In vivo analysis of key elements within the renin regulatory region

Sean T. Glenn,1 Craig A. Jones,1 Li Pan,2 and Kenneth W. Gross1
1Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York; and 2Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts

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Glenn ST, Jones CA, Pan L, Gross KW. In vivo analysis of key elements within the renin regulatory region. Physiol Genomics 35: 243–253, 2008. First published September 9, 2008; doi:10.1152/physiolgenomics.00017.2008.—Renin is responsible for initiating the enzymatic cascade that results in the production of angiotensin II, the major effector molecule of the renin-angiotensin system (RAS). Extensive information on the regulatory region of the renin gene has been derived by transient transfection studies in vitro, particularly using the As4.1 cell line. To verify key factors within the regulatory region of renin in vivo, homologous recombination was used to introduce a green fluorescent protein (GFP) cassette into exon one of the renin gene contained within a 240 kb bacterial artificial chromosome (BAC) to create a construct that has GFP expression controlled by the renin regulatory region (RenGFP BAC). Within the regulatory region of the RenGFP BAC construct we independently deleted the enhancer, as well as mutated the HOX-PBX site within the proximal promoter element. Transgenic lines were generated for each of these BAC constructs and GFP expression was analyzed throughout a spectrum of tissues positive for renin expression including the kidney, adrenal gland, gonadal artery, and submandibular gland. The results described within this manuscript support the interpretation that the renin enhancer is critical for regulating baseline expression where as the Hox/Pbx site is important for the tissue specificity of renin expression.

Particularly critical among these sites is a motif homologous to the consensus cAMP-responsive element (CRE), which has been shown to bind members of the CREB/ATF transcription factor family and an E-box motif that binds the bHLH-leucine zipper transcription factors. Aside from the CRE and E-box motifs, other less critical sites have been found within the renin enhancer. These sites include the Ec and Eb sites, which have been shown to bind RAR, RXR, EAR,2 and other unidentified orphan receptors (7, 14, 20). Also found within the renal enhancer is a NF-Y binding site (19) as well as sites identified within the distal portion including four NFκ binding sites, an Sp1/Sp3-binding site, and an unknown transcriptional binding site (19). It has also been shown that within the enhancer NF-κB can form a complex with proteins binding to the CRE, inhibiting Ren-1c gene expression (23). The CRE has also been shown to be involved in 1,25(OH)2D3-induced suppression of renin expression (26). Zhou et al. (27) have reported that a thiolated kidney enhancer for human renin is required to maintain baseline renin expression in vivo. The mouse renin enhancer has also been shown to be critical for renin expression in response to in vivo stimuli (8).

Outside of the human renal enhancer the proximal promoter region contains many transcription factor binding sites including the TATA box, an Ets-binding site, the HOX/PBX-binding site, a CRE that binds transcription factors such as CREB and ATF1, a putative binding site for ARP-1 (COPU-TFII), and other yet to be identified transcription factor binding sites (1, 25).

Additionally, located between the proximal promoter region and the enhancer of the renin gene in mouse, rat, and human, is a CBF1 site that is involved in the notch signaling pathway (13, 14). Nakamura et al. (9) have identified a CRE (an overlapping CRE and a negative response element), which is also located between the renin promoter and enhancer.

In previous studies we have used a renin reporter transgene that consisted of 4.1 kb of 5′ renin flanking sequence controlling green fluorescent protein (GFP) expression (5). In an effort to more exquisitely and comprehensively ensure control of reporter expression, homologous recombination was used to introduce a GFP cassette into exon one of the renin gene residing within a 240 kb BAC to create a construct that has GFP expression controlled from within the entire natural genomic sequence context for renin. The insertion of GFP into the renin gene, which is centrally located within the bacterial artificial chromosome (BAC), allows large amounts of 5′- and 3′-flanking sequence to act on reporter expression in an effort to gain a more faithful representation of endogenous renin gene expression. Another advantage of using BAC transgenics over conventional plasmid-based technologies is that the more exten-
sive natural sequence content of the BAC helps to insulate transgene expression from influences of surrounding sequence at the insertional site. Within this manuscript two different transgenic lines created using the above mentioned BAC construct will be designated as RenGFP (1 copy) and RenGFP (3-4 copy).

Using homologous recombination, we deleted the renin enhancer from the RenGFP BAC construct, and transgenic lines were created to study the importance of the enhancer to renin expression in vivo. The mutated enhancer transgenic line will be designated as ΔEnh throughout the rest of the manuscript. We also produced transgenic mice with a single base point mutation within each of the homeobox (HOX) and pre-B cell leukemia transcription factor (PBX) half sites that make up the proximal promoter element (PPE) in the RenGFP BAC construct. This line will be designated as ΔHP.

The results reported here support the notion that the renin enhancer is critical for setting the magnitude of baseline expression, and that the Hox/Pbx site that comprises the PPE is an important contributor to tissue specificity of renin expression.

**MATERIALS AND METHODS**

**Transgenic Constructs**

The BAC clone, RP23-88k07, was obtained from BACPAC Resources Center, CHORI (Oakland, CA). This clone was isolated from the RPCI-23 Female (C57BL/6J) Mouse BAC Library (http://bacpac.chori.org/femmouse23.htm). The renin gene is centrally located within the BAC (Fig. 1A). The homologous recombination-based modification methodology developed by Yang and coworkers (24) was used to insert the GFP gene (a gift from N. Muzyczka) (5, 28) into RP23-88k07 so that GFP expression is under the control of all identified and not yet identified regulatory elements found both upstream and downstream of the Ren1c gene. First, an SD/SA element and GFP gene cassette were inserted into the pBV1.IRES.LacZ.PA vector to replace the IRES and lacZ gene using the BamHI restriction sites. Next, a 550 bp fragment containing the Ren-1c promoter sequence was amplified using the primers (HindIII restriction site sequence underlined) Hom-1, 5'-GCGGAAGCTTACCTAGCTTG-GTCTCACAGTCAAAG-3' and Hom-2, 5'-GCGGAAGCTTG-GCTTTCTAAGAGCTGTGTGCCCAG-3', and the fragment was then inserted, using HindIII, upstream of the SD/SA-GFP-PA cassette. A 600 bp fragment containing the first intron of Ren-1c was then amplified using the primers (XhoI restriction site sequence underlined) Hom-3, 5'-GCGGTCTAGATAGACATTAAGTTTTCCTTG-3' and Hom-4, 5'-GCGGTCTAGATCCAATCTTAACTGAAGCCACCAACAAGC-3'. The amplicon was then inserted into the pBV vector downstream of the SD/SA-GFP-PA cassette using XhoI. The combination cassette was then excised by SalI digestion and inserted into the SalI site of the psV1.RecA shuttle vector (24).

The shuttle vector was transformed into the RP23-88k07 BAC containing DH10B bacteria, and selection for resolved BACs (RenGFP) containing the integrated GFP cassette downstream of the renin gene was performed. The BAC construct was then amplified and the resulting BACs were digested with XhoI and SalI. The fragments were then ligated back together using T4 DNA ligase to generate the final BAC construct. This process was repeated three times to ensure that the GFP cassette was stably integrated into the BAC construct. The final BAC construct was then used to transfect NIH 3T3 cells, and the resulting transfectants were screened for GFP expression.

**Fig. 1.** Generation of bacterial artificial chromosome (BAC) green fluorescent protein (GFP) constructs. A: schematic of the RP23-88k07 BAC containing the centrally located renin gene. The expanded view shows the 5' regulatory region of renin, as well as the insertion site of the GFP cassette. B: 5' flanking sequence of the renin gene showing the region deleted in the ΔEnh BAC and the mutated bases within the ΔHP BAC.
Ren-1\textsuperscript{t} promoter was carried out using the homologous recombination methodologies of Yang et al. (24). BACs containing the inserted GFP cassette were confirmed by both restriction analysis using pulsed field gel electrophoresis (PFGE) as well as sequencing through the integration site. The integrated GFP cassette contains two NotI restriction sites, which alter the restriction pattern of the RP23-88k07 BAC.

Deletion of the Enhancer within the RenGFP BAC

The Ren-1\textsuperscript{t} enhancer, containing the CRE, E-box, Ec, and Eb sites, was deleted in the modified RenGFP BAC using the Yang method (24) (Fig. 1B). We first modified the pBV1.IRES.LacZ.PA vector by excising the IRES.LacZ using BamHI; the vector was then reannealed at the BamHI site. A homology arm upstream of the region to be deleted was PCR amplified with the primers (HindIII restriction site sequence underlined), Enh-1, 5\textsuperscript{\prime}-GCCGAGGTTTCCCCCAGATGATCTTCATCTG-GAACAG-3\textsuperscript{\prime} and Enh-2, 5\textsuperscript{\prime}-GCCGGAAGTTTGAGGATTACAGAAGGAACAGGGTG-3\textsuperscript{\prime} and subsequently cloned into the modified pBV1 vector. Next, a homology arm downstream of the region to be deleted was amplified with the primers (XbaI restriction sequence underlined), Enh-3, 5\textsuperscript{\prime}-GCCGCTCTAGAGGTTGTGATAGCGCATGCCCA-TGTTGGAAG-3\textsuperscript{\prime} and Enh-4, 5\textsuperscript{\prime}-GCCGCTCTAGATATAGGTTCCTTGGAGGAG-3\textsuperscript{\prime}. The amplicon was then cloned using XbaI and into the modified pBV1 vector, which also contains the upstream homology arm. The recombination cassette was then excised with SalI and cloned into the SalI site within the shuttle vector pSV1.RecA. The shuttle vector was transformed into the RenGFP containing DH10B bacteria, and selection for resolved BACs was carried out by the Heintz method of homologous recombination.

Confirmation of the correctly deleted enhancer sequence within the RenGFP BAC was validated by PFGE as well as by sequencing of the enhancer region.

Mutation of the Hox/Pbx sites within the RenGFP BAC

To mutate the HOX/PBX sites, the pBV vector with the upstream 550 bp fragment containing the Ren-1\textsuperscript{t} promoter, the SD/SA.GFP.PA, and the downstream region with PacI restriction sites, which alter the restriction pattern of the RP23-88k07 BAC, was used as the template in a Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primers were designed that incorporated the two base mutations in the HOX/PBX site: mHPUP (HOX/PBX site underlined) 5\textsuperscript{\prime}-CCCACAGGCCCTGGGGTAA-3\textsuperscript{\prime} and mHPLW 5\textsuperscript{\prime}-GGCTCTGCTTTTT-3\textsuperscript{\prime}); 245ANALYSIS OF THE RENIN REGULATORY REGION by 10.220.33.5 on May 30, 2017 http://physiolgenomics.physiology.org/ Downloaded from
upper transgenic band and a lower endogenous band. ImageQuant software (Molecular Diagnostics) was used to calculate copy number for each of the transgenic lines using the band from the endogenous renin loci to normalize copy number. Briefly, the software measured band intensities of the transgene insertion, as well as the endogenous renin loci; and we then divided the transgenic band intensity by the endogenous band to extrapolate the transgene copy number. The ΔEnh transgenic line showed two distinct transgenic bands on the quantitative Southern; therefore, the bands intensities were added and then divided by the endogenous signal to determine copy number. The presence of two bands could be due to partial insertion of a BAC or different insertion points within the genome. The RenGFP (1 copy) BAC line has a single copy of the transgene, and the RenGFP (3-4 copy) contains 3-4 copies of the transgene. The ΔEnh transgenic line analyzed contained five copies of the transgene, while the ΔHP line was determined to have a single copy of the transgene.

Husbandry and Production of Transgenic Animals

All mouse protocols were reviewed and approved by the Roswell Park Cancer Institute’s (RPCI) Animal Care and Use Committee (IACUC). Animals were obtained from in-house breeding programs and maintained on a 14:10-h light-dark cycle in the Department of Laboratory Animal Research facilities at RPCI. Mice were provided with standard laboratory chow and water ad libitum. Transgenic mice were produced by pronuclear microinjection of the above constructs according to established methodologies by the RPCI Gene Targeting and Transgenic Core Facility. In brief, fertilized F2 oocytes were derived from an F1 × F1 cross of (C57Bl/10Ros-p/S × C3H/HeRos) F1 mice after superovulation of immature females (3-4 wk of age). Eggs were collected, microinjected, and reimplanted in SW (CD-1) experienced pseudopregnant female mice. Multiple positive founder animals from each BAC transgenic were identified by detection of GFP by PCR assay and analyzed for GFP expression.

Genotyping. DNA for genotyping was prepared from tail biopsies. Primers to detect GFP were located within the GFP structural sequence. These were 5'-agcaagggcagaaagctactg-3' and 5'-ggagctcaggaagtgctgtg-3'.

Both juvenile (11- to 12-day-old) and adult (6–8 wk) mice treated with captopril were given intraperitoneal injections of 10 mg/kg for 3 days and then killed on the fourth day within the policies of IACUC.

In Situ Visualization of GFP

Whole mount fetal embryonic day (e) 16.5 kidney, e13.5–e16.5 adrenal gland, and e14–e16.5 gonadal artery or squashes (tissue flattened between two slides) of whole postnatal (juvenile and adult) kidneys and submandibular glands (SMG) were prepared. Fluorescent stereomicroscopy was performed (model ZX12, Olympus) with mercury bulb illumination through an MGFP excitation/emission filter (Olympus). Images were captured with a Spot RT digital camera and software (Diagnostic Instruments) at identical exposures.

Histology

Indirect immunofluorescence. Rabbit anti-GFP (ab290) was obtained from Abcam Laboratories (Cambridge, MA). Goat anti-renin serum was the kind gift of C. M. Wilson (Southwestern Medical Center, Dallas, TX) and has been extensively characterized (6). Donkey anti-goat Alexafluor 594 (cat. # A11058) and donkey anti-rabbit Alexafluor 488 (cat. # A21206) were obtained from Molecular Probes (Eugene, OR).

Freshly isolated tissues were immersed in 2% buffered formalin containing 20% sucrose overnight or until they sank. These were embedded in optimum cutting temperature compound (O.C.T. compound, Tissue-Tek, Sakura) in a foil mold and snap-frozen. Frozen sections were cut at 5–6 μm and lifted onto silane-coated slides (EM). Before immuno-staining, slides were air dried at room temperature (RT) and immersed in acetone for 2 min and again air-dried. Sections were permeabilized with 0.1% Tween 20 in PBS, rinsed in PBS, and blocked with 0.025% Tween 20–1% normal donkey serum-PBS. Primary antibodies were diluted in blocking buffer, applied to the sections, and incubated at RT for 1 h. Sections were washed two times in PBS for 5 min. Secondary antibody was diluted in blocking buffer, applied to sections, and incubated at RT for 30 min. Sections were again washed two times as above, mounted with Vectashield (Vector Laboratories), and viewed. Images were captured with a Nikon E-300 compound microscope with an X-cite 120 (EXFO) light source for illumination using a Spot RT digital camera and software (Diagnostic Instruments).

Protein Isolation and GFP ELISA

A GFP ELISA kit (Cell Biolabs, San Diego, CA) was used to quantitate levels of GFP in transgenic kidneys. Kidneys from both captopril-treated and nontreated 6- to 8-wk-old male adults were bisected first by cross-section and then again longitudinally to create four morphologically similar pieces. One piece was weighed from each kidney, coarsely minced, and suspended in buffered detergent (50 mM Tris, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM PMSF) at 2 microliters buffered detergent per milligram tissue. To this was added a cocktail of protease inhibitors (aprotinin, leupeptin, pepstatin) to a final concentration of 1 microgram/ml each. Each suspension was sonicated 3–4 times on ice until no visible tissue particles were evident. Kidney suspensions were then microcentrifuged at 15,000 g for 15 min at 4°C, and the supernatant was stored at −80°C until time of assay. Upon thawing, protein was adjusted to 6.5 mg/ml after performing a DC Protein Assay (Bio-Rad, Hercules, CA), and 100 microliters were used per assay. Data were reported as an average of duplicate samples in picograms GFP/microgram of total protein with calculated standard error.

RESULTS

Mutation of Renin Enhancer Within BAC Transgenic

We have previously identified, using the As4.1 cell line, a 242 bp renin enhancer (located at −2866 to −2625 relative to the transcription start site), which is responsible for activating transcription >50-fold in combination with the renin proximal promoter (17). To study the effect of the enhancer on renin expression in vivo a deletion of key elements of the enhancer, which correspond to the CRE, E-box, Ec, and Eb sites, was made in the RenGFP BAC (Fig. 1B). Transgenic animals were made with this construct (designated as ΔEnh), and GFP expression was analyzed at different developmental stages. To confirm accurate reporting of renin expression, we colocalized expression of renin and GFP using immunohistochemistry within the kidney and SMG of the transgenic lines analyzed. As can be seen in Fig. 3, A–C, renin and GFP were expressed in the same cells in kidney of adult mice from the ΔEnh transgenic line. GFP expression within the ΔEnh and RenGFP (1 copy) adult kidneys was at reduced levels compared with the RenGFP (3-4 copy) adult kidney (result discussed below), but for the purpose of confirming accurate colocalization, regions with high GFP expression within the ΔEnh and RenGFP (1 copy) adult kidneys were used for assessment by immunohistochemistry. The RenGFP (1 copy) (Fig. 3, G–I) and the RenGFP (3-4 copy) (Fig. 3, J–L) also showed accurate reporter of the transgene within the RenGFP (1 copy) (Fig. 4, G–I) as well as the RenGFP (3-4 copy) (Fig. 4, J–L).
Gonadal artery has been previously shown to express GFP at e14–e17 of plasmid-based renin GFP transgenic animals (5). In the current study both the RenGFP (1 copy) and RenGFP (3-4 copy) also showed gonadal artery GFP expression (data not shown); however, the ΔEnh line showed no detectable GFP expression within the e14–e16.5 gonadal artery.

Kidneys and adrenal glands from e16.5 ΔEnh mice did not show GFP expression (data not shown), contrary to what is seen in the RenGFP (3-4 copy) transgenic line where strong GFP expression is visualized in the primary and secondary vasculature of the developing kidney, as well as in the adrenal gland parenchyma (Fig. 5A). The RenGFP (1 copy) transgenic line also showed a GFP expression pattern similar to the multiple copy construct; however, the intensity of GFP expression was proportionally weaker (Fig. 5B).

Eleven- to twelve-day-old juvenile animals were treated with the ACE inhibitor captopril, which has been shown to both enhance the intensity of renin production per cell and recruit additional cells to express renin. Kidney squashes from both captopril- and noncaptopril-treated animals were analyzed for GFP expression. Kidneys from the RenGFP (3-4 copy) mice showed strong expression of GFP in the afferent arterioles and JG cells (Fig. 6D). Relative to the untreated kidneys, captopril treatment was associated with an increased level of expression within the JG cells as well as recruitment of additional cells along the vascular network (Fig. 6H). At this stage GFP expression was evident, but at a considerably lower level, in both the RenGFP (1 copy) (Fig. 6C) and the ΔEnh (Fig. 6A) juvenile kidney compared with the RenGFP (3-4 copy) line. Captopril was shown to induce the level of expression within the JG cells as well as recruit additional cells along the vascular network within the RenGFP (1 copy) (Fig. 6G); however, GFP expression was not as strong as what was seen in the RenGFP (3-4 copy) (Fig. 6H). No additional expression of GFP in the juvenile kidney was evident upon captopril treatment of the ΔEnh line using fluorescence stereomicroscopy (Fig. 6E). To confirm the proper function of captopril, control stainings for renin were carried out using immunohistochemistry on the transgenic lines analyzed with captopril treatment and without (Supplemental Fig. S11). Induction of endogenous renin

was evident in all lines studied, confirming efficient ACE inhibition.

To assess whether the enhancer is critical for in vivo renal renin expression in adult kidney, 6- to 8-wk-old adult ΔEnh transgenic animals were treated with captopril, and kidneys from both captopril-treated and nontreated animals were analyzed and compared with the wild-type RenGFP lines. Kidney squashes from the RenGFP (3-4 copy) showed GFP expression with a strong recruitment and enhancement of expression when treated with captopril (Fig. 7, D and H). Kidneys from the RenGFP (1 copy) had a significantly lower GFP expression compared with the multiple-copy wild-type

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<table>
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<tr>
<th>ΔEnh</th>
<th>∆HP</th>
<th>RenGFP (1 copy)</th>
<th>RenGFP (3-4 copy)</th>
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Fig. 5. Fluorescent GFP image of an embryonic day (e) 16.5 kidney (K) and adrenal gland (Ad) from the RenGFP (3-4 copy) (A) and RenGFP (1 copy) (B) BAC animals. Primary, secondary, and tertiary vasculature branching of the kidney is visualized, as well as GFP expression in the adrenal gland.
line (Fig. 7C); however, a strong induction of GFP was evident upon treatment with captopril (Fig. 7G). Kidneys from the ΔEnh BAC line had very low GFP expression in a more limited number of JG cells, and no induction or recruitment was evident when we analyzed kidneys from captopril-treated ΔEnh BAC animals (Fig. 7, A and E) with fluorescence stereomicroscopy.

To increase the sensitivity of analyzing GFP protein present within the captopril-treated and nontreated adult kidneys of the transgenic lines studied, a GFP ELISA was performed (Table 1). Captopril treatment elicited a 9.1-fold and 13.7-fold increase in GFP protein in the RenGFP (3-4 copy) and RenGFP (1 copy) transgenic lines, respectively. The ΔEnh kidney showed a lesser, but evident, 4.7-fold induction of GFP protein when treated with captopril. Although a greater fold induction was seen in the RenGFP (1 copy) kidney compared with the RenGFP (3-4 copy) and ΔEnh kidneys, both the RenGFP (3-4 copy) and the ΔEnh in fact had a higher level of GFP protein present in the captopril-treated and nontreated kidney than the single copy line (Table 1).

Adult SMG from the ΔEnh transgenic line were analyzed for GFP expression. The SMG from the ΔEnh did not exhibit any discernable GFP expression (Fig. 8A), whereas GFP expression was visualized within the RenGFP (1 copy) (Fig. 8C) and RenGFP (3-4 copy) (Fig. 8D) SMGs. Evaluation by fluorescence stereomicroscopy of GFP expression levels in tissues for the various transgenic lines are summarized in Supplemental Table S1.

**Mutation of HOX/PBX Binding Site Within BAC Transgenic**

Renin expression in the As4.1 cell line is dependent on both the PPE as well as the enhancer (11, 15). The PPE has been shown to be a HOX-PBX DNA recognition element (16), preferentially binding Pbx1b and Hox Abd-B paralogs. To study the effect of the HOX-PBX binding site on renin expression in vivo, mutations of two critical bases within the site were generated within the RenGFP BAC (Fig. 1B).

To confirm accurate reporting of renin expression by the ΔHP construct we colocalized expression of renin and GFP using immunohistochemistry within the SMG (Fig. 4, D–F) and kidney (Fig. 3, D–F). GFP and renin were present within the same granular convoluted tubule cell population of the SMG; however, no GFP expression was detectable in the ΔEnh kidney by immunohistochemical methods. The RenGFP (1 copy) (fig. 4 G–I) and the RenGFP (3-4 copy) (Fig. 4, J–L) also showed accurate reporting of the transgene with renin in the SMG.

The ΔHP transgenic line was analyzed for GFP expression within the kidney at different developmental stages. In contrast to the RenGFP mice, kidneys of the ΔHP transgenic animal showed no GFP expression at e16.5 (data not shown), nor in 11- to 12-day-old juvenile s (both captopril treated and nontreated; Fig. 6, B and F), nor in 6- to 8-wk-old adults (captopril and noncaptopril treated, Fig. 7, B and F). The images captured under the fluorescence stereomicroscope show a green autofluorescence found in both GFP-expressing tissue as well as negative tissue (as seen in the ΔHP kidney). This “background” is observed throughout the entire mouse for both GFP-positive and -negative littermates and is not indicative of GFP signal. Using a GFP ELISA, we observed a 4.4-fold induction of GFP protein levels of the ΔHP adult kidney by captopril. However, the levels of GFP protein present in the ΔHP captopril-treated and nontreated kidneys were extremely low compared with the levels determined in the RenGFP transgenic lines (Table 1).
addition, there was also no visible GFP expression by fluorescence stereomicroscopy within the e14–e16.5 gonadal artery as well as in the e13.5–e16.5 adrenal gland of the \( \Delta \text{HP} \) animal (data not shown). In contrast, using fluorescence stereomicroscopy we saw strong GFP expression in the granular convoluted tubule cells of SMG from the adult \( \Delta \text{HP} \) transgenic animals (Fig. 8B).

**DISCUSSION**

Previous in vitro studies, using the renin-expressing As4.1 cell line, have documented the importance of both the renin enhancer and PPE for the expression of renin (17). In this study, a GFP reporter under the control of the \( \text{Ren1c} \) gene centrally located within a BAC was used to make transgenic lines to analyze the roles of the enhancer and Hox/Pbx site (PPE) in vivo. Here it is shown that the enhancer and the PPE are both critical for basal expression of renin within the kidney. The two elements are dependent upon one another to activate renin expression, and when one or the other is mutated there is a loss of this synergistic effect causing a decrease in reporter gene expression.

Pan and Gross (14) have recently reviewed a large body of studies detailing the critical elements necessary for activity of the 242 bp enhancer (−2866 to −2625 relative to the CAP site) of the \( \text{Ren1c} \) gene in vitro. In the \( \Delta \text{Enh} \) transgenic animal the CRE, E-Box, Ec, Eb, and Ea sites were deleted, and their combined effects on GFP expression were analyzed. GFP expression was not detected in the embryonic kidney, adrenal gland, or gonadal artery (data not shown) of the \( \Delta \text{Enh} \) animals, whereas strong GFP signal can be detected in the vascular network of the kidney as well as in the adrenal gland (Fig. 5A) and gonadal artery (data not shown) in the wild-type RenGFP (3-4 copy) animal, as well as, to a lesser extent, in the RenGFP (1 copy) animal at this developmental stage (Fig. 5B). These data support the critical role played by the renin enhancer in the developing kidney, adrenal gland, and gonadal artery. Furthermore, GFP expression within the juvenile and adult kidneys of the \( \Delta \text{Enh} \) animals was greatly reduced compared with kidneys from the RenGFP (3-4 copy) animal. However, GFP expression levels within the RenGFP (1 copy) juvenile and adult kidneys appeared similar to those of the \( \Delta \text{Enh} \) by fluorescence stereomicroscopy. Although it would be expected that the \( \Delta \text{Enh} \) would have a decreased level of GFP expression compared with the wild-type RenGFP (1 copy), the level of GFP protein measured within the \( \Delta \text{Enh} \) adult kidney by ELISA was shown to be greater in both the captopril-treated and nontreated animal compared with the RenGFP (1 copy) line (Table 1). The facts that the \( \Delta \text{Enh} \) animal contains five copies of the transgene compared with the single copy of the RenGFP (1 copy) and that BAC copy number has been shown to be correlated to an

Table 1. Quantitation of renal GFP from adult transgenic animals

<table>
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<tr>
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<th>Captopril Treatment, pg GFP/μg Protein</th>
<th>No Captopril Treatment, pg GFP/μg Protein</th>
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<tr>
<td>RenGFP (1 Copy)</td>
<td>6.07±0.08</td>
<td>0.443±0.007</td>
<td>13.7</td>
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<td>RenGFP (3-4 Copy)</td>
<td>57.5±2.0</td>
<td>6.34±0.07</td>
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<td>( \Delta \text{Enh} ) (5 Copy)</td>
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<td>( \Delta \text{HP} ) (1 Copy)</td>
<td>0.122±0.001</td>
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GFP, green fluorescent protein.
increase in expression level of the transgene (2) could explain why the GFP expression is higher within the ΔEnh kidney.

When the different transgenic lines were treated with captopril, a large induction and recruitment of GFP-expressing cells were seen within both of the RenGFP transgenic lines, but no detectable induction or recruitment of GFP was visualized in the ΔEnh mice by fluorescent stereomicroscopy. From measurement of GFP protein levels within the kidney of the ΔEnh by ELISA, it is clear that there is a 4.7-fold induction with captopril treatment (Table 1). This induction, however, is not as strong as the 13.7-fold and 9.1-fold inductions seen in the RenGFP (1 copy) and RenGFP (3-4 copy), respectively. The RenGFP (1 copy) shows a higher fold increase in GFP protein levels with captopril treatment than the RenGFP (3-4 copy), although the level of protein in the multiple copy transgenic line is higher. This could be due to the fact that the RenGFP (3-4 copy) transgenic line is at a saturation point of recruitment and induction because of its already high expression of the transgene, which could exhaust the molecular machinery required for expression, where as the lower-expressing RenGFP (1 copy) transgenic line may have a greater ability to induce expression and recruit cells because it is starting at a lower level of initial GFP expression.

Interestingly, there was no detectable GFP expression within the adult SMG of the ΔEnh transgenic animal. The importance of the renin enhancer for baseline expression of renin within the kidney, adrenal gland, gonadal artery, and SMG is validated from the analysis of the ΔEnh transgenic line.

A recent study by Markus et al. (8), who generated a knockout of the endogenous renin locus (REKO mouse), supports the notion that the enhancer is critical for renin expression in a spectrum of tissues. REKO mice analyzed for renin protein and mRNA showed significant decreases in the kidney, SMG, adrenal gland, and other tissues. Curiously, Markus et al. also observe that when renin protein and mRNA content were assayed at the level of the whole tissue for kidney, the reduction was considerably less than that observed specifically for JG cells. This apparent paradox may be a consequence of the fact that renin, the rate-limiting step for the RAS, appears to be under negative feedback regulation by ANG II signaling and that the kidney possesses a “reserve” group of cells that are recruited to produce and secrete renin when a physiological challenge is present. REKO mice exposed to a low NaCl diet also observe that when renin protein and mRNA content were assayed at the level of the whole tissue for kidney, the reduction was considerably less than that observed specifically for JG cells. This apparent paradox may be a consequence of the fact that renin, the rate-limiting step for the RAS, appears to be under negative feedback regulation by ANG II signaling and that the kidney possesses a “reserve” group of cells that are recruited to produce and secrete renin when a physiological challenge is present. REKO mice exposed to a low NaCl diet did not show a full response to this stimulus in tissues that are usually affected, indicating that one or more of the transcription factor binding sites within the enhancer could be involved in the response to potent in vivo stimulus (8). A potential explanation for the diminished response to the physiological stimulus could reflect the fact that the mutated enhancer has of itself compromised the promoter, reducing ANG II levels and therefore causing vascular recruitment of renin-expressing cells. This recruitment would in effect exhaust the ability of the kidney to produce additional renin in response to further physiological stimuli.

In the reporter format that we have utilized, ANG II signaling is not compromised and thus there is no stimulus to recruit expression of the reporter or the endogenous renin loci, unlike the situation in REKO mice. However, when the ΔEnh mouse is challenged with captopril, ANG II signaling is perturbed effectively mimicking the phenotype of the challenge seen in the REKO mouse. Even though we have not been able to clearly discern detectable induction of the ΔEnh reporter under captopril treatment using conventional fluorescent stereomicroscopy, we have shown a 4.7-fold induction of GFP protein levels when treating with the ACE inhibitor using a GFP ELISA (Table 1). The fact that the induction of the GFP protein is not visible by fluorescent microscopy but can be detected by the more sensitive GFP ELISA supports the notion that the enhancer, when abrogated, causes a drop in the efficiency of the cell to express the transgene. Therefore, when ANG II signaling is compromised by captopril, an induction of native JG cells, as well as a recruitment of cells expressing the ΔEnh reporter, is in fact present. However, the native and recruited cell population’s ability to efficiently produce the GFP protein is compromised by the loss of the enhancer function, which would normally be directing baseline expression. The decreased response of the transgene when treated with captopril in the ΔEnh kidney, compared with the induction seen in both wild-type lines, validates an important role of the enhancer in response to an in vivo stimulus.

Zhou et al. (27) used homologous recombination to delete the enhancer from a 160 kb P1 artificial chromosome (PAC) containing human renin and then made transgenic mice with this construct. Human renin mRNA levels were shown to decrease by 3- to 10-fold in the mutated enhancer PAC animals compared with mice containing the PAC with the enhancer intact. Captopril treatment of both the wild-type PAC animals (containing the enhancer) and the transgenic lines made with the mutated enhancer within the PAC induced both murine and human renin mRNA. These results, in contrast to those of

Fig. 8. Fluorescent GFP images of the SMG from 6- to 8-wk adult mice from the BAC transgenic animals. A: SMG from ΔEnh. B: SMG from ΔHP. C: SMG from RenGFP (1 copy). D: SMG from RenGFP (3-4 copy).
Markus et al. (8), demonstrate that sequences other than the enhancer may be responsible for regulated expression of human renin. Markus et al. have proposed that the difference between the conflicting results could have to do with either the human enhancer missing a 23-bp sequence at the 3' end of the enhancer, which contributes partially to a 100-fold higher renin expression and plasma level in mouse compared with human, or the possibility that mouse transcription factors do not carry out similar actions on the human renin promoter and enhancer as they do on their natural mouse DNA sequence. Zhou et al. (27) have raised the issue that in fact the REKO mouse used in the Markus et al. (8) studies not only has a deleted enhancer but is also missing 486 bp of flanking sequence, which may be critical for the lack of response to in vivo stimuli they are reporting. Our study has shown that the deleted enhancer indirectly affects the ability of the kidney to respond to captopril, where recruitment of cells to produce the transgene is present, but their ability, as well as the native JC cells’ ability, to efficiently express GFP is diminished due to the abrogated enhancer. A 4.7-fold induction of GFP expression in the adult kidney is measured by ELISA when the animal is treated with captopril. This is not as pronounced as a response as in the wild-type transgenic animals due to the fact that the renin-expressing cells, both JC and recruited, cannot express GFP efficiently due to the mutated enhancer. Therefore the enhancer is partially responsible for eliciting a diminished response to an in vivo stimulus such as captopril. However, since GFP induction is still present within the DeltaEnh when treated with captopril, other sequences, possibly in collaboration with the enhancer, must be responsible for the full response of renin expression to an in vivo stimulus.

Zhou et al. (27) have also shown that an analogous human renin kidney enhancer is required to maintain baseline renin expression in vivo, which is in agreement with what is observed in both the REKO mice as well as the DeltaEnh mice in this study, confirming the importance of the renin enhancer for baseline expression of renin within the kidney.

Expression of RenIc in As4.1 cells is critically dependent on not only the enhancer but also the PPE, which is located at approximately −60 relative to the transcription start site (11, 15). The PPE has been shown to be identical to a consensus AbdB HOX-PBX DNA recognition sequence (16). In vitro studies using the As4.1 cell line have shown that mutation of a single base in either the HOX or PBX half site dramatically reduces the transcriptional activity of RenIc, suggesting that both of the HOX and PBX sites are necessary for renin expression. To test the importance of the PPE in vivo we mutated the two critical bases found in the HOX-PBX binding sequence within the RenGFP BAC and made transgenic lines with the mutated construct. There was no detectable GFP expression in the embryonic adrenal gland or gonadal artery (data not shown). No GFP expression was visualized within the kidney at any developmental stage including e16.5, juvenile, and adult. Captopril treatment of the DeltaHP animals showed no visual induction of GFP within the kidney by fluorescence microscopy. However, a fourfold induction was measured by a GFP ELISA. Even though an induction was detected, the picogram amount of GFP protein in both the captopril-treated and nontreated adult kidneys was substantially lower than what was observed in the RenGFP (1 copy) wild-type animal. GFP expression was observed in the SMG of the adult DeltaHP male animal. Therefore, it can be concluded that the HOX-PBX site that comprises the PPE, while essential for renin expression within the kidney, is not apparently necessary for the expression of renin within the SMG.

There are 39 class I Hox genes found in higher vertebrates, which are organized in four clusters (A, B, C, D) on different chromosomes that arose by gene duplication. Members of a cluster are classified into 13 paralog groups based on sequence similarity and 5'-3' position within a cluster (18). Genes located more to the 3' in the cluster show the most anterior boundaries of expression, whereas more 5' localized genes exhibit more posterior boundaries of expression. Interestingly the HOX binding site within the PPE is a precise match to the preferred recognition sequence of the Abd-B class paralogs, which are 5' within the cluster and have more posterior expression boundaries. Pan et al. (16) have shown that the Hox Abd-B paralog classes 9 and 10 are part of the PPE specific binding complex. While it is conceivable that more anterior Hoxs might recognize the PPE at more anterior sites, the fact that the adenosines, which were disrupted by point mutations in each half site, are critical for all homeodomain binding, secures the notion that Hox or other homeodomain factors are not involved.

We favor an alternative explanation based on the fact that Field et al. (3) has demonstrated that within the SMG of the mouse there are renin transcripts that are initiated further upstream than the primary transcription start site that is found in the kidney as well as other tissues. The organization of the renin gene, containing multiple transcription start sites within the SMG, could allow renin to be differentially regulated by different intracellular signals or transcription factors (21). The current result demonstrating that the Hox/Pbx site is not required for expression of renin within the SMG shown in the present study supports the model that the presence of alternate transcriptional start sites within the renin gene could be responsible for regulating renin expression through different transcription factors within the SMG. Further analysis of the alternate transcriptional start sites and their surrounding regulatory regions is necessary to fully grasp the regulatory mechanisms acting on the control of renin expression within the SMG.

In this study, the importance of the renin enhancer on the baseline expression of renin in multiple tissues has been validated. The enhancer has also been shown to be partially responsible for a diminished response to an in vivo stimulus such as captopril. We have also shown the HOX-PBX site is necessary for cell specificity in the kidney but is not needed in SMG.

NOTE ADDED IN PROOF

While this manuscript was being reviewed, the following article was published: Tanimoto K, Sugiuira A, Kanafusa S, Saito T, Masui N, Yanai K, Fukamizu A. A single nucleotide mutation in the mouse renin promoter disrupts blood pressure regulation. J Clin Invest 118: 1006–1016, 2008. Tanimoto et al. generated a transgenic mouse containing a 155 kb Ren-1' BAC in which the wild-type or mutated form of the Ren-1' promoter could be excised using a Cre/loxP-mediated system. The design of this experiment is compelling because the integration of the Ren-1'-containing BAC, including both the wild-type promoter and the mutated promoter within the genome, is the same and the Cre-mediated excision of the
wild-type or mutated promoter generates two different mouse lines. The mutation made within the RP-2/PPE is identical to the base change we created in the HOX site within our experiments. However, we also mutated a single base within the PBX site within the same construct. From their experiments Tanimoto et al. showed that the RP-2/PPE is absolutely necessary for basal promoter activity of the Ren-I within the kidney. Also within their work they have shown that the RP-2/PPE mutated transgenic mouse maintains normal extra-renal Ren-I gene expression. The results reported by Tanimoto et al., that the RP-2/PPE is essential for kidney-specific renin expression but not extrarenal expression of renin in the SMG, are in parallel to what we report in this article.

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