codon, AUG, is encountered. Stable binding of the preinitiation complex to the AUG codon yields the initiation complex. Subsequent joining of the 60S ribosomal subunit results in the formation of the 80S initiation complex. AUG recognition and the joining of the 60S ribosomal subunit both trigger GTP hydrolysis. The 80S complex contains an aminoacylated initiator tRNA in the P site of the ribosome and an empty A site. It now is competent to catalyze the formation of the first peptide bond. **(B)** Elongation: A ternary complex containing aminoacylated tRNA and the correct anticodon is brought into the A site of the ribosome. Codon–anticodon recognition leads to guanosine triphosphate (GTP) hydrolysis. This allows for conformational changes within the tRNA and the ribosome. Peptide bond formation (deacylation of the P site tRNA and the transfer of the peptide chain to the A site tRNA) then occurs. Translocation of the tRNA and the mRNA is facilitated by a GTPase, eukaryotic elongation factor-2 (eEF2). The ribosome is then ready for the next round of elongation, with a deacylated tRNA in the E site, peptidyl tRNA in the P site, and an empty A site. **(C)** Termination: When a stop codon on the mRNA is encountered in the A site, eukaryotic release factor-1 (eRF1) binds to the ribosome A site and triggers the release of the peptide chain from the tRNA in the P site. eRF3 then binds GTP and promotes dissociation of eRF1 from the ribosome. Hydrolysis of GTP is required for subsequent release of eRF3. The ribosome is then left with mRNA and a deacylated tRNA in the P site. The ribosomal releasing factor, together with eEF2 and GTP, is required to disassemble the complex and prepare the ribosome for a new round of protein synthesis. Much of the mechanism of mRNA, translation factor, and subunit release after peptide chain termination remain to be determined. GDP, guanosine diphosphate.


Met
GTP
2
U A C
IF 1,1A,3,5
40S
43S pre-initiation complex
3′ AAAAAAAAAAAAAAAA
PABP
IF4F
CAP
5′
ATP
ADP + Pi
A U G C G A U A G
Scanning
ADP +Pi
2 GTP
2 GDP
+2Pi
E site
P site
A site
eIF5B
+
60S subunit
80S initiation complex

A


Arg
GTP
EF-1A
G C U
GDP +Pi
Met
U A C G C U
A U G C G A U A G
EF-2
GDP
+ Pi

B


RF-1
RF-3
GTP
GDP + Pi
Factor, tRNA and mRNA release
GDP + Pi
Ribosomal recycling factor
EF-2

C

synthesis by phosphorylation of eIF2a, which blocks GDP-GTP exchange and reconstitution of the functional ternary complex. Paradoxically, the same modification increases the translation of some mRNA that have upstream open reading frames. It appears that the 60S ribosomal subunit dissociates at the stop codon of the first open reading frame and the 40S subunits remain associated with the mRNA and resumes scanning. The 40S subunit must acquire an active ternary complex during scanning to translate downstream open reading frames. The probability of translating the most 3′ open reading frame therefore depends on the distance (scanning time) between the open reading frames and the availability of amino acids within the cell (16).

Internal ribosome entry sites (IRESs) mediate translation initiation independent of the cap structure by recruiting the ribosome directly to an internal position of the mRNA (17). Both structural features and short-sequence elements appear to be involved in ribosome recruitment in eukaryotic IRESs. Exactly how these motifs combine to promote internal initiation remains to be determined. The IRES appears to be a complex RNA scaffold that contains multiple sites for interaction with components of the translational apparatus. Structural domains have been identified that interact with the initiation factors eIF4G and eIF4B (18,19), with eIF3 (20,21), or directly with the 40S ribosome subunit at multiple sites. An IRES is also described that can assemble an 80S ribosome at its initiation codon without the aid of any initiation factors or an initiator tRNA (22). A growing body of evidence exists to support the hypothesis that cellular IRESs are involved in the regulation of gene expression under physiologic conditions during which the efficiency of cap-dependent protein synthesis is greatly reduced. IRESs enable cells to respond to these conditions against the background of a general reduction in protein synthesis.

### Elongation

Each ribosomal subunit has three binding sites for tRNA: designated the A (aminoacyl) site, which accepts the incoming aminoacylated tRNA; P (peptidyl) site, which holds the tRNA with the nascent peptide chain; and E (exit) site, which holds the deacylated tRNA before it leaves the ribosome. The end of the initiation process leaves an aminoacylated initiator tRNA in the P site of the ribosome and an empty A site, which serves to start the elongation process. Aminoacylated tRNA is brought into the A site as a ternary complex with eukaryotic elongation factor-1A (eEF1A) and GTP. Correct codon-anticodon interactions result in conformational changes in the ribosome that stabilize tRNA binding and trigger GTP hydrolysis by eEF1A. This leads to the release of the aminoacyl end of the A site tRNA by eEF1A; the tRNA then swings into the peptidyl transferase site of the large subunit in a process called accommodation. The peptide bond is formed through deacylation of the P site tRNA and the transfer of the peptide chain to the A site tRNA. The ribosome then has a deacylated tRNA in the P site and peptidyl tRNA in the A site. Translocation of tRNA and mRNA is facilitated by eEF2, which is also a GTPase. The ribosome is then ready for the next round of elongation, with deacylated tRNA in the E site, peptidyl tRNA in the P site, and an empty A site ready to receive the next cognate ternary complex (see Fig. 2-1B).

### Termination

Termination begins when a stop codon (UAA, UGA, or UAG) is encountered in the A site mRNA. Stop codons are recognized by eukaryotic release factor-1 (eRF1). The GTPase eRF3 then binds the complex of eRF1 bound to the ribosome. Binding of eRF1 to the ribosome at the stop codon A site triggers the hydrolysis and release of the peptide chain from the tRNA in the P site. Hydrolysis of peptidyl tRNA by eRF1 is required for binding of GTP to eRF3 on the ribosome. This, in turn, leads to a conformational change in eRF3 that has high affinity for ribosomes and the dissociation of eRF1 from the ribosome. Hydrolysis of GTP is required for subsequent dissociation of eRF3 from the ribosome (see Fig. 2-1C) (23,24).

### Localized Translation Regulation

In addition to regulation of the initiation process, mRNA-specific translation regulation also occurs regionally in polarized cells. This is clearly demonstrated in neural tissues where stimulation of synapses induce the polyadenylation and translation of cytoplasmic polyadenylation element-containing, but not cytoplasmic polyadenylation element-lacking, mRNA stored in dendrites (25). This allows the generation of protein gradients emanating from particular positions in cells or the restriction of protein expression to a specific region and is a potential mechanism by which a cell may modulate its response to repeated, directional stimuli.

### RNA Silencing

Small RNA molecules regulate mRNA-specific translation either by translational repression, in the case of microRNA (miRNA) (26–28), or by mediating the degradation of the target mRNA, in the case of small interfering RNA (siRNA) (29,30). The functional difference between miRNA and siRNA (both about ~22 nucleotides in length) depends on the degree of complementation between the small RNA molecule and the mRNA target (31,32). miRNA hybridize by incomplete base paring, usually to several sites in the 3′ UTR of target mRNA. siRNA show perfect complementation to the target mRNA. miRNA and siRNA have distinctly different origins within the nucleus, but they have common RNA-binding proteins (33). It is unclear whether a single type of small RNA-protein complex can mediate both

target mRNA cleavage and translational inhibition (see review by Sontheimer [34]).

### Other Regulators of Messenger RNA Stability

Regulation of the rate of decay is an important control point in determining the abundance of an mRNA species, and decay rates of individual mRNA differ widely and can be differentially affected by environmental cues. Several sequence elements can regulate the rate of turnover of a transcript by attracting specific binding proteins that can either destabilize or stabilize the transcript. The strength of the association of these binding proteins can be modified by changes in the cellular environment. The principal mRNA-degradation pathway begins with removal of the 3′ poly(A) tail. Interaction of the cap proteins and the poly(A)-binding proteins with the translational machinery likely protects the 5′ and the 3′ end of the mRNA from attack by deadenylases and decapping enzymes (35,36). This means that translation and mRNA decay are linked. Support for this comes from studies demonstrating that inhibition of translation initiation destabilized mRNA (37) and inhibition of translation elongation (with cycloheximide) promotes mRNA stability (38). The nonsense-mediated decay pathway further links translation to mRNA turnover. This pathway ensures that mRNA with premature stop codons are not translated. To be recognized as premature, a termination codon must lie upstream of the last intron (39–43). Exon-exon junctional complex proteins, which mark the position of exon-exon junctions in the mature mRNA, may play an important role in surveillance for potentially deleterious nonsense mutations (44).

## POSTTRANSLATIONAL PROCESSING

Although it would appear that the translation of polypeptide hormones is similar to that of other eukaryotic proteins, the posttranslational processing of prohormones is unique. Since the initial discovery of proinsulin (45), it has been evident that the synthesis of polypeptide hormones of the gut involves a series of modification steps after the initial translation of the gene product that are distinct from the biosynthesis of other cellular proteins. These modifications, achieved via a variety of posttranslational processing reactions, may enlarge or diminish the size of the peptide precursor, but, in general, they result in the formation of biologically active and physiologically relevant products. Efforts to determine the nature and mechanisms of peptide hormone posttranslational processing reactions were greatly facilitated by the development of molecular biological techniques that permitted the deduction of peptide precursor sequences. Information on precursor structure has led to the development of molecular probes that can be used to characterize individual processing reactions, as well as patterns of processing reactions for groups of related peptides. Application of these probes to ultrastructural studies has provided information on the cellular compartments in which processing reactions take place. *In vitro* reconstitution experiments have led to the elucidation of some of the mechanisms responsible for the transport of peptide precursors between cellular compartments. Development of techniques to isolate and culture functionally intact peptide-secreting cells has permitted physiologists to examine the sequence and dynamics of the complete posttranslational modification and activation process for given peptides. Many of the enzymes responsible for prohormone processing have now been isolated. Coexpression or deletion, or both, of these enzymes within cells has allowed for elucidation of their activities for multiple prohormone substrates.

Previously, it was thought that proteins exited from cells via two distinct pathways: the constitutive or the regulated secretory pathways (46,47). As has been the case with other biological systems, more recent evidence suggests that there might be overlap between these pathways (48). Generally, however, the constitutive pathway is reserved for those secreted proteins that are not stored in the cell and usually do not undergo extensive posttranslational processing, as seen with the products of fibroblasts and hepatocytes. Proteins secreted constitutively exit the cell soon after synthesis on the ribosome. Polypeptide hormones, however, enter the regulated pathway of secretion in most neuroendocrine cells. These cells are capable of storing secretory products for hours or days in electron-dense secretory vesicles and releasing them on stimulation. The intracellular pathways and organelles involved in this pathway were first defined in studies (49) in the exocrine pancreas demonstrating that polypeptides are initially synthesized on the rough endoplasmic reticulum (ER), transported to the Golgi apparatus, and finally placed into secretory granules (Fig. 2-2). On cell stimulation, these secretory granules or vesicles fuse in a calcium-dependent manner with the cell membrane to release their contents into the extracellular milieu. This chapter reviews the enormous progress made in recent years in elucidating the mechanisms for posttranslational processing of gastrointestinal peptide hormones, and then presents a detailed analysis of one hormone, gastrin.

## TRANSPORT INTO THE ENDOPLASMIC RETICULUM

Polypeptide hormones are synthesized ribosomally from the amino-terminal end and enter the secretory pathway via translocation into the ER. This process is of critical importance to both prokaryotes and eukaryotes. Thus, it has been thoroughly examined by several notable scientists including Gunther Blobel, who won a Noble Prize for his work in this area (50). In summary, the first few amino acids of the preprohormone, translated from the leader sequence of the specific mRNA, are called the signal peptide (Fig. 2-3) (51). This peptide (designated as the presequence in preprohormones) is not secreted under normal circumstances but serves as a means of translocating the newly synthesized and

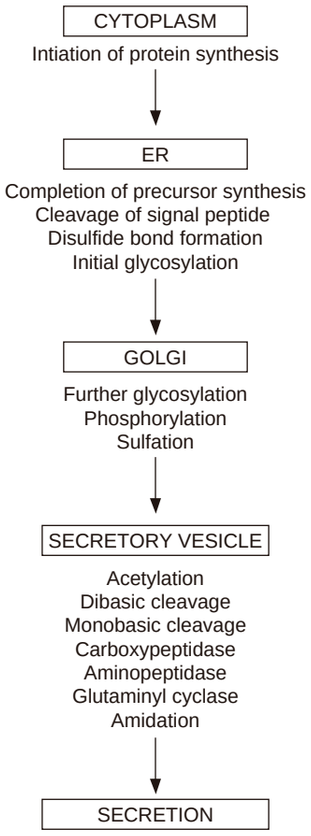



**FIG. 2-2.** Intracellular location of posttranslational processing steps. ER, endoplasmic reticulum.

gradually elongating polypeptide chain into the ER (52–54). After emerging from the ribosome, the signal peptide binds to the signal recognition particle (SRP) in the cytoplasm after chain elongation has produced a preprohormone of approximately 50 to 60 amino acids (55–57). This binding results in an arrest of translation, and the SRP initiates the translocation of the nascent polypeptide by binding to the SRP receptor or docking protein located on the cytosolic side of the ER (58–60). The SRP is then released, and the translocation of the peptide continues through a protein channel across the ER membrane (53,59,61–63). The signal peptide is later cleaved by a specific enzyme (signal peptidase) located on the inner membrane of the ER (64). The individual components of these ER translocation events are described in more detail in the next section.

### Signal Peptides

The signal peptide, or *pre*sequence, constituted by the amino-terminal 20 to 30 amino acids of a newly synthesized polypeptide chain, directs the translocation of the polypeptide into the ER lumen. There seems to be little primary amino acid homology in the signal peptides of the known gastrointestinal hormone precursors. However, three general characteristics are shared: (1) a positively charged amino-terminal region of 1 to 10 amino acids, (2) a central hydrophobic region of 7 to 17 amino acids, and (3) a more polar region that often contains an α helix breaking proline or glycine residue, as well as uncharged residues that determine the cleavage site and complex pattern of amino acids adjacent to the site of cleavage between the signal peptide and the prohormone (65,66). The secondary structure of these peptides can assume several different conformations including α helices and β-pleated sheets, depending on the environment (67). Recently, analysis of the new, extensive protein databases has allowed investigators to accurately predict signal peptides (68). The positively charged amino terminus appears to be important in the release of the SRP once docking of the nascent peptide to the ER has occurred. Mutations in this area that result in a net negative charge interfere with both export and synthesis of secretory proteins in prokaryotes (69), although this does not appear to be the case in eukaryotic systems (70). Mutations that substitute polar or charged amino acids for the amino acids present in the hydrophobic region of the signal peptide result in impaired binding of the nascent peptide chain to the SRP (57). Thus, translation is complete, but export of newly synthesized protein is inhibited (71). Initially, it was thought that conservative substitutions of one hydrophobic amino acid for another (e.g., glycine for valine) did not alter the recognition between the SRP and the signal peptide (72). More recently, others have noted that even small changes in the central hydrophobic core can alter SRP binding (73,74). However, in these cases, translocation across the ER membrane still occurs through an unknown mechanism. SRP binding is not dependent on the presence of a net positive charge at the amino terminus or on any identifiable features at the carboxyl terminus (75,76). There do not appear to be any specific structural requirements for the site of signal peptide cleavage, although the carboxyl-terminal amino acid of the signal peptide usually has a small uncharged side chain such as alanine (65,77) (see Signal Peptidase later in this chapter).

After translocation through the ER membrane pore, the signal peptide can loop back through the membrane. The signal peptide is then cleaved at its c terminus by signal peptidase. However, recent studies have shown that the signal peptide can be cleaved further by a signal peptide peptidase to release the amino terminal fragment into the cytosol (78). In the case of prolactin, this fragment then binds to calmodulin in a calcium-dependent manner (79). Currently, the full biological implications of this finding are unknown. In addition, it is not known if this applies to multiple other peptide hormones.

### Signal Recognition Particle

SRP has three known functions: signal peptide recognition, elongation arrest, and promotion of translocation (55,56, 80–83). This particle consists of six polypeptide components with molecular weights of 72, 68, 54, 19, 14, and 9 kDa,

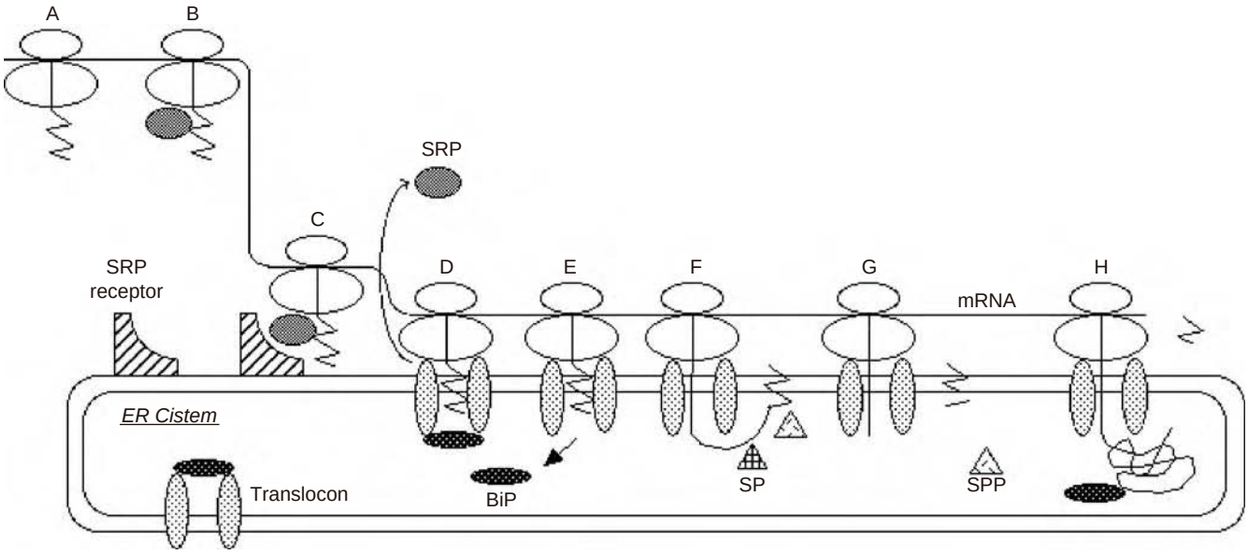



**FIG. 2-3.** Signal peptides. **(A)** Ribosome binds to the messenger RNA (mRNA) and translation begins at the amino terminus. **(B)** Signal sequence emerges from the ribosome and binds to the signal recognition particle (SRP), which induces an arrest of translation. **(C)** SRP-ribosome complex binds to the SRP receptor or docking protein located on the endoplasmic reticulum (ER) membrane. **(D)** The translocon or protein pore binds to the ribosome, releasing the SRP and its receptor. **(E)** With binding of the ribosome to the cytosolic side of the translocon, BiP is released from the lumenal side of the translocon. **(F)** Translation resumes at the carboxyl terminus, and the signal peptide can be reinserted into the ER membrane. **(G)** Signal peptide is cleaved for the prohormone by signal peptidase (SP), and the signal peptide fragment is released into the cytosol via signal peptide peptidase (SPP) cleavage. **(H)** Translation continues until the entire precursor is located within the ER cistern and properly folded, often in association with chaperones such as BiP.

as well as a 7SL RNA (80,84). Each component is held together in a defined tertiary structure by $Mg^{2+}$ ions and is essential for the functions of the SRP. The 19- and 54-kDa proteins exist as monomers, but heterodimers of the 9- and 14-kDa proteins and the 68- and 72-kDa proteins are formed (85). The 54-kDa protein contains a series of amphipathic helices with methionine residues, located predominantly on one face, that appear to be important for the binding to the hydrophobic region of the signal peptide (83,86–88). Interestingly, SRP will not bind to signal peptides that are not tethered to a ribosome, although the peptide region responsible for the SRP–ribosome interaction is not known. It appears that the 54-kDa protein binds to the 7SL RNA through the 19-kDa protein that binds directly to the middle of the RNA strand (88). In addition, the 7SL RNA contains 5′ and 3′ Alu-like elements that bind to each other and the 9/14-kDa protein heterodimer (89–91). The 9/14-kDa protein heterodimer mediates elongation arrest of translation, but plays no role in the translocation process (91,92). The 68/72-kDa heterodimer binds to the middle segment of RNA close to the 19-kDa binding site and appears to mediate the binding of SRP to its receptor (93,94). Thus, the 68/72-kDa heterodimer is not involved in elongation arrest but serves to aid in translocation.

The 54-kDa protein binds GTP in concert with binding to the signal peptide (80,82,95–97). An additional GTP is required on binding of the SRP to the SRP receptor (96). When the SRP/SRP receptor complex associates with the ER membrane or translocon there is a subsequent release of GDP (98). The hydrolysis of GTP releases the SRP from the signal peptide and allows translation to proceed.

### Signal Recognition Particle Receptor

The SRP receptor is located on the cytosolic side of the ER and binds to the SRP–ribosome complex, but not to free SRP as noted earlier. The SRP receptor plays an important role in termination of the elongation arrest and in the translocation of polypeptides into the ER lumen (59). The SRP receptor is a heterodimeric protein consisting of a 30-kDa integral membrane protein (β subunit) and 72-kDa α subunit that possesses domains that are homologous to GTP-binding proteins and the GTP-binding region of the 54-kDa SRP protein (88,95,99–104). The 72-kDa α subunit of the SRP receptor binds to SRP, and GTP is necessary to release SRP from the signal peptide–ribosome complex (95,105).

### Endoplasmic Reticulum Membrane Protein Channel or Translocon

There have been numerous theories about whether the nascent polypeptide chain is transported directly across the

lipid bilayer or in the aqueous environment of a protein channel. Although the initial thought was that the hydrophobic core of the signal peptide would allow for direct translocation across the membrane, there is a large transmembrane channel that is opened by the presence of signal peptides (106–108). The eukaryotic ER translocon is a heterotrimer (Sec61α, Sec62β, and Sec61γ) estimated to have a diameter of about 30 Å (109–111). The size of the pore is too small for folded proteins, thus ensuring that only nascent, unfolded proteins can enter the ER lumen. The lumenal side of the translocon is sealed by a protein, BiP, that aids in protein folding after passage through the membrane (112). On binding of the ribosome to the translocon, the SRP and its receptor disassociate from the complex, allowing resumption of translation (113). This process also seals the pore on the cytosolic side of the ER membrane, releasing BiP from the lumenal side.

## PROCESSING IN THE ENDOPLASMIC RETICULUM

### Signal Peptidase

During translocation, the signal peptide is cleaved from the propeptide by signal peptidase, an integral membrane protein complex on the lumenal surface of the ER. Signal peptidase has been purified from the dog pancreas as a complex of 5 polypeptides with molecular weights of 12, 18, 21, 22/23, and 25 kDa (114,115). The enzyme in the hen oviduct has only 2 subunits of 19 and 22/23kDa (116). The canine and hen 22/23-kDa proteins are glycosylated, and their amino acid sequences are 90% identical (117,118). cDNA encoding the canine 18 (119) and 21 kDa (120) are homologous to 2 yeast SEC11 proteins (121) that are components of the yeast signal peptidase, which contains 4 proteins in total (with molecular weights of 13, 18, 21, and 25 kDa). The 21-kDa protein is absolutely required for enzymatic function in yeast (122,123), raising the question of the exact function of the other proteins. As is the case with processing enzymes, there are great similarities between the yeast and mammalian enzymes (124). Although it appears that the structure of eukaryotic signal peptidases are phylogenetically conserved, the *Escherichia coli* signal peptidase consists of only a single subunit of 323 amino acids (125). Nevertheless, there is some sequence homology between bacterial signal peptidases and subunits of the eukaryotic enzyme. Furthermore, the substrate specificity of the eukaryotic and prokaryotic signal peptidases is similar (126). Eukaryotic signal peptidase has a broad pH optimum and requires phosphatidyl choline as a cofactor (127,128).

Determination of the amino acid sequences that define the substrate specificity of signal peptidase has been difficult because of the enormous structural diversity of signal peptides (129,130). However, there is clearly a hierarchy of preferred substrates for amino acids located at the carboxyl terminus of the signal peptide as follows: Ala>Cys>Gly>Ser>>Thr>Pro>Asn>>Val, Ile, Leu, Tyr, His, Arg, Asp (65,131). Mutations of the signal peptide that increase the number of amino acids between the end of the central hydrophobic domain and the site of cleavage and mutations in the positively charged amino-terminal domain inhibit the cleavage reaction (132–134).

### Disulfide Bond Formation

After peptide prohormones are translocated into the ER lumen, they can undergo intermolecular or intramolecular disulfide bond formation (e.g., proinsulin) (Fig. 2-4). In the case of proinsulin, disulfide bonds are formed before cleavage of proinsulin into its component A and B fragments by removal of the C peptide. Thus, the disulfide bonds that are intramolecular on the prohormone are subsequently converted to intermolecular linkages that cannot be recreated easily after they are reduced. Although spontaneous formation of disulfide bonds of peptides such as somatostatin can occur *in vitro* over a few hours under optimal conditions, *in vivo,* the process occurs either cotranslationally or within seconds after translocation (135). The rapidity of this process suggests that it is catalyzed by an enzyme, the prime candidate being protein disulfide isomerase (PDI) (136–138). In solution, PDI exists as a homodimer ($2 \times 57$ kDa) with a highly acidic isoelectric point (pI) (139). PDI has a broad substrate specificity encompassing relatively small proteins such as insulin, as well as large multidomain proteins such as immunoglobulins (137). PDI also forms the β subunit of a tetrameric enzyme ($\alpha_2\beta_2$) denoted as prolyl-4-hydroxylase, which is responsible for hydroxylation of proline in the formation of procollagen (140). In tissues requiring both PDI and prolyl-4-hydroxylase activities, it appears that the β subunits of prolyl-4-hydroxylase are synthesized in large excess with a fraction being recruited into the prolyl-4-hydroxylase tetramers and the remainder as functional PDI homodimers (140).

Previously, it was thought that glutathione provided the oxidizing equivalents for PDI (141,142). More recently, investigators have identified an ER membrane protein

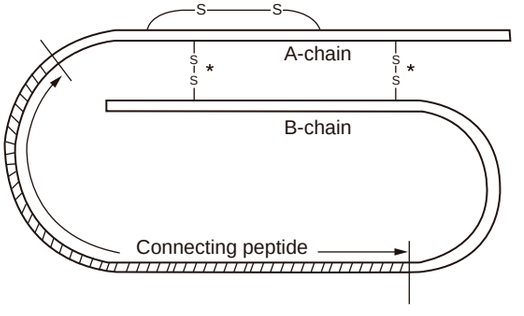



**FIG. 2-4.** Structure of proinsulin. A and B peptides are linked by intramolecular disulfide bonds *(asterisk),* but after the connecting peptide is removed by endoproteolytic cleavage, disulfide bonds are intermolecular. On exocytosis, insulin (A and B peptides) is coreleased with the C peptide.

(Ero 1p) in yeast (143,144) that serves this function. Indeed, Ero 1p directly oxidizes PDI through disulfide exchange (141,145). The reoxidation of Ero 1 involves flavin adenine dinucleotide (FAD) (145).

### Asparagine-Linked N-glycosylation

There are few examples of gastrointestinal peptides with N-linked glycosylation. The primary amino acid sequence of -Asn-X-Thr/Ser, where *X* can be any amino acid except proline, is obligatory for N-glycosylation of asparagine (146). The anterior pituitary glycoprotein hormone family (thyroid-stimulating hormone, follicle-stimulating hormone, and leuteinizing hormone) is the best example of glycosylated hormones. These are dimeric proteins with a common β subunit and different but homologous β subunits that confer specific biological activities. The glycosylation of both subunits is important for their correct assembly into dimers (147). Proopiomelanocortin (POMC) (148) and proenkephalin A (149) are also glycosylated, although the functional significance of this modification is unknown in these peptides. Secretogranin I (also known as chromogranin B) has a single glycosylation site, but it is uncertain whether it is glycosylated *in vivo* (150).

### Protein N-myristoylation

Protein N-myristoylation refers to the cotranslational linkage of myristic acid (C14:0) to the amino-terminal glycine of proteins; protein N-myristoylation is reviewed elsewhere (151). There are no known examples of myristoylated prohormones; however, this modification may play a role in the regulation of a variety of cellular events including posttranslational processing. Examples of N-myristoylated proteins include GTP-binding proteins and the catalytic subunit of cyclic 3′,5′-adenosine monophosphate-dependent protein kinase A.

### Protein Folding

Polypeptides must be folded into a conformation that is compatible with exit from the ER (109,152). Misfolded proteins are tightly but noncovalently bound to a heavy chain binding protein or BiP and retained in the ER (153) until folding is complete and the polypeptide is released on hydrolysis of ATP (154,155). BiP, a member of the heat shock family of proteins (HSP70), binds newly translated and translocated aliphatic single polypeptides and prevents them from folding prematurely (156,157). It is currently unknown whether BiP or other folding proteins (158) are involved in the posttranslational processing of mammalian gastrointestinal prohormones, although BiP is clearly important in the translocation and folding of the yeast prohormone, pro-α factor in the ER (159). Another important folding chaperone is calnexin, but it interacts only with N-glycosylated proteins (158). Because few prohormones are glycosylated, it is hypothesized that this pathway is not involved in prohormone processing. An important factor that should not be forgotten is the role that disulfide bond formation plays in maintaining the folded nature of many polypeptides such as proinsulin.

## TRANSPORT FROM THE ENDOPLASMIC RETICULUM AND THROUGH THE GOLGI

The mechanisms responsible for protein sorting beyond the ER have been the subject of much investigation. Unlike the well-defined sorting of prohormones to the ER lumen through a signal peptide, there is no single unifying mechanism of prohormone transport from the ER and through the Golgi. Two types of sorting mechanisms have been hypothesized. The first is that prohormones are transported in the nonspecific "bulk flow" of contents from the ER to the Golgi in transport vesicles. An alternative hypothesis is that there is some signal contained in the prohormone structure that specifically directs their sorting through the intracellular compartments. This latter hypothesis is the case for resident soluble ER proteins such as BiP and PDI. Investigators noted in the structures of BiP and PDI a carboxyl-terminal consensus sequence KDEL (LysAspGluLeu) (160). Truncated forms of BiP lacking the KDEL sequence are not retained in the ER, but rather are secreted constitutively. In analogous fashion, prohormones destined for secretion but tagged with KDEL are retained in the ER in an unprocessed form (161). The homologous tetrapeptides DKEL, RDEL, and KNEL are all capable of directing ER retention in mammalian cells, whereas the HDEL sequence is used primarily in yeast (162,163). Although the KDEL-tagged proteins could be retained by a KDEL receptor in the ER membrane, it appears that these proteins initially exit the ER and are then recaptured in a salvage compartment at or near the cis-Golgi and returned to the ER (164). A mutant strain of yeast ERD2 (for *E*R *r*etention *d*efective) has been shown to have a defect in the KDEL/HDEL receptor (165). The structure of the *ERD2* gene was then used to aid in the search for a mammalian homologue (166). This powerful technique of identifying genes of fundamental importance to the sorting of proteins in yeast and then using the yeast model to identify a mammalian homologue has been a fruitful approach in the study of peptide hormone processing. The *ERD2* gene encodes a protein of 26 kDa that contains 7 membrane-spanning regions and is highly homologous to a putative human *ERD2*-like gene (167). The mammalian KDEL receptor cycles from the ER to the Golgi and back to the ER, thus retaining lumenal ER proteins within that compartment (168).

Prohormones proceed from the ER to the Golgi stack where they undergo further posttranslation modification. Prohormone movement through the Golgi stack is by bulk flow (169,170) rather than a process mediated by a sorting signal. Bulk transport of soluble ER proteins to the Golgi and through the various Golgi compartments (cis, stack, and

trans-Golgi network [TGN]) was once thought to occur through transport vesicles (171). In this model, the Golgi was a series of stable, disconnected stacks through which proteins were progressively sorted, modified, or "distilled" toward their final destination. Although this model was attractively simple, it now appears that the Golgi is a much more fluid organelle (171–174).

Although the transport vesicle model was indeed attractive, the described vesicles were too small (70 nm in diameter) to transport many secreted proteins. An alternative model is that newly synthesized proteins move from the ER to the cis-Golgi cisternae located near the ER. This newly formed cisternae progresses through the Golgi stack from the ER to the trans-Golgi (175). The transport vesicles in this case merely shuttle enzymes that characterize the various layers of the Golgi back through the cisternae (176–179). Thus, prohormones are transported from the ER to the cis-Golgi and are not transported out of this compartment, but rather are carried forward to the trans side as newer enzymes and proteins are added to the cis-Golgi.

The nature of the ER to Golgi transport has been studied extensively (173). The ER membrane has a fixed number of exit sites from which proteins leave the lumen (180). The ER membranes cause buds that eventually become coated on their outer cytoplasmic surface with dispersed cytoplasmic proteins (coatamers, coat promoter, or COPs) (181–184). The budding ER vesicle is coated with COPII and traps prohormones together with other ER proteins (185–187). The COPII-coated vesicles then uncoat and fuse into a larger vesicular tubular complex (VTC) (185). It appears that the VTC is not continuous with the ER membrane. Eventually, the VTC combines with COPI (188). After fusing with the Golgi membrane (in a GTP-dependent manner) (189–191), the COPI-associated VTCs return ER proteins for recycling. Thus, nonhydrolyzable analogues of GTP such as GTP-γ-s interfere with fusion and block transport through the stack (192). VTCs move from the ER to the Golgi along a microtubular network that is powered by the dynein–dynactin motor (190,193). The fungal metabolite Brefeldin A, which is known to block protein transport through the Golgi stack, blocks the binding of the coatamer complex to budding Golgi membranes (194–197). The finding that Brefeldin A interferes with the posttranslational processing of progastrin suggests that this pathway is involved in the sorting of prohormones, as well as other soluble secretory proteins (198,199).

Proteins secreted via either the constitutive or regulated secretory pathways share a common trail from the ER through the Golgi stack, but they diverge in the TGN where proteins are sorted according to their final destination (169,200–202). The sorting signal for enzymes destined for lysosomes involves a glycosylation reaction that occurs in the Golgi stack to attach mannose-6-phosphate residue proteins. A receptor protein in the TGN specifically binds mannose-6-phosphate–modified proteins (203,204) and directs their sorting to lysosomes. To date, searches for a common sequence (KDEL-like) or posttranslational modifications (mannose-6-phosphate–like) in the structure of prohormones that might direct sorting to secretory vesicles in the TGN have not been successful.

Although investigators have long sought to elucidate "the" Golgi sorting signal in neuroendocrine cells, none has been entirely successful. Indeed, it appears that three different mechanisms may be responsible for prohormone sorting to secretory vesicles. These include sorting signal motifs, aggregation, and membrane or lipid raft binding (205). Initially, the search for a sorting signal was pursued vigorously. In a fashion akin to the signal peptide ("pre" region of preprohormones), investigators sought sequences in the "pro" region of prohormones that, although lacking homology in their primary amino acid sequence, still contain sufficient structural information to direct sorting in the TGN. An α-helical motif with three leucine residues occupying one side of the helix was proposed as such a signal, but this hypothesis was not proved (206). In other studies, a chimeric protein containing the "prepro" region of somatostatin at the amino terminus and a constitutively secreted protein such as γ-globulin at the carboxyl terminus were sorted and processed in the secretory pathway (207). Studies with POMC and somatostatin precursors containing deletions in the "pro" region indicate the presence of sorting information at these sites, as well as in other portions of the peptide (208,209). In contrast, deletion of the "pro" sequence from trypsinogen and renin did not disrupt the routing of these proteins into the secretory pathway (210,211). A study expressed neuropeptide Y (NPY) fragments tagged with green fluorescent protein (GFP). GFP, a jellyfish protein not normally secreted, was correctly sorted, stored, and released from neuroendocrine cells when fused to half of the prepro-NPY sequence or only the signal sequence alone of pre-NPY (212). Thus, it appears that some prohormones are likely sorted by a specific signal found in their "pro" regions, but this does not appear to be a universal finding.

A second sorting hypothesis is selective aggregation (213–215) of prohormones into acidic clathrin-coated secretory vesicles in the presence of high concentrations of divalent cations such as $Zn^{2+}$ or $Ca^{2+}$. Support for this hypothesis comes from observations that specific mutations in the structure of proinsulin that result in inhibition of hexamer formation with zinc also impede processing (48,216). Furthermore, *in vitro* studies have demonstrated that intravesicular conditions (pH 5.2 and 10 mM $Ca^{2+}$) can result in selective precipitation of peptides that exit the cell through the regulated pathology of secretion. This applies to secreted proteins such as secretogranin II but not proteins that are constitutively secreted such as immunoglobulins (217). A heterodimeric protein in adrenal chromaffin granules, termed *glycoprotein III,* can selectively aggregate with two prohormone-processing enzymes carboxypeptidase E (CPE) and a dibasic endoprotease (218). Evidence contradictory to the selective aggregation hypothesis can be found in studies with guinea pig proinsulin, which does not form hexamers with zinc and yet is sorted and processed with high efficiency (219). In addition, in the marine mollusk Aplysia, the egg-laying hormone precursor is processed into two distinct mature hormone products that are sorted into different