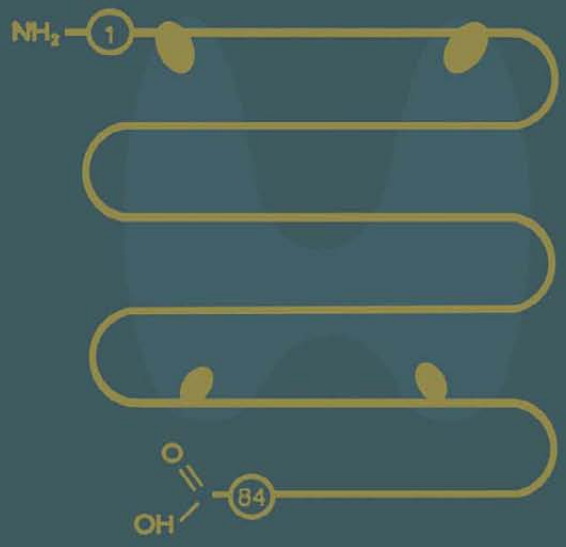

The Parathyroids

Basic and Clinical Concepts

SECOND EDITION

John P. Bilezikian
Robert Marcus
Michael A. Levine

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Preface to the Second Edition

The first edition of *The Parathyroids* was published in 1994. It marked a milestone in the field, carrying on the tradition of Albright and Reifenstein whose 1948 classic *The Parathyroid Glands and Metabolic Disease* established a key role for the parathyroids in calcium homeostasis and metabolic bone disease. In *The Parathyroids*, we assembled a body of knowledge that had been accumulating over a 30-year period. The spectacular pace of discovery placed the tiny parathyroid glands at an epicenter of an enormous research effort in metabolic bone disease. The first edition was used widely and filled an essential gap in reference literature. Over the past seven years, as this field has continued to grow, with newer and greater appreciation of the role of the parathyroids in the overall governance of calcium homeostasis, a second edition appears to be particularly apt.

The second edition of *The Parathyroids* contains chapters that have been extensively revised and expanded and many new chapters as well. The chapters document our new knowledge about virtually every facet of this field and reexamine classic precepts that have stood the test of time. We understand better than ever before the structure and function of the parathyroid hormone gene and protein as well as the regulatory control of parathyroid hormone synthesis and secretion, the physiological and pathophysiological aspects of parathyroid hormone-related protein (PTHrP), the mechanisms of parathyroid hormone and PTHrP action, and the cell biology of PTH and PTHrP. With regard to primary hyperparathyroidism, we now appreciate a spectrum of clinical presentations according to where in the world it is detected. Information about the course of primary hyperparathyroidism with and without parathyroid surgery is also new, as are the molecular genetics, biochemical, and histomorphometric dynamics of primary hyperparathyroidism. Advances in preoperative localization of parathyroid tissue and newer operative approaches to parathyroid gland surgery are noteworthy. The hypoparathyroid disorders are understood better with regard to their molecular genetics, pathophysiology, and mechanism. Finally, newer information is available about how parathyroid hormone can be both a catabolic and anabolic hormone for bone. This newer knowledge has fueled provocative ideas about the pathophysiology of osteoporosis and is heralding a new era in the therapeutics of osteoporosis. The second edition, thus, is still a comprehensive examination of basic and clinical concepts of the parathyroids. It is intended for students, teachers, practitioners, and investigators.

In light of these newer developments in the field, the second edition has been reorganized to provide the reader with information that follows best the changing scientific logic. Fifty-five chapters are divided into seven sections. In Section I, nine chapters are devoted to basic concepts of parathyroid hormone and PTHrP, covering embryology, anatomy, and pathology of parathyroid tissue; gene structure, biosynthesis, and metabolism of PTH and PTHrP; receptors, nuclear targeting, and signal transduction for PTH, PTHrP, and calcium ion; and a comprehensive review of the immunoassays for PTH and PTHrP. In Section II, eight chapters are devoted to the physiological aspects of calcium metabolism and the anabolic and catabolic effects of PTH at the level of bone and bone cells. Five chapters cover in detail all aspects of PTH and PTHrP with regard to traditional and nontraditional target organs. In Section III, 21 chapters are devoted to clinical aspects of primary hyperparathyroidism. Chapters on the growth of normal and abnormal parathyroid cells and the molecular genetics of primary hyperparathyroidism are followed by three chapters that describe different clinical presentations of primary hyperparathyroidism throughout the world. Detailed coverage of bone dynamics and stone disease is followed by information relevant to the medical and surgical management of primary hyperparathyroidism. Also covered are other presentations of primary

hyperparathyroidism: as a malignancy, as an acutely hypercalcemic disorder, and in association with the multiple endocrine syndromes I and II. A chapter on familial hypocalciuric hypercalcemia completes this section. Two chapters in Section IV cover the parathyroids in renal disease. These are followed by six chapters in Section V that focus on special considerations. The first three chapters review the differential diagnosis of hypercalcemia, including syndromes caused by the local and systemic production of hypercalcemic factors such as PTHrP. Jansen's disease, the acute management of hypercalcemia, and hypercalcemia in children are considered in separate chapters. In Section VI, the hypoparathyroid states are reviewed in six chapters, which cover molecular, ionic, and immunological defects in the hypoparathyroid states and the role of hypoparathyroidism in the differential diagnosis of hypocalcemia. In Section VII, the role of parathyroid function in osteoporosis is covered in three chapters describing changes in parathyroid function with aging, parathyroid function and responsiveness in osteoporosis, and the potential of parathyroid hormone as a therapy for osteoporosis.

As was true for the first edition, we recognize that this book is not likely to be read from cover to cover. Thus, each chapter has been written to provide a body of knowledge that can stand alone. The chapters, however, are also liberally cross-referenced to help the reader continue reading more directly related material if desired.

The first edition of this book was dedicated to the memory of Gerald D. Aurbach, whose untimely and tragic death was its catalyst and inspiration. Virtually all the principal authors of the first edition had known and worked with Jerry. We remembered him then for his "wisdom, scientific acumen, investigative skills, and daring insights." We remember him now in much the same way. We were and still are mindful of the role Jerry had not only for us but also for the entire field, which he helped to create. We were his scientific progeny. It is 10 years since Jerry's death, virtually a generation in the world of science. As a result, some of the leading figures in this field have emerged without having had the special privilege of working with or knowing Jerry. The authorship of the second edition has been broadened, therefore, to include the very best in our field, recognizing that although Jerry's legacy is still alive, it now extends to an even broader cross section of the field.

We wish to thank Jasna Markovac of Academic Press, who was instrumental in both the first and current editions of *The Parathyroids.* Mica Haley of Academic Press was also most helpful in attending to the many details required to ensure a rapid turnaround time to final publication.

Enjoy the book.

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Robert Marcus
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Preface to the First Edition

One of us (JPB), dreamed of this book about five years ago. It seemed then that advances in our knowledge of the parathyroids represented nothing less than a 30-year revolution of spectacular progress. We gained knowledge over this period at an explosive pace with a concomitant new appreciation of the basic and clinical ramifications of these four tiny endocrine glands. The major secretory product, parathyroid hormone (PTH), was isolated, sequenced, assayed, and cloned. PTH became one of the first hormones to be shown to utilize cAMP as a second messenger. Regulation of PTH synthesis and secretion by calcium and 1,25-dihydroxyvitamin D was appreciated, as well as the cellular effects of PTH on its two major target organs, bone and kidney. The discovery of parathyroid hormone-related protein (PTHrP) as a cause of hypercalcemia of malignancy and a more general appreciation of PTHrP and PTH as polypurpose factors with many diverse biological effects represent exciting new advances in our field. The recent cloning of a bona fide receptor for both PTH and PTHrP is a tremendous achievement, as is the thinking that both PTH and PTHrP may utilize more than one second messenger pathway, and perhaps interact with more than one receptor. At the clinical level, we have seen a remarkable evolution in the presentation of primary hyperparathyroidism and are beginning to understand molecular features of this disease. Pseudohypoparathyroidism is now appreciated, in its classical form, to be a G protein deficiency disease. Autoimmune and molecular features of hypoparathyroidism have been identified and studied. New knowledge of the pathophysiology of secondary hyperparathyroidism associated with renal failure has had direct impact on management and clinical outcome. PTH is now appreciated to have important anabolic properties in bone that may have implications for its use as a therapeutic agent in osteoporosis. This incomplete summary argues persuasively for how fast and how far this field has advanced.

This is not to say that we were in the dark ages before Aurbach isolated parathyroid hormone. Certainly, it was Fuller Albright who in 1948 correctly pointed out that "back in the dark ages of endocrinology, in the early 1920s, hyperparathyroidism was an unknown fact." It was also Albright who reminded us of the work of Sandstrom, who in 1880, 40 years before the first known cases of hyperparathyroidism wrote, "The existence of a hitherto unknown gland in animals that have so often been a subject of anatomical examination called for a thorough approach to the region around the thyroid gland even in man. Although the probability of finding something hitherto unrecognized seemed so small that it was exclusively with the purpose of completing the investigations rather than with the hope of finding something new that I began a careful examination of this region, so much the greater was my astonishment therefore when in the first individual I examined, I found on both sides at the inferior border of the thyroid gland an organ of the size of a small pea, which judging from its exterior, did not appear to be a lymph gland, or an accessory thyroid gland, and upon histological examination showed a rather peculiar structure."

The first chapters on the parathyroids were indeed written by Albright and a band of spectacular clinical investigators of the 1920s, 1930s, and 1940s. These chapters are recorded in the Albright and Reifenstein classic *The Parathyroid Glands and Metabolic Disease.* We recommend this insightful 45-year-old book as important and provocative reading. *The Parathyroids* is designed to follow the Albright and Reifenstein text. Certainly all endocrinology reference texts routinely include a section on the subject matter of this book. Other texts that are more focused on calcium metabolism provide more information than the standard endocrinology texts on the parathyroids. However, there is no book that is exclusively devoted to a comprehensive examination of basic and clinical concepts

of the parathyroids. As indicated by the size and scope of *The Parathyroids*, it is clear that a book devoted to this subject is worthy and long overdue. It is time for such a book to stand on the endocrine shelf near its anatomical partner, the thyroid gland, which in Werner and Ingbar's *The Thyroid* has had its own literary repository since 1955.

This book is intended for students, teachers, practitioners, and investigators of this field. It covers in a current and concise yet complete manner virtually all that we know about the parathyroids. Thus, it is both a basic and a clinical text. The 51 chapters are divided into a presentation of basic knowledge of the parathyroids and the clinical disorders associated with dysfunction of these glands. Section I, Basic Concepts of the Parathyroids, consists of 22 chapters. Chapters 1–7 cover the embryology, anatomy, and pathology of the parathyroid glands; calcium homeostasis; regulation of parathyroid hormone by dietary calcium and vitamin D; anabolic and catabolic effects of parathyroid hormone; cellular actions of parathyroid hormone on osteoblast and osteoclast function; autocrine and paracrine functions of parathyroid tissue; and the chemistry and biology of parathyroid hormone secretory protein. In Chapters 8–16, parathyroid hormone is considered with respect to the discovery by Aurbach of one of its second messengers, cAMP; regulation of it biosynthesis and metabolism; the parathyroid hormone gene; structure–function analysis of parathyroid hormone and parathyroid hormone-related protein; measurement of parathyroid hormone in the circulation; parathyroid hormone and parathyroid hormone-related protein as polyhormones; receptors for parathyroid hormone and parathyroid hormone-related protein; G proteins as transducers of parathyroid hormone action; biochemical mechanisms of parathyroid hormone action. The book proceeds in Chapters 17–20 to a consideration of PTHrP: its structure, physiological processing, and actions; its causative role in hypercalcemia of malignancy; it skeletal and renal actions; and its measurement in the circulation. Other causes of hypercalcemia, besides PTHrP, and the management of PTH and PTHrP-dependent hypercalcemia complete this section (Chapters 21–22).

Section II, Clinical Concepts of the Parathyroids, begins with an 18-chapter section on primary hyperparathyroidism (Chapters 23–40). This segment is a full exploration of the hyperparathyroid state from theoretical aspects of parathyroid cell growth to the molecular basis of primary hyperparathyroidism. A discussion of the spectrum of parathyroid tumors leads to a consideration of its modern clinical presentations and the course of primary hyperparathyroidism. The change in clinical presentation of primary hyperparthyroidism from a disease of bones and stones and groans to a relatively asymptomatic disorder does not lose sight of a major clinical complication, nephrolithiasis, which is still seen in patients on a regular basis. A chapter devoted to newer markers of bone turnover in primary hyperparathyroidism is followed by a discussion of the histomorphometric features of the disease. Medical and surgical management of primary hyperparathyroidism and the role of preoperative localization techniques are covered completely. Unusual manisfestations of primary hyperparathyroidism include separate discussions of parathyroid carcinoma and acute primary hyperparathyroidism. The MEN syndromes I and II focus on the parathyroids, as does the chapter on familial hypocalciuric hypercalcemia. In Chapters 41 and 42, the parathyroids in renal disease are reviewed with respect to pathophysiology, clinical profile, and management.

Chapters 43–47 cover the hypoparathyroid states with respect to differential diagnosis, autoimmune etiologies, molecular genetics, and a special consideration of the clinical, biochemical, and molecular features of pseudohypoparathyroidism. A separate chapter is devoted to the therapy of hypoparathyroidism.

The last four chapters of the book, Chapters 48–51, cover unusual aspects of the parathyroids: parathyroid function in the pathophysiology of osteoporosis and parathyroid hormone as a potential therapy of osteoporosis. Parathyroid functions in Paget's disease of bone and in magnesium deficiency complete the treatise.

We recognize that few readers will read this book from cover to cover, although many of the chapters are closely interrelated. In order to permit virtually all chapters to "stand alone" but also to be connected to the rest of the book, we have liberally included cross-references to other chapters where appropriate. The reader can thus easily refer to other chapters for more information on a given subject. This design also necessarily calls for some interdigitation between chapters so that the reader in not always required to refer to another chapter but, rather, can get a brief summary in the chapter being read of an area that is covered more completely elsewhere.

If it was true that we needed a book on this subject five years ago when the idea was first germinating, why did it take so long to get it done and what was the impetus for finally accomplishing the task? The first of these two questions has a simple answer. Ideas for books are rather easy to develop but it is quite another matter to mobilize an army of over 90 experts to bring that idea to reality. As is true for so many things, this idea was put on the shelf to be admired for its own sake and to be completed later. The mobilizing impetus and the inspiration for this effort eventually did come. Regrettably, it came in the form of a tragic event in our lives, the death of Gerald D. Aurbach.

The death of Jerry on a street in Charlottesville, Virginia, on November 4, 1991, was random, senseless, and violent. At 64 years of age, Jerry was still alive with love for his work, his family, and his friends. In a moment, we suddenly lost a man who guided the very definition of our field for over 30 years. We lost a man who was our teacher and our friend. We lost a brilliant scientist who was involved in most of the major advances in this field over the past three decades. We lost a man who trained an extraordinary number of us for successful careers in basic and clinical investigation of the parathyroids. We lost a gentle man who consistently brought out the best of us. A summary of the many accomplishments that came from Jerry's laboratory and the trainees, collaborators, and associates who worked with him is depicted in the time-line on pages xxvi–xxvii of this book. It is an extraordinary legacy. The two *IN MEMORIA*, by Bilezikian (*Journal of Bone and Mineral Research* 7:ix–x, 1992) and by Potts and Spiegel (*Journal of Clinical Endocrinology and Metabolism* 75:1386–1388, 1992), speak volumes to his career, to his accomplishments, and to his persona.

In a flash, the dream shelved in the recesses of consciousness and relegated to "when I get to it" became an urgent need. *The Parathyroids* had to be written in the memory and honor of Gerald D. Aurbach, and it seemed altogether fitting that it be written by those who were close to Jerry. We who knew him so well and respected him so much would write a volume for the field. Virtually all of the principal authors of this text fit into that category. Maurice Attie, who also belongs in this book, was tragically killed in a bicycle accident in Philadelphia only a few months after Jerry's death. We remember Maurice and wish that he too were still with us. It is extraordinary that a book designed to be as comprehensive as this could be assembled by a collective authorship whose scientific roots were established by Jerry. His contributions to this field are represented not only by his science but also by his scientific progeny who are the next generation of investigators to study and write about it.

We took up this task with time in mind. *The Parathyroids* had to be published with a short lag time because the book is a timely dedication to Jerry's memory. It had to be published soon because this field is in "fast forward" and if one used the normal publication time for a book of this magnitude, it would run the risk of rapidly becoming outdated. To the credit and thanks to all the authors, virtually all 51 chapters were submitted within a six-month period of time. The dedication of the authors to this task is gratefully acknowledged by us. We also are grateful to Jasna Markovac of Raven Press, who helped to ensure that the process ran as efficiently as possible and whose efforts also were instrumental in ensuring a rapid turnaround time to final publication.

John P. Bilezikian
Robert Marcus
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The Parathyroids

Basic and Clinical Concepts

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CHAPTER 1

Parathyroids

Morphology and Pathology

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INTRODUCTION

The morphologic abnormalities seen in the parathyroid glands are predominantly those related to hyperfunction, i.e., primary hyperparathyroidism. Thus the focus here is on this aspect of parathyroid pathology, because almost all surgical specimens of parathyroid lesions are derived from patients with hyperparathyroidism. Because morphologic abnormalities are an important factor in the surgical treatment of this disease, a review of parathyroid embryologic development, anatomy, and normal histology is included. A brief discussion of parathyroid pathology in hypoparathyroidism is also included, as is discussion of the pathology of the glands in humoral hypercalcemia of malignancy.

DEVELOPMENT OF PARATHYROID GLANDS

In the 8- to 10-mm embryo, the parathyroids begin to develop from the third and fourth branchial pouches. The third branchial pouch gives rise to the thymus and the parathyroid complex. The parathyroids migrate to and remain at the lower poles of the thyroid. Thus, in the usual case, the inferior parathyroids migrating with the thymus come to rest below the parathyroid derived from branchial pouch four (1). Embryologic studies in animals have demonstrated that ablation of the ventral half of the third branchial arch leads to nonformation of the upper parathyroid gland (2). Hoxa3 mutant homozygotes show defects in development and migration pathways of thymus, thyroid, and parathyroid glands; the molecular events underlying the actions of the Hoxa3 genes remain to be determined (3). The fourth branchial pouch, or the fourth–fifth pharyngeal complex, gives rise to the superior parathyroid glands and via the ultimobranchial body to the parafollicular or C cells in the lateral thyroid. The superior parathyroids lie adjacent to the upper poles of the thyroid.

ANATOMY OF PARATHYROID GLANDS

Both the number and the location of the parathyroid glands vary in normal individuals. Variation in location of the glands can lead to problems during surgical exploration of the neck. For example, there may be difficulty in locating the diseased, abnormal parathyroid tissue in patients with hypercalcemia; conversely, surgery on the neck for other reasons, such as thyroid or laryngeal disease, may inadvertently cause trauma or removal of parathyroid glands because of the normal variability in their anatomic position (1,4–7). A report by Lee *et al.* indicates that almost 12% of patients undergoing thyroid resection have one parathyroid gland removed inadvertently (8).

Although from one to twelve parathyroid glands can be found, (1), 84% of normal adults have four parathyroids (4). From 1 to 7% of adults have three glands and 3 to 13% have five glands (1,4–7). The variability of the location of the parathyroid glands is usually greater in the lower parathyroids. The superior parathyroids may be found close to the thyroid capsule or actually within

the thyroid capsule, but they may also be located behind the pharynx or the esophagus, lateral to the larynx, or behind any part of the thyroid. The lower glands, which usually lie near the lower pole of the thyroid, may be found behind the thyroid, in the paratracheal area, or close to or within the thymus in the superior mediastinum. The glands tend to be bilaterally symmetrical in location, with approximately 75% of cases showing such symmetry (4,5).

The parathyroid glands measure between 2 and 7 mm in length, 2 and 4 mm in width, and 0.5 and 2 mm in thickness. They are reniform, soft, and brown to rust in color. However, color varies with fat content, the degree of vascular congestion, and the number of oxyphil cells present (5,9,10). Parathyroid tissue weight varies with sex, race, and overall nutritional status of the individual (11). The combined weight of all parathyroid tissues in a normal adult male is around 120 mg; in females combined tissue weight is around 145 mg. Weights of individual glands range from 3 to 75 mg, with averages of around 35 to 55 mg (5,9–11).

HISTOLOGY OF PARATHYROID GLANDS

Microscopic examination shows that each parathyroid gland is invested by a thin connective tissue capsule that extends into the parenchyma as fibrous septae, dividing the gland into lobules. A rich capillary vascular network is surrounded by nests and cords of parenchymal cells. Small clusters of cells are interspersed with foci of adipose tissue (Fig. 1). However, there is variability in the location and interrelationships between the fat and the parenchymal cells in the parathyroid gland, so that biopsies from specific areas of the parathyroid may be predominantly fat, predominantly parenchyma, or a mixture of these two. In the adult, the parathyroid is composed of chief and oxyphil cells, fibrous stroma that is usually thin and delicate, and variable amounts of fat.

Historically, the ratio of 50:50 cells:fat has been accepted as normal for adults. However, numerous studies have indicated that individuals dying without hormonal dysfunction of any type have parathyroids in which the stromal fat content is significantly less than 50% in most cases. It may be as little as 10%. In fact, numerous studies (11–14) have shown that an approximately 17% fat content is normal in an adult parathyroid gland. Indeed, cell:fat ratios in terms of stromal fat serve little purpose in microscopic interpretation of functional status. Densitometry measurements concur, indicating that parenchymal cell mass accounts for 74% of parathyroid weight (4,14).

The cells that make up the parathyroid glands include chief cells, oxyphils, and clear cells (Fig. 2). These variable cell groups probably represent different morphologic expressions of the same parenchymal cell. The chief cell is polyhedral in shape, poorly outlined, and measures 6 to 8 nm in diameter (15). It has an amphophilic to slightly eosinophilic cytoplasm, a

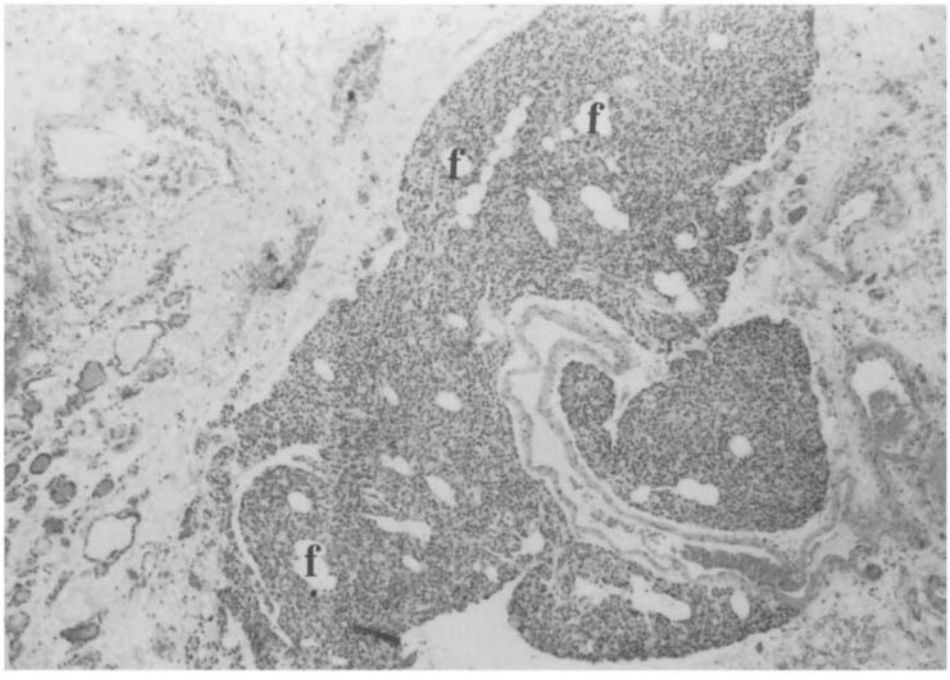

FIG. 1 Normal parathyroid gland adjacent to thyroid (lower left). Note the cellularity of the gland and the relative paucity of fat (*f* clear spaces) in this section. Hematoxylin and eosin, ×50.

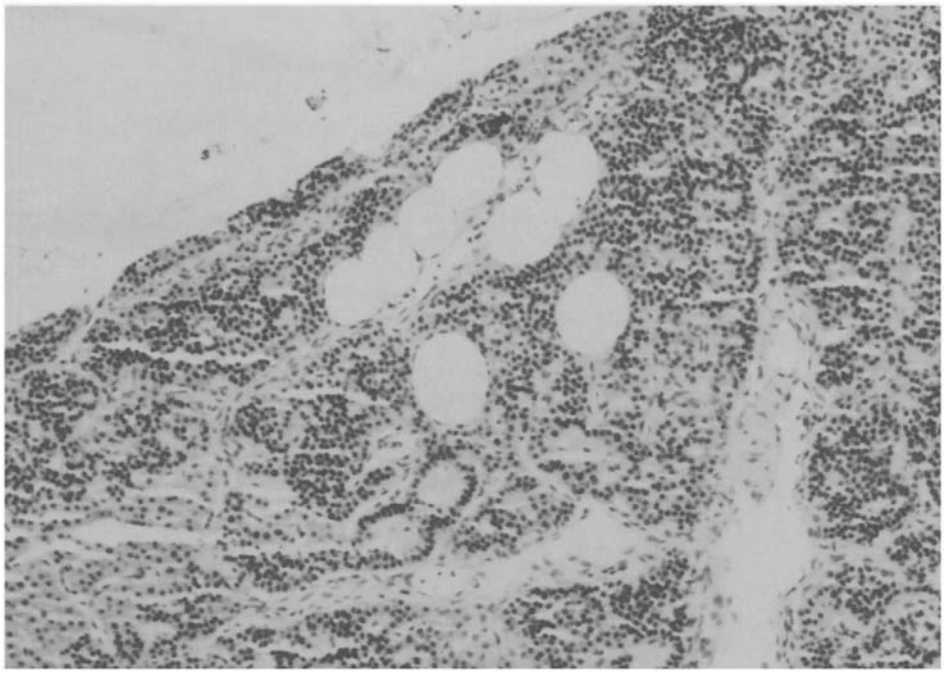

FIG. 2 Parathyroid chief cells (dark cells), oxyphils (larger cells), and fat in normal adult gland. Hematoxylin and eosin, ×150.

sharp nuclear membrane, and well-defined, abundant nuclear chromatin. Clear cells represent chief cells in which there is an excessive amount of glycogen in the cytoplasm.

Oxyphils, which tend to be found initially around the time of puberty and rarely in childhood, apparently increase in number with age and may form small microscopic nodules. The oxyphil cell in the parathyroid, as in other organs, is large, measuring approximately 10 nm in diameter, has a well-demarcated cell membrane, and has eosinophilic granular cytoplasm (Fig. 3). This reflects a marked mitochondrial content (9,10,15).

In contrast to stromal fat content, intracellular fat content may be helpful in defining functional status. Thus, in

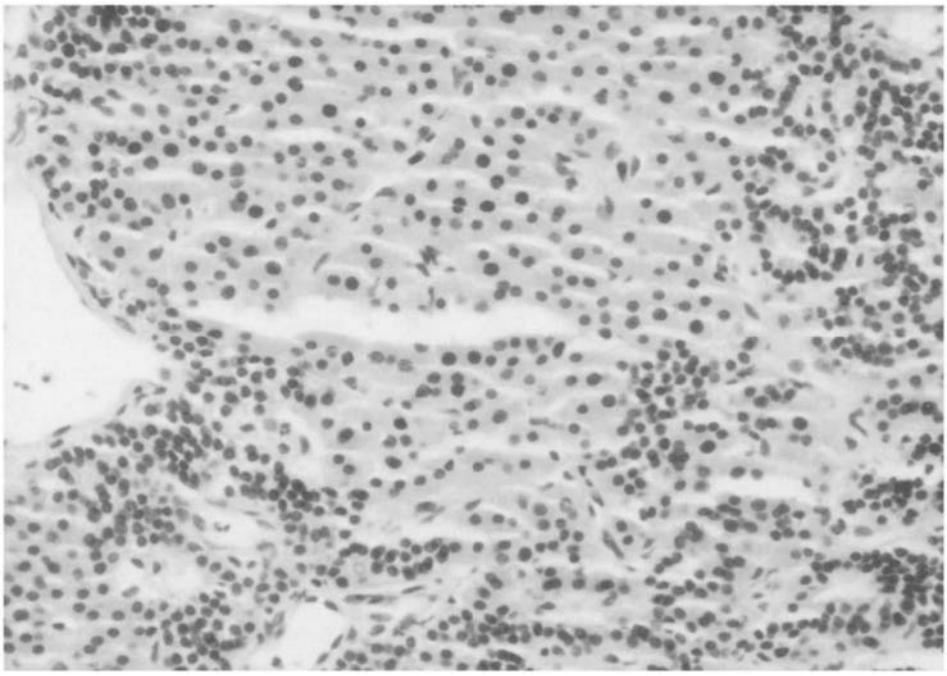

FIG. 3 Cluster of oxyphils in normal parathyroid gland. Hematoxylin and eosin, ×250.

chief cells, which are the predominant cells in the parathyroid, intracellular fat, i.e., intracytoplasmic fat, is found in the overwhelming majority of cells in the euparathyroid state (approximately 80% of cells) (11–14).

Ultrastructurally, the chief cells undergo a cyclic process during synthesis and secretion of parathyroid hormones, with the hormone being synthesized on Golgi apparatus-associated membrane-bound secretory granules. These cells eventually secrete these particles of hormone into the surrounding milieu. Little lipid is present in the active parathyroid cell, which in the euparathyroid state is approximately 20% of the parenchymal cell population (15).

DISEASES OF THE PARATHYROID—PATHOPHYSIOLOGY

Surgical pathologists dealing with the parathyroids almost always evaluate parathyroid tissue in patients who have hypercalcemia. The predominant effect of parathyroid hormone, as noted, is to increase serum calcium. The usual clinical problem is not to distinguish normal from hypercalcemic patients, but rather to distinguish those who have hypercalcemia caused by hyperparathyroidism from those who have hypercalcemia arising from other causes.

Primary hyperparathyroidism is defined as the disease in which, in the absence of a known stimulus, one or more parathyroid glands secrete excess parathyroid hormone, producing hypercalcemia. Serum calcium ranges from 11 to 18 mg/dl, with most asymptomatic patients found in the lower end of the spectrum (16). The prevalence of primary hyperparathyroidism in the United States is estimated to be 1–5 cases per 1000 adults (16). The etiology of the disease is unknown. In a certain number of individuals a history of irradiation to the head and neck may be found, although the magnitude and significance of this association are not clear (17,18). Prinz *et al.* (18) found that 67% of patients in their series with combined thyroid and parathyroid tumors gave a history of irradiation. In some patients, genetics plays a role [multiple endocrine neoplasia (MEN) syndromes; see also Chapter 19] (19–25). Mutations of the *MEN-1* gene (menin) have been identified in some irradiated patients with hyperparathyroidism (22–24).

Pathology of the Parathyroid Glands in Primary Hyperparathyroidism

Three subgroups of pathologic lesions are found in patients with primary hyperparathyroidism: adenoma, multigland hyperplasia, and, rarely, carcinoma.

Parathyroid Adenoma

The parathyroid adenoma is responsible for hyperparathyroidism in 30–90% of cases. The wide range of variation indicates both pathologic interpretation and surgical interpretation of the disease (9,10,26–29). Most researchers believe that 75–80% of primary hyperparathyroidism is caused by a solitary adenoma (9,10,26–29). Evidence supports a clonal origin for parathyroid adenomas. Although older studies using protein polymorphisms indicated that parathyroid adenomas were polyclonal (30,31), many studies (25,32–36) using the techniques of molecular biology show that sporadic parathyroid lesions are monoclonal neoplasms.

Grossly, parathyroid adenomas tend to be located more commonly in the lower glands than in the upper glands. Typically, the adenoma is an oval red–brown nodule that is smooth, circumscribed, or encapsulated. The lesion, which often replaces one parathyroid gland, may show areas of hemorrhage and, if large, cystic degeneration. Occasionally in small adenomas, a grossly visible rim of normal yellow–brown parathyroid tissue may be seen. Weights of adenomas vary from 300 mg to several grams. The size ranges from 1 to over 3 cm (9,10,27,29).

Microscopically, adenomas are usually encapsulated lesions composed of parathyroid chief cells arranged with a delicate capillary network, recapitulating endocrine tumors in general (Figs. 4 and 5). Rarely, lobules are seen, and sometimes nodules may be formed. Stromal fat is usually absent. Unless they are very large, about 50% of adenomas will appear to have a normal rim or even atrophic parathyroid tissue outside the adenoma capsule. The cells in the rim tend to be smaller and more uniform, with stromal and cytoplasmic fat abundant in the rim but absent in the adenoma (9,10,27,29,37). However, the absence of a rim does not preclude the diagnosis of adenoma, because large tumors may have overgrown the preexisting normal gland or the rim may have been lost during sectioning.

In large tumors, zones of fibrosis may be found in addition to hemorrhage, cholesterol clefts, and hemosiderin, as well as occasional areas of calcification. Rarely, lymphocytes will be noted within an adenoma (38). Thymic tissue may be found in association with an adenoma or an adenoma may be found within the thymus. There may be atypical cells in an adenoma. Most cells comprising the lesion have relatively small, uniform, dark nuclei. Usually focally, bizarre multinucleated cells with dark, crinkled nuclei can be seen. These nuclei probably represent degenerative changes rather than malignant or premalignant potential. It has been stated that mitotic activity is never found in a parathyroid adenoma and that such activity should suggest the

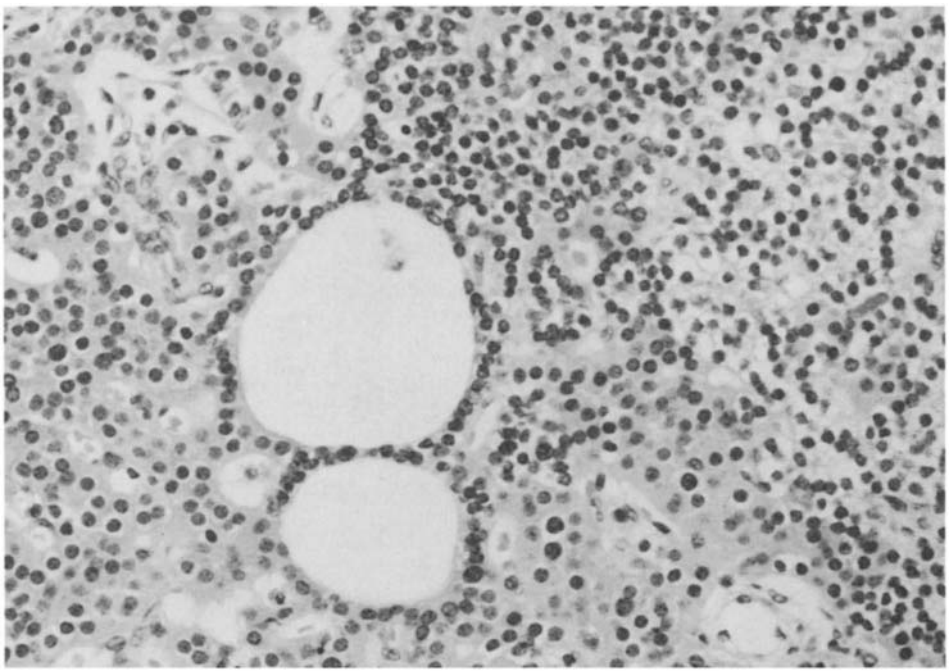

FIG. 4 Parathyroid adenoma. Almost all chief cells; no fat present. Hematoxylin and eosin, ×200.

possibility of a malignant neoplasm. This particular diagnostic area, however, is fraught with difficulty and is under debate at the present time. The nonadenomatous glands in a patient with a parathyroid adenoma may show normal to increased cytoplasmic fat content and normal weight (9,10,13,14).

In about 10% of cases microscopic examination of biopsies from "normal" glands will show areas of hypercellularity, so-called microscopic hyperplasia. Although this may represent a true parenchymal cell increase, the difficulty in defining "normal," or more likely sampling errors, probably accounts for this (39–41). Oxyphilic or oncocytic adenomas do occur and can function. These tumors tend to be larger than chief cell adenomas and the serum calcium levels tend to be minimally elevated (42–47).

Because of the embryologic migration patterns, parathyroid adenomas can occur in ectopic locations.

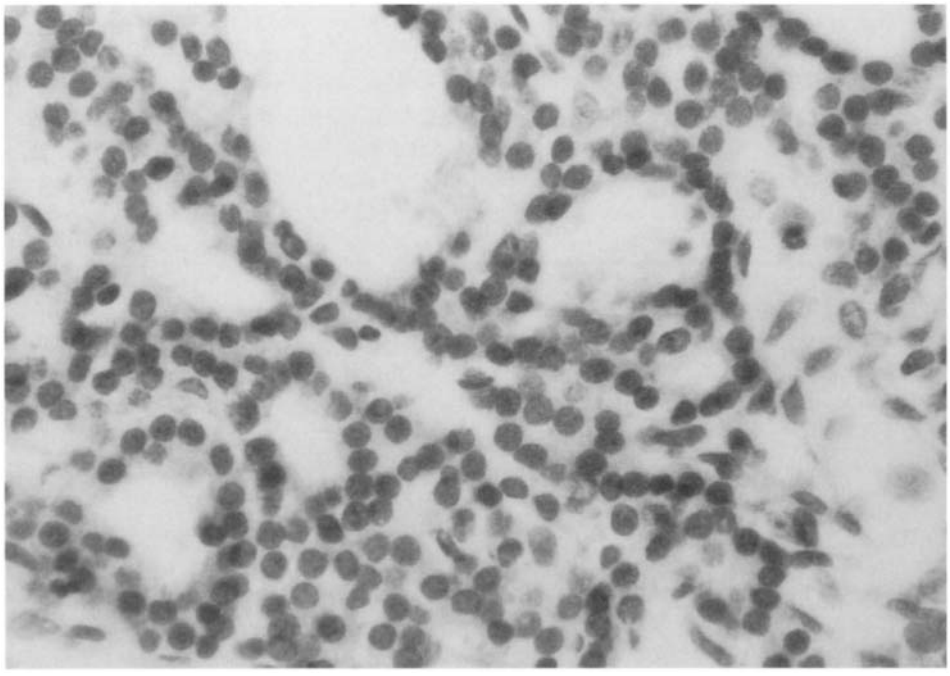

FIG. 5 Parathyroid adenoma with follicle formation; rarely, this is mistaken for thyroid tissue, especially on frozen section. Hematoxylin and eosin, ×300.

Thus, when hyperparathyroidism occurs in such an individual and no adenoma or abnormal glands are identified in the neck, ectopic locations that should be considered include the mediastinum, with or without associated thymic tissue, behind the esophagus, or even intrathyroidal (48–54). Double adenomas, if they occur, are very rare (55–57). Most patients who have so-called double adenomas will, over a period of time, have recurrent hyperparathyroidism and in fact have four-gland hyperplasia. The diagnosis of double adenoma can be made only if two glands are enlarged and histologically abnormal; the remaining glands are normal, there is no family history of parathyroid disease, and permanent cure of hypercalcemia follows excision of only two enlarged glands (55–65). Indeed, heterogeneous size of four glands in primary hyperplasia may account for some cases interpreted as "double adenomas" (65).

Primary Parathyroid Hyperplasia

Primary parathyroid hyperplasia is divided into two main groups: chief cell hyperplasia, which is common, and water clear cell hyperplasia, which occurs less commonly (9,10,29). Chief cell hyperplasia accounts for 15% of hyperparathyroidism in most series, although some reports indicate that about half of primary hyperparathyroidism is produced by hyperplasia. The reasons for this probably lie in discrepancies in pathologic interpretation. About 30% of patients with chief cell hyperplasia have familial hyperparathyroidism or one of the syndromes of multiple endocrine neoplasia (9,10,29,66–73). Grossly, all four glands are enlarged equally or nonequally. If unequal in size, the lower glands are usually larger. Occasionally one gland will be much larger than the others and will convey the surgical impression of an adenoma. The weight of all four glands ranges from 150 mg to over 20 g, but usually is in the range of 1 to 3 g (9,10).

Microscopically, diffuse chief cell hyperplasia may be characterized by solid masses of cells with minimal to no fat. Usually almost all cells are chief cells, with rare oxyphils. Nodular or pseudoadenomatous hyperplasia consists of circumscribed nodules of chief, transitional, or oxyphil cells, each nodule devoid of fat, and with there being little fat in the intervening stroma. Usually in hyperplasia there is no rim of normal tissue. Bizarre nuclei are rarely found in primary hyperplasia. Mitoses may occasionally, however, be identified (9). Therapy in this disease is directed to the removal of all parathyroid tissue, with or without autotransplantation.

Clear cell (water clear cell) hyperplasia is very rare and is the only condition of the parathyroid in which the superior glands are larger than the lower. Total weights of such parathyroids always exceed 1 g and usually range from 5 to 10 g. The glands are irregular and show pseudopods and cysts; a distinct mahogany color is seen grossly. Histologically, the glands are composed of diffuse sheets of clear cells without any mixture of other cell types. No rim is present (9,10,74–76). An interesting association of clear cell hyperplasia with the blood group O allele has been reported (77).

Parathyroid Carcinoma

Parathyroid carcinoma accounts for approximately 1% of primary hyperparathyroidism (78–94). There is clinically an unusual scenario with an almost equal sex ratio, which is uncommon in parathyroid adenomas and usual hyperplasias, in which women predominate. The incidence of benign hyperparathyroidism appears to increase with age; however, patients with parathyroid carcinoma tend to be somewhat younger and are almost always symptomatic with very high levels of serum calcium. Very rarely, parathyroid carcinoma can occur in the setting of familial endocrine disease (95–99) or as a complication of secondary parathyroid hyperplasia (100–104). Most of the latter cases occur in patients with renal failure (12 cases were documented in 1999) (104).

Clinically, patients with parathyroid carcinoma show high calcium levels (up to 15 mg/dl). Many have polyuria, polydypsia, nausea, vomiting, weight loss, and constipation. They may also have bone pain, renal stones, and other symptoms related to hypercalcemia. An important clinical clue is the presence of a palpable mass in the neck on physical examination. The mass may be clinically thought to be an adenoma of the thyroid (9,10,78–94).

Parathyroid carcinomas tend to be large tumors (average weight 12 g) and characteristically show a histology with trabecular arrangement of tumor cells divided by thick fibrous bands, with capsular and blood vessel invasion in the presence of mitotic figures (Fig. 6) (9,10,105). The cytology may be clear or rarely oxyphilic; nuclear atypia may be seen or may be absent (9,10,102,106). Because mitotic figures are almost never found in a benign parathyroid adenoma, their presence in tumor cells should raise the suspicion of malignancy. However, this has been called into question and parathyroid tumors with mitotic activity may in fact be benign. As a note of caution, long-term follow-up in the reported series is quite limited, and there is a long natural history to parathyroid carcinoma, so the answers are not all in yet (107,108). Mitotic activity in secondary hyperparathyroidism is not to be equated with malignancy, and mitotic activity may occasionally be found in primary hyperparathyroidism as well (9).

The presence of capsular invasion is not equated with malignancy because large parathyroid adenomas

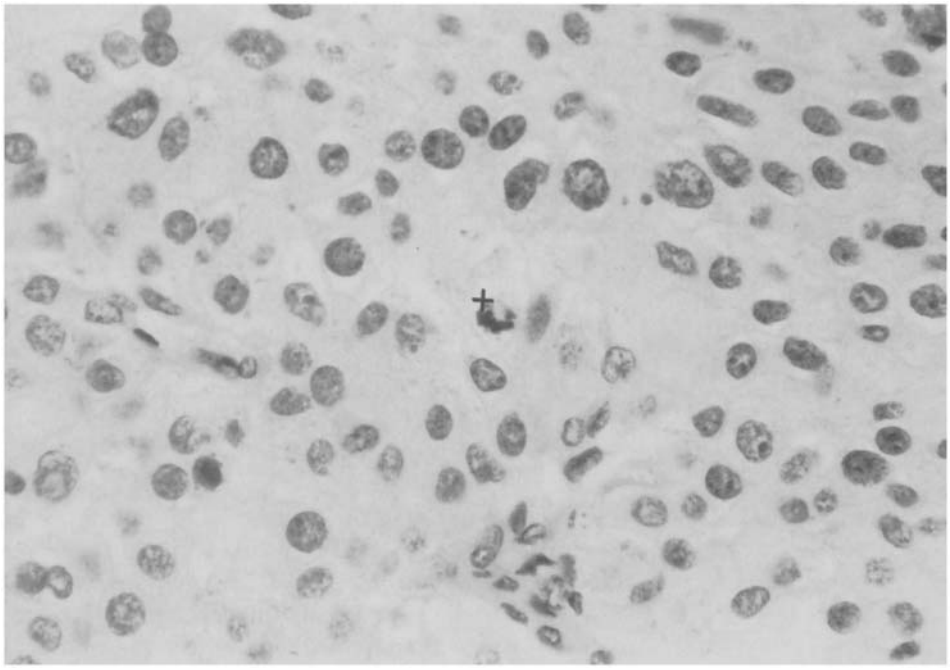

FIG. 6 Parathyroid carcinoma; note mitosis (+). This tumor recurred three times locally and eventually metastasized to the lungs. Hematoxylin and eosin, ×300.

may have undergone prior hemorrhage, with consequent fibrosis and trapping of tumor cells within the capsule. Vascular invasion is difficult to define except if seen outside the vicinity of the neoplasm. An important clue to the diagnosis of parathyroid carcinoma is the surgical finding of adherence and/or invasion into local structures, which should raise the suspicion of a carcinoma (9,10,78–94,102). Metastases at the time of presentation are unusual, but may be found in the regional lymph nodes. There may also be local invasion into nerves, soft tissue, and the esophagus. Rarely, nonfunctioning parathyroid carcinomas have been described. These lesions tend to be large and composed of clear or oxyphil cells (83,109,110).

The prognosis of parathyroid carcinoma is usually one of an indolent malignancy. Metastases may occur in up to one-third of cases and are found in regional lymph nodes, bone, lung, and liver. Many patients survive long periods of time, however. Multiple recurrences are known to occur over a 15- to 20-year period (9,10,78–94,102). The severity of the symptoms due to metastatic disease is directly related to tumor burden, because this is related to parathyroid hormone produced (111).

Some solitary parathyroid tumors show features that suggest carcinoma, such as large size, fibrous bands, etc., but not all of the characteristics of malignancy are present. We use the term "atypical adenoma" for these lesions; short-term follow-up suggests they are benign, but long-term studies are needed in this subset of lesions.

Multiple Endocrine Neoplasia Syndromes

The syndromes of MEN-1 (Wermer's syndrome) and MEN-2 (Sipple syndrome) are associated with pathologic changes in the parathyroids. In MEN-1, pathologic changes similar to adenomatous or pseudoadenomatous chief cell hyperplasia as described above are found (9,10). In MEN-2, the parathyroids tend to show a diffuse hyperplasia, but occasionally one gland is involved, suggesting an "adenoma." In this syndrome the hyperparathyroidism is considered to represent a genetically determined event and not a response to hypercalcitoninemia (9,10). Parathyroid abnormalities are much less common in other variants of MEN-2 syndromes.

Familial hyperparathyroidism shows the pathologic alterations of chief cell hyperplasia similar to Wermer's syndrome; in familial hypercalciuric hypercalcemia, mild parathyroid hyperplasia has been described (9,10).

Unusual Lesions of the Parathyroid

Parathyroid Cysts

Cysts of the parathyroid glands are unusual and may present and be misinterpreted clinically as thyroid nodules (112–124). They occur more frequently in women than in men, usually are large, ranging from 1 to 6 cm, and may be located in any parathyroid gland, although most are found in the lower glands. Occasionally they may be found in the mediastinum, mimicking superior/anterior mediastinal masses (114,121,123).

Grossly, these cysts are almost always unilocular and smooth walled and contain water fluid with a high parathyroid hormone content. Histologically, they are lined by one layer of clear epithelium containing glycogen. The cyst wall is fibrous, with fragments of smooth muscle and nests of normal parathyroid tissue. It is unclear how these cysts arise. Microcysts are found in about half of normal parathyroids and might possibly enlarge by accumulation of secretions, or may fuse and produce grossly visible cysts. The cysts may arise from embryologic remnants of pharyngeal pouches in the neck undergoing cystic degeneration and entrapping portions of parathyroid tissue. Many investigators believe, however, that parathyroid cysts represent degenerated parathyroid adenomas, and in some cases, in fact, that the cysts are associated with hyperparathyroidism (Fig. 7) (117–119). However, this is uncommon and only a few functional cysts have been reported. It may be that different parathyroid cysts have different origins, although pathologically they resemble one another.

Cytologists may encounter parathyroid cysts during attempts to aspirate thyroid nodules (112,124). The cyst fluid can be assayed biochemically for parathyroid hormone to confirm the diagnosis.

Lipoadenoma–Hamartoma of the Parathyroid

These tumors present as masses that histologically are composed of parathyroid cells arranged in nests, similar to normal parathyroid but intimately associated with large areas of adipose tissue (125–128). The lesion may be functional or nonfunctional and usually is circumscribed but rarely encapsulated. In unusual examples a rim of normal parathyroid tissue is present at the periphery. In some instances at least one other histologically normal parathyroid has been recognized. Also, in some, there is an unusual myxomatous stroma, and other mesenchymal elements including metaplastic bone may be found. Wolff and Goodman (125) suggest the term "parathyroid adenomas with stromal component." More than three-quarters of the reported cases functioned, although with relatively low levels of hypercalcemia. We have studied a woman presenting with an orbital brown tumor due to a parathyroid lipoadenoma that weighed over 10 g (128).

Parathyromatosis

In rare instances of hyperparathyroidism due to primary hyperplasia, nests of hyperplastic parathyroid cells are found in the neck, outside of hyperplastic glands (129–131). In the individuals for which this has been reported, these nests were discovered at the first neck exploration, so that spillage during prior surgery could be excluded. In each of these patients there was no evidence of malignancy. It has been postulated that during embryologic development nests of pharyngeal tissue containing parathyroid cells might be scattered throughout the adipose tissue of the neck and mediastinum. Normally these nests are inconspicuous. However, in the process of diffuse hyperplasia of the parathyroids, all functioning tissue may become hyperplastic and appear as separate fragments on histologic evaluation.

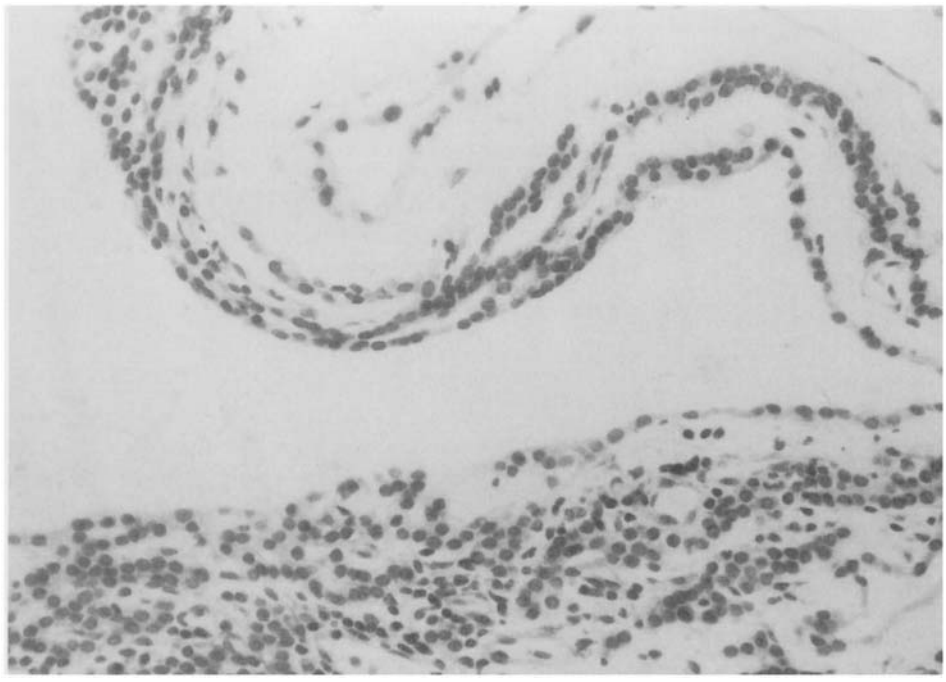

FIG. 7 Parathyroid cyst. This tumor presented with hypercalcemia and extended from the lower neck to the upper mediastinum. It was 6 cm in size, but most of the lesion was cyst. However, about 10–15% was solid parathyroid tissue, making this a cystic adenoma. Hematoxylin and eosin, ×150.

Infarction of Parathyroid Adenomas

Twelve documented cases (132) of adenomas that spontaneously infarcted have been reported. This phenomenon is associated with remission of hypercalcemia. The etiology of the infarction is unclear in most cases, although some have been associated with the intake of certain drugs that may predispose to vascular damage, thrombosis, or hemorrhage. Therapeutic infarction can also result in cure of the metabolic abnormalities (85).

INTRAOPERATIVE ASSESSMENT OF PARATHYROIDS—THE BANE OF THE SURGICAL PATHOLOGIST

In normal parathyroid glands, 80% of the cells are in the nonsecretory phase and contain intracytoplasmic fat (12,13,15). Therefore, is the fat stain useful in distinguishing hyperplasia from adenoma, because all hyperfunctioning glands should be fat depleted? The advocacy of fat stains (Sudan IV or Oil Red O) on parathyroid tissue removed at surgery has come into vogue. The scenario is as follows. A sample of an enlarged parathyroid gland is sent for frozen section and by hematoxylin and eosin stain it is hypercellular with little or no stromal fat. Thus it either represents an adenoma or a hyperplastic gland and is not normal. A biopsy of a second parathyroid is frozen and is normocellular or minimally hypercellular. Fat stain shows abundant cytoplasmic fat in the latter biopsy; hence this is a normal gland. The enlarged gland, which shows minimal to no fat, represents an adenoma. Many authors have cautioned, however, that the fat stain cannot be the sole procedure on which to base a diagnosis, because although the fat stain is helpful, it is helpful in only about 80% of cases and must be considered as an adjunctive technique in light of gross findings, gland weight, and size, and cannot be relied on by itself (133–141). We have found it useful to perform a rapid (30-second) toluidine blue stain on frozen sections of parathyroid tissue. The intracellular fat is well defined by this stain and it is faster to perform and interpret compared to Oil Red O (Lyle S, *et al.*, unpublished observations, 2000).

Another rapid technique that may prove useful for intraoperative assessment is density gradient measurements (142). There is an almost linear relationship between density and parenchymal content of parathyroid tissue and thus such a technique can assess parenchymal cell mass. The technique is to take a sample of the gland and weigh it, and take a small piece from the center and a piece from the rim, determining their densities in a 25% mannitol solution. Abnormal parathyroid tissue sinks because of decreased fat and high parenchymal mass. Wang and Ryder (142) have found that this is a simple test to be used by the surgeon in the operating room for distinguishing normal from abnormal glands.

In the intraoperative assessment of parathyroid pathology it cannot be stated strongly enough that there must be close communication between the surgeon and pathologist during the operation. The pathologist needs to be apprised of the gross findings and cannot work in a vacuum. What is recommended is as follows: the largest parathyroid gland found is resected *in toto*, then the pathologist weighs it, measures it, and examines it histologically. If the gland shows diffuse growth of chief cells and perhaps a normal-appearing rim, a lack of fat, and bizarre nuclei, a diagnosis of presumed adenoma can be rendered. If the histology is that of hypercellularity but criteria for adenoma are not seen, biopsy of at least one more gland is needed, and, in fact, in many centers pathologists prefer to have the largest abnormal gland and at least a biopsy of one more gland. Weight ratio of parenchymal cells to fat, and normal or abundant intracytoplasmic fat content in the second gland, strongly support that the first gland is an adenoma (133–141).

The success rate of identifying parathyroid tissue by frozen section is over 99% (143); distinguishing one-gland from multigland disease is much more problematic.

OTHER TYPES OF HYPERPARATHYROIDISM

Secondary Hyperparathyroidism

Secondary hyperparathyroidism is usually due to renal disease and is relatively common in the age of hemodialysis and renal transplantation. The role of the surgical pathologist in the evaluation of secondary hyperparathyroidism is basically to identify parathyroid tissue at the time of frozen section to allow for the surgeon to remove portions of this tissue for autotransplantation. Secondary hyperparathyroidism is really no different histopathologically from primary hyperparathyroidism (144–146). Mitotic activity may occasionally be found in such glands. Usually all four glands are enlarged, although one or two glands may be of very great size.

Transplantation of parathyroid tissue is successful in the majority of cases and occasionally part of this tissue may be removed if hyperfunction again becomes a problem (147,148). Such lesions will have small nests and islands of vascularized parathyroid tissue growing in muscle or fat, usually having been implanted in the arm (149,150).

Tertiary Hyperparathyroidism

Although the existence of tertiary hyperparathyroidism has been questioned, most authors believe it represents the autonomous function of one parathyroid gland that develops in the face of long-standing secondary hyperparathyroidism (151). The pathology resembles that of secondary hyperparathyroidism, although one of the four glands is usually disproportionately enlarged.

Familial Hyperparathyroidism

In addition to the multiple endocrine neoplasia syndromes, in which hyperparathyroidism is often a prominent clinical problem, familial parathyroid hyperplasia without other endocrine lesions has been reported. The lesions in all of these patients resemble those of primary chief cell hyperplasia (9,10,152–154), although a ribbon pattern of cell growth may be prominent in MEN-1.

Familial Hypocalciuric Hypercalcemia

Familial hypocalciuric hypercalcemia, inherited as an autosomal dominant gene, is manifested clinically by familial occurrence, moderate to minimally elevated serum calcium, and reduced urinary calcium excretion (see Chapter 41). The parathyroid glands appear normal to mildly hypercellular, and subtotal parathyroidectomy fails to reverse the hypercalcemia. The defect appears not to be in the parathyroid glands (155,156).

SPECIAL STUDIES AND THE PARATHYROID

Cytology

Because most parathyroid lesions are not palpable, direct biopsy of a parathyroid tumor by fine-needle aspiration (FNA) is unusual. However, on occasion, parathyroid lesions present clinically as thyroid nodules or are large enough to be clinically evident. The FNA features of parathyroid adenoma include cellular fragments of epithelial cells arranged around vascular cores, an organoid or trabecular architecture, and microacini. Parathyroid chief cells contain uniform round nuclei; groups of oxyphilic cells are helpful in defining the tissue as parathyroid. If available, immunostains for parathyroid hormone may help (157).

Proliferative Markers

Attempts at using immunocytochemical markers (158–164) of proliferation index (M1B1 for cell cycle-associated Ki-67 antigen) for distinguishing between parathyroid adenomas and hyperplasia have met with varied success (159). Whereas statistically significant differences are found between normal (suppressed "rim") parathyroid tissue and hyperfunctioning glands, similar proliferative indices are noted between adenomas and hyperplasias (159,160). Loda *et al.* (160) identified higher numbers of labeled nuclei in adenomas than in hyperplasias by proliferating cell nuclear antigen (PCNA) immunostaining. The labeling index of individual cases of parathyroid tumors shows so much overlap that it cannot be used to distinguish benign from malignant lesions (161–164).

Flow Cytometry and the Parathyroid

Several studies of DNA content have shown that aneuploidy may be found in parathyroid adenomas, and even in hyperplasia, as well as in carcinomas. Approximately 70% of parathyroid carcinomas, 30% of adenomas, and 30–50% of chief cell hyperplasia glands have aneuploid DNA populations (165–171). As in proliferations of other endocrine organs, the finding of aneuploid cell populations does not ensure a diagnosis of malignancy (169–173).

Clonality

Modern molecular biology techniques, primarily using restriction fragment-length polymorphisms, have shown that most (if not all) parathyroid adenomas are monoclonal proliferations (25). In addition, about 40% of primary hyperplasias and 60% of secondary hyperplasia (secondary to chronic renal disease) are clonal. Different laboratories utilizing different probes as markers confirm these findings (25,174–176). The biologic meaning of these results is unclear.

Genetics

The *PRAD1* oncogene has been implicated in parathyroid tumorigenesis. *PRAD1* (for parathyroid adenoma), which encodes cyclin D1, results from a chromosome inversion that occurs as a dominant clonal event in some parathyroid adenomas. The inversion is created by a break in the vicinity of the parathyroid gene on the short arm of chromosome 11 (band 11p15), another break in the long arm (band 11q13), rotation of the center piece around the axis of the centromere, and rejoining (177). Cyclin D overexpression can be detected immunohistochemically in 18–38% of parathyroid adenomas, and in 91% of carcinomas (178,179).

The retinoblastoma (Rb) gene is a tumor suppression gene that has growth inhibitory effects in the cell cycle. Inactivation of the Rb gene has been associated

with loss of an Rb allele by molecular analysis, and immunostaining for Rb protein may assist in the distinction between parathyroid adenomas and carcinomas (179–183). However, caution must be used in interpretation of the results, because some parathyroid carcinomas do not show loss of Rb protein and a few adenomas do (181,182).

Studies of parathyroid neoplasms (benign and malignant) have not shown p53 mutations in such lesions (184). In another study of parathyroid tissues, there were significant differences between p27 protein expression in parathyroid hyperplasia, adenomas, and carcinomas, suggesting that this cell cycle protein may be useful in distinguishing between these two conditions (185,186).

HUMORAL HYPERCALCEMIA OF MALIGNANCY, OR ECTOPIC PARATHYROIDISM

Hypercalcemia without bone metastasis in nonparathyroid malignancies may be found in association with a malignant tumor. Hypercalcemia is relieved by excision of the tumor and returns with its recurrence. This paraneoplastic endocrine syndrome is due in many cases to a peptide that resembles parathyroid hormone but is distinctly different. The factor responsible for the syndrome of humoral hypercalcemia of malignancy, which is due to parathyroid hormone-related protein (PTHrP), is discussed in other chapters in this volume. PTHrP binds to parathyroid hormone receptors on bone and kidney and mimics the actions of parathyroid hormone. The tumors most commonly associated with this syndrome include squamous carcinomas arising in a number of primary sites, including lung, vulva, esophagus, and head and neck, and clear cell cancers, especially of renal and ovarian origin (187–191). The parathyroid glands appear normal or atrophic histologically.

HYPOPARATHYROIDISM

The most common parathyroid pathology found in patients with hypoparathyroidism is four normal glands. Unfortunately, they often have been surgically removed from the patient! Accidental excision of normal parathyroid glands during the course of neck surgery, especially thyroid surgery, is an uncommon but unfortunately not a rare event (8). In addition to actual excision of the glands, injury to their vascular supply may cause their infarction, or they may be so damaged that they become functionally absent.

Infiltration

Impaired parathyroid function caused by infiltration of parathyroid glands has been described in hemochromatosis, amyloidosis, and metastatic carcinomas. These are all rare causes of hypoparathyroidism (192).

Radiation

Rarely, patients are reported who have developed hypoparathyroidism after radioactive iodine treatment for hyperthyroidism. The presumed mechanism is radiation damage to and fibrosis of the parathyroids (193).

Autoimmune Parathyroid Destruction

Lymphocytic infiltration of parathyroid tissue, with subsequent autoimmune destruction of the glands, is probably the most common cause of hypoparathyroidism (noniatrogenic cause). It may occur as an isolated event or in association with autoimmune diseases of other endocrine organs, i.e., thyroid, adrenal, or ovary (194–197).

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CHAPTER 2

Parathyroid Hormone Biosynthesis and Metabolism

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INTRODUCTION

The parathyroid hormone gene has many jobs. It must encode a peptide that can bind to and activate receptors on target tissues. Equally importantly, the amount of parathyroid hormone (PTH) produced must be carefully controlled to maintain the blood level of calcium within a narrow range. Nature's solution to these problems has involved the specific synthesis of PTH primarily in the parathyroid chief cell, a cell designed to sense the blood level of calcium. In the chief cell, synthesis and secretion of the hormone can be carefully regulated. Furthermore, the structure of the hormone is designed for rapid metabolic degradation, even in the absence of receptor binding. In this way, the rapid turnover of the hormone can assure that blood levels of hormone change quickly in response to changes in hormone secretory rate. This rapid metabolism of hormone is required of a system designed to respond quickly to sudden changes in the amounts of calcium entering and leaving the bloodstream. Studies over the past two decades have shown that the sequences of PTH and its precursors are designed to steer the hormone through the chief cell's secretory pathway, to direct the hormone's binding to receptors, and to assure rapid metabolism of the hormone. More recent studies have begun to unravel the mechanisms whereby synthesis of PTH is regulated in the chief cell. Descriptions of the structure of the PTH gene and a summary of the current understanding of how this structure allows the gene to accomplish its multiple functions are presented in this chapter.

BIOSYNTHESIS OF PARATHYROID HORMONE

PTH is synthesized as part of the larger precursor molecule, preproparathyroid hormone (preproPTH). Only trace amounts of this full-length precursor are found in parathyroid chief cells, because the "pre," or signal, sequence is cleaved from the amino terminus while the protein is being synthesized (see Fig. 1). As the signal sequence emerges from the ribosome, it binds to a signal recognition particle, an RNA–protein complex that recognizes signal sequences on most secreted proteins. The signal recognition particle then binds to a receptor on the rough endoplasmic reticulum (docking protein) and directs the nascent preproPTH molecule to a protein-lined channel, through which the preproPTH molecule is transported. A signal peptidase located on the inner surface of the membrane of the endoplasmic reticulum then cleaves off the signal sequence, leaving the intermediate precursor, proparathyroid hormone (proPTH) in the cisternae of the endoplasmic reticulum. ProPTH then travels via a series of vesicles to and through the Golgi apparatus (see Fig. 2). In the Golgi, the short, amino-terminal "pro" sequence is removed, leaving the mature PTH molecule. PTH is then concentrated in dense core secretory vesicles; these vesicles fuse with the plasma membrane and release PTH in response to a decrease in extracellular calcium. The hormone secreted is predominantly the intact 84-residue PTH molecule, though a variable fraction made up of carboxy-terminal PTH fragments is secreted, as well.

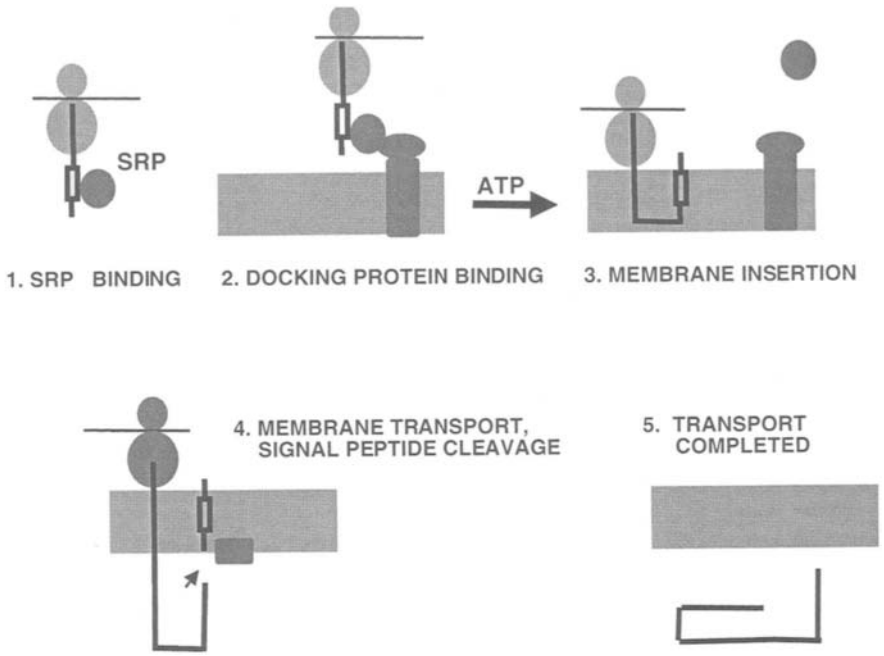

FIG. 1 The signal, or pre, sequence directs the nascent polypeptide to the apparatus for transport across the membrane of the endoplasmic reticulum. SRP, Signal recognition particle.

Function of the Pre (Signal) Sequence

The specific sequences of each of the three portions of the preproPTH molecule are responsible for directing the hormone through the complicated pathway of transport and cleavage. The known preproPTH sequences from human (1), bovine (2), rat (3), pig (4), chicken (5,6), and dog (7) tissues share a 25-residue pre sequence and a 6-residue pro sequence (see Fig. 3). Each pre sequence contains a hydrophobic stretch of amino acids preceded by a positively charged residue. The signal sequence ends with a small amino acid at the last and third-to-last positions. These characteristics are typical of most signal sequences. The preproPTH signal sequence was first discovered (8) when parathyroid gland mRNA was translated in a cell-free extract devoid of endoplasmic reticulum. Directed mutations have demonstrated the importance of each of the regions of

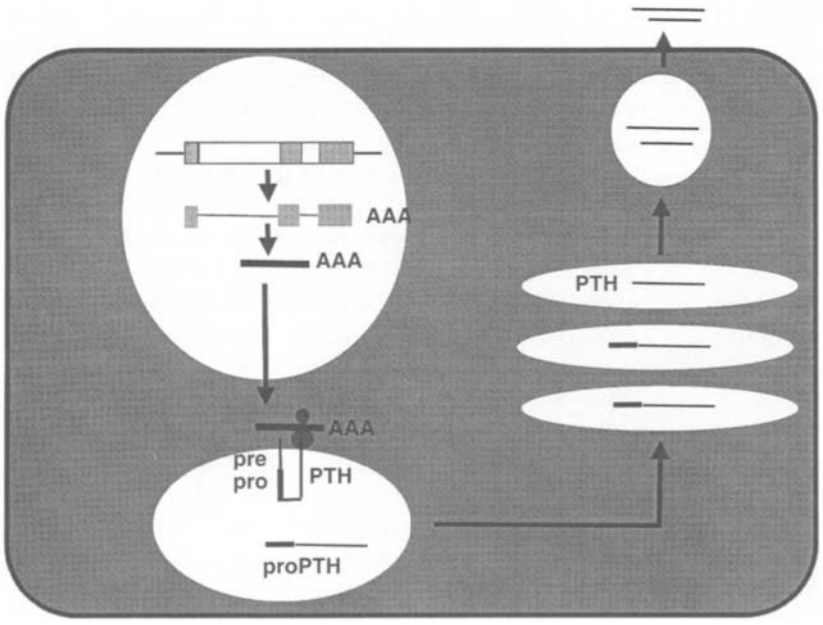

FIG. 2 Multiple cleavages occur during the intracellular transport of PTH.

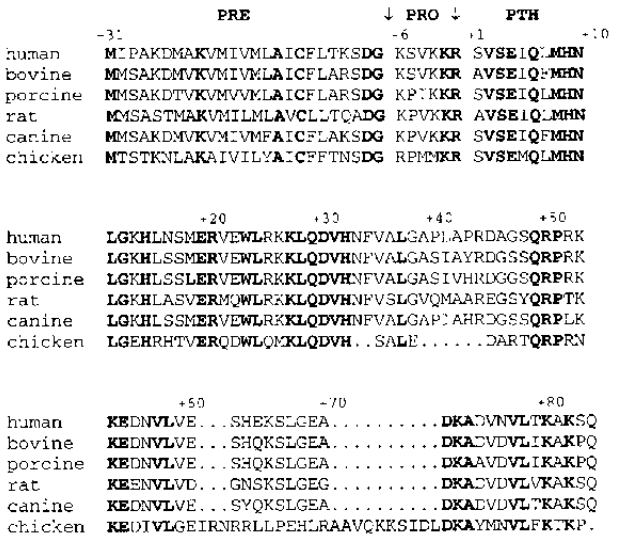

FIG. 3 Amino acid sequences of preproPTH from mammalian and avian species. Residues −31 to −7 constitute the pre sequences; residues −6 to −1 constitute the pro sequences. Dots represent residues found in chicken PTH without corresponding residues in the mammalian sequences. Amino acids are indicated by the single-letter code: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile, L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

the preproPTH signal sequence for normal signal function (9–11). Further, when a synthetic prepro peptide was added to a cell-free extract, it blocked the transport and cleavage of preproPTH by microsomal membranes (12). Most strikingly, a point mutation was found in the signal sequence of a preproPTH gene in a family with inherited hypoparathyroidism (13). A point mutation at residue 18 changed the cysteine to arginine and thereby inserted a charged residue into the hydrophobic core of the signal sequence. When this mutant preproPTH was expressed in cell-free extracts or in cultured cells, the precursor was inefficiently transported and cleaved (14).

Function of the Pro Sequence

The signal sequence of preproPTH, thus, resembles the signal sequences of other secreted proteins and performs the important role of directing the protein across the membrane of the endoplasmic reticulum and into the secretory pathway. The function of the pro sequence is less well established. In all known preproPTH sequences, the pro sequence is six residues long. The first is always positively charged, the third is hydrophobic, and the last two residues are Lys-Arg. This pattern closely resembles that found in rat proalbumin (Arg-Gly-Val-Phe-Arg-Arg) and that predicted to be present in the pro sequence of preproparathyroid hormone-related peptide (Arg-Arg-Leu-Lys-Arg). ProPTH was first discovered as a large PTH-related molecule that was the predominant form of the hormone found in parathyroid cells after pulse labeling with radioactive amino acids (15,16). Subsequent chase incubations demonstrated that the proPTH was converted to PTH in about 15 minutes; this correlated in time with transport to the Golgi (17). After this time, no trace of the pro peptide or possible fragments could be found in the cell or medium (18). These data strongly suggest that the pro sequence serves an exclusively intracellular function, probably involved in movement through the secretory pathway. Wiren *et al.* (19) tested this hypothesis by deleting the DNA sequences encoding the pro hexapeptide from cloned cDNA encoding human preproPTH and by subsequently expressing the cDNA in cell-free protein-synthesizing extracts and in intact rat pituitary GH4 cells. The mutant precursor functioned abnormally in both expression systems. The precursor crossed the membrane of the endoplasmic reticulum inefficiently, and, consequently, the subsequent cleavage of the signal sequence was inefficient. Cells secreted PTH but also secreted a molecule slightly bigger than PTH. Sequence analysis showed that the abnormal protein included the last two residues of the signal sequence. Thus, the removal of the pro sequence resulted in imprecise and inefficient function of the signal sequence. The pro sequence of preproPTH should be considered part of the functional unit responsible for transport and cleavage of the precursor on its entry into the secretory pathway. This result is not surprising. In other precursor proteins, the sequences immediately distal to the signal sequence can affect signal sequence function. One can speculate that the constraints on this region conflict with the constraints on the amino terminus of the mature PTH molecule. The PTH receptor, for example, requires very specific residues at the amino terminus of PTH for subsequent activation of adenylyl cyclase. The experiments of Wiren *et al.* show that these residues cannot be placed immediately distal to the signal sequence. The pro sequence can be considered a linker region that allows efficient signal sequence function and physically separates the signal sequence from the mature hormone sequence, which has its own and separate evolutionary constraints. The possibility that the pro sequence has additional functions, such as the promotion of proper folding of the PTH molecule in the endoplasmic reticulum, has not been rigorously examined.

The enzyme responsible for cleavage of the pro sequence of proPTH has not yet been characterized, but a number of arguments suggest that the protease, furin (or a close relative), is the cleavage enzyme (20).

Furin is a subtilisin-like enzyme that is located in the Golgi cisternae of probably all mammalian cells. The enzyme cleaves sequences like the pro sequence of rat proalbumin, which ends in dibasic residues and is preceded by other basic residues. Unlike the related PC2 and PC1 proteases, which are found in cells with secretory granules, furin cleaves precursors in cells like hepatocytes, which have no secretory granules. Cleavage by furin probably explains why proPTH, in contrast to proinsulin, for example, is cleaved normally when the hormone is synthesized in all sorts of cells, from parathyroid chief cells to fibroblasts and kidney cells (21,22). One can only speculate as to why proPTH, which is normally synthesized virtually exclusively in specialized parathyroid chief cells, uses an enzyme designed for cleavage of proteins secreted from nonendocrine cells. One plausible explanation is an evolutionary argument. The parathyroid hormone gene may well be derived from the gene encoding parathyroid hormone-related peptide (PTHrP). The PTHrP gene is widely expressed, both in cells with secretory granules, such as parathyroid chief cells and neurons, and in cells without secretory granules, such as smooth muscle cells. Therefore, it would be expected that the pro sequence of proPTHrP would be designed for cleavage by an enzyme expressed in most cells. The pro sequence of proPTH may well share this property because of its evolutionary heritage, even though proPTH is normally expressed only in cells with a secretory granule apparatus.

Intracellular Roles of the Mature PTH Sequence

Like the prepro sequence, portions of the mature PTH molecule serve to facilitate intracellular handling of PTH (23). Shortened versions of preproPTH are not stable in transfected cells. When the human preproPTH cDNA was modified to encode preproPTH(1–40) (in which the numbers refer to the mature PTH sequence), the signal sequence functioned, and proPTH(1–40) was produced in transfected cells. The proPTH(1–40) was not further cleaved to PTH(1–40), however. Instead, it was degraded intracellularly; no PTH peptides were secreted from the cells. A similar, though less dramatic, defect in secretion was exhibited by preproPTH(1–52). These short precursors were long enough for the signal sequence to direct them into the secretory pathway, but they were unstable and were not transported through the entire pathway. These results may partly explain the role of the carboxy-terminal portion of the PTH molecule. One function of the full 84-residue protein may be to allow stable and efficient transport through the secretory apparatus. Because all secreted peptides are synthesized as rather large precursors, this need for a minimal length of translation product may be a general one for secreted proteins. Of course, this "length" requirement for PTH does not preclude other functions for the carboxy-terminal portion of PTH, such as binding to a distinct PTH receptor (24).

Even the 84-residue PTH molecule is not completely stable in the parathyroid chief cell. PTH(1–84) is concentrated in secretory vesicles and granules that contain the proteases, i.e., cathepsins B and H (25,26). This colocalization of proteases and PTH may explain the observation that the hormone secreted by calves *in vivo* under conditions of hypercalcemia consists largely of carboxy-terminal fragments of PTH (27). Secretion of fragments of PTH was studied in detail by Habener *et al.* (28) and Chu *et al.* (29). These workers noted that the degradation of newly synthesized PTH is influenced by the level of extracellular calcium. Few fragments were secreted when the gland was stimulated *in vitro* by medium containing low levels of calcium. In contrast, most of the hormone secreted under conditions of hypercalcemic suppression consisted of fragments. Thus, calcium regulated the amount of available intact PTH by causing the intracellular degradation of hormone. This effect could have been caused by the activation of a PTH-degrading pathway. Alternatively, the intracellular degradation rate might have been constant; the decrease in total degradation of PTH associated with low calcium levels might simply have resulted from rapid secretion of hormone and the concomitant shorter time of exposure to the intracellular degradation mechanism.

Phorbol ester treatment of parathyroid cells *in vitro* has also been shown to result in the secretion of an increased fraction of PTH fragments, both in high and low calcium concentration conditions (30). Phorbols are either activating a proteolytic mechanism or may be selectively stimulating secretion from secretory granules containing a high proportion of PTH fragments. The physiologic correlate *in vivo* of this action of phorbol esters has not yet been established. In any case, the parathyroid gland has the capability of varying the fraction of PTH secreted as the biologically active, intact molecule. This seemingly wasteful capability makes it possible for the gland to vary quickly and dramatically the amount of biologically active hormone secreted. This regulatory capability provides a rationale for the intracellular instability of the hormone.

To sum up, it can be seen that all portions of the preproPTH molecule have intracellular functions. The prepro region is required for efficient introduction of the hormone to the secretory pathway. The carboxy-terminal region of the mature hormone is required for efficient and stable transport of PTH through the secre-

tory pathway. Inherent instability of even the full-length hormone provides a regulatory mechanism that allows extracellular calcium to alter rapidly the amount of active hormone available for secretion.

THE PARATHYROID HORMONE GENE

The genomic DNAs encoding human (31), bovine (32), rat (33), and chicken (34) preproPTH have been cloned; the complete sequences of the human (35) and bovine (32) genes have been determined. Each gene contains three exons separated by two introns (see Fig. 4). The introns vary in size from species to species, though the first intron is invariably large, and the second intron in the human, bovine, and rat genes is about 100 base pairs in length. This length is close to the minimum length that can be recognized by the splicing machinery. The introns interrupt the sequences encoding mRNA at precisely the same locations in each species. The first exon contains most of the 5′ noncoding sequence. The second exon encodes most of the prepro sequence; the second intron comes in the middle of the triplet encoding the lysine residue that precedes the dibasic cleavage sequence Lys-Arg found at the end of the known pro sequences. The third exon encodes the Lys-Arg sequence, the mature PTH sequence, and the 3′ noncoding region of the gene.

The human and bovine genes are preceded by two functional TATA boxes that determine the two closely spaced start sites of the human and bovine transcripts. The rat and chicken genes are preceded only by one TATA box, found in a position equivalent to the second TATA box in the human and bovine genes. Though both start sites of transcription are used in the human and bovine genes, no conditions have been found that favor the use of one start site over the other. No data suggest that the two transcripts have importantly different stabilities or translatability, but such questions have not been exhaustively studied.

The 5′ noncoding regions of each gene extend approximately 120 base pairs. The 3′ noncoding regions of each gene vary substantially in length, from the bovine at 227 base pairs to the chicken at more than 1600 base pairs. The 3′ noncoding region binds proteins that may regulate the stability of the preproPTH mRNA (36,37).

The human, rat, and bovine PTH genes are represented only once in the haploid genomes of each species. The human PTH gene is located on the short arm of chromosome 11 at band 11p15 (38–40). A series of restriction fragment length polymorphisms (41,42) have made it possible to show that the human PTH gene is linked to the genes encoding catalase, calcitonin, H-ras, insulin, and β-globin (43). Two other polymorphisms have been identified through the use of denaturing gel electrophoresis (44). All of these polymorphisms have proved useful in defining the inheritance of specific alleles of the PTH gene in families with calcium disorders (45).

Several features of the PTH gene suggest that the gene is related to that encoding PTHrP (46–48). Most importantly, the major coding exon of both genes starts precisely at the same nucleotide, one base before the codons encoding the Lys-Arg residues of the pro sequences of each hormone. After the Lys-Arg sequences, the PTH and PTHrP amino acid sequences are identical in 8 of the next 13 residues. Further, the PTHrP gene is located on chromosome 12, a chromosome known to encode many genes that resemble genes on chromosome 11; for this reason, the chromosomes are thought to have arisen by an ancient duplication event (49). One can speculate that the PTH gene may represent a variation of the PTHrP gene; the PTH hormone takes advantage of the PTHrP receptor in order to regulate calcium metabolism. If this hypothesis is correct, then the gene had to change in order to assure expression primarily in the parathyroid chief cell and to assure appropriate regulation by modulators such as extracellular calcium and 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$]. A hypothalamic peptide called TIP39 (50) has been found to activate the PTH2 receptor and to be distantly related in sequence to PTH and PTHrP. The structure of the TIP39 gene has not yet been reported. This gene may represent a third member of the PTH gene family.

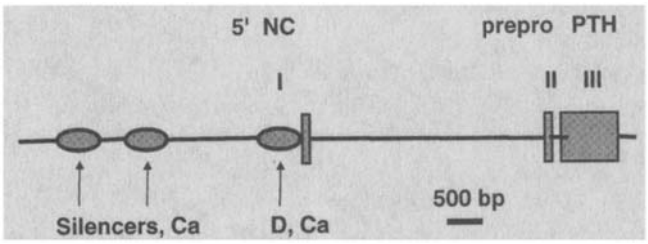

FIG. 4 The parathyroid hormone gene. NC, Noncoding.

REGULATION OF PTH BIOSYNTHESIS

The minute-to-minute stability of the level of blood calcium depends on the regulation of PTH secretion by calcium. Longer term homeostasis depends on several other levels of control (see Fig. 5). The number of parathyroid chief cells is carefully regulated; when appropriately stimulated, the parathyroid glands can

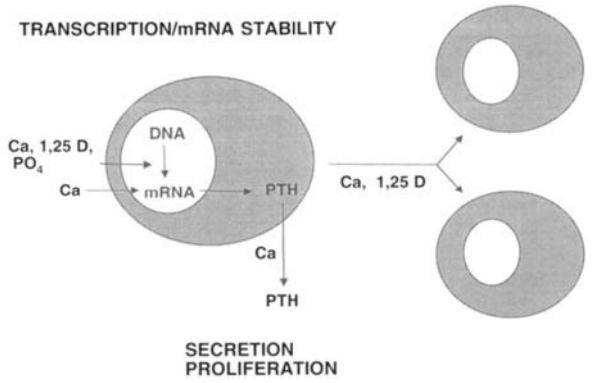

FIG. 5 Levels of parathyroid cell regulation.

increase in size dramatically. The parathyroid chief cell is uniquely designed to express the PTH gene; the state of differentiation of the chief cell can, therefore, influence the rate of PTH biosynthesis. Specific blood-borne signals, most notably calcium and $1,25(OH)_2D_3$, regulate the activity of the PTH gene, as well. These several levels of regulation of PTH biosynthesis are examined in the following discussions (see also Chapter 18).

Regulation of Parathyroid Cell Number

Little is known about the regulation of parathyroid cell number. The relatively uniform morphology of chief cells suggests that all chief cells have the potential to divide, if appropriately stimulated, but the alternative hypothesis that a subset of chief cells has the unique, stem-cell-like capability to proliferate has not been evaluated. Further, there is the general impression that parathyroid cells are long-lived, because mitoses are seldom seen in normal glands of mature animals, because the observed rate of apoptosis is low, and because hyperplastic glands only slowly decrease in size after stimulation. Nevertheless, specific studies to define potential modulators of chief cell longevity have not been performed. Despite this paucity of information, the dramatic hyperplasia of parathyroid cells in patients and animals with renal failure demonstrates the likely roles of calcium, phosphate, and $1,25(OH)_2D_3$ in regulating parathyroid cell proliferation. Dietary manipulation alone can similarly lead to chief cell hyperplasia. Naveh-Many and Silver (51), for example, used flow cytometry to count parathyroid cells and showed that 3 weeks of a calcium- and vitamin D-deficient diet fed to weanling rats led to a 1.7-fold increase in parathyroid cell number. These investigators subsequently studied the mechanism of the increase in parathyroid cell number caused by hypocalcemia, hyperphosphatemia, vitamin D deficiency, and uremia *in vivo* (52). They found that hypocalcemia and uremia led to increases in parathyroid cell proliferation, whereas hypophosphatemia led to decreases in parathyroid cell proliferation. Administration of $1,25(OH)_2D_3$ for 3 days had no effect on parathyroid cell proliferation. None of these conditions led to changes in the rate of parathyroid cell apoptosis. Further studies of the effects of calcium in the uremic model suggest that calcium works by acting on the same calcium-sensing rector that mediates the actions of calcium on PTH secretion. The calcimimetic compound NPS R-568, like calcium, suppressed parathyroid cell proliferation in uremic rats (53).

The possibly independent roles of calcium and $1,25(OH)_2D_3$ in the regulation of parathyroid cell proliferation have not been studied extensively. *In vivo,* these variables are difficult to manipulate independently in the intact animal. One particularly instructive *in vivo* model, the vitamin D receptor knockout mouse, has been studied, however (54,55). These mice develop hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism in the days and weeks after weaning. When the hypocalcemia and hypophosphatemia are prevented by a diet high in calcium, phosphate, and lactose, the hyperparathyroidism and parathyroid gland enlargement are prevented. Because these mice lack vitamin D receptors, they must be able to regulate parathyroid cell number without using the genomic actions of $1,25(OH)_2D_3$. Presumably, the direct effects of normal calcium and phosphate are sufficient to prevent parathyroid cell replication.

In studies of cultured parathyroid chief cells, it has been possible to vary the levels of calcium and $1,25(OH)_2D_3$ separately. Several groups have shown that $1,25(OH)_2D_3$ can regulate parathyroid cell proliferation *in vitro.* Whether the cells were grown in the presence of serum (56,57) or serum-free growth factors (58), administration of $1,25(OH)_2D_3$ decreased their rate of proliferation. Studies of the effects of calcium on parathyroid cell proliferation *in vitro* have yielded differing results. Several studies (59–61) have shown that lowering of calcium leads to increased cellular proliferation. Other studies of dispersed, early-passage chief cells have demonstrated no effect of calcium on the rate of cell proliferation, however (57,58,62).

Though extracellular levels of calcium and $1,25(OH)_2D_3$ can be independently regulated *in vitro,* it is hard to be sure that parathyroid cells in culture respond to modulators of proliferation in this setting in the same way that they do *in vivo.* Thus, though the combined effects of low calcium and low levels of $1,25(OH)_2D_3$ to stimulate parathyroid cell proliferation are well established, the individual roles of calcium, phosphate, and $1,25(OH)_2D_3$ *in vivo* remain uncertain.

Cell-Specific PTH Gene Expression

Expression of the parathyroid hormone gene occurs almost exclusively in the parathyroid chief cell. [Expression has also been noted in the rat hypothalamus (63).] Thus, genes required for parathyroid chief cell differentiation are possible candidates for genes that might regulate the PTH gene as well. These genes, identified through the study of knockout mice, include *hoxa3* (64,65), *pax9* (66), and glial cells *missing 2* (67). The *hoxa3* and *pax9* mutant mice lack a range of branchial arch derivatives, whereas the glial cell *missing* knockout mouse exhibits highly selective parathyroid cell deficit. When the chief cell is disrupted by neoplastic transformation, the regulation of PTH gene expression can be altered. For example, parathyroid cancers may stop synthesizing PTH completely (68). Presumably, specific DNA sequences associated with the PTH gene respond to the environment of the chief cell to activate gene expression. Because no well-differentiated cell line expressing the PTH gene has been established, it has been difficult to determine the sequences responsible for chief cell-specific PTH gene expression. Occasional "experiments of nature" have provided important clues, however. Very rarely, human nonparathyroid tumors have been found to produce PTH ectopically, for example. In one case that was studied carefully (69), the PTH regulatory region upstream from the gene was disrupted in tumor cells. Presumably, this gene rearrangement allowed the gene to be expressed in nonparathyroid cells by providing new regulatory signals or abolishing normal silencing mechanisms found upstream of the gene. Further, in a subset of parathyroid adenomas, the entire upstream portion of the PTH gene along with the first, noncoding exon are separated from the rest of the gene and rearranged adjacent to the *PRAD1* gene (70). As a consequence of this rearrangement, the *PRAD1* gene (encoding cyclin D1), a regulator of the cell cycle, is dramatically overexpressed. These observations suggest that the PTH gene upstream region contains sequences that stimulate gene transcription in parathyroid chief cells. Further analysis of the sequences that determine chief cell expression of the PTH gene must await studies of transgenic animals or the establishment of well-differentiated parathyroid chief cell lines.

Modulators of PTH Gene Expression

The effects of calcium on PTH gene expression were first demonstrated in experiments using primary parathyroid cells in culture. Russell *et al.* (71) found that high levels of calcium resulted in a decrease in PTH mRNA levels over a several-day period. In those studies, no difference was noted between the effects of low and normal levels of extracellular calcium. The decrease in PTH mRNA levels in response to high calcium levels could be reversed by lowering the calcium level; thus, the suppressive effect of calcium was not an irreversible, toxic effect. These *in vitro* observations have been confirmed by Brookman *et al.* (72), who noted a slight increase in PTH mRNA under low calcium level conditions at one time point. Subsequent studies by Russell *et al.* (73) showed that the rate of transcription of the PTH gene in nuclei of dispersed bovine parathyroid cells fell within 6 hours in response to high levels of extracellular calcium. The rate of transcription of the actin gene was unchanged; therefore, the effect of calcium was shown to be specific.

The lack of parathyroid cell lines that produce PTH has hampered the search for DNA sequences responsible for the transcriptional effects of calcium noted in cultured parathyroid cells. Okazaki *et al.* (74) have identified short sequences several thousand base pairs upstream from the start site of PTH gene transcription that may well be important for calcium regulation, however. These investigators identified the region by showing that several short sequences in the region could decrease gene transcription from many different promoters, including the PTH gene promoter (75). Further, when the level of extracellular calcium was varied, after transfection of fusion genes containing a short oligonucleotide from this region, high calcium levels further suppressed transcription from genes containing the sequence but had no effect on control plasmids. Intriguingly, almost identical sequences were found in the gene encoding rat atrial natruiretic polypeptide, another gene negatively regulated by calcium. This DNA sequence could also confer calcium sensitivity to a fusion gene in fibroblast transfection experiments. Though these experiments are very suggestive, further studies will be required to show that the regulatory region can confer calcium sensitivity in its normal location far upstream from the PTH gene transcription start site. Ultimately, studies using well-differentiated parathyroid cells will be required.

Two groups have studied the acute effects of changes in blood calcium on PTH mRNA levels in the intact rat. Both showed that acute lowering of blood calcium (with phosphate, calcitonin, or EDTA) led to a prompt increase in PTH mRNA levels (76,77). Elevations in blood calcium, in contrast, led to no change in PTH mRNA levels after 6 hours (76) and to a slight decrease in PTH mRNA levels after 48 hours (77). The parathyroid gland apparently, then, in the normal state, rests near the bottom of the calcium dose–response curve. The gland is well equipped to increase PTH production, but poorly prepared to decrease production in the

face of hypercalcemia. Subsequent studies showed that changes in PTH mRNA caused by hypocalcemia *in vivo* are not caused by a transcriptional mechanism, but rather are caused by changes in mRNA stability (37).

$1,25(OH)_2D_3$ has been shown to be an important regulator of PTH gene transcription in studies both *in vitro* and *in vivo*. Silver *et al.* (78) used primary parathyroid cells in culture to show that exposure to $1,25(OH)_2D_3$ led to a decrease in PTH mRNA levels. This work has been confirmed by studies of Karmali *et al.* (79) and Brown *et al.* (80). Russell *et al.* (81) then showed that $1,25(OH)_2D_3$ lowers the PTH gene transcription rate as early as 2 hours after exposure of cells to $1,25(OH)_2D_3$. Similarly, in intact rats, intraperitoneal injections of $1,25(OH)_2D_3$ rapidly led to decreased transcription of the PTH gene and decreased PTH mRNA levels (82). The doses of $1,25(OH)_2D_3$ were so low that blood calcium did not change; the precise blood levels of $1,25(OH)_2D_3$ required to suppress PTH gene transcription acutely *in vivo* have not been established, however.

The effects of low levels of $1,25(OH)_2D_3$ have not been studied extensively in intact animals. Such studies are difficult to interpret, because of confounding effects of vitamin D deficiency on blood calcium and parathyroid cell number. Weanling rats fed a vitamin D-deficient diet for 3 weeks had a modest increase in their PTH mRNA levels (51). This increase occurred with no apparent decrease in blood calcium levels.

In the intact organism, calcium and $1,25(OH)_2D_3$ seldom vary independently; consequently, the effects of changes in both parameters simultaneously have important physiologic relevance. When rats were made acutely hypocalcemic with phosphate and were at the same time given $1,25(OH)_2D_3$ intraperitoneally, the suppressive effect of $1,25(OH)_2D_3$ reversed the effect of hypocalcemia and led to a decrease in PTH mRNA (76). In contrast, when rats were fed a low-calcium diet for 3 weeks, blood calcium levels decreased and blood $1,25(OH)_2D_3$ levels increased dramatically. In this setting, PTH mRNA levels rose severalfold; thus, the effects of low calcium levels were more influential than the effects of high $1,25(OH)_2D_3$ levels. The partial vitamin D resistance of the parathyroid gland in the setting of hypocalcemia makes sense physiologically: in that setting the action of vitamin D to increase intestinal calcium absorption is needed, but the action to inhibit PTH synthesis is not. Sela-Brown *et al.* (83) studied the mechanism of hypocalcemia-induced resistance to vitamin D action on the parathyroid gland. They showed that hypocalcemia *in vivo* induces nuclear accumulation of calreticulin, a calcium-binding protein, in parathyroid chief cells, and that calreticulin can interfere with the actions of the vitamin D receptor on a negative vitamin D response element in transfected cells *in vitro*.

In experimental uremia, the double stimulus of hypocalcemia and low levels of $1,25(OH)_2D_3$ has consistently led to increases in PTH mRNA (84,85). Administration of $1,25(OH)_2D_3$ could reverse this increase. This effect of $1,25(OH)_2D_3$ is likely to contribute importantly to the decrease in PTH blood levels seen in dogs with experimental uremia (86) and in dialysis patients (87).

A series of transfection studies and DNA binding assays have been used to identify DNA sequences in the PTH gene responsible for modulating transcription of the PTH gene in response to $1,25(OH)_2D_3$. When a fusion gene containing 684 base pairs (bp) of DNA upstream of the human PTH gene was introduced stably into rat pituitary GH4 cells, expression of the gene was specifically suppressed by $1,25(OH)_2D_3$ (88). Three groups have identified DNA sequences upstream of the PTH gene that bind to $1,25(OH)_2D_3$ receptors *in vitro*. Filter binding assays showed that $1,25(OH)_2D_3$ receptors can bind to bovine PTH gene sequences between -485 and -100 bp upstream from the transcription start site (89). Subsequently, gel mobility-shift assays were used to identify a specific 26-bp sequence, located 125 bp upstream from the start site of transcription of the human PTH gene, that binds $1,25(OH)_2D_3$ receptors (90). When this short sequence was linked to a reporter gene and expressed in pituitary GH4 cells, $1,25(OH)_2D_3$ decreased expression of the reporter gene. This suppression of transcription was even greater when the number of $1,25(OH)_2D_3$ receptors in the GH4 cells was increased by cotransfection of a $1,25(OH)_2D_3$ receptor expression vector. The human negative $1,25(OH)_2D_3$ (vitamin D) response element (VDRE) contains one copy of a motif found in two copies in the mouse osteopontin gene, a gene up-regulated by $1,25(OH)_2D_3$. Negative VDREs have also been identified in the chicken (91) and rat (92) PTH genes. These sequences closely resemble positive VDREs and have been shown to bind heterodimers of the vitamin D receptor and RXR, just as positive VDREs do. Subtle differences in binding interactions may explain why these particular VDREs in the PTH gene can act as negative VDREs with vitamin D receptor–RXR heterodimers (93).

Until recently, the effects of phosphate on the parathyroid cell were thought to be indirectly mediated by the hypocalcemia associated with increases in blood phosphate. The rapid actions of changes in phosphate levels *in vivo* on PTH secretion work through such a mechanism. However, studies using intact rat parathyroid glands *in vitro* demonstrate that changes in phosphate levels can, after several hours, lead to changes in PTH secretion (94,95). PTH mRNA did not change in these studies *in vitro*, but analogous studies performed in intact rats demonstrated that phosphate, in the setting of apparently constant levels of calcium and

$1,25(OH)_2D_3$, increases PTH mRNA by a posttranscriptional mechanism (96).

Though calcium and $1,25(OH)_2D_3$ are certainly the most important physiologic regulators of PTH gene transcription, other circulating factors are likely to modulate PTH gene transcription as well. The PTH gene contains a consensus cyclic AMP response element that can function in the context of a fusion gene in transfection experiments (97). Thus, hormones that stimulate adenylyl cyclase may increase PTH gene transcription. Glucocorticoids have been shown to increase PTH mRNA in dispersed, hyperplastic human parathyroid cells (98) and to abolish the decrease in PTH mRNA in response to $1,25(OH)_2D_3$ in dispersed bovine parathyroid cells (79). These cell culture studies need to be confirmed by studies *in vivo* to determine their physiologic significance. In ovariectomized rats, estradiol administration led within 24 hours to a fourfold increase of PTH mRNA (99). Estrogen receptors were identified in rat parathyroids. These observations may have important implications for an understanding of postmenopausal osteoporosis and hyperparathyroidism. The possibility that the effect of estrogen on PTH mRNA levels is a direct effect on the parathyroid gland needs to be tested by studies using cultured parathyroid cells.

Peripheral Metabolism of PTH

Intact PTH is rapidly cleared from the circulation with a disappearance half-time of approximately 2 minutes (100–103). Removal of PTH from the blood occurs mainly (60–70%) in the liver but also in the kidneys (20–30%) and, to a much lesser extent, in other organs (100,102,103). Clearance of PTH by the liver is mediated mainly by a high-capacity, nonsaturable uptake by Kupffer cells and is followed by rapid and extensive proteolysis (104). Renal clearance occurs almost entirely by glomerular filtration. The hormone is also reabsorbed by the renal tubules and then extensively degraded, so that little or no intact PTH appears in the final urine (102). A large membrane-bound protein, megalin, binds PTH (but not carboxy-terminal fragments of PTH) in the lumen of the proximal tubule to initiate this reabsorption (105). In both the liver and kidney, as in bone, some PTH is removed by high-affinity binding to cell surface receptors, but this constitutes only a small fraction (<1%) of overall PTH clearance (102,106,107). Thus, it appears that the main role of the liver and kidney in PTH metabolism is rapid removal and degradation of circulating biologically active hormone, assuring that the concentration of hormone available to receptors in target tissues is dictated exclusively by the rate at which PTH is secreted by the parathyroid glands.

This simple first-order model, in which secreted intact PTH that is not bound to specific high-affinity cellular receptors is rapidly cleared by peripheral organs, was found to be inadequate when it was recognized that multiple species of immunoreactive PTH molecules are present in the circulation (108,109). Early observations with region-specific immunoassays *in vivo* and in medium from perfused organs *in vitro,* followed later by direct analysis of the peripheral metabolism of intravenously administered radioactive PTH, confirmed that intact PTH is not only rapidly cleared from the blood but also is rapidly cleaved by endoproteases to a series of carboxyl-terminal (C) fragments, some of which reenter the circulation (100,102,103,108,110,111). The chemical identities of these fragments have not been established definitively. Microradiosequencing of radioactive fragments recovered from blood of rats following intravenous administration of [^{125}I-Tyr-43]bPTH or [^{3}H-Tyr-43]bPTH has shown that PTH peptides produced by cleavages between residues 33–34, 36–37, 40–41, and 42–43 are the predominant large "signature" C fragments (103,104,107,110). These fragments exhibit apparent molecular masses of 4000–7000 Da and it is not certain that they all extend entirely to the original C terminus (i.e., to residue 84) of the intact hormone. Also, because fragments shorter than PTH(43–84) would not have been detected by these radiosequencing analyses, it is possible that such shorter fragments also exist in blood. Other evidence, derived from detailed analysis by region-specific immunoassays of circulating forms of PTH, points to the presence in blood of shorter, "midmolecule" fragments, presumably derived from proteolysis within both the amino (N-) and C-terminal portions of the intact hormone (108,112,113).

In vitro, isolated hepatic Kupffer cells, but not hepatocytes, generate C fragments that are chemically identical to the major circulating form (104,114). Also, liver ablation, but not nephrectomy, blocks the production of circulating C fragments that otherwise are produced by proteolysis of administered radiolabeled intact PTH (103). These findings suggest that hepatic Kupffer cells are responsible for both the rapid clearance and the extensive proteolysis of PTH that occur in the liver. Moreover, Kupffer cells appear also to be the source of the major circulating C fragments identified so far that result from postsecretory metabolism of PTH. In the kidney, extensive proteolysis of PTH occurs also, but the kidney does not contribute significantly to the circulating pool of PTH C fragments (100,103,110). Studies of the cellular enzymes responsible for these cleavages are most consistent with a role for lysosomal enzymes of the cathepsin B/D family (107,115).

Quantitatively, less than 10–20% of secreted intact PTH is converted to circulating C fragments by

peripheral metabolism (100,103,110). On the other hand, C fragments comprise between 50 and 90% of total circulating PTH immunoreactivity (102,107,108,112,113,116). This disparity results from at least two factors. First, the clearance of C fragments, which does not occur in the liver but rather proceeds mainly via glomerular filtration in the kidney, is significantly slower than that of intact PTH (102,117). Second, it is now clear that C fragments are secreted along with intact PTH by the parathyroid glands, which thus constitute an independent source of these fragments (115,116,118–120). Remarkably, the major C fragments secreted by the parathyroids are chemically identical (at their N termini) with the principal circulating products of peripheral metabolism of PTH (115,119). The impact of these factors is especially obvious in renal insufficiency, in which delayed renal clearance (up to 100-fold) of C fragments, combined with their accelerated generation, both within the hyperplastic parathyroid glands and during peripheral metabolism of overproduced intact hormone, leads to massive accumulation of C fragments vs. intact hormone in the circulation.

Metabolism of PTH, both peripherally and within the parathyroid glands, involves cleavages within the region PTH(33–43), which, at least potentially, could generate biologically active N-terminal fragments. Accordingly, considerable interest has focused on the possibility that such circulating N fragments might result from PTH metabolism and, further, that the overall rate or pattern of PTH proteolysis might be regulated physiologically to modulate the production of such fragments. Circulating amino-terminal PTH fragments have been demonstrated occasionally, almost exclusively in the setting of renal failure or hyperparathyroidism, or both, but it has been difficult to exclude postcollection proteolysis as the explanation in these circumstances (107,108,121). Moreover, immunochemical analyses of normal plasma have not provided convincing evidence of circulating N-terminal PTH fragments. More recently, direct analysis of the fate of the N terminus of PTH has been possible using [^{35}S]methionine to radiolabel biologically active hormone to high specific activity within the N-terminal region of the molecule. These studies in normal rats have shown that N-terminal PTH fragments produced by isolated Kupffer cells *in vitro* or by the liver or kidney *in vivo* are rapidly degraded *in situ* and do not reenter the circulation, at least in concentrations above 50 f*M* (100). Similar investigations have provided evidence that peripheral metabolism of PTH is not regulated physiologically in response to alterations in serum or dietary calcium, vitamin D intoxication, or parathyroid status (101,122). In contrast, secretion of C fragments by the parathyroid glands is strikingly influenced by extracellular calcium, in that hypercalcemia increases the ratio of secreted C fragments to intact hormone whereas the opposite occurs in hypocalcemia (108,116,118,119). These alterations in the intracellular proteolysis of PTH are reflected by corresponding changes in the predominant immunoreactive forms of PTH in the circulation observed during hypercalcemia and hypocalcemia (108,112).

The extensive metabolism of PTH(1–84) to fragments of PTH that accumulate in the circulation but cannot activate the PTH/PTHrP receptor has created an obstacle to the measurement of biologically active PTH in the circulation by immunologic methods. A series of two-site immunoassays, in which antibodies to the amino-terminal region and carboxy-terminal region of PTH must both bind the ligand simultaneously to register as a PTH molecule, have revolutionized the clinical usefulness of PTH assays (123). However, later generation versions of these assays have demonstrated unanticipated complexity in the PTH fragments present in the circulation (124–126). Some two-site PTH assays, but not others, recognize a large PTH fragment, roughly the size of PTH(7–84). This fragment is normally only a small fraction of the circulating PTH, but it can represent as much as 50% of the PTH recognized by some two-site PTH assays in the presence of renal failure. The fraction of immunoreactive PTH represented by the large PTH fragments is also increased in hypercalcemic states. Though PTH fragments missing the amino terminus of PTH are not expected to activate the PTH/PTHrP receptor, the possibility that these fragments might antagonize the actions of PTH(1–84) or might have unique activities of their own is currently being explored. The precise structure, site of origin, and biologic activities of these fragments, thus, are important questions for the future.

In summary, the extremely rapid peripheral clearance and proteolysis of intact PTH play an important role in limiting the duration of hormone action and in assuring that the secretory activity of the parathyroid glands is the overriding determinant of the circulating concentration of biologically active PTH. Whereas a small percentage of degraded PTH molecules reappears in the blood, these are exclusively composed of large carboxyl-region and midregion fragments that are devoid of classic PTH bioactivity. Additional fragments are released directly by the parathyroid glands, reflecting an intraglandular mechanism involved in calcium regulation of hormone secretion, but these too are biologically inactive. It remains possible that such carboxyl-region and midregion PTH fragments may exert novel biologic actions in some tissues via receptors other than those known to be activated by intact PTH or N-terminal PTH fragments, but there is little evidence at present in support of a critical physiologic role for such fragments.

CONCLUSION

The blood level of PTH is regulated at several levels, each designed to respond to different challenges to calcium homeostasis. Over short time frames, the regulation of PTH secretion by calcium, coupled to the rapid metabolism of the hormone, assures that the blood level of PTH can adjust to sudden changes in calcium flux. Turnover of PTH within the parathyroid gland decreases under hypocalcemic conditions; this adjustment provides rapid increases in available hormone. Over a longer time frame both calcium and $1,25(OH)_2D_3$ regulate PTH biosynthesis. It is, of course, reasonable that $1,25(OH)_2D_3$ should regulate PTH biosynthesis, because the need for PTH can be expected to be great in face of vitamin D deficiency, no matter what the instantaneous level of blood calcium. Though the physiologic studies are not extensive, the synthetic machinery seems designed particularly to respond dramatically to decreases in blood calcium and $1,25(OH)_2D_3$ levels. Over a longer time period, calcium and, perhaps to a greater extent, $1,25(OH)_2D_3$ regulate the number of parathyroid cells. This is a relatively crude and slow process. The slow turnover of parathyroid chief cells suggests that the parathyroid gland is not meant to rely on frequent changes in parathyroid cell number, but uses this method of amplifying its signal only when other alternatives prove insufficient. This perspective on parathyroid control, with its emphasis on multiple levels of regulation, provides a framework for understanding the alterations caused by disease and may suggest therapeutic strategies as well.

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CHAPTER 3

Parathyroid Hormone-Related Protein

Gene Structure, Biosynthesis, Metabolism, and Regulation

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was identified in the course of the search for the agent responsible for the paraneoplastic syndrome, humoral hypercalcemia of malignancy. Malignancy-associated hypercalcemia, recognized as a clinical entity since the mid-1920s, was originally assumed to be related to local erosion of the skeleton by resident metastatic lesions (1–4). However, in 1941, Fuller Albright proposed the elaboration of a humoral factor by a neoplasm as an alternative mechanism to explain the reversal of hypercalcemia and hyperphosphatemia after removal of the primary tumor in a patient with renal carcinoma (5). It is now recognized that local osteolytic mechanisms account for only approximately 20% of cases of malignancy-associated hypercalcemia and that in the majority of the remaining 80%, hypercalcemia results from elevated circulating levels of PTHrP (1–4,6).

The biochemical purification of PTHrP and the subsequent cloning of the cDNA by several laboratories in the late 1980s revealed that PTHrP and parathyroid hormone shared striking similarity at the aminotermini of the respective mature peptides: 8 out of the first 13 amino acids are identical, a further 3 represent conservative changes, and the two proteins share considerable conformational similarity through residue 34 (Fig. 1). This extensive homology accounts for the ability of PTHrP to bind to and activate classic parathyroid hormone (PTH) receptors in bone and kidney and consequently to generate the paraneoplastic effects on calcium and phosphate metabolism that occur in humoral hypercalcemia of malignancy (1–4,7–9).

The similarity between PTHrP and PTH extends to their overall genomic organization as well. Their two genes share a similar organization and similar positioning of intron–exon boundaries (Fig. 1). Furthermore, the human PTHrP gene maps to the short arm of chromosome 12, where it is flanked by lactate dehydrogenase B and the K-*ras* protooncogene, and the PTH gene is located on a homologous region of chromosome 11 with lactate dehydrogenase A and H-*ras* (7–9). These two chromosomes are thought to have arisen through a tetraploidization event that occurred some 200 to 300 million years ago (10). Both PTHrP and the PTH/PTHrP receptor sequences have been identified in teleost fish and the respective proteins have been found in the cartilaginous elasmobranchs (11–13), thus it seems quite possible that PTHrP represents the ancestral gene and that PTH evolved subsequent to the gene duplication, as a response to the demands placed on calcium regulation by the emergence of bony skeletons in fish or by the adaptation to a terrestrial environment by early amphibians.

GENE STRUCTURE: PROMOTER ELEMENTS, SPLICE SITES, AND TRANSCRIPTIONAL TERMINATION SITES

From their common origin, the PTHrP and PTH genes have clearly diverged. In humans the PTHrP gene

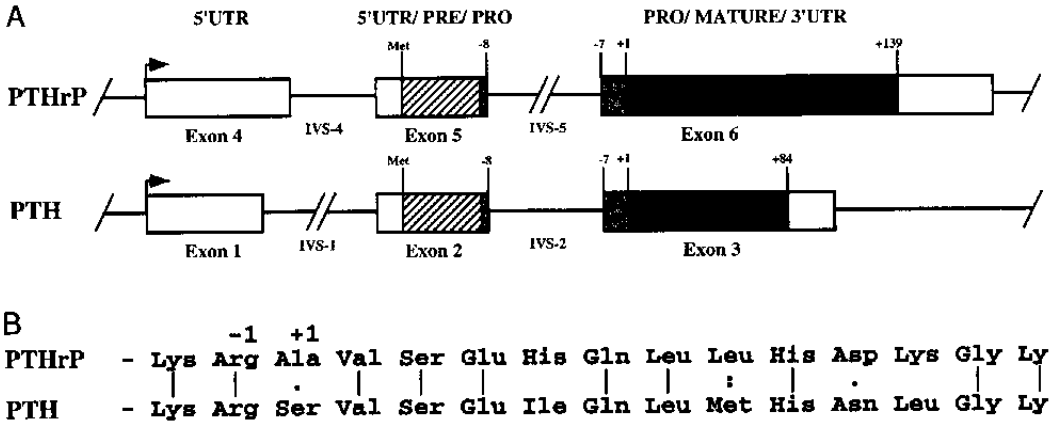

FIG. 1 Comparison of parathyroid hormone-related protein (PTHrP) and parathyroid hormone (PTH). (A) Gene structure. The principal human PTHrP and PTH exons and introns are aligned relative to the coding regions. Exons are boxed, the 5′ and 3′ untranslated regions (UTRs) are white, the pre segment is hatched, the pro segment is shaded, and the mature peptide is black. Amino acid residues are numbered relative to the first residue of the mature peptide. (B) Amino acid sequence. The sequences of human PTHrP and PTH are aligned from the last two residues of the pro segment (encompassing the main cleavage site) through the first 13 residues of the mature peptide. Identity is indicated by vertical connecting lines and mismatches are scored according to the degree of evolutionary distance, with two vertical dots indicating greater similarity than one dot. In the rat and bovine PTH sequences, the first amino acid of the mature peptide is identical to that in PTHrP (Ala). From data in Mangin *et al.* (14).

is by far the larger and more complex of the two, spanning more than 15 kilobases (kb) and containing eight exons (Fig. 2; various exon numbering systems have been employed by different research groups; for the sake of simplicity, a sequential 1 through 8 nomenclature is used here). Three promoters have been functionally characterized (14–18). The first (P1) lies just upstream of exon 1 and contains a canonical TATA sequence; a second TATA-containing promoter (P2) resides some 2.7 kb downstream within a small, 45-bp intron between exons 3 and 4. An additional promoter element has been identified immediately upstream of exon 3, but does not contain a TATA sequence and instead resembles the GC-rich initiator element found in many housekeeping genes. In addition to the use of multiple promoters, the transcriptional diversity of PTHrP gene expression is also mediated by alternative splicing of primary transcripts (19–22). The 5′ end of the gene contains four noncoding exons that may be variably incorporated into the mature, processed mRNA and the resultant splicing pattern appears to be largely dependent on promoter usage. For transcripts initiating from P2, for example, the first exon (exon 4) is uniformly spliced to exon 5, whereas for transcripts initiating from the initiator element, the first exon (exon 3) is always spliced directly to exon 5, omitting exon 4. For transcripts initiating at P1, however, two possible patterns exist: exon 1 may be spliced to exon 2 (in which case splicing exon 2 to exon 3 and exon 3 to exon 5 is obligatory) or exon 1 may instead be spliced directly to exon 3 (in which case splicing exon 3 to exon 5 is obligatory and exon 2 is omitted). However, because all of these 5′ alternative exons represent untranslated regions (UTRs), the functional consequences of any particular splice choice are presently uncertain.

Still further diversity of PTHrP mRNA expression in humans is generated by alternative splicing at the 3′ end of the gene, which contains three possible terminal exons, each encoding a distinct carboxy terminus for the PTHrP protein (19–22). The splicing pattern here appears to be generated in large part by the site of transcription termination (Fig. 2). Transcripts terminating immediately 3′ of exon 6 possess no 3′ splicing alternatives and translation necessarily yields a 139-residue mature peptide. Transcripts that terminate following exon 7 have only one possible 3′ splice (exon 6 to exon 7), which, if available, will presumably be executed by default during mRNA processing. Translation of this mRNA product then yields a mature peptide of 173 amino acids. Only for transcripts terminating beyond exon 8 does there appear to be a splicing choice (either exon 6 to exon 7 or exon 6 to exon 8, with translation of the latter giving a 141-residue product), although complete removal of intronic sequences would necessarily render obligatory the exon 6 to exon 8 splice. The operative mechanism here, therefore, seems to be one of alternative transcription termination (and polyadenylation), rather than alternative splicing. In theory, the simplest means of generating equal amounts of each of the three alternative