Expression of a renin/GFP transgene in mouse embryonic, extra-embryonic, and adult tissues

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Abstract

Jones, C. A., M. I. Hurley, T. A. Black, C. M. Kane, L. Pan, S. C. Pruitt, and K. W. Gross. Expression of a renin/ GFP transgene in mouse embryonic, extra-embryonic, and adult tissues. Physiol Genomics 4: 75–81, 2000.—A reporter construct was assembled with 4-kb of renin 5′-flanking sequence fused to humanized green fluorescent protein (GFP) cDNA. Transgenic mice carrying this construct were produced and assayed for GFP expression. In the adult, expression was detected in juxtaglomerular (JG) cells of the kidney and granular convoluted tubular cells of the submandibular gland. Furthermore, treatment of mice with captopril induced GFP expression in renal vascular smooth muscle cells. During embryogenesis, GFP expression was first detected at embryonic day E13 in the adrenal gland and Wolffian duct. Expression was also seen in the developing renal vasculature as early as E14 and remained detectable through birth. Renal GFP expression became restricted to JG cells in adults. Fetal adrenal and gonadal arteries also expressed GFP. In the placenta, GFP was observed in giant cell trophoblasts, consistent with reports of renin expression in chorionic cells of both humans and mice. We conclude that 4 kb of renin 5′ flank is sufficient to direct multiple known renin expression patterns. Furthermore, the renin-GFP construct characterized here will provide a useful vital reporter for renin expression.

Renin is an aspartyl protease most commonly recognized for its systemic role in regulation of arterial pressure and electrolyte balance through its participation in the renin-angiotensin system (RAS). Under normal physiological conditions, circulating active renin is processed and secreted from juxtaglomerular (JG) cells within the adult kidney. In mice, several other adult tissues also express detectable levels of renin mRNA including the gonads, female adrenal gland, male submandibular gland, and placenta. These tissues exhibit complex renin gene-specific hormonal and developmental expression patterns (for reviews see Refs. 15 and 27).

JG cells are located at the distal end of afferent arterioles, proximal to the glomerulus, where they are in a unique position to sense fluctuations in humoral factors and systolic pressure. This localization is the end result of progressive restriction of renin-expressing cells along the intrarenal arteries during development (9, 16). In mice, renin expression is detected by in situ hybridization at embryonic day E14 in the earliest observable intrarenal arteries and is associated with the newly formed branches of the renal arterial tree (24). However, as renal vascular smooth muscle cells differentiate during organogenesis and then postnataally, they stop producing renin until only JG cells, a modified smooth muscle cell, is observed to express renin. That renin expression is functionally important during kidney organogenesis is supported by the observations that pharmacologic and genetic disruptions of RAS components result in aberrant renal morphology (7, 20, 34). In addition, other reported fetal sites of renin expression are adrenal gland, the testes (16), and gonadal arteries (18), as well as placental tissue (22, 25).

Under pathophysiological conditions such as stenosis of the ureter or renal artery, reactivation of renin expression occurs in a subset of medial cells of larger renal arteries (4, 5, 17, 23). A similar reactivation is produced by pharmacologic or genetic disruption of RAS function. Additionally, renin-positive cells have been found associated with renal vascular adventitia in angiotensin converting enzyme (ACE) knockout mice (12).

Although vertebrates usually have a single renin gene, some strains of mice contain a duplicated renin locus designated as Ren-2, which is closely linked to the Ren-1 locus (1, 2). Strains with both loci exhibit approximately equal steady-state levels of Ren-1 and Ren-2 mRNA in the kidney. However, specific differences exist in the relative levels of these two gene products in extrarenal tissues (reviewed in Ref. 15). Genetic and transgenic analyses indicate these differences appear to be mediated by cis-acting sequences located close to the structural gene (reviewed in Ref. 26).

We have previously constructed reporter transgenes consisting of SV40 large T antigen (TAg) fused to 5′ flanking sequence from Ren loci. While mice expressing
a Ren-1/TAg transgene were not viable, mice expressing high levels of transgene with the Ren-2 construct were obtained (29). Using these mice, a cell line capable of expressing its endogenous renin gene (As4.1) was developed by transgene targeted oncogenesis (28). These cells have been used to identify sequences required for directing renin expression (21). Reporter constructs utilizing roughly 4 kb of Ren-1 5’ flanking sequence were found to confer high-level cell-specific expression in this cell line in contrast to noncognate cell lines. To test whether this flanking sequence was sufficient to confer high-level cell-specific expression to appropriate tissues in situ, this region was fused to green fluorescent protein (GFP). In this report we demonstrate expression of this construct in As4.1 cells as well as fetal, placental, and adult transgenic tissues.

MATERIALS AND METHODS

DNA construct. The Ren-1/GFP construct (Fig. 1A) was assembled in a three step process. First, renin promoter region was isolated as an Xho I fragment from p-117CAT (21) and cloned into the Xho I site in pGEM4Z. Next, a fragment spanning −4100 to −117 was isolated by BamH I digestion from pRI-4.1CAT (21) and was cloned into the BamH I site of promoter-pGEM4Z. Last, an Xho I-Sal I fragment was isolated from pTRUF5, a gift from N. Mucyczka (35). This fragment contained a humanized GFP structural sequence flanked by an SV40 splice donor-splice acceptor and an SV40 poly-A site and was cloned into the Xho I site of the second intermediate construct above. The structure of the construct was confirmed by sequencing.

Animals. All manipulations of mice were done in accordance with policies of the Institute Animal Care and Use Committee. Animals were obtained from in-house breeding programs and maintained on a 12:12-h light-dark cycle. Mice were provided standard laboratory chow and water ad libitum. Captopril treatment was according to established protocol and consisted of substituting 0.5 mg/ml captopril in water for 1 wk of ad libitum consumption instead of water (30). Transgenic mice were produced by zygotic microinjection of the construct according to established methodologies (13).

Recipient BCF₂ zygotes were derived from a cross of C57Bl/10Ros-p²d × C3H/HeRos F1 mice after super-ovulation of immature females (3–5 wk of age). Eggs were collected, microinjected, and reimplanted into Ha/ICR experienced female mice. Positive animals were identified by PCR assay. Females for timed pregnancies were checked for vaginal plugs the morning after potential copulations. Evidence of plugs was designated as day E0.

Polymerase chain reaction. The primers used to screen DNA from tail biopsies for the presence of GFP were located within the GFP structural sequence. The upper primer was (5’ ageaaggcgaggaactgtaactg 3’), and the lower primer was (5’ ggtgccaggttaagttgttctg 3’). The resulting amplification product was 594 bp.

Cell culture and transient transfection. The renin-expressing As4.1 cell line (ATCC no. CRL2193) was previously established in this laboratory from a kidney tumor of a transgenic mouse harboring a Ren-2 5’ flank/SV40 T antigen hybrid transgene (28). It expresses high levels of the endogenous renin gene, Ren-1. As4.1 cells were propagated in DMEM containing 10% FBS. At 72 h prior to transfection, As4.1 cells were subcultured into Opti-MEM reduced serum media supplemented with 2% FBS and 1 mg/ml ALBUMAX II. The switch of media from DMEM + 10% FBS to the supplemented reduced serum media is optimal for cell attachment post-electroporation and expression of transfected constructs in the As4.1 cells. Cells were transfected by electroporation using a BRL Cell Purator and BRL electroporation chambers (0.4 cm electrode spacing). Conditions for electroporation were as follows: 2 × 10⁷ cells were resuspended in 1.0 ml of 1× HeBS (25 mM HEPES, pH 7.05, 140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, and 6 mM glucose) with total plasmid DNA concentration of ~ 25–50 µg/ml, plus 250 µg/ml of sonicated salmon sperm DNA added as carrier. The cells were then exposed to a single electric impulse of 300 V at a capacitance setting of 1,180 μF and subsequently transferred to dishes containing supplemented reduced serum media plus penicillin (100 U/ml) and streptomycin (100 µg/ml).

Microscopy. Confocal microscopy was performed at the Microscopy Facility at Roswell Park Cancer Institute using a confocal scanning microscope (Bio-Rad model MRC 600) with

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an argon laser source to generate excitation at 488 nm and observed emission at 520 nm.

Fluorescent stereomicroscopy (FSM) was performed (model SZX12, Olympus) with a zoom range of 8.4–108. Illumination was provided by a lamp housing (model U-ULH, Olympus) with a 100-W mercury bulb. GFP was visualized through an MGFP excitation/emission filter (Olympus). Images were captured with a Spot RT digital camera and software (Diagnostic Instruments).

RESULTS AND DISCUSSION

We have previously shown that sequences residing within 4 kb of Ren-1 5′ flanking sequence could confer high-level cell-specific expression in As4.1 cells in contrast to nonexpressing cells. For example, in vitro cellular transfection demonstrated that a distal enhancer element (located −2866 to −2625 from the transcription start site) working in conjunction with a proximal promoter element (located around −60) results in high-level cell-specific expression of renin/chloramphenicol acetyltransferase (CAT) or renin/luciferase reporter constructs. These assays also suggest a negative regulatory element(s) resides between the promoter and enhancer (3, 21).

Initially, we sought to determine whether this region, when linked to GFP coding sequence, would direct detectable levels of fluorescence in As4.1 cells. As can be seen in Fig. 1, b and c, As4.1 showed robust expression when transiently transfected with this construct. This assay affirmed that the flanking sequence was functional with the GFP sequence and might be capable of driving high-level, cell-specific expression as a transgene in the whole organism. Therefore, transgenic mice were generated with this reporter construct and screened for fluorescence.

Adult tissues. Since kidneys are the primary source of active, circulating renin, adult kidneys were exam-

Fig. 2. Detection of GFP signal in adult tissues. Micrographs of wide-field (a) and confocal (b) epifluorescent illuminations of +/- Ren-1/GFP kidneys from untreated mice, and micrographs of wide-field (c) and confocal (d) epifluorescent illuminations, respectively, of +/- Ren-1/GFP kidneys from mice treated with captopril. Note that the epithelioid juxtaglomerular cells appeared more fluorescent than the ringlike morphology of vascular smooth muscle cells induced to express GFP. Submandibular gland (SMG) and sublingual gland (SL) wide-field micrographs with bright-field (e) and epifluorescent (f) illuminations. The notation “+/−” indicates one copy of the transgene is present, whereas “−/−” denotes no transgene is present.

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ined. Detectable GFP fluorescence was evident at JG sites (Fig. 2, a and b). This is consistent with the distribution of renin expression as detected by either immunohistochemical or in situ hybridization assays (31).

The ability of the Ren-1/GFP transgene to respond to physiological induction was assessed by treatment of the mice with captopril. Captopril is an ACE inhibitor, which has been previously shown to induce expression of renin in renal vascular smooth muscle cells. Captopril treatment resulted in widespread and intense induction of GFP expression along afferent, interlobular, and some arcuate arteries (Fig. 2, c and d), consistent with the effect of ACE inhibitors on endogenous mouse and rat renin expression (8, 27).

Submandibular gland (SMG) is also a known site of renin expression in mice (32, 33). This expression is androgen responsive and is easily detected in male SMG but not in the adjacent sublingual glands. This was also the case for Ren-1/GFP, as can be seen in Figs. 2, e and f, where GFP was detected in the granular convoluted tubules of the SMG. The different levels of GFP signal evident for male and female SMGs (data not shown) are consistent with the sexual dimorphism observed for endogenous renin expression (6).

Although a comprehensive survey of adult tissues was performed, no definitive fluorescent signal was detected at any site other than the kidney and SMG. Sites surveyed included, but were not limited to: adrenal gland, gonad, striated muscle, cardiac muscle, bladder, brain, selected nonrenal vasculature, intestine, lung, stomach, and liver. Despite careful examination, we could not detect fluorescence in Leydig cells of fetal testes, a known renin-expressing site. This is not necessarily unexpected in this tissue or other tissues with low per-cell renin expression. Detection of scattered renin-positive Leydig cells in testes required long exposures using in situ hybridization assays with radiolabeled probes (19). One possibility is that low transgene signal occurs which is not distinguishable above background noise.

**Fetal tissues.** The temporal pattern of renin expression in the developing renal vasculature in rats and mice is well documented (9, 16). To determine how closely expression of the Ren-1/GFP transgene mimics the pattern of expression for the endogenous gene, we followed renin-directed GFP signal throughout urogenital organogenesis. No GFP expression was seen when fetuses as early as day E12 were screened by FSM. However, GFP signal was observed in E13 fetuses. Brightly fluorescent cells were found in cords of the adrenal gland and in male mice in the remnant mesonephric duct (developing epididymis and vas deferens) connecting the testes to the urethra (Fig. 3a). No fluorescent signal was observed in the kidney or any other structure at this time point.

GFP expression in the kidney was first detected at E14 in cells of the renal and intrarenal arteries (Fig. 3b). Both brightly and weakly fluorescent cells were observed in the adrenal gland, some of which could still be observed arrayed in cords. Adrenal and gonadal arteries were faintly observable at this stage as well (Fig. 3, b and c).

Bright fluorescence in intrarenal vascular cells was first observed at day E15. Primary vessels branched from the hilus and radiated toward the cortical zone (Fig. 3d). These vessels did not reach to the periphery of the developing kidney. A few short, fluorescent, secondary vessels branched from the primary vessels. Both types of vessels contained cells of varying intensity. Most of the cells of these vessels had an epithelial-like morphology and fluoresced brightly except in areas proximal to the hilus, which were beginning to exhibit circumferential morphology typical of vascular smooth muscle cells. Regions of brightest fluorescence were frequently observed along the primary vessels at the junctions of secondary branch formation. In addition, fluorescent cells were detectable in the walls of the renal artery. The adrenal gland continued to express GFP, but with a smaller percentage of brightly fluorescent cells than seen at E14.

The next stage examined was day E16. The primary renal vessels had more and longer GFP-positive secondary branching vessels than observed at E15 and were beginning to develop tertiary branches (Fig. 3e). Otherwise, E16 kidney vasculature appeared similar to the previous stage. The renal artery was larger at this stage and had many positive cells in the wall. Male and female gonadal arteries continued to express GFP (data not shown). These brightly fluorescent cells were circumferentially arranged around the vessel and did not have the epithelial appearance of some of the intrarenal vascular cells. While cells at the end nearest the aortic branch point were brightly fluorescent, GFP expression became less frequent and of weaker intensity as the artery progressed toward the gonads. Adrenal glands were less fluorescent than at E15.

Continued growth of the kidney by day E17 made it necessary to prepare squashes of the kidneys to better reveal vascular structural features (Fig. 3, f and g). Secondary vessels extended through kidney segments and were now observed to be sprouting brightly fluorescent branches. Again, the brightest fluorescence was seen in cells with an epithelial morphology in the more distal branches, whereas circumferential ring morphology was more evident along the rest of the vessel walls. The renal artery was larger and branched at the hilus and exhibited many fluorescent cells. GFP
in adrenal arteries was still observable at this time point while the adrenal gland was negative. In addition, gonadal arteries along the posterior abdominal wall expressed GFP. These could be followed to ovaries with branching to the oviduct and to the testes with branching to the epididymis (Fig. 3, h and i).

In neonatal mice, GFP expression was not detectable in the renal arteries from hilus to aorta when observed in situ. However, small, positive collateral arteries could be observed entering the kidney around the hilus (Fig. 3j). Squash preparations of the kidneys revealed little if any fluorescence in the largest vasculature, although expression was detected in cells of other vessels when prepared by this method (Fig. 3k). Gonadal arteries were also positive at this time point, whereas GFP expression was not detectable in adrenal glands (data not shown).

We conclude that fetal expression of the Ren-1/GFP construct accurately followed the spatial and temporal expression pattern of endogenous renin, notably in the metanephric kidney vasculature, adrenal gland parenchyma, and gonadal arteries (9, 16, 18). As has been detailed in previous reports on endogenous murine renin genes, renin reporter expression was progressively restricted to more distal regions of the renal arterial tree during development. Likewise, transgene signal paralleled the rise and fall of Ren-1 mRNA in fetal adrenal gland (16). We also note Ren-1/GFP expression in the developing adrenal artery, which has not been previously reported to express renin.

**Extra-embryonic tissues.** Finally, we have found that Ren-1/GFP transgene is expressed in the placenta (E12, Fig. 3, l and o; and E17, Fig. 3, m and n; the earliest and latest stages examined). In these experiments, crosses were set up so that the dam was transgene negative and the sire was transgene positive. Thus positive placental cells must be of fetal origin. GFP observed in this tissue was present in a layer lying between the fetal and decidual components of the placenta. These cells continued over the edge of the placenta so that they could also be seen on the periphery of the umbilical side. Fluorescent cells were minimally 100 μm in diameter with nuclei at least 30 μm in diameter (data not shown). This cell morphology and location corresponds to the giant cell trophoblast layer and is consistent with identification of chorionic tissue as one of the main extrarenal sites of renin expression in humans (22). Since the transgene was passed from the sire, fluorescence could only be of fetal origin, ruling out the cells as being decidual. Conversely, when the opposite cross was set up (dam transgene positive, sire negative), fluorescent cells were only in placentas of transgenic fetuses. No fluorescence was associated with decidual or myometrial cells in this later cross when checked at days E1, E3, E6, E10, and E15.

Chorionic trophoblasts, decidual cells, and macrophages of human choriocarcinoma (10, 14, 22), as well as decidual and entodermal epithelial cells of mouse (25), have all been identified by immunohistochemical methods to contain renin in previous studies. Although renin mRNA in human decidual macrophages and chorion was identified using RT-PCR, other studies utilized protein level detection methods (either by renin activity assay or immunohistochemistry; Ref. 11). Indirect localization of renin gene expression by the presence of its protein product must be considered in light of the following caveats: 1) detection at these sites could be due to uptake by the cells after release from adjacent tissues; 2) renin antiserum cross-reactivity with other highly similar aspartyl proteases such as cathepsin D must be rigorously controlled; and 3) cathepsin D has been shown to mimic renin activity by cleaving angiotensin I from angiotensinogen (11). Visualization of Ren-1/GFP reporter, which exhibits verified and correct expression at other known sites, has an advantage over the above approaches for identifying bona fide sites of renin gene expression.

**Summary.** In this report we have shown that 4.1 kb of Ren 5’ flanking sequence is capable of directing multiple known renin expression patterns. These include androgen- and captopril-responsive expression, tissue- and cell-specific expression, and the progressive restriction of expression in the renal vasculature during development. This suggests that renin flanking sequence, when fused to GFP, will provide a useful vital reporter function for renin expression. Generating high-level expression of GFP in various renin-expressing cells may enable other methods of characterizing such cells. For example, it should be possible to utilize FACS sorting of GFP-expressing cells to isolate renin-expressing cell populations from complex populations of fetal or adult tissues. This could enable comparison of their respective complements of RNA through examination on mouse cDNA arrays. Information gained this way should help to elucidate cellular mechanisms of renin expression.

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