Role of Molecular Biology in Hypertension Research
State of the Art Lecture
Victor J. Dzau, Martin Paul, Norifumi Nakamura, Richard E. Pratt, and Julie R. Ingelfinger

In this article we will examine the potential impact of molecular biology on hypertension research. We will review the available molecular techniques, which include gene cloning, transient and stable expressions, as well as the use of transgenic animals. To facilitate our discussion, we will focus primarily on research of the renin gene. Renin provides a useful model that illustrates the power of biotechnology in providing detailed structural and biochemical information on a complex protein that exists in low quantities in vivo. Studies of its messenger RNA and gene expression have resulted in an improved understanding of the biology of the renin system and in generating new hypotheses. These approaches can be generalized to studies of other vasoactive hormones, contractile protein, and other gene products related to cardiovascular regulation. To elucidate the role of a specific gene in genetic hypertension, we will discuss the use of genetic markers in cosegregation or linkage analysis. Finally, we will examine the potential of transgenic animals in the study of regulation of gene expression in the whole animal and the contribution of selective genes to hypertension. We believe that molecular biology complements the biochemical and physiological approaches and provides new opportunities for furthering our concept of hypertension mechanisms. (Hypertension 1989;13:731-740)

Blood pressure is regulated by multiple control mechanisms. Abnormalities in the regulation of one or several of these control systems may result in a transient or a sustained elevation in blood pressure. For instance, renal, endocrine, neurogenic, cardiogenic, and vascular mechanisms have each been demonstrated to participate in the development of experimental hypertension as well as in the pathogenesis of secondary forms of human hypertension. However, the etiology and pathogenetic mechanisms of essential hypertension are still enigmatic. Although many altered physiological responses and biochemical parameters have been reported, both in patients with essential hypertension and in animal models of genetic hypertension, it remains unclear which of the various reported changes are primary (i.e., causative) and which are secondary (i.e., the result of hypertension). Physiological and biochemical events are usually modulated by multiple related factors at multiple biological levels (i.e., organ, cellular, subcellular, biochemical, and molecular). An understanding of the mechanisms of hypertension necessitates a knowledge of the regulation and integration of control processes at these various levels. The purpose of this review is to examine how molecular and cellular biological research can contribute to and enhance our understanding of the mechanisms of the control processes and their potential role in the pathogenesis of hypertension.

Since multiple biologically active substances are involved in the cellular and subcellular events involved with blood pressure regulation (Figure 1), the study of such substances by means of molecular and cellular techniques can provide an understanding of these regulatory processes (Table 1). For example, the cloning of the gene that encodes an important vasoactive polypeptide would provide useful information concerning its gene structure, organization, and regulation. The cloning of the complementary DNA (cDNA) for this vasoactive polypeptide should yield knowledge of its amino acid structure and rapidly lead to specific molecular probes for the studies of its gene expression. Once the candidate gene or its cDNA are available, one can introduce it into heterologous cell lines, which then will express the polypeptide in high quantities,
providing a reproducible and abundant source for purification and for structure-activity studies. Furthermore, an understanding of the posttranslational biosynthetic processing and secretion of the polypeptide can be derived from studies of these transfected cells. It is clear that an understanding of the physiology of a biologically important polypeptide also requires an understanding of the regulation of its gene expression at both the transcriptional and posttranscriptional levels. Additional insight can be obtained through the localization and quantitation of the specific messenger RNA (mRNA) encoding a polypeptide, which is important to cardiovascular regulation under physiological and pathophysiological conditions, especially if such experiments are performed in parallel with biochemical and physiological studies. Finally, the understanding of the inherited basis of hypertension will require the application of the principles of genetics and molecular biology. We will now review each of these areas to examine their potential impact on hypertension research.

Table 1. Molecular Approaches to Research in Hypertension

| 1. | Clone a candidate gene |
| 2. | Clone complementary DNA |
| 3. | Transfection and expression of cloned gene or complementary DNA in heterologous cells |
| 4. | Localization and regulation of messenger RNA |
| 5. | Regulation of gene expression |
| 6. | Posttranslational processing and secretion of polypeptide |
| 7. | Genetics of hypertension |
| 8. | Transgenic studies |

Table 2. Application of Cloning of Candidate Gene

| 1. | Analysis of structure, organization, and localization of gene |
| 2. | Knowledge of evolution of gene |
| 3. | Information of putative regulatory elements of gene expression |
| 4. | Transfection and expression of cloned gene in heterologous cells |
| 5. | Studies of transcriptional control of gene expression |
| 6. | Restriction fragment length polymorphism (RFLP) |
| 7. | Production of transgenic animals |

Molecular Cloning of Genomic DNA

The structure, organization, and localization of a candidate gene can be studied once the gene is cloned (Table 2). These studies employ methodologies such as restriction enzyme analysis, sequencing, and hybridization experiments. Information derived from such studies is helpful in understanding the evolution of the gene and the control of its expression. Several genes that encode vasoactive substances important for blood pressure regulation, including renin, angiotensinogen, vasopresin, atrial natriuretic factor, and endothelin, have been the subject of investigation. To focus our discussion, we will examine in detail the information derived from studies of the renin-angiotensin system.¹

Human,²⁻⁴ rat,⁵ and mouse⁶ renin genes have been cloned and sequenced. The human renin gene is approximately 12.5 kb in size. Like most genes in higher eukaryotes, the coding sequences (exons) of renin are interrupted by stretches of noncoding sequences (introns) whose function remains unclear. The human renin gene contains 10 exons and nine
intron. Once the sequence of a gene is known, one can examine its degree of identity (homology) with genes that encode proteins that have similar properties, functions, and structures. This exercise of finding homologies may provide insight into the functional domains of the proteins and the evolution of a particular gene and related genes. For example, renin, along with pepsin and cathepsin D, is a member of the aspartyl proteinase family. Significant homologies in the gene structures as well as the protein sequences exist between renin and these other aspartyl proteinases. This is best illustrated by the comparison between human renin and pepsin, as described by Hobart and coworkers. Remarkable similarity exists between the intron and exon structures of the genes for these two related proteins. Comparisons of the primary sequences for these two aspartyl proteinases demonstrate that both the size and sequence of their domains are well conserved. The three-dimensional model of renin suggests that it is a protein with two symmetrical lobes. It is interesting to note that the domains in the first half of the protein can be paired with those in the second half based on their respective secondary structure and their symmetrical positions relative to the substrate binding cleft. Thus, the domain of exon 2 can be paired with exon 6, exon 3 with exon 7, and so forth. This pairing of domains and symmetry can be seen with other aspartyl proteinases and has led to the suggestion that the symmetry resulted from a duplication of an ancestral gene that comprised four exons. It has also been proposed that the intron encoding the signal peptide was subsequently added as exon 1 as these enzymes evolved into secretory zymogens. The unique exon 5A, which encodes for 3 amino acids in the human renin gene (absent in the rat and mouse), must be an even more recent addition whose function is unclear.

The existence of two renin genes (Ren-1 and Ren-2) in some mouse strains (e.g., DBA/2J and SWR/J) has raised the question as to when the renin gene was duplicated in the mouse. Ren-1 and Ren-2 are 95% identical, which suggests a divergence 2-7 million years ago. Consistent with the divergence of mouse and rat 10-20 million years ago, rat renin exhibits only 85% identity with Ren-1 and 81% identity with Ren-2. Thus, the data suggest that the two renin genes in the mouse resulted from a recent duplication of the Ren-1 gene that occurred after the speciation of rats and mice.

A knowledge of the regulatory elements of an eukaryotic gene may be gained from an analysis of the nucleotide sequence of the 5' flanking region and the introns. A comparison of the sequence, using available computer data banks, with other well-characterized genes may allow the development of hypotheses and speculations concerning the existence of regulatory elements that can then be experimentally tested by means of transient expression systems. With this approach, the molecular basis of the regulation of gene expression can be elucidated, as will be discussed in detail later in the section on gene expression.

Cloning of Complementary DNA and Its Application

Complex proteins or polypeptides, which exist in low quantities in tissue or plasma, are frequently difficult to purify, and sufficient quantities are usually not available for detailed structural studies. cDNA cloning and sequencing can provide important information on the primary amino acid sequence, which is difficult to obtain by means of biochemical methods. For example, submandibular gland renin was purified to homogeneity in 1972, but the primary amino acid sequence of renin of mouse submandibular gland, by means of biochemical methodology, was not reported until 1982. On the other hand, the molecular cloning of the cDNA of mouse submandibular gland renin was accomplished in 1982, and the primary amino acid sequence was immediately deduced from the cDNA sequence. Similarly, human renin was completely purified in the late 1970s, but the primary amino acid sequence has only been derived from the nucleotide sequence of the cDNA.

A detailed analysis and comparison of renin from several species was possible after the renin cDNA from these different species became available. Studies of mature mouse renin indicate that it exists as a two-chain polypeptide that is derived from a single-chain precursor by the removal of an internal dibasic residue (Arg-Arg). The available biochemical evidence suggests that mature rat and human renal renins are glycosylated single-chain polypeptides. In support of this, the primary amino acid sequences of renins, as deduced from the nucleotide sequence of the cDNA, show that amino acids to which sugars can be linked exist in rat and human renins but not in the mouse submandibular renin and that neither rat nor human renins contain the dibasic residues that correspond to the internal cleavage site of mouse renin.

Comparison of the cDNA-derived amino acid sequences of mouse and human renin with that of the other aspartyl proteinases revealed a high degree of homology. This allowed a prediction of the three-dimensional structure of renin based on the structure of other aspartyl proteinases, which had been determined by x-ray crystallography. Like other aspartyl proteinases, the catalytic site of renin is formed as a cleft between two lobes of the enzyme molecule. The substrate binding cleft accommodates 7-8 amino acids of angiotensinogen. However, there are also some remarkable differences between renin and other aspartyl proteinases at the edge of the active-site cleft. Here, renin appears to contain highly basic residues. In addition, renin contains a unique "flap," not present in other aspartyl proteinases, that may be important in determining the substrate specificity. These data improve our understanding of renin-substrate reaction and enhance the design of inhibitors of the enzyme.
cDNA sequence analysis can provide insight into the biosynthetic processing of a protein. Most secretory proteins are synthesized as a preproform. The preregion allows for the insertion of the protein into the endoplasmic reticulum, whereas the preregion keeps the protein in an inactive form. Based on the consensus sequences for preregions, precleavage sites, and proactivation sites, a model for the biosynthetic processing of a polypeptide can be predicted, once the amino acid sequence is deduced from the cDNA sequence. For example, amino acid and cDNA sequence analyses of mouse submaxillary gland renin suggest that this enzyme is synthesized in the preproform, which contains an 18-amino acid signal sequence and a 45-amino acid prosegment. cDNA sequence analysis of human renin suggests that the human enzyme is also synthesized as preprorenin with a 20-amino acid signal sequence and a 46-amino acid prosegment. To examine directly the posttranslational processing, fresh tissue or cultured cells for pulse-labeling experiments are required. However, such studies are usually hampered by lack of availability of fresh tissue or cell lines that express the polypeptide of interest; this has also been a problem in the studies of renin biosynthesis. Another approach is to transfect the candidate gene or cDNA into established cultured mammalian cell lines. The resultant cells that contain the candidate gene or cDNA, stably integrated into the host cell genome, will provide a permanent cell line with consistent properties for studies of biosynthesis and secretion. Such an approach has been used for studies of human renin biosynthesis. We have transfected successfully mouse fibroblast (L. 929), Chinese hamster ovary (CHO), and mouse pituitary tumor (AtT-20) cells. Studies from these cells demonstrate that human renin may be secreted by at least two cellular pathways. Transfected AtT-20 cells release preprorenin by constitutive secretion, but mature renin is secreted by a regulated mechanism that leads to renin release from storage granules. The secretion of the former is not influenced by 8-bromo-cyclic adenosine monophosphate (cAMP), but the release of the latter is significantly stimulated. The more tedious and time consuming pulse-labeling experiments of human kidney have provided results similar to those presented here for the AtT-20 cells. In the human kidney, prorenin is either rapidly secreted by the constitutive pathway or is slowly converted by an intracellular process to mature renin. The constitutive secretion of prorenin provides a potential explanation for the observation that prorenin constitutes a large (70-90%) percentage of the total renin in human plasma. The similarity in the processing of renin by the AtT-20 cells to that of the human kidney indicates that these cells are a suitable model system for the studies of human renin biosynthesis. The results presented here demonstrate the usefulness of transfected cells in studying peptide. In vitro mutagenesis of the coding region of a candidate gene before transfection allows the opportunity of identification and characterization of the signals required for proper intracellular sorting of the candidate polypeptide.

The expression and secretion of a complex polypeptide or protein such as renin from transfected cell lines have provided an important and reproducible source of large quantities of the protein for purification. Thus, purified prorenin or renin may be used as reagents for a variety of studies, which include crystallization and three-dimensional structural studies, development of renin inhibitors, and in vivo studies of renin metabolism.

**Messenger RNA Expression: Tissue Specificity and Regulation**

The regulation of a candidate gene in diverse tissues can be studied by the determination of its RNA transcript by means of hybridization analysis techniques such as Northern blots, slot blots, solution hybridization, and S1 protection assay. Such methods can reveal the levels of a specific mRNA in response to certain stimuli. For example, renal renin mRNA levels increase with sodium depletion, β-adrenergic agonist administration and renal ischemia. These responses are usually associated with increases in renin secretion rate as well. Thus, an approach that combines hybridization and biochemical methods allows an evaluation of gene expression, synthesis, and secretion during various conditions. Synthesis-secretion coupling can also be studied to yield information concerning the temporal sequence of physiological response to various stimuli.

The measurement of mRNA levels reflects a steady-state concentration, which is the net difference between the rate of RNA transcription and degradation. The control of mRNA level can take place at either site. Methods such as nuclear runoff may be used to study transcriptional rate versus RNA stability. With use of such techniques we have recently shown that angiotensin stimulated proto-oncogene c-fos mRNA expression in vascular smooth muscle cells and that this induction is a result of increased c-fos transcription rate.

**Localization of Messenger RNA Expression**

With use of hybridization techniques, the distribution of the expression of a specific mRNA can be determined. Before the availability of molecular biological methodology, biochemical data had suggested that renin existed not only in the kidney but also in multiple extrarenal tissues. However, these data were met with some skepticism, since the reninlike activity might be due either to nonrenin aspartyl proteinases (e.g., cathepsin D) or be derived from plasma contamination of the tissue. The demonstration of specific mRNA within a tissue or organ is evidence for local gene expression. Thus,
surveys of various tissues or organs for mRNA of interest may settle questions of local production. In a series of studies that used cDNA probes, we examined coexpression of angiotensinogen and renin mRNA in a number of tissues in both rat and mouse.29 Our results demonstrate that both of these mRNAs are expressed in multiple tissues of both species, which implies the possibility of local angiotensin production. These data have resulted in the hypothesis that local renin-angiotensin systems exert autocrine-paracrine influences on the regulation of local tissue functions. Locally synthesized angiotensin II could also play a role in intracellular function since nuclear angiotensin II receptors have been found in responsive tissues and since angiotensin II has been shown to increase both RNA and protein synthesis.30,31 These possibilities are currently being examined in a number of laboratories.

The cellular distribution of mRNA expression within a tissue can be determined by in situ hybridization histochemistry,32-35 which allows for the identification of the cell type that expresses a specific mRNA. In this method, native mRNA within a tissue is hybridized to a specific cDNA, complementary RNA (cRNA), or oligomeric probe radiolabeled with α-32P, α-35S, or α-3H. For example, we examined both renin and angiotensinogen gene expressions in the rat kidney by in situ hybridization histochemistry. With use of cRNA probes to both rat renin and angiotensinogen, we demonstrated that angiotensinogen mRNA was localized predominantly in the proximal renal tubules, with considerably lesser amounts in distal tubular segments, blood vessels, and glomerular tufts, whereas renin mRNA was localized predominantly in the juxtaglomerular apparatus area, with lower amounts demonstrable in the glomerular tufts. These findings provide powerful evidence for a complete tissue renin-angiotensin system within the kidney, which could possess unique regional functions. For example, we hypothesize that a proximal tubular renin-angiotensin system might directly modulate processes such as tubular transport of sodium, bicarbonate, and water. Other regional functions of a tissue renin-angiotensin system within the kidney, including modulation of glomerular contractility and hemodynamics, regional renal blood flow, and glomerular-tubular feedback, would seem possible.

**Tissue Specific Regulation of Messenger RNA Expression**

A growing body of data demonstrate the existence of tissue-specific renin and angiotensinogen gene expression. As mentioned above, we have shown that sodium depletion and β-adrenergic stimulation increases renin expression in kidney. These manipulations also increase renin mRNA expression in heart and adrenal but not in testicular or submandibular tissue. On the other hand, both androgen and estrogen increase extrarenal renin expression but not renal renin mRNA.36-40 Angiotensinogen gene expression is also differentially regulated in various tissues. For example, sodium depletion stimulates renal, cardiac, aortic, and adrenal angiotensinogen mRNA levels but not hepatic and adipose angiotensinogen mRNA levels.41 In contrast, dexamethasone stimulates hepatic, adipose, and renal angiotensinogen mRNA but not aortic smooth muscle angiotensinogen mRNA levels.42

The tissue-specific regulation of the local renin-angiotensin system expression may have important physiological functions. The renin-angiotensin system in tissues involved with cardiorenal homeostasis is stimulated by sodium depletion. Increased local angiotensin may enhance angiotensin-mediated function such as sodium retention, vasoconstriction, and increased cardiac contractility. In contrast, the renin-angiotensin system in other tissues not involved with the circulation does not respond to changes in sodium homeostasis but rather may be involved with other local functions (such as hormone regulation or reproduction).

**Regulation of Gene Expression**

Changes in tissue mRNA concentration may be due to regulation of gene expression at the transcriptional level. In most eukaryotic genes, the 5' flanking region contains essential regulatory elements. These sequences are termed cis-acting elements. The most common control sequences are the following: 1) Promoters, which are specific sites of transcriptional initiation on a DNA template. RNA polymerase recognizes and binds to these sequences and initiates transcription of the mRNA. 2) Enhancers, which are sequences that influence promoter activity and thereby gene expression. These sequences act independent of distance or orientation. The mechanism by which an enhancer increases promoter activity is not well understood. 3) Regulatory elements, which are sequences that are influenced by external signals such as cAMP or steroid hormone.

The combined action of these elements would result in the proper tissue-specific expression and regulation of a gene. Let us examine the information derived for the renin gene with this approach. Based on the high degree of homology of the human 5' flanking region to that of the mouse and rat genes, one would anticipate similarities in the regulation of renin gene expressions among these species. One would expect to find sequences responsible for the tissue-specific expression and regulation in the 5' homologous regions. Analysis of the 5' flanking region reveals the presence of 11 well-conserved blocks of sequence homology in the first 500 base pair in human, rat, and mouse genes. These conserved blocks are located at similar positions, average 9-29 nucleotides in length, and mostly are over 80% homologous. Besides the major 476 base pair insertion in the mouse gene, few deletions or insertions are found among the human, rat, and mouse genes. Therefore, cis-acting elements.
have remained presumably intact. Within these sequences the presence of consensus sequences for cAMP-, glucocorticoid-, estrogen-, and progesterone-responsive elements have been observed. In addition, sequences homologous to the core sequences of the polyoma and SV40 viral enhancers are also present. One also anticipates the presence of an angiotensin regulatory element whose consensus sequence has not yet been reported. The direct documentation and identification of these sequences in the human renin gene is an active area of research.

How can one investigate the transcriptional regulation of a gene? One approach is the use of reporter genes. Various regions of the 5' flanking sequences can be fused to the gene for chloramphenicol acetyl transferase (CAT) for transient expression experiments. This protein is easily assayed and has no mammalian counterpart. Cloning of the human and mouse regulatory regions 5' to CAT and introduction of these constructs into mammalian cells in culture allow the determination of promoter and enhancer activities. This approach can provide useful insight regarding the functionalities of the various regulatory regions of a gene in vivo. Expression of human gene as measured by CAT activity was limited to cells that retain a degree of differentiation in culture and that originated from tissues known to synthesize renin in vitro. Rat vascular smooth muscle cells and human primary chorionic or choriocarcinoma cells were those that tested positive.43,44

Once the expression system is established, one can examine the various functional regulatory elements of the gene by deletion analysis. Deletion of selective putative regulatory sequences with use of endonucleases can result in the loss of responsiveness to a regulatory stimulus. For example, in the human renin gene there are two putative cAMP-responsive elements as identified by computer analysis. We deleted selectively these sequences and demonstrated a loss of cAMP-stimulated CAT expression. This approach allowed us to define and localize the cAMP-responsive element in the human renin gene. The mechanism by which cAMP regulates gene expression has not been completely elucidated but may involve cAMP-dependent phosphorylation of specific DNA binding proteins. This hypothesis is under study in our laboratory with use of mobility shift DNA binding assay.45 Our preliminary data suggest that cAMP activates several nuclear proteins that bind to DNA fragments in the 5' flanking region of the human renin gene (N Nakamura, DW Burt, VJ Dzau, unpublished observations).

Also of interest is the presence of negative control elements that inhibit gene expression. Such elements appear to exist in the renin gene. When mouse Ren-1d gene is compared with the chicken lysozyme gene,46 the negative control region of the lysozyme gene, which contributes to the tissue specific regulation of this gene, shows significant local homologies, particularly in four regions. We have recently observed that an insertion element of 160 base pair in length is present in the Ren-2d gene, which interrupts this potential negative control sequence.47 The suggestion that a negative control element may be interrupted in the Ren-2d gene supports the model of renin gene control proposed by Field and Gross,48 who suggested that the Ren-2d gene fails to respond to negative control in the submandibular gland whereas Ren-1d does respond. This would explain the reduced level of Ren-ld gene expression in the submandibular gland (100-fold) relative to that of the Ren-2d gene. This experiment of nature, which probably resulted from a mutational insertion, demonstrated that a loss of inhibition of expression can occur if such insertion interrupts a functional inhibitory genetic element. On the other hand, if such an insertion interrupts an enhancer cis-acting element, a reduced level of expression may be seen. These detailed analyses of the regulatory elements of a gene may be important in the understanding of aberrant gene expression. Mutational insertions and deletions of selective sequences may result in abnormal expression of genes responsible for blood pressure regulation. Thus, the genetic basis of certain forms of hypertension may be elucidated by such an approach.

Genetics of Hypertension

Studies of Human Hypertension

Epidemiological studies and studies of twins suggest that a large proportion of the phenotypic variation in blood pressure is genetically determined. The results from all the major studies in which statistical analyses are applied to determine the mode of genetic transmission of blood pressure indicate that the inheritance of blood pressure is polygenic. In other words, the observed variability in blood pressure can be accounted for by the sum of the effects of variation in many genes. The nature of the genes responsible for the genetic variability of blood pressure has not been elucidated. Although multiple abnormalities in physiological responses and biochemical parameters have been described in human essential hypertension, it has not been possible to determine which, if any, of these abnormalities is primary. With the recent application of recombinant DNA technology, the identification of the altered gene or a closely linked genetic marker for a disease trait has become possible. One approach used to study the genetics of hypertension is the use of restriction fragment length polymorphism (RFLP). Genomic DNA is isolated from human peripheral white blood cells and digested with a restriction endonuclease. The resultant DNA fragments are separated by agarose electrophoresis, transferred to nitrocellulose by capillary blotting,49 and hybridized with a specific DNA probe to study the gene fragments of interest. Specific fragments of certain sizes can be produced from a candidate gene with restriction endonuclease digestion. A variation
in the structure of a gene fragment may result in a change in the migration pattern of that fragment (i.e., fragment length polymorphism). Thus, the genetic variation is demonstrated as heritable DNA restriction fragment length patterns that differ between individuals. An RFLP may lie within the defective candidate gene. It may be localized to an intron (noncoding region) or, less commonly, to the regulatory region or the coding element of the gene. In the latter two conditions, its presence may provide a molecular basis for genetic hypertension. More likely an RFLP does not lie within the defective gene but is simply located close by. Once an RFLP is identified, linkage analysis may be performed to link the inheritance of a genetic sequence marker with the inheritance of a certain phenotype (i.e., hypertension). Once the linkage of an RFLP to disease is established, the RFLP can become an important surrogate marker for the identification of those subjects at risk for the disease (e.g., hypertension). Genetic linkage analysis requires the study of families in which the disease occurs over several generations and is clearly an inherited trait. The DNA from individuals in these families can be analyzed to see if any RFLP is linked to or cosegregates with the disease. The analysis of RFLP has been a useful tool to explore alterations in genes encoding insulin,\textsuperscript{50} a and \textit{b} globin,\textsuperscript{51} and others. In some diseases, such as diabetes mellitus, it has been difficult to link the RFLP to the phenotypic expression of disease, but in others such as sickle cell anemia,\textsuperscript{52} Huntington's chorea,\textsuperscript{53} and Duchenne's muscular dystrophy,\textsuperscript{54} such linkage analyses have led to the development of an important screening test.

In a complex disease such as hypertension, several approaches are commonly used. One of these is the candidate gene approach. In other words, the DNA probes for known genes involved with blood sinogen, or atrial natriuretic factor, can be used to examine the occurrence of RFLP and its linkages to phenotype of hypertension in large family pedigrees. Another method is to use multiple "anonymous" markers (RFLP). In this case, multiple probes are used to look for any RFLP that is linked to the phenotypic trait. Recently, a detailed map of the location of multiple RFLP on all 23 human chromosomes has been published. Taking advantage of this map, these markers can assist in identifying the defective gene by a mathematical analysis that uses interval mapping and simultaneous searching.\textsuperscript{55} This approach anticipates the complexities that may be encountered in searching for markers of complicated disease such as genetic heterogeneity, incomplete penetrance, or gene interaction. By following the inheritance of the RFLPs in the family with hypertension, the probability that an individual also inherits the diseased gene can be determined. Furthermore, since the RFLP is located on the same chromosome as the defective gene, localization of the exact chromosome, with use of chromosome specific markers, can be determined. The technique of "chromosome walking" can move from the marker locus to the defective gene locus. The defective gene can be identified, cloned, and its sequence compared with the normal gene. Proof that the defective gene is responsible for disease can be obtained through experiments of transfection and expression as well as through the studies of transgenic mice (as will be described later). In summary, genetic markers such as RFLP may be useful in the identification of subjects at risk for hypertension. In addition, these markers are useful in the localization and identification of the defective gene, in the elucidation of the molecular and cellular basis for hypertension, and in developing preventive and therapeutic strategies (Table 4).

Analysis of Genetic Hypertension in Animals

Studies in genetically hypertensive rats have improved our understanding of human hypertension. Models of genetic hypertension include the spontaneously hypertensive rats (SHR), the Dahl salt-sensitive (DS) and salt-resistant (DR) rats, and the Milan hypertensive rats. A popular approach is to compare the SHR with the related normotensive strain, the Wistar-Kyoto (WKY) rat. Many investigators compare a biochemical or physiological trait between the SHR and WKY rats and suggest that a strain difference in the trait may be closely related to the blood pressure difference. Unfortunately, these studies do not provide causal relation. Frequently strain differences in a trait may be due to a genetic drift or are secondary to the alterations in blood pressure. A more appropriate approach is to examine whether these traits cosegregate with an

<table>
<thead>
<tr>
<th>Table 4. Strategies in Study of Genetics of Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Identification of genetic markers (RFLP) and intermediate phenotypes</td>
</tr>
<tr>
<td>2. Genetic linkage analysis with use of family pedigrees</td>
</tr>
<tr>
<td>3. Localization of chromosome of defective gene</td>
</tr>
<tr>
<td>4. Identification and cloning of defective gene</td>
</tr>
<tr>
<td>5. Elucidation of molecular basis of genetic hypertension (transfection and expression; transgenic animals)</td>
</tr>
<tr>
<td>6. Development of genetic markers of heritable hypertension for preventive measures</td>
</tr>
<tr>
<td>7. Target therapeutic strategy</td>
</tr>
</tbody>
</table>
increment of blood pressure in genetically segregated populations. To propose that a given trait (biochemical, physiological, or RFLP) may be relevant for explaining a blood pressure difference between the two strains of rats, the following criteria must be met: 1) a difference in the trait between the two strains has to be demonstrated; 2) the trait must follow Mendelian inheritance; 3) the trait must cosegregate within an increment of blood pressure significantly different from zero in F2 population from the cross of F1 hybrids; and 4) there must be a logical biochemical-physiological link between the trait and blood pressure.

In the case of genetic markers, the identification of an RFLP that cosegregates with increment in blood pressure fulfilling to the paradigm of Rapp should either identify the defective gene or be located close to the defective gene. Localization of the RFLP and identification of the defective gene can be determined with the same approach as that described for the human studies earlier. Thus, the variant gene can be characterized and tested for its relevance to the pathogenesis of hypertension. Recently, the use of such an approach by Rapp et al. identified RFLP associated with the renin gene in Dahl rats. Further analysis showed a deletional mutation in the first intron of the renin gene in the DS rats that appears to cosegregate with increments in blood pressure. Thus, one may expect that the genetic basis of selective models of animal hypertension will soon be elucidated with use of these methods.

Another approach is to construct congenic strains by multiple backcrossing (up to seven cycles) to obtain strains that might approach theoretical ideal. An ideal control strain will be genetically identical to the hypertensive strain except at the genetic locus influencing blood pressure. This approach has been discussed in some detail in a recent paper. Once such strains are available, the same approach of genetic analysis can be used in the understanding of the basis of hypertension.

Studies of Blood Pressure Regulation With Use of Transgenic Animals

The recent development of the transgenic animal technique has allowed the creation of potentially interesting mutations in genes of physiological importance and has aided the analysis of the phenotypic effects in whole animals. Mutant genes can be constructed in vitro from cloned genes of interest and reintroduced into germ lines of mice through microinjection of early embryos. The embryos are then reimplemented into the uteri of pseudopregnant females and will hopefully develop into live mice. The advantage of transgenic animals is the introduction of selected mutations of the gene of interest into a homogenous genetic background. The inbred lines of animals produced should permit us to address a number of important questions about the mechanisms that regulate the expression of a gene that is introduced and the response of other components of the blood pressure control system to the perturbation initiated by the abnormal function of the introduced gene. The spectrum of in vitro mutagenesis and alterations include: 1) Constructions that place the normal structure gene under control of inappropriate regulatory elements. Thus, the gene can be turned on rapidly and the effects of its overexpression on blood pressure and tissue functions can be examined. 2) Constructions that modify the structure of the polypeptide by deletion or modification of the genetic sequences that encode for the specific structure. Thus, these transgenic animals will synthesize an abnormal polypeptide and the specific effect on blood pressure can be studied. 3) Constructions that may transform specific cell types in situ in the whole animal. In this case, tissue-specific promoters will be linked to oncogenes and reintroduced into the whole animals. Thus, tissue-specific expression and expression-induced pathology can be studied. Furthermore, the cell types of interest with respect to blood pressure regulation can be immortalized and established in cell culture for detailed studies of mechanisms of gene regulation.

These approaches have recently been employed in the studies of atrial natriuretic factor and renin transgenic mice. Sigmund and coworkers have recently produced transgenic mice in which the renin gene promoter sequences are fused to SV40 tumor antigen. These animals developed kidney and subcutaneous tumors that were mainly of mesenchymal and vascular nature. In addition, diffuse hyperplasia of arteries and arterioles were observed in the kidneys. These findings document the tissue sites of renin gene expression and may be important models for the studies of the effect of excessive renin production on the development of hypertension. In addition, these transgenic animals may provide a potential source of transformed cells for tissue culture studies of renin gene expression. With use of a similar approach, Field produced transgenic mice that had atrial natriuretic factor promoter sequences and the SV40 tumor antigen. These animals developed atrial hyperplasia, tumors, and severe atrial arrhythmias. Such pathology may be helpful in comprehension of the mechanisms of atrial arrhythmias and the physiological effects of excessive atrial natriuretic factor production and ultimately may help in establishment of a useful animal model for the development of antiarrhythmic drugs.

In summary, transgenic animals provide a unique opportunity to examine the regulation of gene expression in the whole animal as well as the contribution of selective genes to the development of genetic hypertension. Such animals have a unique homogeneous background except for an alteration or mutation in the gene of interest. This approach is useful in evaluation of the relevance of a variant gene as identified in the studies of cosegregation analysis.
In this article, we have attempted to examine the potential impact of molecular biological techniques to hypertension research. These techniques complement the biochemical and physiological approaches and should greatly enhance our understanding of the mechanism of hypertension.

References

20. White BA, Bancroft JC: Cytoplasmic dot hybridization: Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J Biol Chem 1982;257:8569
60. Field LJ: Atrial natriuretic factor-SV40 T antigen transgenes produce tumors and cardiac arrhythmias in mice. Science 1988;239:1029–1033

KEY WORDS • molecular biology • gene regulation • renin-angiotensin system • genetics
V J Dzau, M Paul, N Nakamura, R E Pratt and J R Ingelfinger

Hypertension. 1989;13:731-740
doi: 10.1161/01.HYP.13.6.731

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/13/6_Pt_2/731

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/