J Am Soc Nephrol 10: 2614–2619, 1999

Micropuncture Analysis of Tubuloglomerular Feedback Regulation in Transgenic Mice

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Abstract. Micropuncture methods have been used widely as a means to define the function of single tubules and study the functional connection between tubules and afferent arterioles (so-called tubuloglomerular feedback [TGF]). Transgenic mouse strains have become a new research tool with the potential of shedding new light on the role of specific gene products in renal tubular and vascular function. The micropuncture approach has therefore been adapted to studies in the mouse kidney. Although the data presented here support the feasibility of using this technique in the mouse, technical improvements are desirable in the areas of anesthesia, ureteral urine collections, blood collections, volume replacement, and functional stability for extended time periods. During ketamine/inactin anesthesia, TGF responses could regularly be elicited in wild-type mice. In contrast, changes in loop flow did not alter stop-flow pressure in angiotensin II type 1A receptor and angiotensin-converting enzyme knockout mice. Infusion of angiotensin II in subpressor doses partially restored TGF responsiveness in angiotensin-converting enzyme knockout animals. Normal TGF responses compared to wild type were found in nitric oxide synthase I and thromboxane receptor knockout mice. Using free-flow micropuncture techniques, the proximal-distal single-nephron GFR difference was found to be augmented in aquaporin-1 and Na/H exchanger-3 knockout mice, suggesting TGF activation in these strains of mice. These results support an essential role of angiotensin II in TGF regulation mediated through the angiotensin II type 1A receptor. Chronic nitric oxide synthase I and thromboxane receptor deficiency did not change TGF responsiveness. Aquaporin-1 and Na/H exchanger-3 deficiency enhances TGF suppression of TGF probably by volume depletion-mediated TGF sensitization. The use of micropuncture methodology in transgenic mice combines old and new research tools in a way that promises to yield important new insights into single-nephron function in physiologic and pathophysiologic conditions.

Addition of micropuncture and *in situ* microperfusion techniques to clearance-based methodology has been instrumental in advancing studies of renal function from the level of the whole organ to that of the individual nephron. This shift in paradigm has been the foundation of a quantitative description of the filtering and transport functions of many parts of the nephron, and has led to an appreciation of the intersegmental and inter-regional heterogeneities that exist within the kidney. The micropuncture approach has also provided definitive proof for the existence of the functional connection between tubules and glomerular vessels that is generally described as tubuloglomerular feedback (TGF) (1). Although two *in vitro* systems have been developed to investigate this phenomenon, variations of the micropuncture technique are still the most productive and efficient way to investigate TGF regulation of GFR (2,3). In recent years, mice with genetic alterations have been generated that have the potential of providing new ways for studying questions of integrated organ physiology and pathophysiology. However, full utilization of these animal models requires advances in the development of methods suitable to the small size of the mouse. As part of this effort, we have made an attempt to adapt the micropuncture approach to studies of the mouse nephron with the specific aim of examining the effect of various gene knockouts on TGF regulation of nephron function.

Problems in Micropuncture Specific to Its Use in Mice

Sufficient evidence is now available to assert that the application of micropuncture techniques in mice is feasible (4,5). Nevertheless, several problems remain that require further technical improvements. First, maintenance of an appropriate level of anesthesia is unequivocally more difficult in mice than in rats. For reasons that are not entirely clear, the therapeutic window is much narrower than in rats. Inactin, the preferred anesthetic for rats, is not suitable as a single drug, but inactin given intraperitoneally in combination with ketamine (given intramuscularly in our laboratory) can induce the desired depth of anesthesia without lowering BP inappropriately. Mainte-

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1046-6673/1012-2614
Journal of the American Society of Nephrology

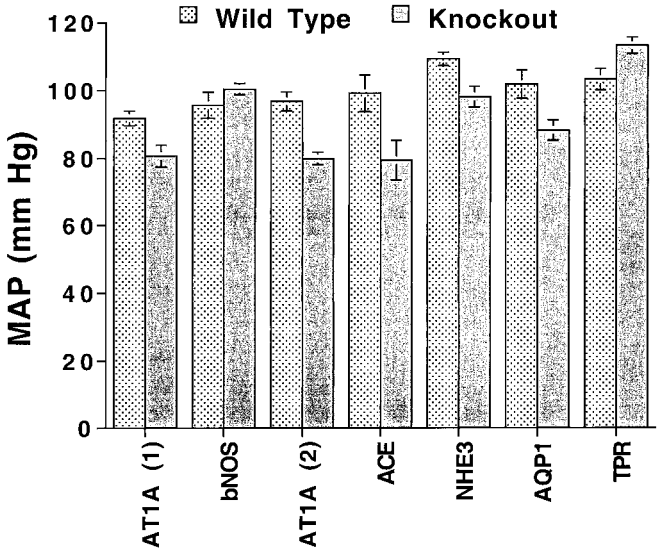

Figure 1. Mean arterial BP (MAP) in seven experimental series performed in our laboratory on six strains of control and mutant mice. Genes deleted by knockout mutations are indicated on the x-axis (5,7) (unpublished observations).

nance of anesthesia over several hours usually requires complementary doses of intramuscular ketamine. The use of gas (isoflurane) anesthesia has been suggested as a possible way to better control the depth of anesthesia (6). Second, we encountered substantial difficulties in our attempts to assess the overall function of the kidney undergoing micropuncture by collecting urine through a catheter placed into the ureter close to the renal pelvis. Although we have successfully accessed the ureter in the mouse with fine catheters, urine collections for extended periods of time have often been interrupted by partial or total blockade of the catheters with crystals of unknown nature. The surreptitious development of hydronephrosis leads to a gradual decline in renal function that eventually compromises micropuncture analysis of superficial nephron function. To avoid this problem, we currently collect bladder urine despite the loss of sided analysis and the much greater urinary tract and catheter dead space. Third, blood collections for clearance measurements are limited by the available volume of approximately 1.5 ml of blood for an average-size mouse. We collect blood through a short femoral artery catheter into 5-μl microcaps that are sealed and spun to yield about 2.5 μl of plasma. This is sufficient for determination of radioactive markers (^{125}I-iothalamate or labeled inulins) and probably of electrolytes. A terminal blood sample can of course be larger and may be of potential use for enzyme and hormone measurements. To reduce the number of blood samples, we restrict the total experimental time not including the time needed for cannulations and kidney preparation to about 2 h. Fourth, volume replacement during micropuncture experiments has long been recognized as an important source of data variations. Because the extracellular fluid reservoir is only about 5 ml, states of fluid disequilibria, either volume expansion or volume depletion, are more rapidly produced in the mouse than in the rat. We are currently infusing mice at a rate of about 1.3 ml/h $\times$ 100 g body wt with saline containing 2.25 g/dl bovine serum albumin. Hematocrit and urine flow rate are useful, but probably not very sensitive indices of stability of the extracellular volume state. Finally, more work is needed to establish that renal functional parameters remain reasonably stable over the required time period of 2 or more hours. In a recent study in a strain of Na/H exchanger-3 (NHE3) knockout mice, we noted a significant decline in GFR with time causing a significantly lower GFR in the second compared to the first hour of the experiments (unpublished results).

BP in Anesthetized Mice

Mean arterial BP in anesthetized and laparotomized mice of the six knockout strains studied in our laboratory thus far are shown in Figure 1. In wild-type animals, MAP ranged from 91.8 $\pm$ 2.2 to 109.5 $\pm$ 2.0 mmHg with a tendency for BP to be somewhat higher in the later experimental series (5,7) (unpublished data from our laboratory). Using the tail-cuff method, arterial BP values of wild-type mice of the same strains have been reported to be about 5 to 15 mmHg higher, although it is not always indicated whether these measurements reflect systolic or mean BP or how large the pressure amplitude is (8–11). For reasons that are not entirely clear, it appears that the extent of the BP-lowering effect of laparotomy/anesthesia in mice exceeds that typically observed in rats. Although it is possible that relative hypotension is caused by loss or shifts in

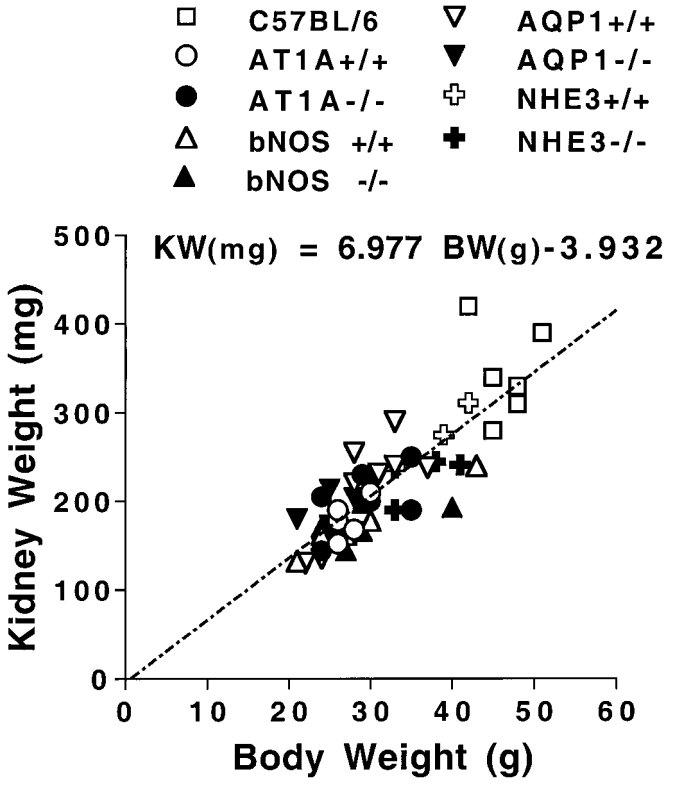

Figure 2. Relationship between kidney weight (KW, mg) and body weight (BW, g) in control mice (C57BL/6) and in four strains of wild-type and knockout mutant mice used in our laboratory.

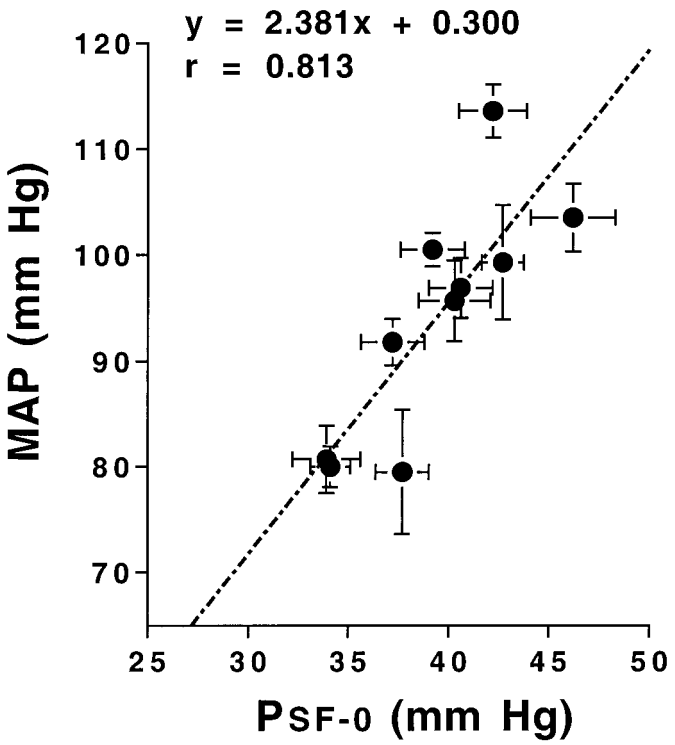

Figure 3. Relationship between MAP and stop-flow pressure in the absence of loop perfusion (P_{SF-0}) in five strains of wild-type and knockout mice used in our laboratory (5,7) (unpublished observations). Vertical and horizontal lines indicate SEM.

blood volume, it is more likely that it reflects the central effects of the anesthetic on the cardiovascular system. As also depicted in Figure 1, the effects of specific gene alterations on BP previously described in conscious mice were also detected during anesthesia. Consistent with these earlier measurements in conscious animals, BP in both AT_{1A} and angiotensin-converting enzyme (ACE) knockout mice was lower than in wild-type littermates (5). Nevertheless, reliable micropuncture data can be generated as long as arterial pressures are about 85 to 100 mmHg and are comparable between control mice of different strains. As judged from the relationship between kidney and body weight, none of the mutations studied in our laboratory appears to affect kidney growth out of proportion to body weight (Figure 2).

TGF Responses

TGF was assessed by measuring the response of stop-flow pressure (P_{SF}) to changes in loop of Henle flow rate. Changes of early proximal P_{SF} accurately reflect changes in glomerular capillary pressure and have been used extensively to characterize the TGF response pattern in rats (1). Adaptation of this technique to mice did not require major modifications. However, recordings of longer duration were more difficult than in rats because tubules tended to be more leak-prone as a result of greater BP irregularities. P_{SF} at zero flow was around 40 mmHg not markedly different from values found in rats. Because P_{SF} at zero flow is poorly autoregulated, it is directly dependent on arterial BP (Figure 3). Therefore, in mice with knockout mutations in renin-angiotensin system genes, P_{SF} at zero flow was at the lower end of the range observed in our studies.

AT_{1A} Knockout Mice

To assess the role of angiotensin II type 1A (AT_{1A}) receptors in macula densa control of vascular resistance, we examined TGF responses in a strain of AT_{1A} knockout mice generated by Ito *et al.* and in their wild-type littermates (9). At zero loop flow, mean P_{SF} in wild-type mice was 37.2 ± 1.6 mmHg (arterial pressure 92 ± 2 mmHg). Increments in loop flow to 15 nl/min or higher caused P_{SF} to reach the minimum value of about 29 mmHg (5). Compared with rats, the TGF function was shifted to lower flow rates with $V_{1/2}$, the tubular flow rate associated with half-maximal reduction of P_{SF}, being 8.7 ± 0.4 nl/min. In contrast, in mice homozygous for the AT_{1A} mutation, P_{SF} did not significantly change in response to increases in loop perfusion rate. In mice heterozygous for the AT_{1A} receptor mutation, P_{SF} fell with increasing loop flow rates, but the magnitude of the response was significantly smaller than in wild-type animals. Overall, P_{SF} fell by a maximum of about 9 mmHg in AT_{1A} +/+, by 5.1 ± 0.4 mmHg in AT_{1A} ±, and by only 0.64 ± 0.3 mmHg in AT_{1A} −/− animals (Figure 4). Since the TGF response magnitude depends on arterial pressure, it is conceivable that the lower arterial pressure in AT_{1A} −/− mice contributed to the TGF attenuation (12,13). However, TGF responses were blunted in the knockout mice regardless of the pressure of the individual mice, suggesting that the BP reduc-

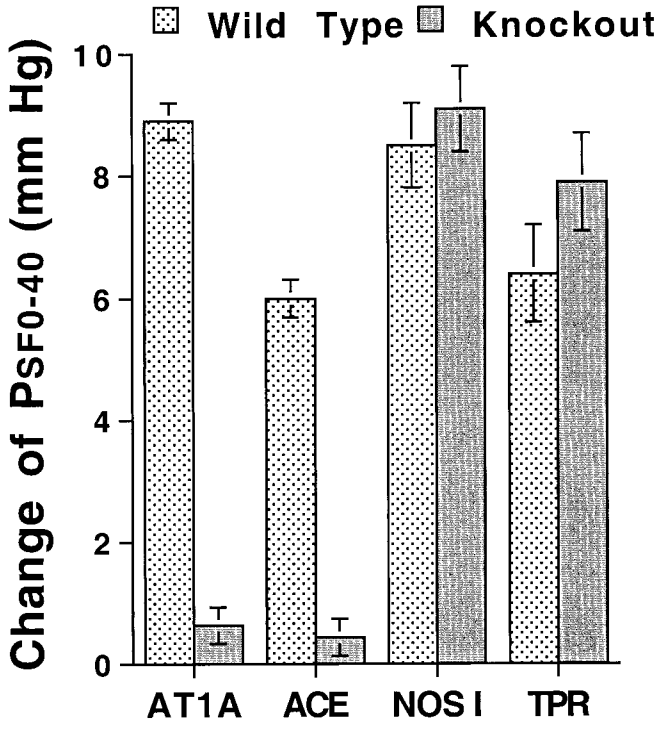

Figure 4. Change of stop flow pressure (P_{SF}) in response to an increase in loop perfusion rate from 0 to 40 nl/min in four strains of wild-type and knockout mutant mice (SEM indicated by vertical lines).

tion is not sufficient to explain the absence of TGF-mediated vasoconstriction (5). These results emphasize the critical role of angiotensin II in determining TGF response magnitude and demonstrate that this effect is mediated through the AT_{1A} receptor.

ACE Knockout Mice

Interference with the renin-angiotensin system by a null mutation in the ACE gene caused a TGF phenotype similar to that seen in AT_{1A} knockout mice. In ACE knockout animals from a colony of the ACE mutants generated by Krege *et al.*, TGF responses were severely blunted (10). Whereas P_{SF} fell by 9.2 ± 0.9 mmHg ($n = 16$) in ACE +/+ mice in response to an increase of loop of Henle perfusion rate from 0 to 40 nl/min, it fell by only 0.4 ± 0.3 mmHg in ACE −/− null mutants ($n = 10$) (Figure 4). Interestingly, TGF responses were also blunted in ACE ± heterozygous mice in which the mean decrease of P_{SF} was 2.25 ± 0.5 mmHg, a value significantly lower than that found in ACE +/+ animals. When angiotensin II was infused at rates that did not significantly increase arterial BP, TGF responsiveness in both homozygous and heterozygous mutants was restored to some extent, although the magnitude of the TGF response did not reach normal values. It has been argued that by stabilizing NaCl delivery to the collecting duct system, TGF is a mechanism that protects against a loss of salt during random fluctuations of arterial pressure that are not caused by alterations in extracellular fluid volume. It is of note that both AT_{1A} and ACE knockout mice identified in our studies as chronic models of absent TGF regulation have reduced arterial BP where the demand to control distal salt delivery is less urgent. It is conceivable that the low BP at least in part reflects a state of relative volume depletion and that absence of TGF contributes to the generation of this state.

Nitric Oxide Synthase I Knockout Mice

There is strong experimental evidence to suggest that acute inhibition of nitric oxide synthases (NOS) causes augmentation of TGF responses, and this has been interpreted as an expression of a tonic TGF-suppressing effect of nitric oxide formed by NOS I in the macula densa (14,15). It therefore was of interest to examine the effect of chronic and specific absence of NOS I on the TGF response magnitude using mice with a null mutation in the NOS I gene generated by Huang *et al.* (16). Mean arterial BP was comparable between wild-type and knockout mice (99.6 ± 1.7 mmHg, $n = 7$, compared to 96.6 ± 3.8 mmHg, $n = 6$). As shown in Figure 4, TGF responses of P_{SF} to changes in loop perfusion rate were not different between wild-type and homozygous NOS I mutants. In NOS I +/+ mice (13 tubules), mean P_{SF} at zero loop flow was 40.3 ± 1.8 mmHg, falling to 31.9 ± 1.8 mmHg at a flow of 45 nl/min (Δ 8.5 ± 0.7 mmHg). The flow rate causing half-maximal responses, $V_{1/2}$, was about 12 nl/min. In NOS I −/− animals (18 tubules), mean P_{SF} at zero flow was 40.7 ± 1.7 mmHg, falling to a steady-state value of 31.9 ± 2 mmHg at 45 nl/min (Δ 9.1 ± 0.7 mmHg). $V_{1/2}$ was about 13 nl/min. Thus, chronic absence of macula densa NOS was not associated with significant changes in TGF activity, an observation in contrast to previous results showing augmentation of TGF responsiveness during acute inhibition of NOS activity (14,15). Normal TGF responses in the absence of macula densa NOS may indicate the influence of compensatory factors. For example, it is possible that the TGF-enhancing action of NOS absence was counterbalanced by a reduction in renal renin production (8). Alternatively, other NOS isoforms in addition to NOS I may contribute to the TGF-enhancing effect produced by acute pharmacologic NOS inhibition.

Thromboxane Receptor Knockout Mice

The vasoconstrictor prostaglandin thromboxane (TP) has been implicated in TGF on the basis of the observation that the acute administration of inhibitors of TP receptors or of TP synthesis reduced the magnitude of TGF-induced vasoconstriction (17). To explore the effect of chronic TP receptor deficiency, we have studied TGF in mice with a targeted null mutation in the TP receptor gene generated recently by Thomas and Coffman at Duke University (18). In response to a saturating flow rate, P_{SF} fell from 42.2 ± 1.7 to 35.9 ± 1.4 mmHg in wild-type mice, and from 46.2 ± 2.1 to 38.3 ± 2 mmHg in TP receptor knockout mice. Thus, chronic TP receptor deficiency did not cause a diminution of TGF responses (Figure 4). Presence of functional TP receptors in the area of the juxtaglomerular apparatus was examined by including 50 μM of the TP mimetic U46619 in the tubular perfusate. As in rats, luminal U46619 augmented TGF responses from 6.4 ± 0.4 to 10.2 ± 0.9 mmHg in wild type. In contrast, U46619 had no effect on the TGF response magnitude in knockout animals. Our data suggest that the amount of TP generated at the level of the juxtaglomerular apparatus in mice may be insufficient under control conditions to activate TP receptors.

Proximal-Distal Single-Nephron GFR Difference

Previous evidence has established that the functional connection between macula densa and afferent arteriolar cells is constitutively active and causes a tonic suppression of GFR (1). This feature of the TGF regulatory system needs to be considered in micropuncture determinations of single-nephron GFR (SNGFR). Fluid collections in the proximal tubule eliminate the TGF signal and the resulting SNGFR is therefore a non-steady-state value that reflects the acute withdrawal from TGF suppression. During distal collections, on the other hand, the macula densa is unperturbed, and the resulting SNGFR is a steady-state value. On the basis of about 40 experimental series in rats in which proximal and distal collections were performed in the same nephron, proximal SNGFR was found to exceed distal SNGFR on average by 16% (1). The magnitude of the proximal-distal SNGFR difference is a measure of the net GFR-suppressing effect of the TGF system at the time of measurement. Rather than evaluating SNGFR responses to loop perfusion, we have used this simple approach to gain insight into the operation of the TGF system in two strains of knockout mice in which the existence of relative volume depletion and therefore activation of the TGF system seemed

possible. Micropuncture analysis of tubule function was performed in aquaporin-1 (AQP1) knockout mice generated by Ma *et al.* (19), as well as in NHE3 knockout mice generated by Schultheis *et al.* (11). In both strains of mice, evidence was obtained to suggest that TGF causes enhanced suppression of steady-state GFR. Proximal SNGFR values were found to be nearly identical in NHE3 wild-type and knockout animals, but SNGFR was significantly lower in the knockouts when measurements were made in the distal tubule (20). Essentially the same observation has been made in AQP1 knockout animals (21). Thus, the proximal-distal SNGFR difference is enhanced in both strains of knockout mice. Both of these strains are characterized by relative extracellular volume depletion, and activation of the renin-aldosterone axis has been demonstrated in the NHE3 knockouts by Schultheis *et al.* (11). Thus, it is most likely that a shift in the sensitivity of TGF much like that produced by angiotensin II infusion is responsible for the resetting of steady-state GFR (1). Kidney GFR was found to be reduced in both AQP1 and NHE3 knockouts compared to wild type, both when expressed per gram kidney weight or in absolute terms.

Genetic Background

Information that is relevant to the function of the adult organism can be expected from transgenic mice only when the mutation is not developmentally lethal and does not cause major structural alterations in an organ of interest. In addition, there is growing appreciation of the fact that specific phenotypes observed in mutant mouse strains may be caused by unrecognized differences in the genetic background rather than by the mutation itself (22,23).

Successive brother-sister inbreeding of wild-type and mutant mice of the F2 generation is efficient, but will eventually generate new inbred strains in which the genetic background of wild-type and mutant animals is considerably different. Thus, wild-type animals may no longer be an appropriate control for the mutants, since random segregation of alleles can occur that lead to differences in modifying genes and possibly to alterations in the phenotype of the mutation. Furthermore, the genotype of these inbred strains is complex and unknown, and it would therefore be difficult to reproduce and compare results obtained in such animals with results from other laboratories. It is also possible that inbreeding leads to disadvantageous allele combinations that may affect the well-being and vigor of inbred colonies. Standardization of the genetic background is optimal if the mutation is kept in a congenic background, which can be achieved by multiple backcrossings of heterozygous hybrids. The phenotype of interest can then be studied in F1 generation animals produced by crossings of heterozygous animals of the two congenic strains. Unfortunately, the generation of congenic mice is time consuming and costly.

Another potential problem that can affect the phenotype of the mutant is related to the fact that in homozygous mutants, the region around the targeted locus contains the genes from the embryonic stem (ES) cells, whereas in wild-type animals it contains the background from the other parental strain. It is possible to generate wild-type animals in which the genes in the target region come from the ES background by identifying a polymorphism in the targeted locus that permits discrimination between ES and C57 origin. It should be noted that a mutation causing a robust phenotype will probably be relatively independent of the confounding influence of genetic background differences. For example, the interference of null mutations in either AT_{1A} or ACE genes with the TGF response observed in our studies was rather invariable despite the fact that genetic background differences between wild-type and mutant animals must have been considerable.

The development of embryonic stem cells from a strain of mice that is more viable than the 129 strain and their use in the generation of transgenic animals would appear to be the ideal solution to the problem of genetic background differences. For now, generating and distributing congenic strains and if necessary defining the genetic background by using microsatellite markers (Research Genetics, Huntsville, AL) distributed over the mouse genome appear to be available means to avoid erroneous conclusions (24).

Acknowledgments

Work in the laboratory of the author was supported by National Institutes of Health Grants DK 37448, DK 39255, and DK 40042. The experimental work referred to in this article was done at the University of Michigan in collaboration with Drs. J. Briggs, T. Traynor, T. Yang, Y. G. Huang, A. Smart, M. Ford, and J. N. Lorenz. We are indebted and grateful to the following researchers for providing transgenic animals: Drs. T. M. Coffman, M. I. Oliverio, and D. Thomas, Duke University (AT_{1A} and TPR mutant mice); Drs. J. H. Krege and O. Smithies, The University of North Carolina (ACE mutant mice); Drs. P. L. Huang and M. C. Fishman, Massachusetts General Hospital and Harvard University, and Dr. O. A. Carretero, Henry Ford Hospital (NOS I mutant mice); Drs. T. Ma and A. S. Verkman, University of California, San Francisco (AQP1 mutant mice); and Drs. P. Schultheis and G. Shull, University of Cincinnati (NHE3 mutant mice).

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