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COMMENTARY

STRATEGIES TOWARDS A TRANSGENIC MODEL OF ESSENTIAL HYPERTENSION

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The generation of genetically modified animals by transgenic technology has proven to be a surprisingly versatile resource for researchers, providing an increasing number of new tools for biological investigation. As well as permitting the analysis of gene function and regulation *in vivo*, modifications of the techniques are being used to suppress or abolish the expression of specific genes, and further refinements have permitted the ablation of specific cell-types and the development of differentiated cell lines from tissue-specific tumours. In hypertension research, where many important questions have been frustratingly difficult to address by previously available methods, the advances afforded by transgenic studies have already been significant and are likely to be even more profound in the future. With the further development of these techniques, it may be possible to produce new and more representative models of essential hypertension.

Despite progress in the treatment of essential hypertension and in our understanding of some of the mechanisms involved, the underlying causes remain unknown. One of the central aims of hypertension research is to identify the genes involved in predisposition to the development of this disease. Of at least equal importance is to identify the biochemical, morphological and physiological abnormalities which result in high blood pressure. A third aim, which will become increasingly important as evidence implicating specific genes becomes available, is to relate lesions at the genetic level to abnormalities at the biochemical, morphological and physiological levels. Both classical and molecular genetics have been utilized in attempts to identify the genes important in hypertension. Evidence based on variance analysis of blood pressure heritability has suggested that between two and five genes could be involved in genetic hypertension in the rat [1, 2] although essential hypertension in humans may be more complex. Restriction fragment length polymorphism (RFLP†) and linkage studies will be crucial to the determination of these genes, but such studies will not necessarily indicate the mechanisms by which hypertension is caused. The development of genetically hypertensive strains of animals, such as the spontaneously hypertensive rat (SHR) and Dahl hypertensive rats, has been of great value in probing mechanisms of cardiovascular regulation, compensation and disease. However, since the underlying genetic defects are still unknown, there is a fundamental problem in differentiating between primary and secondary abnormalities in these strains. In the case of the SHR at least, there is an even more basic problem of defining which parameters are abnormal, since there is likely to be considerable genetic drift between the SHR and the Wistar–Kyoto (WKY) strain from which the SHR was originally derived.

Our ability to investigate the links between specific genetic lesions and subsequent mechanisms leading to disease has recently been improved greatly by transgenic animal technology, and the power of this experimental approach has already been demonstrated (see below). The ability to construct animal models with precisely defined genetic lesions opens a new dimension in hypertension research. The so-called candidate genes, whose importance in blood pressure regulation has been shown by physiological experiments, can now be investigated at the genetic level. There are many such candidate genes, but we will discuss here mainly the renin, angiotensinogen and atrial natriuretic peptide (ANP) genes, since these have been the genes most studied in transgenic experiments. This will serve to illustrate many of the approaches that are possible for other genes, but we will also discuss some specific approaches that may be useful for investigating particular genes in transgenic animals, where appropriate.

Gene regulation

Transgenic animals have found their widest application in the study of gene regulation. In hypertension research, the analysis of regulation of many of the genes of interest, including renin, has been hampered by a lack of appropriate cell lines. Even when cell lines are available, it is generally better to analyse gene regulation *in vivo*, as cell lines often poorly reflect the cells from which they

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† Abbreviations: RFLP, restriction fragment length polymorphism; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto; ANP, atrial natriuretic peptide; SMG, submaxillary gland; PDGR, platelet-derived growth factor; ES, embryonic stem; and HSV-tk, Herpes Simplex Virus thymidine kinase.

were originally derived. To facilitate analysis of expression in transgenic animals, the promoter in question can be fused to a reporter gene, such as bacterial *Lac*-Z or the SV40 T-antigen oncogene. The expression of bacterial *Lac*-Z can be detected by a rapid colorimetric assay, and expression of T-antigen can be detected by mRNA assay, immunohistochemistry, or altered morphology (ranging from diffuse hyperplasia to overt tumour formation) of the expressing tissue. To investigate the possible effect of regulatory sequences within a gene or in the 3′-flanking region, the gene may be introduced as an intact genomic fragment. In this case, analysis of expression usually entails the more time-consuming RNAse Protection assay, to differentiate expression of the transgene from its endogenous counterpart. In either case, the effect of systematic deletions of the putative regulatory sequences can be analysed in transgenic animals. These approaches help to identify regulatory sequences involved in tissue-specificity and in hormonal regulation. The combination of mouse genetics and *in vivo* molecular dissection is greatly under-utilized in hypertension-related studies and although the rat has distinct advantages for studies in physiology (see below), the mouse is presently unequalled in the study of gene regulation.

A number of transgenic studies have addressed the tissue-specific regulation of renin expression in the mouse, most notably those of Gross and colleagues [3–5], Rougeon and coworkers [6, 7], and Brammar and coworkers [8]. Substantial progress in the elucidation of regulatory sequences required for renin gene expression was only possible because of existing work on isolation and analysis of the mouse renin genes, which was facilitated by classical mouse genetics. The mouse provides a useful model to investigate tissue-specific regulation, having three renin alleles with different patterns of expression. Strains of mice with one renin gene, such as Balb/c carry the *Ren*-1^c allele, whereas strains with two renin genes, such as DBA/2, contain a *Ren*-1 allele (*Ren*-1^d) and a second renin gene, *Ren*-2^d. *Ren*-2^d differs from the two *Ren*-1 alleles most notably by being highly expressed in the submaxillary gland (SMG). (For a detailed review of the structure and expression of the mouse renin genes, readers are referred to the recent review by Sigmund and Gross [9].) The analysis of renin gene regulation in transgenic mice has led to some surprising findings. Whereas a genomic *Ren*-2^d transgene containing 2.5 kb of 5′ sequences conferred typical *Ren*-2^d tissue-specificity when introduced into *Ren*-1^c mice (including high, androgen-responsive SMG expression) [6], the same amount of *Ren*-2^d 5′ sequence, when joined to a heterologous cDNA (T-antigen), failed to express in SMG or juxtaglomerular cells [7]. These results suggest that important regulatory sequences for tissue-specificity are also present within the *Ren*-2^d gene, or downstream of it. Sigmund *et al.* [5], however, found that a transgenic construct consisting of 4.5 kb of *Ren*-2^d 5′ sequences fused to the T-antigen cDNA was expressed in accordance with the normal tissue-specificity of *Ren*-2^d. This indicates the presence of additional regulatory signals between 2.5 and 4.5 kb upstream of the initiation site and suggests redundancy of regulatory sequences, i.e. sequences located upstream are equally capable of directing tissue-specific expression as sequences within or downstream of the *Ren*-2^d gene. This indicates a fascinating and unexpectedly complex molecular basis for renin expression.

A similar transgenic approach could also be used to address the physiological regulation of kidney renin, e.g. to identify the regulatory sequences effecting renin gene expression in response to changes in sodium status [10] and blood pressure [11]. An important first step in this direction has already been made by Miller *et al.* [8], who introduced the *Ren*-1^d gene into mice normally carrying only *Ren*-1^c. The transgene, which included 5 kb of 5′- and 4 kb of 3′-flanking sequences, was expressed in kidney and its expression shown to be modulated appropriately by altering dietary salt intake. Further transgenic experiments, incorporating progressive deletions from this construct, may lead to identification of the sequences involved in the salt response. Taken further, this could lead to identification of the *trans*-acting factors and second messenger system involved in the response of renin synthesis to salt balance. The transduction mechanisms responsible for this response have proven difficult to define by physiological and pharmacological experiments, and this "reverse" approach, made possible by transgenic technology, could provide an important new approach to the problem.

Generation of specific cell lines

As described above, transgenic experiments have already led to a broad mapping of the location of regulatory sequences for renin. Much further work, however, will be required to define more accurately the *cis*-acting sequences involved, and to characterize them by footprinting and gelshift assays with nuclear protein extracts from various tissues. Tissue-culture experiments in clonal cell lines such as AtT-20 and JEG-3 have already led to the identification of a cyclic AMP responsive element and a negative regulatory element in the mouse and human renin genes [12–14]. Primary cultures of chorion have been used to examine the responses of the human renin gene to forskolin, phorbol ester and calcium ionophores [15]. However, these studies would be facilitated by the availability of renin-expressing cell lines from tissues such as kidney and SMG. One major benefit of transgenic technology is the potential to produce new cell lines through the introduction of appropriate gene constructs into the germline. The recent development of a stable, renin-producing juxtaglomerular cell line by Sigmund *et al.* [16] represents a major advance in this area. The cell line was derived from kidney tumours which developed in transgenic mice harbouring the SV40 T-antigen oncogene under control of the 4.5 kb mouse renin (*Ren*-2^d) promoter. Unlike previous attempts to generate clonal juxtaglomerular cell lines [17], this cell line retains the ability to store renin in granules, activate prorenin, and then to secrete active renin. In addition, the secretion of active renin is regulated, while prorenin is secreted

constitutively. This cell line should be particularly useful in identifying and isolating the *trans*-acting factors required for expression of renin. If these factors can be purified and/or cloned, it would obviously be of great interest to use the *trans*-acting factors themselves as substrates in transgenic experiments: this would provide a powerful means of directing tissue renin expression, and thus of investigating its role in cardiovascular regulation.

Steinhelper *et al.* [18] have also used transgenic mice to develop a specific cell line of great interest in cardiovascular research. Expression of an ANP-T antigen fusion gene in transgenic mice led to atrial tumours which could be propagated by transplanting into syngeneic mice. Cardiomyocytes derived from tumour transplants were able to proliferate in culture yet were highly differentiated by functional and structural criteria. These criteria include organization of cells into sheets linked by gap junctions, the ability to undergo spontaneous rhythmic action potentials and contractions, and expression of cardiac actin, myosin and ANP. The ability of these cells to proliferate in culture is particularly notable, since adult cardiomyocytes have been found to have a very limited ability to undergo cell division [19]. The cells were able to be maintained for four passages in culture, and it is not yet known whether they will be capable, with appropriate culture conditions, of indefinite proliferation in culture. If conditions permitting such proliferation, whilst retaining differentiated features, can be established, the resulting cell line will obviously be an important resource in the study of gene regulation and many other aspects of cardiac cell biology.

Physiological studies using transgenes

Of particular interest recently have been experiments in which the introduction of a transgene has led to the development of a hypertensive or hypotensive phenotype. Ohkubo *et al.* [20] developed lines of transgenic mice carrying either the renin or angiotensinogen genes from the rat. Neither type developed high blood pressure, but when mice were bred from these to contain both of the rat transgenes, a mild degree of hypertension resulted (systolic pressure about 150 mm Hg). The failure of the renin-only transgenic to develop hypertension is expected, since rat renin cannot cleave mouse angiotensinogen [21]. Since in mice it is angiotensinogen rather than renin that is rate-limiting [22], the failure of the substrate-only transgenics to develop hypertension is a little surprising, but could be due to feedback suppression of renin. Unfortunately, renin levels were not reported. Recently, Kimura *et al.* [23] also developed a transgenic mouse carrying the rat angiotensinogen gene (under the control of its own promoter), and found that elevated blood pressure did result. In the experiment of Ohkubo *et al.*, both transgenes were under the control of a heterologous (metallothionein) promoter, and were expressed in liver, kidney, brain, intestine and pancreas. It was not clear where the major site of exogenous renin expression was, nor whether active or inactive renin was secreted. If renin was being produced in sites where it is normally not expressed, it would be interesting to know whether these cells can activate and secrete renin. Mullins *et al.* [24] introduced the mouse *Ren*-2^d gene into the rat, resulting in severe hypertension. Interestingly, plasma and kidney renin levels were suppressed, as was plasma angiotensin II. The hypertensive phenotype is somewhat surprising in view of previous findings that the introduction of this gene into single renin gene mice was not associated with hypertension (Mullins J, unpublished data). In the transgenic rats with this gene, the circulating renin-angiotensin system is suppressed, yet the blood pressure is completely normalized by treatment with converting enzyme inhibitors. Two of the most conspicuous parameters in these rats are high adrenal renin expression, and high plasma prorenin levels. Further experiments are already underway to examine interactions between the adrenal renin-angiotensin system and corticosteroids, and to test the possible role of adrenal renin and plasma prorenin in blood pressure control. These provocative findings provide an example of how the transgenic approach can generate new physiological hypotheses.

Apart from kidney and adrenal expression, the transgene was expressed in a number of sites, including small intestine, thymus, aorta and brain. All sites of expression in the rat were bona-fide sites of expression in the mouse, indicating that the transgene contained the sequences necessary for tissue-specific expression. Thus the design of this experiment benefited from the previous mouse transgenic work which broadly characterized the regions required for tissue-specificity of renin. The importance of transgene design is that the subsequent expression, and physiological effects, are interpretable in terms of tissue-site and level of expression. This will become even more important in the future, as transgenic experiments become more refined: the expanding understanding of the determinants of gene regulation will enable experiments to be increasingly focussed to specific hypotheses. In the case of renin, gene regulation is complex, but the preceding work is now paying off in that important regulatory regions are being identified.

Similar studies to those performed for renin and angiotensinogen can be envisaged for other proteins and peptides involved in cardiovascular regulation, such as endothelin, vasopressin angiotensin 1 converting enzyme and ANP. The effect of overproduction of ANP has already been examined in transgenic animals [25]. ANP precursor was expressed constitutively under control of the transthyreitin promoter, in the mouse liver. ANP is thought to be activated within the secretory granules of atriocytes, normally, and it is unclear how activation occurred in this model, since hepatocytes lack secretory granules. However, the resulting increase in circulating lower molecular weight (i.e. activated) ANP paralleled a chronically reduced blood pressure.

In the future, transgenic studies will play an important role in understanding how these and other agents regulate cardiovascular function. In certain areas transgenic experiments will be crucial, allowing us to address questions not otherwise amenable to direct investigation, such as the effect of a gene product in specific tissues, and the effect of

abnormally high levels at certain times, such as during embryonic and early post-embryonic development.

Another potentially interesting area for transgenic studies is that of growth factors. It would be of great interest to generate a model in which vascular hypertrophy was a primary defect. Much present interest centers on the role of platelet-derived growth factor (PDGF) and other protein growth factors in the etiology of vascular smooth muscle hypertrophy: PDGF is secreted in a regulated manner by endothelial cells, and is known to stimulate proliferation of cultured vascular smooth muscle cells [26]. Further, there is evidence that secretion occurs preferentially into the basal subendothelial compartment [27]. Subendothelial cell proliferation occurs in a variety of disease processes including malignant hypertension, and hypertrophy of vascular smooth muscle cells is an established feature of animal models of hypertension. However, it is difficult to determine the significance of this, if any, for the development or maintenance of hypertension. If it were possible to induce vascular changes by specific over-expression of growth factors, it would be of great interest to monitor any associated changes in blood pressure.

Targeting gene expression

Transgenic experiments to date have been useful in investigating the effects of over-expression of specific genes, but it is also of interest to perform experiments which produce under-expression or non-expression of a particular gene. This would make it possible to model the effects of mutations which lead to suppressed expression or ineffective expression resulting from an inactive gene product. One approach to achieving suppression of expression is via antisense targeting. This involves designing a transgene such that its mRNA product is complementary in sequence to the target mRNA. Annealing of the two mRNAs causes interference with translation of the target mRNA, by a mechanism which is not yet understood. This is a relatively new application of transgenic technology, and there are very few reports at present of successful antisense targeting. One of the problems appears to be the requirement for high level expression of the antisense RNA in order to significantly reduce the amount of free target mRNA. It is possible that antisense experiments will be most suitable for targeting low-abundance mRNAs. However, the antisense approach has already been used successfully to decrease nerve myelination in mice, using a transgene complementary to a region of myelin basic protein mRNA [28]. One could similarly envisage experiments to decrease the expression of potentially anti-hypertensive genes such as sodium-potassium ATPase or ANP and thus test their role in blood pressure regulation. An additional refinement to this technique may be the incorporation of ribozyme sequences into antisense constructs. This would confer the transgene-derived mRNA with an enzymic activity capable of specifically cleaving the target mRNA molecule, and may make it far more effective at suppressing expression of the target.

At best, the antisense approach is probably only capable of partial suppression of gene expression. The only route to complete suppression is to "knock out" the gene of interest using homologous recombination [29]. This involves the mutation or removal of a particular gene in cultured embryonic stem (ES) cells by homologous recombination with a transfected DNA construct. The targeting construct contains regions homologous to the target gene, permitting specific recombination, and also a selectable marker (e.g. neomycin gene) so that the cells which have recombined can be selected. Following injection into blastocysts, the multipotent ES cells can contribute to all tissues including the germ-line. Chimaeric mice result, which can then be bred to obtain mice homozygous for the null or mutated allele. Homologous recombination at present is far from being a "textbook" procedure, and very few laboratories have been able to achieve successful knockouts. Although no genes of primarily cardiovascular interest have as yet been deleted from the genome in this manner, there is considerable interest in the potential application of this technology to hypertension research. The development of a rat ES cell line, which would allow knockouts to be performed in rats, would add great impetus to this, and is thus becoming a priority in hypertension research.

All these techniques are likely to have an increasing impact on many areas of medical research in coming years: they represent a further approach to manipulating gene expression, increasing the repertoire of techniques available to model diseases with a genetic component.

Tissue-ablation

Using cytotoxins such as diphtheria toxin or ricin under the control of a tissue-specific promoter, it is now possible to ablate specific cell-types in the developing embryo [30]. Since such constructs may be lethal, the recent development of an attenuated diphtheria gene may prove useful [31]. Alternatively, conditioned ablation using the Herpes Simplex Virus thymidine kinase gene (HSV-tk) allows the researcher to control the time during development at which cell ablation is induced via the administration of gancyclovir [32]. Gancyclovir is phosphorylated by HSV-tk to form a toxic substrate, but is a poor substrate for mammalian thymidine kinase and is thus relatively innocuous in cells not expressing HSV-tk. Using these techniques it should be possible, in the future, to examine the role of specific cell populations thought to be important in blood pressure regulation by ablating them during development.

Transgenic model of essential hypertension?

The foregoing has summarized briefly the general means by which transgenic studies are being applied to the study of cardiovascular regulation, and especially how they have facilitated the study of areas which were previously difficult to investigate, such as gene regulation and localized tissue actions of cardiovascular agents. Thus, transgenic studies will have a major role in building our overall understanding of cardiovascular regulation, and this will surely lead to new concepts in mechanisms which

lead to decompensation of homeostatic mechanisms and thus to cardiovascular disease.

As we have seen, transgenic experiments have already provided new animal models of hypertension. The mouse developed by Ohkubo *et al.* harbouring the rat renin and angiotensinogen genes, develops hypertension in association with elevated plasma angiotensinogen (renin levels were not reported). In the case of the rat developed by Mullins *et al.* which harbours the mouse renin gene, the hypertension is associated with low plasma renin and slightly elevated aldosterone, yet responds sensitively to converting enzyme inhibition. As such there are parallels to the low-renin subset of essential hypertension in humans. It would of course be dangerous to extrapolate from this similarity, especially as we do not yet know the mechanism of the hypertension in the transgenic rats. The exciting aspect of this model is that, as the mechanisms that lie between expression of the transgene and the development of hypertension are unravelled, they are certain to yield new information about mechanisms by which CEI-sensitive hypertension can develop in association with basal suppression of circulating renin. Direct relevance to humans remains to be seen, but there is a realistic possibility of having a framework on which to make testable hypotheses about low-renin essential hypertension.

The complexity of essential hypertension presents a formidable challenge. Many physiological and biochemical abnormalities have been described, and the difficulty of identifying the primary abnormalities is compounded by the interaction of factors at multiple levels, from organ to cellular and molecular. The enormity of the challenge in modelling essential hypertension is also due to the variation which exists within this disease entity. As well as being polygenic and consequently varied in its phenotypic expression, the interplay with environmental influences such as salt, alcohol, fat intake, physical activity and even psychological stress further acts to create a spectrum of presentations and blur the distinctions between subtypes. A number of different classifications have been made, according to plasma renin activity (low-renin, normal-renin and high-renin), etiological factors where known (renovascular, renal parenchymal, acromegaly, etc.), salt-sensitivity or non-sensitivity, and hemodynamic characteristics (vasoconstrictive- or volume-dependent). Not surprisingly, such a complex disease pattern is associated with a sizable collection of possible underlying causes. Present evidence supports a possible underlying role for a number of diverse entities, including components of the renin-angiotensin system, sodium and calcium transport systems, peripheral sympathetic receptors, central regulatory systems and lipoprotein metabolism. In some of these cases, it is not yet possible to identify a putative gene. In cases where candidate genes have been identified, the effect may be mediated via loss of function, as from a deleterious mutation, or by aberrant regulation leading to under- or over-expression, or inappropriate expression. For example, a defective allele of sodium-potassium ATPase has been tentatively linked to hypertension in the salt-sensitive Dahl rat strain by RFLP studies [33]. In cases such as this, the ability of the candidate gene to cause hypertension, and the mechanisms whereby this occurs, are amenable to investigation in transgenic experiments.

Finally, how might essential hypertension itself, rather than certain aspects of it, be modelled? One approach would be to combine candidate transgenes in a single model. As more transgenics are developed, it will be possible to breed them in order to combine transgenes within a single animal. Thus it will be possible to study interactions—a given transgene which may not be deleterious itself may, in combination with another transgene, cause hypertension. We have already seen an example of this in the case of the introduction of rat renin and angiotensinogen genes into the mouse. Neither transgene alone disturbed blood pressure, but when combined their effect was sufficient to cause hypertension. Further experiments based on this approach could lead to fascinating insights into how different components interact in blood pressure regulation, and tell us much about the inherent limitations of cardiovascular regulatory mechanisms. Taken further, an accumulation of specific genetic lesions may even start to approximate essential hypertension in its broad range of abnormalities and diversity of presentation. In addition, it may be possible to combine anti-hypertensive genes such as ANP, and demonstrate attenuation of the hypertension. The presence of such genes may be involved in determining the range of severity that exists in essential hypertension. Use of the antisense approach could allow under expression of certain genes to be investigated in the context of over-expression of some other genes, further extending the range of possibilities for genetic modelling of hypertension.

It is by no means certain that such an approach will, in fact, eventually mimic part or all of the range of phenotypes collectively grouped under the label of essential hypertension. Already, however, we have reason to be confident that the transgenic approach will bring exciting insights into the ways by which cardiovascular regulatory systems interact in health, and the mechanisms by which certain interactions occur to overcome homeostasis and lead to disease.

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