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Cellular and Molecular Biology of the Renin-Angiotensin System

Edited by
Mohan K. Raizada, M. Ian Phillips, Colin Sumners

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PREFACE

EARLY STUDIES ON HYPERTENSION

Research in the field of hypertension was initiated in 1834 by Richard Bright, a noted physician who observed that hypertrophy of the heart often accompanies kidney disease. Following the disclosure of Bright's observation, techniques for measuring blood pressure were designed, allowing scientists to link cardiac hypertrophy to renal hypertension. In 1898, the discovery of renin by Tigerstadt and Bergmann, and the demonstration that this kidney extract caused vasoconstriction, spotlighted the kidney, in addition to the adrenal medulla, as a hypothetical origin of hypertension.

The next phase of hypertension research began in 1939, when Page, Helmer, and Kohlstaedt maintained that renin itself was not a direct vasoconstrictor, but an enzyme that formed another substance, which they called angiotonin. At the same time the Page team was performing these renin studies, the Braun-Menendez group in Buenos Aires was isolating an identical substance, a small dialyzable material from the renal vein, which they named hypertensin. Page and Braun-Menendez later agreed to call the substance angiotensin. Almost two decades passed before angiotensin was purified and its structure elucidated. In 1956, during the purification stage, Skeggs and his group of investigators discovered angiotensin converting enzyme (ACE), and in the 1950s two peptides, angiotensin I and angiotensin II, were synthesized. When these peptides later became available in large quantities, their widespread supply allowed many teams of investigators to develop a higher level of understanding of the "renin-angiotensin system." Part of this understanding included the concept that angiotensin is not only a vasoconstrictor, but also a major participant in the processes of sodium homeostasis, catecholamine release, and thirst enhancement. Throughout this stage of investigation, Irvine Page continuously cautioned against the attitude of his clinical/scientific contemporaries, who maintained that hypertension was not a disease but a perfusion of the body controlled by several factors in dynamic equilibrium, each affecting the others.

Despite the vast amount of information about the physiology of the renin-angiotensin system acquired during the 1950s and 1960s, the majority of clinicians and scientists maintained that renin was involved only in "renal hypertension," which represents only one aspect of the total picture of the human hypertensive system. This single-aspect concept was challenged by the development of angiotensin antagonists and ACE inhibitors. Because these inhibitors were shown to lower blood pressure in patients with hypertension of unknown etiology, many who doubted that the renin system played a role in forms of essential hypertension became convinced of the involvement of angiotensin, even when circulating renin levels were not elevated. Evidence supporting the more encompassing concept of angiotensin-dependent hypertension was the discovery of renin-angiotensin system components in many tissues of the body, and the demonstration that by blocking one or more of the components of the system, blood pressure became lowered.

EXPLOSION OF KNOWLEDGE

This monograph presents the major achievements in the field of renin-angiotensin investigation during the last decade. So far, various components of the renin-angiotensin system have been sequenced and the genes cloned. Because the angiotensin receptor(s) has so far been particularly resistant to purification, it has not been directly sequenced. The gene(s) for the AT_1 receptor, however, have been sequenced and cloned, an accomplishment that allows an understanding of the structure and function of the elusive receptors.

The synthesis of potent nonpeptide angiotensin II antagonists has decisively established the existence of various types of angiotensin receptors and has stimulated the search for the genes of these receptor types and subtypes. Detailed studies have been performed on the signal transduction mechanisms for the AT_1 receptor, but despite the sophistication and intricacy of this work, the function of the AT_2 receptor is not yet clearly understood.

Since the experiments of the 1960s, which demonstrated that angiotensin II binds to the cellular plasma membrane, angiotensin binding has been assumed to cause a receptor conformational change that results in the activation of a second-messenger system. This hormonal system, as well as various ion channels that, in turn, are activated or inactivated by mechanisms within the hormone system, is contained in the cellular plasma membrane. This plethora of information, now becoming available through the use of methodologies carried out at the molecular and cellular levels, is beginning to explain the role of angiotensin and its metabolites throughout the organ systems. The consolidation of this current information presented in this monograph offers an exciting treatise to the scientists involved in blood-pressure control mechanisms.

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Renin

Chapter 1

SUBCELLULAR SORTING AND PROCESSING OF PRORENIN

T.L. Reudelhuber, C. Mercure, L.L. Théroux, W.N. Chu, J.D. Baxter, and N.G. Seidah

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I. INTRODUCTION

Nearly one hundred years ago, Tiegerstedt and Bergman reported that extracts of kidney induced an increase in blood pressure when injected into rabbits.[1] This activity, which they named renin, was subsequently found to be a proteolytic enzyme which increased the blood pressure by generating the decapeptide angiotensin I, that is subsequently cleaved by angiotensin converting enzyme (ACE) to form the octapeptide angiotensin II (Figure 1). Angiotensin II (AII) modulates blood pressure directly by inducing vasoconstriction and it stimulates the release of aldosterone, which in turn causes sodium retention and potassium loss.[2] In addition, it has recently become apparent that AII has growth factor-promoting activity[3-5] which may be critical in the contributions of the renin-angiotensin system (RAS) to the pathological changes associated with heart failure, kidney failure, and chronic hypertension.[6-10] For these reasons, the RAS has become an important target for treating a number of cardiovascular diseases. While ACE inhibitors have been widely used for treating these pathological states,[6,8-10] new and potent inhibitors of both renin[11-15] and antagonists of AII receptors (see Chapter 9) may soon join these compounds in the treatment of a broad variety of diseases.

Within the circulation, renin is the rate-limiting component of the RAS. The release of renin into the circulation results from a number of intracellular steps, including organelle targeting and site-specific processing of the precursor to renin, prorenin. Both of these processes may eventually offer new targets for pharmacological interventions for controlling RAS activity. In this chapter we outline current knowledge regarding the intracellular processes that govern the ultimate release of active renin, in the context of recent information about the mechanism of secretion of other mammalian proteins.

II. BRIEF OVERVIEW OF MAMMALIAN SECRETORY PROCESSES

All mammalian messenger RNAs are translated into proteins on cytoplasmic ribosomes. Those proteins ultimately destined for insertion into the lumen of the endoplasmic reticulum (ER), lysosomes, Golgi apparatus or endosomes, and proteins which are secreted or are integrated into nuclear, lysosomal, or plasma membranes are synthesized on and integrated into the rough endoplasmic reticulum (RER). For these proteins, this first level of "sorting" occurs via the interaction between an amino-terminal hydrophobic peptide (the signal peptide) on the initial translation product with a "signal recognition particle" (SRP), which in turn binds to a high-affinity receptor on the membranes of the RER.[16] As translation proceeds, the nascent peptide chains are inserted into the lumen of the ER where the first chemical modifications to the protein occur: the signal peptide is cleaved off, disulfide bond formation is initiated between cysteine residues, and oligosaccharide

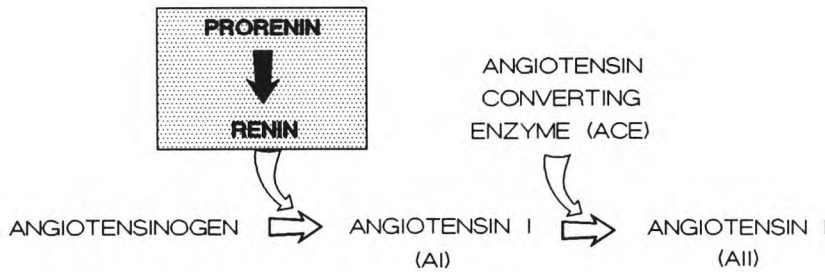


FIGURE 1. Schematic representation of the renin-angiotensin system. The shaded box depicts the area of focus of this chapter.

addition begins on asparagine residues contained in the sequence Asn-X-Ser/Thr (N-linked glycosylation). In addition, the growing peptide chain begins to fold into a native conformation with the help of "molecular chaperones," such as BiP/GRP78.[17] Such proteins are critical for the assembly of oligomeric protein complexes and may recognize specific amino acid sequences in incorrectly folded polypeptides to target them for destruction in the ER.[18,19]

Vesicles budding off from the ER transport the native protein to the first compartment of the Golgi lamellae, the so-called cis-Golgi (Figure 2). Proteins destined to remain in the ER are recaptured at this point by interaction of a specific amino acid sequence (Lys-Asp-Glu-Leu or KDEL) with the KDEL receptor.[20] Both the transport of proteins from the ER through the Golgi stacks and the recycling of proteins back to the ER occurs via vesicular transport in a process that requires hydrolysis of GTP.[20,21] Proteins destined for further transport through the medial and trans-lamellae of the Golgi apparatus may undergo additional chemical modifications including further processing of N-linked carbohydrate residues, sulfation, α-amidation, O-glycosylation, phosphorylation, lipid attachment, and proteolytic processing.[22-24] All of these modifications occur through recognition of specific peptide sequences or through specific positioning of amino acids in the target protein. The trans-most cisternae of the Golgi apparatus (commonly referred to as the trans-Golgi network or TGN) is the site of another major sorting decision (Figure 2): proteins are segregated depending on whether they are destined for inclusion in lysosomes, direct secretion or integration at the plasma membrane (constitutive secretion), or storage in dense core secretory granules for secretion in response to stimuli (regulated secretion).[25-28]

Targeting of proteins to the lumen of lysosomes requires two intracellular recognition events.[28,29] The first is the phosphorylation of mannose residues on N-linked oligosaccharides. In cathepsin D, a lysosomal aspartyl protease, a lysine residue and a noncontiguous 27-amino acid peptide sequence contained in a surface loop of the protein are required for recognition by the phosphotransferase.[30,31] The second recognition event is between the mannose-phosphorylated protein and a mannose-6-phosphate receptor located in

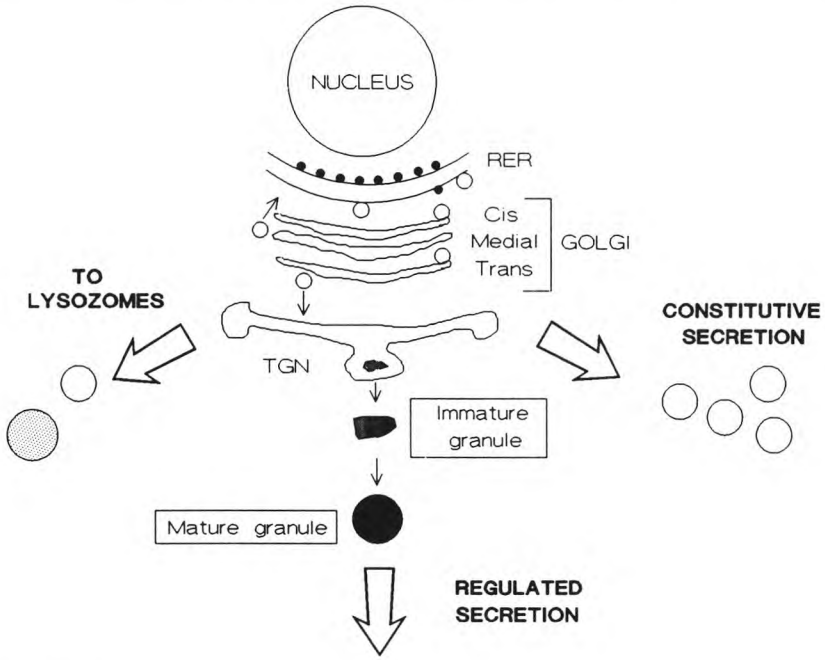


FIGURE 2. Schematic representation of the mammalian secretory pathway. Open arrows represent the major sorting pathways from the trans-Golgi network (TGN).

the TGN.[28] Recent evidence suggests that peptide signals may also play a role in targeting of mammalian lysosomal proteins to the proper intracellular compartment,[28] as they do in the lysosomal equivalent (the vacuole) of yeast and plants.[32]

Proteins are secreted from the cell by two major pathways. The first involves direct secretion from the TGN. Because there is no evidence of intracellular storage of proteins secreted by this route, it has been called the constitutive secretory pathway (Figure 2). Data suggest that this pathway constitutes the bulk flow or "default" pathway of protein secretion[33,34] and it is the dominant secretory pathway in most eukaryotic cell types. Proteins move to the plasma membrane through the constitutive pathway in microtubule-anchored low-density vesicles[35] with a relatively rapid transit time on the order of minutes to hours.[36]

Some specialized endocrine and neuroendocrine cells may also export proteins by the regulated secretory pathway[20,34,37] (Figure 2). In this pathway, certain proteins are sorted at the TGN to dense core secretory granules, where they are stored until the cell receives a signal which triggers their release. Little is known about the precise molecular signals that target proteins to dense core secretory granules. However, using the model system of mouse

pituitary AtT-20 cells, Moore and co-workers obtained the first evidence that cells from different tissues use a common mechanism to sort proteins to secretory granules.[38] AtT-20 cells sort endogenous proopiomelanocortin (POMC) to secretory granules, process POMC to adrenocorticotrophic hormone (ACTH) and other peptides and release them in response to cAMP. When transfected with an expression vector for human proinsulin, these cells sort and process the encoded prohormone correctly and release human insulin in response to a secretagogue.[38] AtT-20 cells have subsequently been shown to correctly sort proteins destined for secretory granules from various species and tissues of origin and to exclude from granules proteins which are known to be secreted in a constitutive manner (Table 1). Further, fusion of a secretory granule-targeted protein with a protein secreted by the constitutive pathway results in secretory granule targeting of the fusion protein.[39,40]

Taken together, these results suggest that the signals for targeting of proteins to dense core secretory granules are (1) contained within the targeted protein; (2) universal (i.e., recognized across species and tissue barriers); and (3) dominant (i.e., not a bulk flow mechanism). Surprisingly, in spite of this apparent universal nature of the sorting machinery, there exists no extended sequence homology between proteins sorted to granules. There is, in fact, no

TABLE 1
Secretion of Exogenous and Endogenous Proteins by Mouse Pituitary AtT-20 Cells

Protein Expressed	Source		Regulated Secretion	Ref.
	Tissue	Species		
Proneuropeptide Y	Intestine	Human	+	131
Proinsulin	Pancreas	Human	+	38
Proenkephalin	Brain	Human	+	132
Prothyrotropin releasing hormone (TRH)	Hypothalamus	Human	+	133
Trypsinogen	Pancreas	Rat	+	134
Growth hormone	Pituitary	Human	+	36
Prosomatostatin	Hypothalamus	Rat	+	40
Procholecystokinin (CCK)	Small intestine	Pig	+	135
Provasopressin/neurophysin	Hypothalamus	Rat	+	136
Proatrial natriuretic factor (ANF)	Heart	Rat	+	137
Prorenin	Kidney submaxillary gland	Rat, mouse, human	+	105–108
Laminin	Ubiquitous	Mouse	−	134
Vesicular stomatitis virus G protein	—	Viral	−	36
Immunoglobulin (kappa light chain)	Lymphoid cells	Mouse	−	138
Angiotensinogen	Liver	Rat	−	139

consensus regarding the actual mechanism of sorting. One hypothesis is that proteins destined for secretory granules interact with a specific secretory granule receptor in much the same way as proteins are sorted to lysosomes. While evidence for the existence of such a "sortase" in dog pancreatic microsomes has been reported,[41] there has been no further confirmation of its role as a sorting receptor.

Another hypothesis advanced to explain the segregation of granule targeted proteins in the TGN is selective aggregation.[34,42] Many of the proteins which end up in dense core secretory granules show a tendency to aggregate in the presence of calcium and a slightly acidic environment, conditions thought to be present in immature secretory granules.[42-44] This proposed mechanism is also consistent with microscopic studies demonstrating aggregates in the trans-Golgi in the process of being encapsulated by membrane.[42] Regardless of the actual mechanism of sorting, the segregated protein is first packaged into a small, relatively electron-lucid "immature" granule which is often characterized by a clathrin "patch."[43] The maturation process for secretory granules is poorly understood but may involve fusion of immature granules and progressive condensation of the granule contents by formation of dense para-crystalline aggregates.[43] Mature secretory granules reside at or near the plasma membrane at "docking" sites[34] and fuse with the plasma membrane in response to an extracellular signal. The transit time of proteins in the regulated pathway is longer than that for the constitutive pathway, being in the range of hours to days, and depends on the timing of a stimulus for exocytosis.[36,45]

Sorting can also occur to two additional secretory pathways in specialized mammalian cells. The first is a recently demonstrated "basal" pathway in which presumably immature secretory granules fuse with the plasma membrane, resulting in a seemingly constitutive pattern of release of proteins which would normally be destined for dense core secretory granules.[46,47] The second is the secretion or membrane anchoring of proteins selectively by either the apical or basolateral membranes of polarized cells. This sorting mechanism, which has been most extensively studied in the targeting of polymeric immunoglobulin receptor to the basolateral surface of epithelial cells, requires a short peptide sequence adjacent to the membrane-spanning domain of the receptor.[48] The finding that thyroglobulin, a regulated secretory protein, is also segregated to the apical surface of thyroid epithelial cells has led to the suggestion that some relationship between the targeting signals for the two secretory pathways may exist.[49]

Cell-type specific proteolytic processing of proteins also plays an important role in the mammalian secretory pathway. Numerous hormones, bioactive peptides, and proteases are synthesized as precursors and are processed in the secretory pathway to the biologically active forms that are subsequently secreted.[50-53] The intracellular site of processing for these precursors may vary significantly, however. For example, proalbumin is a constitutively secreted protein and is processed to albumin in the Golgi lamellae of hepatocytes.[54]

In contrast, insulin appears to be activated only after its sequestration into secretory granules and is stored in these granules in the active form.[55] Atrial natriuretic factor (ANF), on the other hand, seems to be packaged in atrial cardiocyte secretory granules as the prohormone, and is activated only upon release of the granule.[56,57] These processing enzymes can be exquisitely selective in the sites they cleave in individual substrates. For example, while proalbumin and proinsulin are cleaved at sites that contain a pair of basic amino acids,[50,58] pro-ANF is cleaved at a site containing only a single basic amino acid.[57] Indeed, not all potential processing sites within a given substrate are cleaved. POMC is processed differentially in the anterior and intermediate lobes of the pituitary, in spite of the fact that all of the processing events occur at pairs of basic amino acids.[52,59] It is easy to see why prediction of endoproteolytic cleavage sites in complex mammalian proteins based on sequence comparison has not been very successful to date. Thus, processing specificity may be determined by a combination of cellular distribution of different processing enzymes, the particular architecture of the substrate, and effects on the chemistry of the reaction by the intracellular compartment or environment.

While there have been many attempts to purify and characterize processing enzymes from mammalian tissues and cell lines, a major advance was made in this field with the cloning of the gene which encodes the processing enzyme responsible for cleaving yeast pro-(alpha) mating factor.[60] Surprisingly, this enzyme, called Kex2, was also found to correctly cleave both proalbumin and POMC when expressed in mammalian cells.[61,62] Using sequence information from the yeast Kex2 gene, several related sequences have been identified in mammalian cells, including furin, PC1 (also called PC3), PC2, and PACE4.[53,63] Whereas furin and PACE4 are expressed ubiquitously in tissues and cell lines, PC1/PC3 and PC2 have to date only been detected in neural and endocrine cells.[53,64]

III. SECRETION OF PRORENIN AND RENIN IN WHOLE ANIMALS AND TISSUES

The genes encoding mouse, rat, and human renins have been cloned and characterized.[65-69] The genomic organization and deduced amino acid sequence of human renin confirm its close relatedness to other aspartyl proteinases. Human and rat genomes contain only one renin gene[67-70] and while all inbred strains of mice carry the Ren-1 structural gene (expressed at high levels in the kidney), some strains contain a second closely linked gene (Ren-2) which is expressed at high levels in the submaxillary gland (SMG; see Chapter 2). In all cases, these genes encode protein precursors of 400 to 406 amino acids.[70-72] A signal peptide encoded at the amino-terminus directs the nascent polypeptides to the ER. Upon insertion into the ER, the signal peptide is removed (Figure 5) to generate prorenin and posttranslational modification begins. There are two consensus sequences for N-glycosylation in human

renin and three such sites in rat and mouse (Ren-1) renal renin.[70,72,73] These sites may be modified to different extents, resulting in multiple species of renin and prorenin that can be separated by either isoelectric focusing or lectin affinity chromatography.[74,75] In human renin, mannose residues on the carbohydrate side chains may also be partially phosphorylated,[76] creating a classical lysosomal targeting signal. The importance of the glycosylation of prorenin and renin is most obvious in clearance of the protein from the circulation,[77,78] although some evidence has accumulated that glycosylation may also affect intracellular transit time,[79] efficiency of intracellular sorting,[80] and stability[81] (protease sensitivity?) of prorenin. There is no direct evidence that prorenin undergoes post-translation modifications other than N-glycosylation. However, rat prorenin can be fractionated into multiple isoelectric species even after enzymatic deglycosylation.[82] In contrast, recombinant human prorenin in which the glycosylation sites have been eliminated by protein engineering migrates as a single isoelectric species.[143] It is currently uncertain whether this difference is explained by incomplete removal of carbohydrate side chains by the glycosidase, as has been demonstrated for human renin,[83] or whether rat and human prorenins undergo different post-translational modifications.

Evidence to date suggests that glycosylated prorenin is analogous to the circulating "big" or "inactive" renin and can be present at 3 to 5 times the level of "active" renin in the circulation of humans.[84] While circulating active renin is derived almost exclusively from the kidney, numerous nonrenal tissues in humans secrete prorenin.[84,85] In rats, the case seems a bit different: although several extrarenal tissues contain prorenin mRNA and/or protein[86] (see Chapter 4), nephrectomy results in the disappearance of both renin and prorenin from the circulation.[87] Thus, the kidney is clearly capable of releasing both prorenin and renin. How is this accomplished and what determines the relative proportions of the two proteins secreted? Much of what we understand about this process has derived from ultrastructural studies on the cells that secrete renin from the kidney.

The juxtaglomerular apparatus of the kidney is the primary site of synthesis for circulating renin. Juxtaglomerular (JG) cells are modified smooth muscle cells which make up approximately 0.1% of the cellular mass of the adult kidney.[88] While JG cells resemble other neuroendocrine cells in their rich cytoplasmic content of dense core secretory granules (Figure 3), they are distinguished from these other cell types by two rather striking characteristics. First, rhomboid, para-crystalline structures can be seen budding off from the TGN and in membrane-bound structures within the cytoplasm (Figure 4A). Second, the secretory granules of JG cells are atypical and display many similarities to lysosomes. At the ultrastructural level, the electron-dense matrix of these granules is sometimes seen to contain multiple vesicular inclusions and membrane fragments (Figure 4C) and some micrographs suggest that these granules are capable of micropinocytosis and autophagy of other cellular organelles.[89,90] In addition, JG cell granules are immunoreactive to antibodies

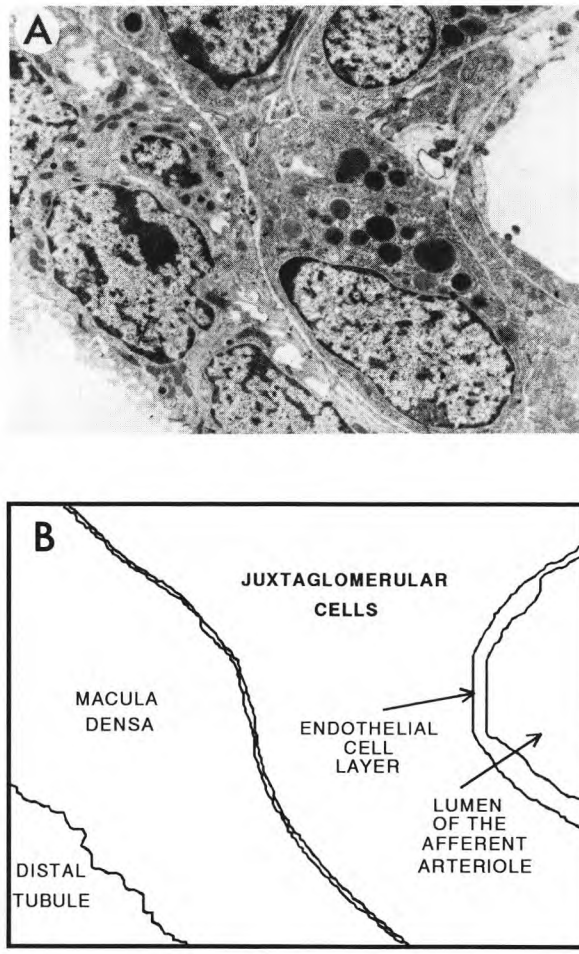


FIGURE 3. Ultrastructure of the juxtaglomerular (JG) apparatus in the 5-day-old mouse kidney. Tissue was fixed in 2% glutaraldehyde and post-fixed in 2% osmium tetroxide. Thin sections were stained in uranyl acetate and lead citrate. (A) Electron micrograph of the JG apparatus. Note the abundant rough endoplasmic reticulum and dense core secretory granules in the JG cells. Original magnification × 2500. (B) Schematic representation of the micrograph depicted in panel A, showing the location of the various cell types in the JG apparatus.

against a number of lysosomal enzymes, including acid phosphatase, β-glucuronidase, arylsulfatase, *N*-acetyl-β-glucosaminidase, and cathepsins B, D, H, and L.[90,91] These results, combined with the apparent lack of any recognizable, classical lysosomal structures, have led some investigators to suggest that JG cells do not contain the type of secretory granules seen in many endocrine cell types, but rather have adapted lysosomes for the processing and secretion of prorenin.[90]

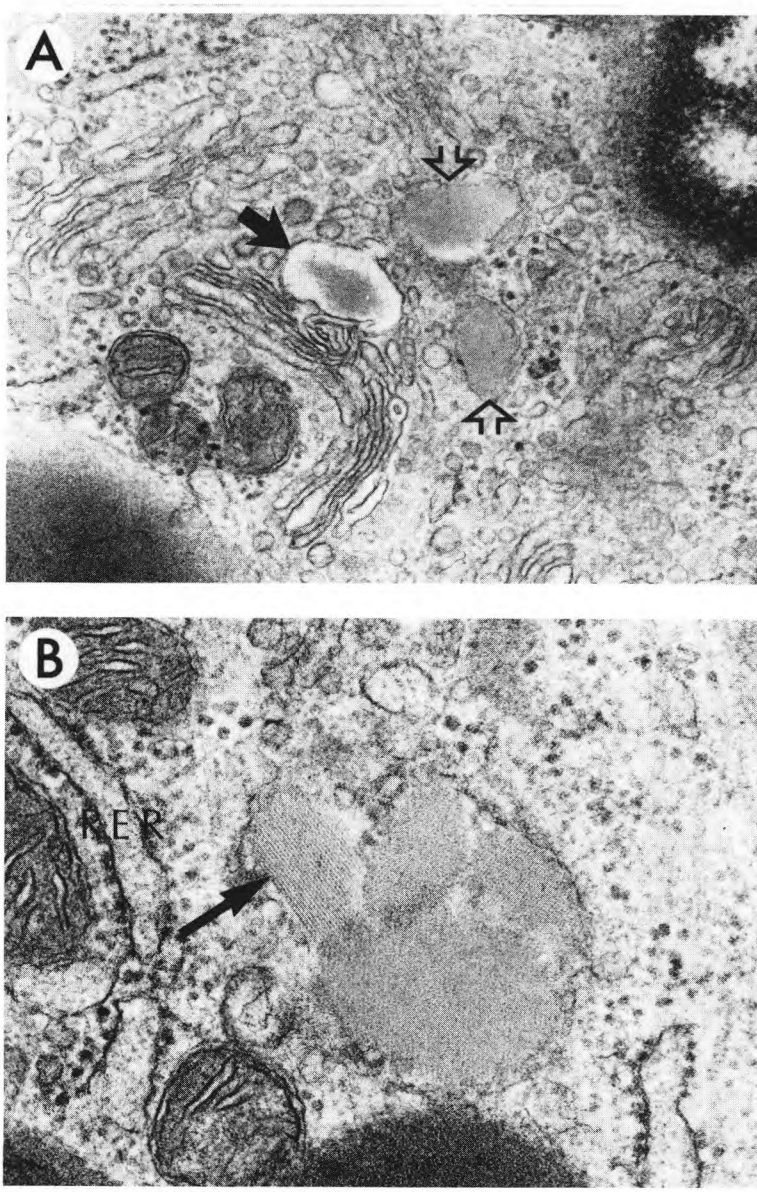
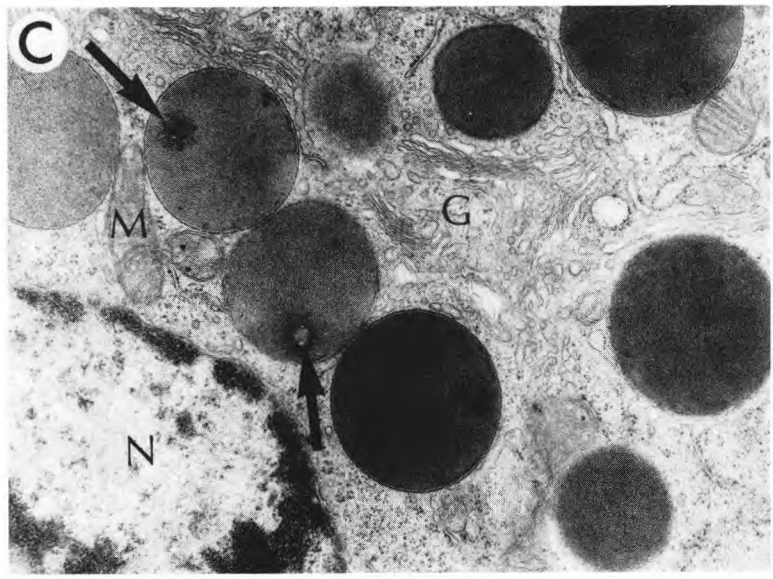


FIGURE 4. Detailed ultrastructure of the JG cells of 5-day-old mice. (A) Emergence of a para-crystalline protogranule (solid arrow) from the TGN. Note the presence of two membrane-encapsulated protogranules in the cytoplasm (open arrows). Original magnification × 46,600. (B) Detail of an immature secretory granule. Note the nonaligned crystalline structures of the recently fused protogranules which will eventually become lost as the granules mature. RER, rough endoplasmic reticulum. Original magnification × 73,200.



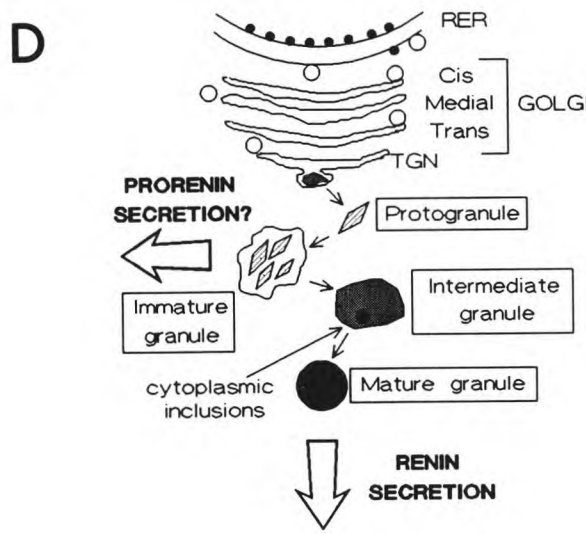


FIGURE 4 *(continued)*. (C) Diversity of granule densities in JG cells. Arrows point to cytoplasmic inclusions in intermediate granules. G, Golgi apparatus; M, mitochondrion; N, nucleus. Original magnification × 17,200. (D) Schematic representation of the proposed routes of prorenin and renin secretion in JG cells. See text for details.

Using antibodies to various portions of the human prorenin prosegment, Taugner and colleagues have demonstrated that the para-crystalline structures budding off from the TGN of JG cells contain prorenin.[92] These structures (Figure 4A), which have been called protogranules, subsequently fuse together to form a membrane-bound structure with a relatively amorphous, low-density content, which has been termed the "immature" or "juvenile" secretory granule (Figure 4B). In some micrographs these immature granules can be seen to fuse directly with the plasma membrane,[90] which would presumably result in the release of prorenin and would correspond, therefore, to the "basal" pathway of protein secretion described above. Immature granules which are not released from the cell become progressively more electron dense and have been referred to as "intermediate" or "mature" granules, depending on their apparent degree of condensation (Figure 4C).

Evidence suggests that conversion of prorenin to active renin begins in the immature secretory granule. Antibodies specific for the prosegment of human prorenin stain predominantly protogranules and immature granules and show little or no staining of intermediate and mature granules.[92] In contrast, an antibody which reacts with both prorenin and renin stains all of these granular structures.[92] The role of the secretory granule in processing prorenin is further supported by biochemical studies: granule fractions purified from kidney homogenates contain predominantly active renin.[93] In addition, renin is secreted more slowly than prorenin from human renal cortical slices and with kinetics that are consistent with its storage in secretory granules.[94] Finally, pharmacologic stimuli which cause a release of secretory granules result in an acute and preferential release of active renin into the circulation.[95]

Amino-terminal sequencing of renin isolated from human kidney lysates suggests that activation occurs by the proteolytic removal of a 43-amino acid prosegment from the amino-terminus of prorenin.[96] This processing site follows a pair of basic amino acids in human renin (Figure 5). Processing of prorenin in the mouse SMG occurs at the analogous position,[97] but processing of rat renal renin seems to occur after a threonine residue which is located 7 amino acids toward the carboxy-terminus relative to the analogous site in human renin[82,98] (Figure 5). The processing site for mouse renal renin is currently unknown. Rat renal and mouse SMG renins subsequently undergo an additional internal processing event, converting "one-chain" active renin to a "two-chain" molecule[82,97,98] in which the two halves are held together by a disulfide bridge (Figure 5). Notably, while renal renins appear to be processed within secretory granules, mouse SMG renin may be processed to "one-chain" renin within the Golgi and only a portion of the protein is further processed in granules to yield the "two-chain" protein.[99] Thus, while renins from mice, rats, and humans share many similarities in protein structure and function, differences exist in the way these proteins are modified within the secretory apparatus.

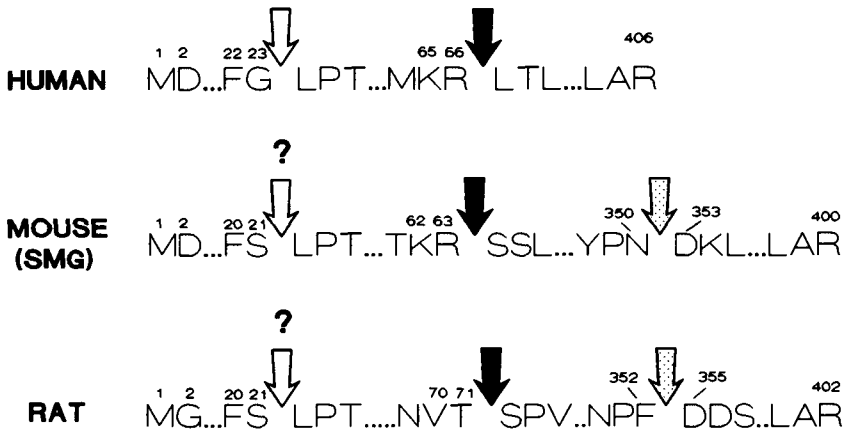


FIGURE 5. Proposed cleavage sites for human and rat renal renins and mouse submaxillary gland renin (SMG). Numbering is from amino acid 1 of preprorenin.[71,82,97,99,115] Open arrows, signal peptide cleavage site; closed arrows, cleavage of the prosegment; stippled arrows, cleavage to generate "two-chain" renin. Question marks indicate that assignment has only been made by homology to human prorenin.

IV. MODELS OF PRORENIN SORTING AND ACTIVATION

Clearly, a critical determinant in the exclusive ability of renal JG cells to secrete active renin is the proteolytic cleavage of prorenin to generate renin. However, since ultrastructural and biochemical studies suggest that this processing is granule specific, the sorting of prorenin to dense core secretory granules is also a crucial step in the secretion of active renin by the kidney. Investigations of the molecular mechanisms of prorenin processing and sorting in the kidney would be facilitated if large quantities of renal JG cells were available for study. Several approaches have been used to isolate and characterize JG cells, including density-gradient enrichment of primary cell preparations[100] and culture of cells from human renin-secreting tumors.[101] To date, these attempts have largely been frustrated by two major problems: the relative paucity of JG cells in the kidney (less than 0.1% of the cell mass) and the tendency of tumor-derived JG cells to dedifferentiate in culture.[102] In an alternative approach, Sigmund et al.[103] have used renin gene fragments to target expression of a viral oncogene to renin-producing cells of transgenic mice. Initial reports suggest that cells derived from a renal tumor in such mice express active renin and contain secretory granules.[104] However, more extensive characterization will be required to determine whether these transformed cells will retain sufficient terminal differentiation to be useful for studying all of the intracellular steps in renin biosynthesis.

In the absence of suitable quantities of JG cells, efforts have largely been directed at characterizing the biochemical properties of various prorenin-processing enzymes and at using model cell systems which correctly sort and process prorenin to obtain a better understanding of these two important intracellular processes.

A. SORTING DETERMINANTS IN PRORENIN

One possible explanation for the seemingly exclusive ability of JG cells to secrete active renin could be that only this particular cell type recognizes sorting and processing signals contained on prorenin. This hypothesis has been directly tested by transfection of a number of cell types with expression vectors encoding human, mouse, and rat prorenins. Transfection of Chinese hamster ovary (CHO) cells, which contain only a constitutive secretory pathway, with an expression vector encoding human preprorenin leads to secretion of prorenin[105,106] (Figure 6). As expected, this prorenin accumulates in a linear fashion in the transfected culture supernatants and secretion is not stimulated acutely by secretagogues. In contrast, transfection of AtT-20 cells, which contain secretory granules and process endogenous POMC, leads to secretion of both prorenin and renin. In addition, while prorenin accumulates in culture supernatants constitutively, treatment of the transfected cells with a secre-

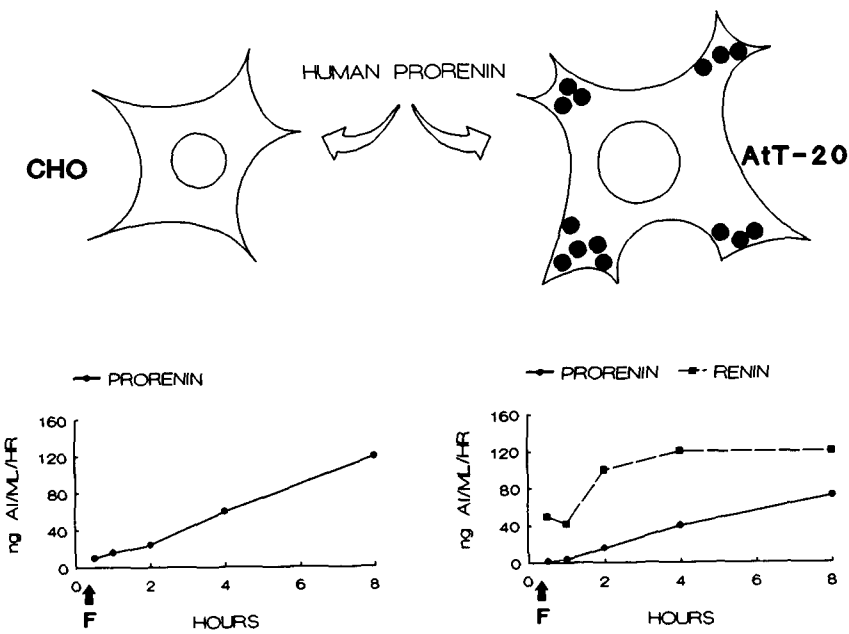


FIGURE 6. Pattern of secretion of prorenin and renin from transfected AtT-20 cells. F, point of addition of secretagogue to the cultures. Data from Fritz et al.[105]

tagogue causes a selective and acute release of active renin[105,106] (Figure 6). This result is characteristic of proteins secreted by the regulated secretory pathway[45] and implies that a portion of the prorenin is sorted to dense core secretory granules where it is processed and stored for later release. Mouse SMG[107] and renal prorenins,[108] as well as rat prorenin,[108] are also sorted to the regulated secretory pathway in transfected AtT-20 cells. Human prorenin is also sorted to the regulated secretory pathway in PC12 rat pheochromocytoma cells[109] and rat somatomammotrophic GH_4 cells,[110] although in these two cases, prorenin is not activated in granules. These results imply that sorting and activation of prorenin can be seen in cells other than JG cells and that prorenin must contain primary or higher-order structural information which directs the cell to carry out these processes.

What is the nature of the secretory granule sorting signal on prorenin? Although the physical segregation of proteins destined for secretory granules occurs at the TGN,[25] commitment to this pathway could theoretically take place as early as insertion of the nascent protein into the ER. However, replacement of the native signal peptide of human prorenin with a signal peptide from a constitutively secreted immunoglobulin M (IgM) did not impair the sorting of human prorenin to the regulated pathway in AtT-20 cells.[111] Likewise, eliminating the glycosylation sites on human prorenin did not prevent its targeting to secretory granules but, instead, increased the percentage of prorenin activated (sorted?) in AtT-20 cells.[111] The dispensability of the carbohydrate residues for granule sorting is also evidenced by the fact that mouse SMG prorenin (which is naturally nonglycosylated) is sorted to granules in AtT-20 cells.[107] The processing of prorenin to renin also is not required; prorenin molecules containing mutations in the paired basic amino acids at the native processing site cannot be activated in AtT-20 cells, but are nevertheless sorted to the regulated pathway and released as prorenin in response to secretagogues.[112] In addition, regulated secretion of prorenin is seen in transfected PC12 and GH_4 cells which contain granules but apparently lack of processing enzyme capable of activating prorenin.[109,110] Finally, the prosegment can also be deleted from human prorenin and the resulting "prerenin" is secreted in a regulated manner in both PC12 and AtT-20 cells.[109,111,113] By deduction, a sorting signal would appear to be located within the protein domain corresponding to active renin.

To further characterize this sorting sequence, we have constructed fusion proteins between a portion of an immunoglobulin constant region (which is constitutively secreted) and fragments of human prorenin and have examined the targeting of these fusion proteins to the regulated secretory pathway in AtT-20 cells (Figure 7A). The results indicate that human prorenin contains a peptide at the extreme amino-terminus of its prosegment that can direct the fusion protein to the regulated secretory pathway (Figure 7B). How do these results fit with the finding that the prosegment can be deleted without preventing correct sorting? The simplest explanation is that prorenin contains more than one domain involved in secretory granule targeting (Figure 7C).

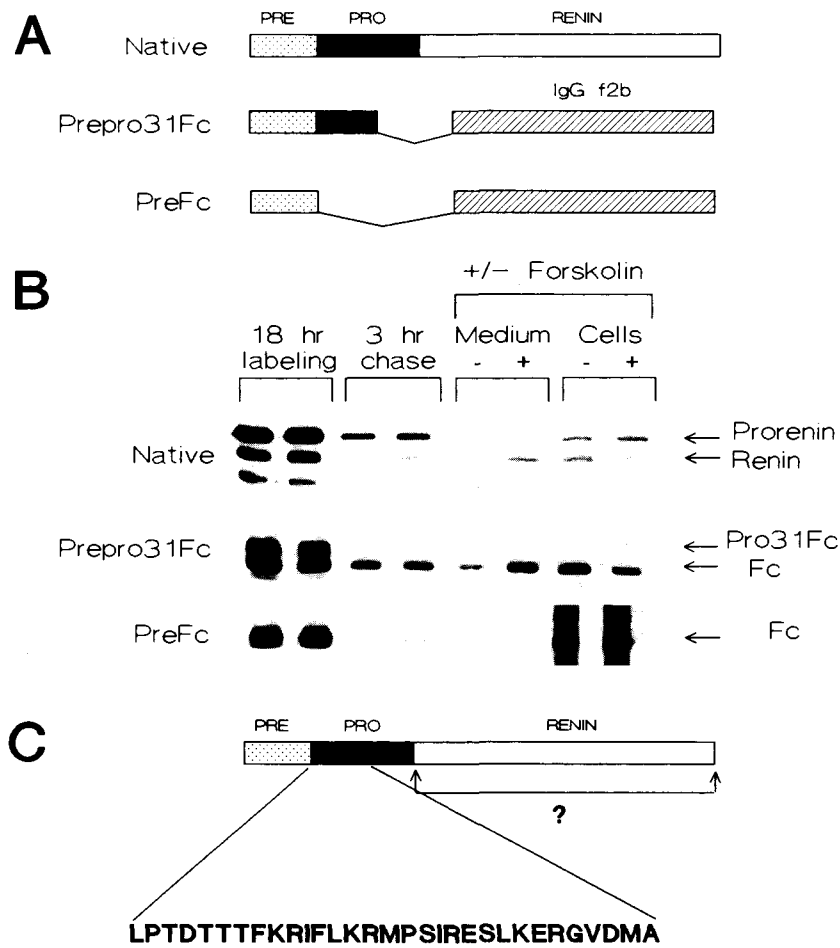


FIGURE 7. Location of a secretory granule-sorting peptide in the prosegment of human prorenin. (A) Construction of the fusion proteins. (B) Plasmids containing either native preprorenin or the fusion proteins were transfected into AtT-20 cells and the expressed proteins were assayed by pulse-chase studies for unstimulated (−) and forskolin (10 μM)-induced release (+) of the products, as described in Chu et al.[111] (C) Location of secretory granule-sorting peptides in human prorenin.

As previously mentioned, there is no obvious linear homology between the putative sorting peptide in the human prorenin prosegment and peptide sequences contained within other proteins destined for the secretory granule such as POMC, growth hormone, insulin, and ANF.[140] This result suggests that potential homologies in secretory granule-sorting peptides may either be in secondary or tertiary structure rather than the linear amino acid sequence. Alternatively, multiple sorting receptors may exist or a given sorting receptor

may have the capacity to interact with a wide spectrum of binding sequences, thereby restricting homologies to subsets of secretory granule-sorted proteins. Discrimination between these possibilities will likely have to await the further characterization of minimal sorting peptides in both prorenin and other proteins secreted by the regulated pathway.

Is the use of an endocrine cell model a reasonable approach to identifying sorting determinants to the lysosome-like granules contained in JG cells? In this regard, it is interesting to note that up to 80% of human prorenin expressed in *Xenopus* oocytes (which do not contain dense core secretory granules) is directed to lysosomes.[76] Elimination of N-linked glycosylation sites in human prorenin by site-directed mutagenesis appears to eliminate lysosomal uptake of prorenin in injected oocytes and leads to the secretion of the prorenin,[114] but does not inhibit targeting of human prorenin to dense core secretory granules in AtT-20 cells.[80] Secretory granule targeting of the nonglycosylated prorenin in the mouse SMG must also occur in the absence of carbohydrate signals, suggesting that the lysosomal and granular targeting signals on prorenin are distinct and separable. Nevertheless, while only 5 to 6% of prorenin expressed in mammalian cells acquires phosphomannosyl residues,[76] it is an intriguing possibility that renal prorenins make use of both granular and lysosomal signaling mechanisms to ensure their efficient sorting to the lysosome-like secretory granules in JG cells. Clearly, it will be imperative to test for the function of any potential prorenin-sorting peptides in the JG cells of intact animals.

B. PROCESSING OF PRORENIN

Amino-terminal sequencing of renin isolated from human kidney lysates suggests that the cleavage of prorenin is highly specific and occurs at a pair of basic amino acids (Lys-Arg), resulting in removal of a 43-amino acid prosegment from the amino-terminus of prorenin.[115] There are 6 additional pairs of basic amino acids in human prorenin,[73] including Lys-Lys, Arg-Arg, and Arg-Lys within the body of renin, two Lys-Arg pairs in the prosegment, and the Lys-Lys-Arg triplet in the body of renin, none of which appear to be cleaved in the kidney. What determines this cleavage site selectivity?

Many proteases are capable of activating human prorenin *in vitro,* including trypsin, plasmin, tissue and plasma kallikreins, and cathepsin B.[84,116,117] While some of these enzymes cleave prorenin with the correct specificity,[118] most are likely to be physiologically irrelevant due to their tissue distribution. An exception to this rule is cathepsin B, which was recently purified as a prorenin-processing activity from human kidney lysates.[117,119] While cathepsin B is a lysosomal enzyme which is expressed in a broad variety of cell types,[120] it appears to co-localize with renin not only in the lysosome-like granules of JG cells,[90,91] but also in the more classical secretory granules of prorenin-containing human pituitary lactotrophs.[121] *In vitro,* cathepsin B cleaves human prorenin with the correct specificity and with a K_s in the nanomolar range.[117]

For these reasons, cathepsin B has been proposed as the renal prorenin-processing enzyme. In a similar effort, an enzyme capable of processing mouse SMG prorenin has been isolated from submaxillary glands.[122] This enzyme, which has been called PRECE, was subsequently revealed to be identical to the mGK-13 gene product[123] (also known as the epidermal growth factor-binding protein type B), a member of the kallikrein gene family. While PRECE can activate mouse SMG prorenin to generate "one-chain" renin, it is unable to carry out the second cleavage to yield the "two-chain" form (Figure 5).[122] In addition, kidney glandular kallikrein cannot activate mouse SMG prorenin and PRECE is unable to activate mouse renal or human prorenins.[122] Recently, a second enzyme capable of converting mouse SMG prorenin to "one-chain" renin has also been isolated from mouse submaxillary gland.[124] Thus, while it has been possible to purify and characterize candidate prorenin-processing enzymes by classical biochemical techniques, the occasional promiscuity displayed by processing enzymes *in vitro* and the tendency for cellular colocalization to be misleading regarding function[125,126] has complicated the unequivocal identification of prorenin-processing enzymes. For this reason, it is imperative that these studies be complemented with genetic or other experiments that specifically block the actions of the putative protease *in vivo* before a specific role in prorenin activation can be confirmed.

AtT-20 cells transfected with a human preprorenin expression vector also cleave prorenin at the same site as that reported for renin purified from human kidney lysates.[105] One hypothesis to explain the cleavage site selectivity displayed by enzymes *in vitro,* AtT-20 cells, and the kidney is that primary and/or higher-order structural determinants on prorenin render the native processing site uniquely sensitive to proteolytic cleavage. This hypothesis was directly tested by introducing single amino acid mutations in human preprorenin surrounding the natural cleavage site and expressing the resultant recombinant proteins to proteolytic activation either by trypsin or by the endogenous processing enzyme in AtT-20 cells.[127] The results suggest that amino acids in addition to the pair of basic amino acids surrounding the cleavage site affect the ability of both trypsin and the AtT-20-processing enzyme to cleave prorenin (Figure 8). Notably, while a proline at position -4 is essential for processing of human prorenin in AtT-20 cells and is correlated with predicted formation of a β-turn at this position, other site-directed mutations suggest that this structural feature in addition to a pair of basic amino acids is not sufficient to lead to proteolytic activation of prorenin.[127] In contrast to the case with human prorenin, neither mouse renal prorenin[108] nor rat prorenin[108,128] are processed at the analogous positions in transfected AtT-20 cells. In both cases, mutagenesis of the natural substrates has demonstrated that this is also due to the particular arrangement of amino acids immediately adjacent to the native processing site (Figure 9).

Is the AtT-20 cell-processing enzyme identical to the enzyme which activates prorenin in mammalian JG cells? The answer cannot be unequivocally positive, since AtT-20 cells are unable to cleave mouse or rat renal

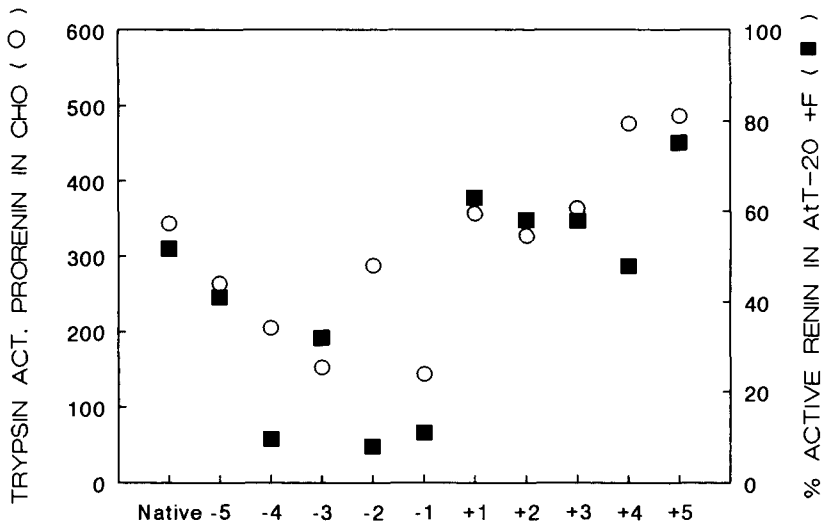


FIGURE 8. Effect of mutations on activation of human prorenin. Abcissa denotes the position (relative to amino acid 1 of human renin) at which the native amino acid has been changed to alanine. Left ordinate, trypsin-activatable prorenin (ng AI/ml/h) in culture supernatants of transfected CHO cells (○). Right ordinate, active renin secreted from transfected AtT-20 cells in the presence of 10 μM forskolin (■). Data from Chu et al.[127]

	SEQUENCE	ACTIVATION
Mouse Ren2	...V F T K R↓S S L T...	++
	...V [R] T K R S S L T...	+++
	...V F T K R [P] S L T...	-
Mouse Ren1	...V F T K R P S L T...	-
	...V F T K R [S] S L T...	+++
Rat	...E F I K K S S F T...	-
	...E F I K [R] S S F T...	+++
Human	...Q P M K R↓L T L G...	++
	...Q P M [R] R L T L G...	+
	...Q P M K [K] L T L G...	-
	...Q P M [R] [K] L T L G...	-
	...Q P M [A] R L T L G...	-
	...Q P M K [A] L T L G...	-
	...Q [A] M K R L T L G...	-
	...Q [F] M K R L T L G...	-
	...Q [G] M K R L T L G...	-

FIGURE 9. Summary of the effect of processing site mutations on the generation of active renin in transfected AtT-20 cells.[108,127] The native sequences of the analogous region of mouse, rat, and human prorenins are represented in bold type. Mutations induced are represented by shaded boxes. Where the native processing site is known, it is denoted by an arrow.

prorenins.[108,128] Nevertheless, it should be remembered that the processing site of rat renal renin is not in the same position as that of human renin[82,98] and the amino-terminus of mouse renal renin has not yet been determined. Therefore, it is possible that species-specific differences exist in the processing enzymes which activate prorenins. Could the AtT-20 enzyme be related to the human renal prorenin-processing enzyme? The first step in answering this question is to identify the human prorenin-processing enzyme in AtT-20 cells. Transfection of secretory granule-containing GH_4 cells with an expression vector encoding human prorenin results in the secretion of prorenin into culture supernatants, confirming the lack of a prorenin-processing enzyme in these cells[110] (Figure 10). When prorenin is cotransfected into GH_4 cells with an expression vector encoding the mammalian subtilisin-like endoprotease PC1, the cells are rendered capable of selectively processing prorenin at the identical site as that reported for the kidney. Interestingly, this processing event does not occur in cells devoid of secretory granules (CHO and BSC-40) and is not due to any apparent differences in processing of PC1 in these cell types.[128] When tested in a similar assay, neither furin,[110] PC2[128] nor human cathepsin B[141] are able to process human prorenin. Mutations which inhibit the processing of human prorenin in AtT-20 cells also inhibit processing of human

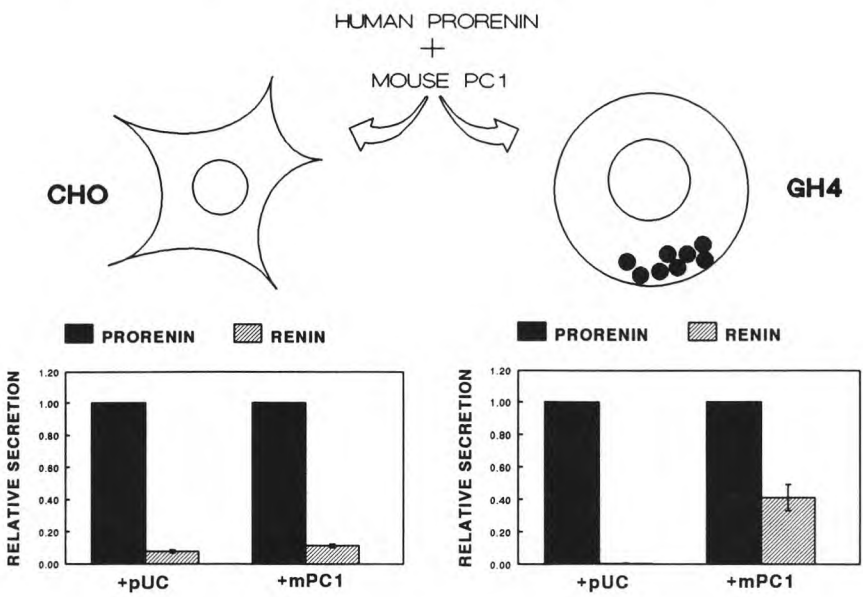


FIGURE 10. Cell type-specific processing of human prorenin by the endoprotease PC1.[128] CHO cells (lacking granules) and GH_4 cells (containing granules) were cotransfected with expression vectors encoding human prorenin and either carrier plasmid DNA (+pUC) or an expression vector encoding mouse PC1 (+mPC1). Cell culture supernatants were tested for prorenin and renin content 48 h after transfection.

prorenin by PC1.[142] This finding, combined with the fact that PC1 is expressed at high levels in AtT-20 cells,[64] makes it likely that the human prorenin-processing activity in AtT-20 cells is PC1. Whether or not the human homolog of PC1/PC3 colocalizes with prorenin in human JG cells remains to be determined.

V. FUTURE DIRECTIONS

What have we learned from studies to date concerning the sorting and processing of prorenins? First, the regulated secretory pathway in JG cells exhibits unique features, particularly with regard to its content of para-crystalline structures containing prorenin and the striking similarity of its secretory granules to lysosomes. In spite of these unique characteristics, prorenins contain information which direct unrelated neuroendocrine cells to sort them to the dense core secretory granules. Several questions remain however: are the sorting signals identified in model cell systems functional in JG cells? Is the secretory granule sorting event mediated by interaction of prorenin with a "sortase" receptor or by aggregation of prorenin in the TGN? Are there unique features of the sorting process for prorenin in the kidney which would make it amenable to pharmacologic intervention in an effort to control the production of circulating renin?

The second lesson we have learned from these studies is that processing of prorenin can be species-, organelle-, and substrate-specific. In the case of rat renal renin, processing occurs at a different amino acid position than that for human renin. In addition, mouse SMG prorenin is processed in the Golgi by an enzyme which will not cleave renal prorenin. Human prorenin can be activated *in vitro* by a widely distributed lysosomal protease (cathepsin B), but active renin is only secreted in the circulation from the kidney. In spite of these complexities, *in vitro* processing studies and model cell culture systems are giving us information which is useful in the ultimate characterization of the processing of prorenin in the kidney. *In vitro* assays are allowing the purification and characterization of candidate processing enzymes. In addition, prorenin molecules containing site-directed mutations provide a powerful tool to distinguish between processing activities, as different proteases appear to require different amino acids in the immediate area of the processing site. Finally, transfection studies in cultured cells will determine whether candidate processing enzymes carry out their putative function in the appropriate cellular compartment. In combination, these approaches will provide extremely useful information in designing experiments to directly test the activity of potential processing enzymes in JG cells.

Nevertheless, the lack of established cultures of fully differentiated JG cells in which prorenin sorting and processing determinants can be directly tested remains a major impediment in this field. For this reason, novel approaches to the development of JG cell cultures, such as by targeted oncogenesis in transgenic animals, should remain a high priority. In the meantime,

targeted expression of native and mutated prorenin molecules to the JG cells of transgenic mice, which have the demonstrated ability to activate and secrete both rat and human prorenin,[129,130] should provide an alternative means of further characterizing the intracellular processes which determine the secretion of active renin.

ACKNOWLEDGMENTS

Because of the breadth of the subject matter covered in this review, it was not possible to cite all pertinent references. Rather, we have attempted to cite recent reviews and illustrative examples to guide the reader in a more in-depth analysis; we apologize to any of our colleagues whose contributions have not been cited for this reason. The authors wish to thank Vivianne Jodoin for typing the manuscript and Drs. Gaetan Thibault and Djamel Ramla for critical reading of the manuscript.

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Chapter 2

THE REGULATION OF RENAL AND EXTRARENAL RENIN GENE EXPRESSION IN THE MOUSE

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I. INTRODUCTION

From the earliest observations indicating the existence of a renal pressor substance,[1] we have come to appreciate that renin is an aspartyl protease which participates in the regulation of systemic blood pressure and electrolyte balance through its fundamental role in the renin-angiotensin system (RAS).[2] Classical systemic renin is produced and secreted by modified intrarenal arterial smooth muscle cells (juxtaglomerular [JG] cells) in response to appropriate physiological and neurological signals.[3-6] Clearly, a detailed understanding of the mechanisms governing how these signals are transduced within the JG cell to regulate the expression and elaboration of renin would assist our comprehension of the biology of the RAS and its role in arterial pressure regulation. Unfortunately, while considerable insight into general features of regulation has been gained from functional studies of the cell *in situ*, direct analysis of fundamental processes, such as gene transcription, has been hampered by the relative paucity of renin-expressing cells in the kidney and the lack of suitable cell culture models.

In addition to the unresolved issues of renal renin expression, it has also become apparent that renin or renin-like activities or immunoreactive renin can be detected at a number of extrarenal sites.[7-14] While the variety of these sites and the precise role subserved by renin expression at these extrarenal sites is not yet clear, the provocative observation that such expression can be found in association with other components of the classic RAS has prompted speculation on, and interest in, the existence of extrarenal tissue-renin-angiotensin systems.[15-19]

Indeed, it was the fortuitous discovery of unusually high levels of a renin-like activity at one extrarenal site, the submandibular gland (SMG) of mice,[20,21] that has proved to play a paramount role in providing molecular access to the renin genes.[22-28] Inbred strains can be divided into two classes: those that produce high levels of SMG renin, and those that produce significantly lower levels.[21] The two expression phenotypes serve to define two alleles, Rnr^s and Rnr^b, at this locus.[29] Rnr^s (after the type strain SWR) can exhibit SMG renin levels that correspond to as much as 2% of SMG protein.[20,21] Moreover, these abundant levels of renin protein are paralleled by high levels of mRNA.[30,31]

It was this copious abundance of a renin mRNA that facilitated development of the first cDNA clones and ultimately recovery of the corresponding genomic sequences encoding the mouse renin gene(s).[22,23,25,32,33] The availability of these cloned recombinant probes from mouse has permitted in turn isolation of the homologous sequences from other mammals and provided the tools necessary to conclusively demonstrate for the first time primary expression of renin mRNA in a spectrum of other tissues.[13,30,33-45]

Cloning of the mouse renin (*Ren*) sequences was also instrumental in definitively establishing that gene duplication provided the molecular basis for the high renin salivary phenotype and ultimately, the demonstration that

the structural genes for renin, lie coincident with the *Rnr* locus on chromosome 1.[22,46,47] We now know that all mice have the *Ren-1* locus which encodes the classical circulating enzyme and has homologs in other species as well. The locus in mouse has two alleles: *Ren-1*^{c} after type strain C57BL/6 which is found in strains with a single renin gene and a low salivary renin phenotype (*Rnr*^{b}), and *Ren-1*^{d} after type strain DBA/2 which is found in strains harboring the renin gene duplication and exhibiting the high salivary renin phenotype (*Rnr*^{s}).[48] The duplicated locus is termed *Ren-2* and is found only in strains with the high salivary renin phenotype.

The species-specific duplication of the renin genes in mouse has been the bane as well as the boon of renin research in this organism. The multiplicity of renin genes in this system has complicated analysis of expression and made it incumbent upon investigators to develop methods to distinguish and quantitate gene-specific expression patterns. Moreover, the existence of the duplication locus has always raised the specter that somehow the mouse was a different and unacceptable model from which to generalize on renin expression and regulation. It may well be, however, that understanding the basis for the differences which superficially appear to distinguish the mouse from other organisms, for example the rat, may in fact provide a key to deriving a deeper understanding of renin's biological role(s) in general. In any event, currently much of our detailed knowledge of renin expression and regulation stems from work performed in mice, and it is evident that the mouse continues to offer an experimentally manipulable system with which to address fundamental issues of renin gene regulation. This reflects its tractability as a classical genetic system as well as the continuing development of a host of sophisticated modern molecular genetic tools, such as transgenic technology and targeted recombination strategies. These combined approaches permit rigorous evaluation of the role and regulation of single or multiple components in complex physiological systems, such as the RAS.

This chapter will review: (1) cellular sites in adult mice where renin mRNA accumulates; (2) spatial, developmental, and hormonal aspects of the differential expression of murine renin genes at renal and extrarenal sites; (3) current knowledge of murine renin gene and transcript structure; (4) what is presently understood about renin gene structure/expression correlations; (5) the use of transfected cells and transgenic mice to identify cis-acting regions of DNA involved in regulating renin expression; and (6) the development of cognate cell lines for examining regulation of renin expression.

II. RENIN GENE EXPRESSION

Since renin is present in the circulation and periarterial fluid, it is important to distinguish whether the presence of renin at a given site is due to uptake from extracellular fluid or serum vs. that actually synthesized at the site.[49,50] For instance, in murine kidneys, renin was detected by immunocytochemistry in cells of the afferent arteriole (JG cells, myoepithelioid cells)

and in cells of the proximal tubule.[51-53] However, *in situ* hybridization assays using a renin cDNA probe located renin mRNA in the former cell types but not in the latter, thereby eliminating proximal tubule cells as a site of primary renin synthesis.[54-57]

A variety of direct approaches employing cDNA probes have been used to screen for the primary expression of renin mRNA in other tissues where renin had reportedly been localized. These investigations were able to confirm or expunge tissues as sites of renin transcription. Numerous reports have now clearly demonstrated the primary expression of renin mRNA in: kidney, adrenal gland, submandibular gland, testes, ovary, and coagulating gland using classical Northern blot assays.[30,33,36,37,42,43,58,59] These results demonstrate that the levels of renin mRNA in these tissues are relatively abundant. In addition, however, substantial controversy exists over whether renin mRNA is present in heart and brain as previously reported.[38,39,42,60] Other sites of renin expression have been detected using the highly sensitive but not quantitative polymerase chain reaction (PCR) assay.[45,61,62] These include brain, heart, hypothalamus, spleen, thymus, lung, prostate, and liver. The significance of these results remains unclear and therefore, for the purposes of this review, we will discuss only those sites where renin mRNA is relatively abundant.

A. DIFFERENTIAL RENIN EXPRESSION IN THE ADULT MOUSE

A priori, with the existence of multiple loci in the mouse, it became necessary to clarify the individual contributions of each gene to the expression pattern in each tissue. The *Ren-1*^{c}, *Ren-1*^{d}, and *Ren-2* genes encode highly similar yet unique renin transcripts. By taking advantage of minute sequence differences between them it proved possible to develop methods to discern the tissue specificity of each. These studies have revealed highly complex gene-specific patterns of differential expression. Details of these methods have been previously described.[30,43] The following is a summary of differential renin gene expression in renal and extrarenal tissues.

Kidney — Renin expression in the kidney has been shown to be roughly equivalent among *Ren-1*^{c}, *Ren-1*^{d}, and *Ren-2*.[30] Under normal physiological conditions, the expression is limited to a population of modified smooth muscle cells of the afferent arteriole proximal to the glomerulus (myoepithelioid or JG cells).[4,52,63] The population of cells expressing renin mRNA, as well as the renin mRNA levels in JG cells, can be modulated.[56,58,64-71] Both are induced by conditions of physiological stress such as sodium depletion, pathophysiologic stress such as uretal and renal artery obstruction, as well as by pharmacologic intervention with angiotensin-converting enzyme inhibitors. *In situ* hybridization assays localizing renin transcripts to specific cells have shown that vascular smooth muscle cells in the afferent arterioles and interlobular arteries can be recruited into a renin-expressing phenotype. These vascular smooth muscle cells have been termed intermediate cells because their ultrastructural appearance contains elements of both vascular smooth muscle cells and fully transformed JG cells.[4,5]

Adrenal gland — In adult adrenal glands, *Ren-1*^{d} and *Ren-2* expression is equivalent and higher in females than in males.[35,43] Expression occurs in the X-zone and *zona fasciculata* but undergoes shifts of cell specificity during the estrus cycle (see below). *Ren-1*^{c} is not detectable in adult adrenal glands of either sex at this site by Northern blot or *in situ* hybridization assay.[31,35]

Salivary glands — Submandibular gland *Ren-2* expression is 100-fold higher than *Ren-1*^{c} expression in male SMG as detected by primer extension analysis.[30] Miller et al.[42] reported *Ren-1*^{d} was detectable but was at very low levels by RNase protection assay. In this tissue, renin is expressed by the granular convoluted tubule cell (GCT, a glandular epithelial cell which makes up 20% of the cellular population) and becomes detectable by *in situ* hybridization at puberty. In the female SMG, renin transcripts accumulate to an approximately 5-fold lower level than is seen in males.[22,37,72] The sublingual gland also expresses renin mRNA which has been located to the striated ductal cell by *in situ* hybridization. These cells were similar to GCT cells in that they appeared to have comparable levels of transcripts on a per cell basis. Male sublingual glands of mice with *Ren-1*^{d} and *Ren-2* genes exhibited high levels of renin mRNA, while in females, transcripts were detectable by treatment with androgen. *In situ* hybridization did not detect any cells with renin transcripts in the parotid gland.[31]

Gonads — Renin expression in Leydig cells of the testes is roughly equivalent for *Ren-1*^{c} and *Ren-2* with *Ren-1*^{d} slightly in excess.[36,43] Renin expression in the ovary is equivalent between *Ren-1*^{d} and *Ren-2*.[45] Low levels of *Ren-1*^{c} were also detected by Sigmund and Gross.[111] The renin-expressing cell type in the mouse ovary has not been identified.

Coagulating gland — Renin gene expression is high for *Ren-1*^{c} but undetectable for *Ren-1*^{d} and *Ren-2*.[43,59] Renin expression in this tissue is limited to the glandular epithelial cells.

B. DEVELOPMENTAL SHIFTS IN RENIN EXPRESSION

In addition to the observed adult patterns of murine renin expression, developmentally regulated expression occurs during organogenesis in the metanephric kidney of mice and rats,[51,53-55,73] the mouse adrenal gland,[14,54] and also in subcutaneous tissues of mice and rats.[74] In the murine fetal kidney, expression of *Ren-1*^{c}, *Ren-1*^{d}, and *Ren-2*, as detected by *in situ* hybridization, is first observed 14.5 days post coitum (pc).[31,54] By 15.5 days pc, renin expression is clearly visible in cells surrounding the lumens of early-forming intrarenal arteries. This expression shifts with the newer portions of the elongating arteries, while the more mature portions of the vascular tree lose the ability to express renin. Expression becomes progressively restricted so that by one week of age, the expression sites become similar to those seen in adult kidney.

Fetal adrenal gland expression is characterized by accumulation of high levels of renin transcripts in both males and females. This stands in contrast to the adult adrenal gland where expression in females of *Ren-1*^{d} and *Ren-2*

genes is higher than in males. Interestingly, *Ren-1*^{c}, which is not detectable in adult adrenal gland, is expressed at comparable levels to *Ren-1*^{d} and *Ren-2* at this time. Again, expression is first visible by *in situ* hybridization at 14.5 days pc.[31,54] Expression is located throughout the entire gland except for the outermost cell layers, as judged by accumulation of silver grains over the tissue. By 16.5 days, expression appears less intense, becomes limited to the cortical region and is clearly absent in the medulla. *Ren-1*^{c} expression disappears by birth, reflecting a developmental downregulation of steady-state levels of renin transcripts while detectable renin expression persists in strains with both *Ren-1*^{d} and *Ren-2*.[35]

Renin expression can also be detected by *in situ* hybridization in the testes during fetal development.[54] Accumulation of silver grains in this fetal tissue is lower relative to the kidneys and adrenal glands. The testes, along with the kidney and the adrenal gland derive from the same limited region of the intermediate mesoderm.[75,76] This leads one to speculate that perhaps some event predisposes cells derived from this embryonic tissue with the potential to express renin, provided the cells then follow defined paths of differentiation.

Renin expression in murine subcutaneous sites also appears to be developmentally regulated.[74,77] The expression of an SV40 large T antigen reporter gene under the control of renin regulatory elements in transgenic mouse fetuses first suggested the presence of renin at this extrarenal site. The reporter gene was expressed at all fetal sites known to express renin and in a mesenchymal cell type amid the muscle layers directly beneath the developing dermis. Interestingly, the fetal expression pattern of the reporter gene is remarkably similar to the pattern of angiotensin II receptors reported by Zemel et al.,[78] suggesting a possible developmental role for the RAS. The levels of expression were below the limit of sensitivity of *in situ* hybridization. However, Northern blot analysis of decapitated and eviscerated fetal carcasses revealed the presence of renin mRNA consistent with localization to extra-visceral tissue. Sigmund et al.[74] were able to show that transcripts from either the *Ren-1*^{c} or the *Ren-1*^{d} allele accumulated to higher levels than transcripts derived from the *Ren-2* gene.

C. HORMONAL INFLUENCES ON RENIN EXPRESSION

Renin expression in mouse SMG has been shown to be androgen and thyroxine responsive.[20,29,46,79] Wilson et al.[29] investigated renin activity in SMG of female mice with the *Ren-1*^{d}/*Ren-2* genotype. Onset of activity was observed around puberty (3 to 4 weeks of age), reaching maximum basal levels around 7 weeks of age. The basal activity in females treated with dihydrotestosterone was found to be increased 4- to 5-fold compared with untreated females, which is comparable to the levels in the male gland. Likewise, *Ren-1*^{c} exhibits androgen inducibility. Nuclear runoff transcription assays show androgen responsiveness to be a result of increased transcriptional

activity, as opposed to merely increased message stability.[72,80,81] Administration of the thyroid hormone thyroxine has the same effect as dihydrotestosterone, resulting in an approximately 5-fold increase in the accumulation of renin mRNA.[72,80,81]

Another interesting fluctuation in renin expression which appears to be under hormonal influence has been observed in the adrenal gland of some closely related inbred strains carrying *Ren-1*^{d} and *Ren-2*.[35] Female mice of this genotype exhibit shifts in renin expression between the X-zone and the *zona fasciculata* of the cortex as the animal cycles through estrus. The adrenal gland at various stages of the estrus cycle was examined by *in situ* hybridization and revealed that in proestrus, renin transcripts are evident in both the X-zone and the *zona fasciculata*. During the next stage, estrus, transcripts are found only in the *zona fasciculata*. At metestrus, expression is evident in the X-zone but not the *zona fasciculata*. In diestrus, expression at both locations is evident, with noticeably higher accumulation of transcripts in the *zona fasciculata*. Differential primer extension revealed that the level of *Ren-1*^{d} and *Ren-2* remained equivalent at each stage of the estrus cycle. The molecular mechanisms regulating this change in cell specificity remain unclear.

D. INTERSPECIES CONSERVATION OF EXTRARENAL RENIN EXPRESSION

The important role of renal renin expression is widely conserved in vertebrate animals (see Nishimura[82] and Wilson[83] for reviews). The relevance of extrarenal renin expression is not as clear but conservation of expression at a given site suggests these sites may also have an important function. It seems probable that common sites of extrarenal expression found across mammalian species serve an important function in order to have persisted over an evolutionary time scale. Table 1 lists extrarenal tissues where renin mRNA has reportedly been detected by Northern or *in situ* analysis in mice, rats, and humans.

Renin expression in some extrarenal sites is apparently unique to the mouse, raising questions as to its functional relevance. Examples of this are the SMG and the coagulating gland.[84,85] In addition, there are differences in cell specificity among species. For instance, renin expression in the rat adrenal gland is restricted to the zona glomerulosa.[8] On the other hand, renin expression in the mouse adrenal gland is confined to the inner cortical zones, X-zone, and *zona fasciculata*.

III. PHYSICAL STRUCTURE OF THE MOUSE RENIN GENES

A. GENE STRUCTURE

Extensive genetic linkage information has permitted the formation of a relational map of mouse chromosome 1. Using the linkage information, the

TABLE 1
Comparison of Mouse, Rat, and Human Extrarenal Sites of Renin mRNA Synthesis

	Ren-1^c	*Ren-1*^{d}	*Ren-2*	Rat	Human
Submandibular gland	A	Negative[a]	A	Negative	No data
Adrenal gland	F	F, A	F, A	A	A
Coagulating gland	A	Negative	Negative	Negative	No data
Testes	F, A	F[b], A	F[b], A	A	No data
Ovary	A	A	A	A	No data
Subcutaneous tissue	F	F	F	F	No data
Chorion	No data	No data	No data	No data	Positive

Note: F, Present in fetal tissue; A, present in adult tissue.

[a] Detectable by RNase protection assay.
[b] Relative contributions of *Ren-1*^{d} and *Ren-2* not determined.

position of the renin structural genes has been established in relation to other chromosomal markers (Figure 1A).[86] Comparison of the three mouse renin genes revealed they have the same intron-exon arrangement and that this region spans a distance of roughly 10 kb (Figure 1B).[87] Sequence comparison of the respective cDNAs revealed that the coding regions of *Ren-1*^{c} and *Ren-1*^{d} are 99% identical, while *Ren-1*^{d} and *Ren-2* are 97% identical.[88] Interestingly, the *Ren-2* gene does not encode any of the potential N-linked glycosylation sites encoded in the *Ren-1* gene (and found in the renin-1 polypeptide).[26,87,89] The lack of glycosylation could explain the thermolability of renin seen in the SMG of mice with the duplicated gene. The mouse, rat, and human renin genes share significant structural organization and sequence similarity with each other. The renin gene coding regions in mouse and rat are approximately 88% identical, while between mouse and human renin genes they are approximately 78% identical.[28] These genes are members of the aspartyl protease family.[87]

The 5′ flanking regions of the mouse renin genes exhibit significant homology.[90-93] The renin genes are homologous for 150 bases upstream from the transcription start site (designated as +1), preserving the TATA boxes (−23 to −29) and a region of alternating purine pyrimidine bases (−30 to −45). Renin promoters do not contain a CAAT box but do have an AT-rich region conserved in mice and humans at approximately −60.

B. DNA INSERTIONS ASSOCIATED WITH MOUSE RENIN LOCI

The availability of rat and human genomic renin sequences permitted other interspecies comparisons of renin genes. These studies revealed that 5′ flanking regions of the mouse renin genes exhibit significant segmental homology with each other and regions of rat and human renin.[90-92,94,95] The segmental nature is due to a number of genetic events including not only the

duplication of the gene, but also the presence of numerous insertional elements in the 5′ and 3′ flanking regions of the genes (Figure 1C).[90-93,95-99] Several of these elements are well-characterized repetitive sequences, namely B1, B2, and a partial intracisternal A particle (IAP). Others are anonymous insertions which are arbitrarily referred to as M1, M2, M3, and M4 (M, mouse). At least M1 is known to be repetitive in the mouse genome. The presence or absence of these insertions has helped distinguish between the mouse renin genes and contribute to the segmental homology of the 5′ flanking DNA through their breakup of the primordial flanking regions (Table 2).

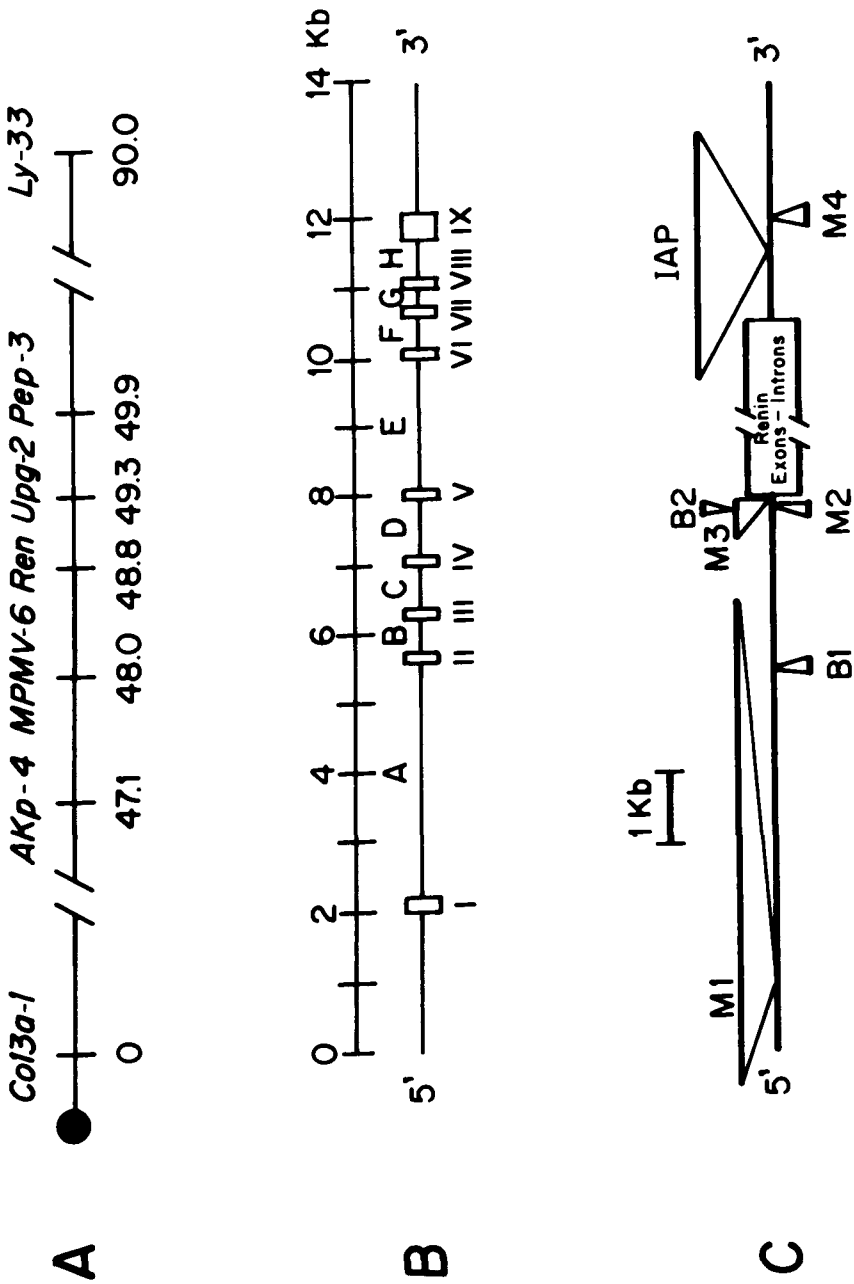
The combined approaches of sequence analysis, southern blotting, and pulse field gel electrophoresis (PFGE) studies have permitted comparison of the murine renin genes. It has been found that *Mus hortulanus,* a wild derived variant with two renin loci, lacks M1, M2, M4, and the IAP found in DBA/2J while possessing the M3 and B2 insertions.[94,95] The lack of the insertions in *M. hortulanus* also suggests the insertional events probably occurred after the duplication event in the inbred strains. These genomic inserts have been proposed to have potential influences on renin gene expression. However, *M. hortulanus,* which lacks many of the inserts, showed the same patterns of tissue-specific expression as is seen in DBA/2J. One exception where a lack of insertions in *M. hortulanus* correlates with altered expression as compared with DBA/2J, is in the adult adrenal gland, where no mRNA from either renin gene is detected.

C. STRUCTURE AT THE DUPLICATION LOCI

The unique pattern of tissue-specific expression in mice carrying the *Ren-1*^{d} and *Ren-2* loci and in those with the solitary *Ren-1*^{c} locus has led to efforts to characterize the physical structure of the duplicate locus. Genetic analysis indicates that the two loci are tightly linked.[22,98,99] PFGE helped to determine that *Ren-2* lies upstream of *Ren-1*^{d}, that the respective coding regions are separated by approximately 21 kb and that the two genes are transcribed in the same direction (Figure 1D).[98,99] Abel and Gross,[99] using PFGE and sequence information, determined the precise site of the recombination event that resulted in the gene duplication. Duplication apparently occurred through nonhomologous recombination. The analysis by PFGE also identified clusters of rare cutting restriction enzyme sites (or HTF islands) in the vicinity of the renin gene. HTF islands have been shown to be associated with 5′ regions of many vertebrate genes. *Ren-1*^{d} and *Ren-2* genes have an HTF island at homologous positions in their 3′ flank which apparently has been duplicated along with the *Ren-2* locus. Additional HTF islands are located 21 kb upstream and 65 kb downstream of the locus.

D. TRANSCRIPT STRUCTURE

In the kidney, the size of the mature mouse renin mRNA is approximately 1450 bases.[33] Additional higher molecular weight species of renin mRNA have been detected in SMG and coagulating gland by northern blot analysis.


A
Col3a-1
AKp-4 MPMV-6 Ren Upg-2 Pep-3
Ly-33
0
47.1
48.0
48.8
49.3
49.9
90.0
B
0
2
4
6
8
10
12
14 Kb
5'
3'
A
B
C
D
E
F
G
H
I
II III IV V VI VII VIII IX
C
1 Kb
5'
3'
M1
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M3
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M4
IAP
Renin
Exons Introns

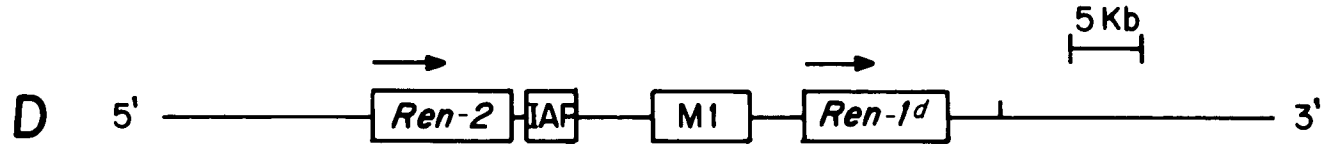

FIGURE 1. (A) Composite linkage map of mouse chromosome 1 illustrating the *Ren* locus and the placement of the closest known flanking loci in cM relative to the centromere. The two loci on the centromeric side of the renin locus *(Ren)* are alkaline phosphatase 4 *(AKp-4)* and modified polytropic murine leukemia virus-6 *(MPMV-6)*. The two loci distal to the *Ren* locus are urinary pepsinogen 2 *(Upg-2)* and peptidase 3 *(Pep-3)*. The collagen-3-α-1 *(Col3a-1* locus is most proximal to the centromere while lymphocyte antigen-33 *(Ly-33)* is most distal. (Adapted from Seldin et al.[86]) (B) Structure of the transcribed region of murine renin genes. This diagram illustrates the approximate arrangement and size of the exons (roman numerals) and introns (capital letters) of the mouse renin genes. (C) Location of murine insertional elements. This representation illustrates the approximate size and location of the insertional elements relative to the murine renin coding sequences. Refer to Table 2 for gene-specific insertions. (D) The renin locus containing the duplicate gene. This schematic illustrates the placement of the duplicated gene relative to the progenitor gene. Arrows indicate direction of transcription.

TABLE 2
Approximate Size and Location of the Insertional Elements Associated with the Mouse Renin Locus

Insertion	Size	Location	Ref.
M1	7.0 kb	−3.1 kb of *Ren-1*^{d}	98
M2	143 bp	−110 bp of *Ren-2**	92,97,98
M3	500 bp	−80 bp of *Ren-1*^{c}, *Ren-1*^{d}, *Ren-2*	97
M4	300 bp	+1.5 kb of *Ren-1*^{d}	98
B1	180 bp	−1.5 kb of *Ren-1*^{c}, *Ren-1*^{d}, *Ren-2*	96
B2	200 bp	Within the M3 element of *Ren-2*	90
IAP	3.5 kb	+1.0 kb of *Ren-2**	92

Note: A (−) indicates upstream distance from exon I if intervening insertions are not present. A (+) indicates downstream distance from exon IX. * Indicates the element is not associated with the *Ren-2* gene of *Mus hortulanus*.

S1 nuclease protection and primer extension assays have demonstrated the utilization of additional upstream transcriptional start sites.[90] These encode an open reading frame which potentially adds 23 amino acids to the N-terminus of the translated products. It remains unclear if this open reading frame is ever utilized.

IV. STRUCTURE/EXPRESSION CORRELATES

As has been shown, the mouse renin genes exhibit an array of complex expression patterns. Recent reports have speculated that the structural variations noted above may be responsible for several of the gene-specific expression patterns.[93,97,98,100] An opportunity to correlate specific structural features with gene expression patterns is afforded by comparing naturally occurring genetic variants (such as *Mus hortulanus*) with inbred strains (such as DBA/2).[95] Also, genetic crosses as well as transgenic analysis have demonstrated that the gene-specific expression differences are mediated by closely linked sequences in cis.[35,43,46] However, the available information does not satisfactorily limit which regions control the variable expression patterns of the renin genes. Therefore, in order to define the specific identity of the regulatory DNA sequences controlling these variations of expression, investigators have employed direct tests of recombinant DNA constructs in expression assay systems.

A. EXPRESSION ASSAY SYSTEMS

To identify regulatory DNA sequences, it is necessary to systematically examine the effects of discrete regions with an assay system which can directly measure the effects of linked DNA sequences on expression from a particular promoter. These fall into two categories, transfection into established cell

lines, and more recently the ability to insert genes via transgenesis.[88,101-103] Each system has its own advantages and disadvantages.

The transgenic approach provides the opportunity to examine expression of transgenes temporally in all tissues, with ensuing physiological feedback regulation. However, this method is time consuming, expensive, and labor intensive. Furthermore, the integration of the transgene into a chromosome is a random event and therefore the site of insertion cannot be controlled. The chromosomal environment around the integration site can have significant influences on transgene expression; a position effect. Therefore, it becomes necessary to examine multiple independent founder lines for each transgenic construct to determine whether the pattern of transgene expression is being controlled by elements of the transgene or by endogenous flanking elements.

The transfection approach allows the rapid testing of many different DNA constructs, as will be illustrated below. This should facilitate systematic examination of DNA sequences derived from large regions known to regulate expression. Ideally, the assay cell line should elaborate the trans-acting factors which promote the transcription of the endogenous renin gene (a cognate cell). Until recently, there have been no suitable established cell lines available for fulfilling this criterion. Previous attempts at establishing cells which express renin *in vitro* have been unsuccessful because the resulting cells often lose the ability to express renin.

B. TRANSGENIC ASSAYS FOR IDENTIFICATION OF CIS-ACTING ELEMENTS

To date, several groups have undertaken informative studies utilizing transgenic animals containing various renin genes and constructs. Initial experiments centered on reconstructing two renin gene type mice from a single transgene on a *Ren-1*^{c} genetic background. Tronik et al.[104] used a *Ren-2* transgene with 2.5 kb of upstream flanking sequence, the exon-intron region, and 3 kb of downstream flanking sequence. The *Ren-2* transgene was expressed in a quantitative tissue-specific manner; and *Ren-2* expression in the SMG was inducible by androgen. Mullins et al.[35,105] performed similar studies using a *Ren-2* transgene with a more extensive upstream and downstream flanking sequence (approximately 5 kb of 5′ flank, the exon-intron region, and approximately 10 kb of 3′ flank). They found qualitatively similar results to those reported with the less extensive *Ren-2* transgene. This group was also able to extend these observations to the adrenal gland, where they showed that the estrus cycle-specific effects on *Ren-2* expression could be partially reconstituted in the transgenic mice. In a similar set of studies Miller et al.[42] examined the expression of a *Ren-1*^{d} transgene (spanning approximately 19 kb with approximately 5 kb of 5′ flank, the exon-intron region, and approximately 4 kb of 3′ flank) in a *Ren-1*^{c} genetic background. They showed that the expression differences between *Ren-1*^{c} and *Ren-1*^{d} were encoded in cis. All these studies are in agreement with genetic studies and support the notion