Differential expression of the closely linked KISS1, REN, and FLJ10761 genes in transgenic mice

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Submitted 9 December 2003; accepted in final form 31 December 2003

Nistala, Ravi, Xiaoji Zhang, and Curt D. Sigmund. Differential expression of the closely linked KISS1, REN, and FLJ10761 genes in transgenic mice. Physiol Genomics 17: 4–10, 2004. First published January 6, 2004; 10.1152/physiolgenomics.00205.2003.—We previously reported the development and characterization of transgenic mice containing a large 160-kb P1 artificial chromosome (PAC) encompassing the renin (REN) locus from human chromosome 1. Here we demonstrate that PAC160 not only encodes REN, but also complete copies of the next upstream (KISS1) and downstream (FLJ10761) gene along human chromosome 1. Incomplete copies of the second upstream (PEPP3) and downstream (SOX13) genes are also present. The gene order PEPP3-KISS1-REN-FLJ10761-SOX13 is conserved in mice containing either one or two copies of the REN locus. Despite the close localization of KISS1, REN, and FLJ10761, they each exhibit distinct, yet overlapping tissue-specific expression profiles in humans. The tissue-specific expression patterns of REN and FLJ10761 were retained in transgenic mice containing PAC160. Expression of REN and FLJ10761 were also proportional to copy number. Expression of KISS1 in PAC160 mice showed both similarities and differences to humans. These data suggest that expression of gene blocks encoded on large genomic clones are retained when the clones are used to generate transgenic mice. Genomic elements which act to insulate genes from their neighbors are also apparently retained.

human genome; mouse genome; gene expression; mouse model; regulation of gene expression; transgenic mice

THE REN GENE EXHIBITS an exquisitely restricted temporal and spatial profile of expression in mammals (28). The main site of renin synthesis leading to circulating protein is the kidney where its expression is limited to a few epithelioid cells at the distal end of the afferent arteriole proximal to the glomerulus. These juxtaglomerular cells make up less than 0.1% of the cellular mass of the kidney and have the ability to regulate REN transcription, synthesis, and processing over a two-order of magnitude range depending on the physiological status of the organism (6). The pattern of expression and the transcriptional responses to physiological cues is strongly conserved in mammals. Mechanisms must therefore exist to faithfully control the temporal and spatial expression of this important gene.

To examine the mechanisms regulating expression of REN, we previously generated a transgenic mouse model containing a 160-kb P1 artificial chromosome (PAC160) (29, 30). This large clone contains the REN gene and ~75 kb and 70 kb of 5' and 3' flanking sequence, respectively. Expression of REN is restricted to the kidney, but in mice containing a high number of transgene copies, expression is also evident in the brain, lung, and placenta (17). In the kidney, the REN transgene is only expressed in juxtaglomerular cells, and its synthesis is highly regulated in response to cues that normally upregulate (ACE inhibition, low-sodium diet) or repress (high-sodium diet, pressor and suppressor infusion of ANG II) expression of the gene. Double transgenic mice containing PAC160 and a systemically expressed human angiotensinogen (hAGT) gene exhibit a 20 mmHg increase in systemic arterial pressure, which is much lower than double transgenic mice expressing a poorly regulated human REN transgene (29). Indeed, human REN expression in the kidney is strongly suppressed in the PAC160/hAGT double transgenic mice. All the current data suggest that the PAC160 transgenic mouse is the most faithfully accurate model for studying the regulation of human REN expression generated to date.

One of the most interesting findings from the PAC160 mouse model is that expression of REN is proportional to transgene copy number and immune from position effects on expression. This rarely occurs when small transgenes containing cDNAs are expressed in mice. Copy number proportional expression generally indicates an ability to organize chromatin at the local level (7, 11). This insulates the transgene from effects caused by integration near other strong regulatory elements controlling genes located at or near the site of insertion. The formation of a “minilocus” of expression may be due to the presence of dominant control regions or nuclear matrix attachment sites in the transgene. Although such elements have yet to be mapped near REN, a strong enhancer of transcription located 12 kb upstream of the gene has been identified and partially characterized (25–27, 37). This enhancer was identified on the basis of homology to a similar sequence 2.6 kb upstream of the mouse renin gene which can strongly transactivate the gene in renin-expressing cells (22). It remains unclear whether this enhancer has properties similar to dominant control regions.

In this study we asked whether other genes are present on the PAC160 transgene, whether they are expressed in PAC160 transgenic mice, and whether their expression pattern is similar or dissimilar to REN. We demonstrate that PAC160 contains complete copies of the tumor suppressor gene KISS1 and the hypothetical gene encoding an ethanolamine (choline) kinase-like enzyme FLJ10761. These genes exhibit a distinct but partially overlapping pattern of tissue-specific expression in PAC160 mice that emulates their expression in human tissues.

MATERIALS AND METHODS

Transgenic mice. Transgenic mice containing PAC160 were previously described in detail (29, 30). All mice received standard mouse chow (LM-485; Teklad Premier Laboratory Diets, Madison, WI) and...
water ad libitum unless specified. Placental samples were obtained after killing pregnant mice when they were close to full term. Care of the mice used in the experiments met the standards set forth by the National Institutes of Health in their Guidelines for the Care and Use of Laboratory Animals, and all procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

Analysis of nucleic acids. Total RNA was prepared using either the guanidium thiocyanate (GITC) or the Tri-Reagent protocol as described previously (29). Northern blot analysis was performed according to standard protocols using 15 μg of total RNA isolated from each tissue. The presence of intact ribosomal RNA bands and equality of loading between lanes was checked by staining with methylene blue. Custom-made Northern blots were obtained from Clontech (Palo Alto, CA). Each lane had 1 μg of poly(A)" RNA, which was normalized, based on the signal for a housekeeping gene. Prehybridization and hybridization was carried out according to the manufacturer’s instructions.

RT-PCR was performed to generate FLJ10761 and KISS1 using the following primers: FLJRT-UP (GAATGAGGTCAGAAACTTCCAG), FLJRT-DN (TCATTGTGCCAGAGGATGGGCTTG), KISS1-UP (AATTCTAGACCCACAGGCCA), and KISS1-DN (GCATGCTCTGACTCCTTTGGG). RT-PCR bands were gel purified (Wizard Promega kit) and ligated into pCR2.1 using protocols from the manufacturer (Invitrogen). The renin cDNA probe was described previously (29). The cloned PCR products were used as probes on Northern blots.

RESULTS

REN is centered on PAC160 ~75 kb from the SP6 end (left end of Fig. 1) and 70 kb from the T7 end (right end of Fig. 1). At the inception of this project, only a cursory sequence of unordered fragments was available for this section of human chromosome 1. Originally therefore, we verified the presence of KISS1 and FLJ10761 on PAC160 by PCR amplification of several exons from each gene and mapped the position of several linked STS marker information was obtained from a combination of NCBI and Ensembl (D1S2480, D1S1725, D1S2717, D1S2668, D1S387, and D1S510) databases. Primers were obtained from Genosys (Sigma-Genosys Biotechnologies). Sequencing was performed at the DNA core facility at the University of Iowa. Sequencing results were analyzed using DNASTAR software package. Sequence analysis of the PAC ends was performed using purified PAC DNA and the SP6 and T7 primers.

Fig. 1. Schematic map of renin locus. A schematic representation of human chromosome 1 extending upstream and downstream of the renin locus is shown. Each gene is color coded, and the approximate location of exons is indicated by the vertical bars. The direction of transcription is indicated below each gene. The PEPP3 and SOX13 genes extend further upstream and downstream of the boundaries of PAC160. The position of prominent restriction sites ShaI (S) and NorI (N) are indicated. The approximate positions of the kidney (KE) and chorionic (CE) enhancers and the FLJ10761 CpG island are indicated.

Fig. 2. Expression of the REN gene. Tissue-specific expression of the REN gene in humans (top) and PAC160 mice (bottom) is shown. B, brain; H, heart; Sk, skeletal muscle; C, colon; Ty, thymus; Sp, spleen; K, kidney; Lv, liver; I, intestine; Pl, placenta; Lg, lung; PBL, peripheral blood lymphocytes; Sg, salivary gland; Pa, pancreas; K−, kidney from nontransgenic littermate.
PEPP3 and SOX13 as flanking genes (Fig. 1). Sequence analysis of the ends of the PAC160 insert revealed that both PEPP3 and SOX13 are incomplete. Only the terminal 6 (of 23) exons of PEPP3 are present, whereas all of SOX13 is present except the 5' flanking region and exon 1. As expected therefore, neither gene is expressed in transgenic mice containing PAC160 (data not shown).

Examination of the mouse chromosome 1 sequence (October 2003 freeze) confirmed the gene order Pepp3–Kiss1–Ren1c–Sox13. Listed in the database between mouse Ren1c and Sox13 is an expressed sequence tag (EST; RIKEN cDNA, NM_175443) that has 67% overall identity and 85% identity within the 5’-most sequence with human FLJ10761, suggesting synteny in this region of human and mouse chromosome 1. Detailed analysis of the sequence of a BAC clone encoding the Ren locus from the 129/SvEv mouse strain (accession no. AC024068) further confirms that the gene order Pepp3, Kiss1, Ren, FLJ10761, and Sox13 is highly conserved. Interestingly, the 129/SvEv strain, like several other strains, contains a duplication at the Ren locus and encodes two Ren genes, Ren2d and Ren1d (1). Consistent with previous reports that Ren2 is the duplicated gene, and given the close proximity of Ren and FLJ10761, it is interesting to note that this strain contains a partial duplication of the FLJ10761 gene, suggesting the downstream breakpoint of the duplication occurred within FLJ10761 (~1,140 bp downstream of exon 2 within the intron before exon 3). The gene order in the 129/SvEv strain is therefore Pepp3, Kiss1, Ren2d, incomplete FLJ10761 (exon 1–2), Ren1d, complete FLJ10761, and Sox13.

Given the close proximity between KISS1, REN, and FLJ10761, in particular REN and FLJ10761, we asked whether they exhibited similar or disparate tissue-specific expression profiles and whether that pattern would be retained in transgenic mice containing multiple copies of PAC160. Expression of REN, KISS1, and FLJ10761 was first examined in human tissues using Clontech's preprepared multiple tissue Northern blots and then in tissues from PAC160 transgenic mice. FLJ10761 expression was confirmed in four different transgenic lines, whereas KISS1 was confirmed in two of these transgenic lines.

As anticipated, expression of REN was abundant in human kidney and was detectable in placenta (Fig. 2). In PAC160 mice, REN was abundantly expressed in kidney and placenta and, in the transgenic mice shown here containing a high

Fig. 3. Expression of the FLJ10761 gene in human tissues. Tissue-specific expression of the FLJ10761 gene in humans is shown. Pr, prostate; Ts, testis; O, ovary; I, intestine; PBL, peripheral blood lymphocytes. See legend to Fig. 2 for other abbreviations.

Fig. 4. Expression of the FLJ10761 gene in PAC160 mice. Tissue-specific expression of the FLJ10761 gene in PAC160 mice is shown. See legend to Figs. 2 and 3 for tissue type abbreviations; +, transgenic; −, nontransgenic littermate. Two lines of mice are shown both of which have a high number of transgene copies.
number of transgene copies, also in brain and lung. Expression of REN in the brain and lung has been previously reported by us and others (14, 31, 35).

Interestingly, despite the observation that FLJ10761 lies only a few kilobases downstream of REN, it exhibits a distinct tissue-specific expression profile with abundant expression in the liver, kidney, and testes, and moderate expression in pancreas, ovary, and prostate (Fig. 3). The pattern of FLJ10761 expression in four lines of transgenic mice was remarkably similar to the pattern detected in human tissue with high-level expression evident in liver, kidney, and testes (Fig. 4). Some differences in relative abundance of FLJ10761 mRNA was detected among tissues from different lines (i.e., relative abundance in liver and kidney in lines 7217/2 and 6919/1).

On the contrary, KISS1 expression in humans was only abundantly detected in placenta, with much lower levels evident in heart, liver, skeletal muscle, and spleen (Fig. 5). Although in much smaller abundance than placenta, the transcripts detected in skeletal muscle and testes appear larger than the placental transcript, whereas a much smaller transcript was detected in heart, liver, and kidney. In transgenic mice, expression was evident in the kidney, brain, and lung, a finding reproduced in two different transgenic lines (Fig. 6). There was no detectable endogenous Kiss1 mRNA in these samples. Expression of KISS1 was also evident in placenta from PAC160 mice, but at a much lower level than anticipated (Fig. 7). The low signal in the nontransgenic placenta samples most likely reflects expression of the endogenous mouse Kiss1 gene, which is 85% identical to human KISS1.

Given the copy number proportional expression of REN in PAC160 mice previously reported by us (29), we determined whether expression of FLJ10761 would be similarly proportional to copy number. Expression of REN and FLJ10761 was examined in kidney and liver from five different lines classified as having low (L), medium (M), or high (H) copy number of the PAC transgene (Fig. 8). Although expression of 28S rRNA was constant in all samples, expression of both REN and FLJ10761 was stronger in mice containing the medium and
high number of copies than in mice containing only 1–2 copies of PAC160. Copy number proportional expression of FLJ10761 was evident in both kidney and liver, whereas REN expression was only evident in kidney.

**DISCUSSION**

The precision by which a transgenic animal model closely emulates a physiological or pathophysiological process depends largely upon the quality and quantity of transgene expression. Important questions to be asked in any transgenic experiment include: Is the transgene expressed in the correct spectrum of cells and tissues? Is the level of expression physiological or pharmacological? Whereas intronic sequences are known to be important for the overall processing and level of transgene expression (3), ultimately, it is the promoter and regulatory sequences which govern the pattern of temporal and spatial expression observed in the animal. Transgenes consisting of promoters lacking important regulatory elements can exhibit ectopic and variegated expression, and few transgenes exhibit copy number proportional expression (reviewed in Ref. 16). We show herein that a large transgene consisting of the REN locus and surrounding loci from human chromosome 1 exhibits copy number proportional expression of REN and the next downstream gene FLJ10761.

Copy number proportional expression of transgenes in mice is the exception not the rule. Copy number proportional expression is most prevalent in mice which contain large transgenes consisting of genomic DNA (exons and introns) and sequences having the ability to manipulate chromatin locally (11, 33). Grosveld and colleagues (10) described a series of DNase I hypersensitive sites in genomic DNA surrounding the β-globin locus which constitute a dominant (or locus) control region (DCR or LCR). Core sequences containing the DNase hypersensitive sites can confer copy number proportional expression in transgenic mice (34). Given the copy number proportional expression observed in mice containing PAC160, it becomes attractive to hypothesize that an LCR may exist near the REN locus. That expression of FLJ10761 was also proportional to copy number begs the question of whether a single LCR or multiple LCRs are present in PAC160.

What is the nature of the renin LCR? The β-globin LCR consists of a series of hypersensitive sites and binding sites for both ubiquitous and erythroid cell-specific transcription factors (34). The renin enhancer, first identified by Gross and colleagues (22), is located about 2.6 kb upstream of the mouse renin gene. A homologous sequence is located about 12 kb upstream of the human renin gene (KE in Fig. 1) (25, 37). This sequence contains the binding sites for at least 10 different transcription factors including NF-Y, RAR, RXR, CREM, CREB, USF-1, USF-2, Ear2, Sp1, and Sp3 (15, 20, 21, 25–27).

By analogy with β-globin, it is possible that the renin enhancer acts as an LCR. Using homologous recombination in bacteria to subtly alter PAC160 (19), we have generated a new transgene (PAC160ΔKE) identical in all respects except it lacks the...
kidney enhancer. We are currently examining the expression and regulation of REN, FLJ10761, and KISS1 in mice containing the altered construct to assess its importance as an LCR.

Expression of FLJ10761 in PAC160 mice closely emulated its normal pattern of expression in humans, that is, high-level expression in the kidney, liver, and testes. It is intriguing that FLJ10761 is located only 3.7 kb downstream of renin yet exhibits high-level expression in the liver where renin is not expressed. A similar situation was reported for the chicken lysozyme (cLys) locus. Just 207 bp downstream of cLys is the cGas41 gene, which exhibits a vastly different expression pattern than cLys (4). Interestingly, cLys and cGas41 are separated by a CpG island, which is commonly associated with I the promoters of housekeeping genes (8) and 2 origins of replication (2). Interestingly, a NotI restriction site, which is rich in CpG dinucleotides (GCGGCCGC) and present in some CpG islands, is located in the first intron of FLJ10761 (Fig. 1).

Moreover, the UCSC Human Genome Browser places a CpG island around the transcription start site for FLJ10761 (Fig. 1), suggesting the presence of a “natural expression boundary” between REN and FLJ10761. Nevertheless, recall that both REN and FLJ10761 share copy number proportional expression, suggesting that other sequences may be present in PAC160 that act as a chromatin organizer.

Further analysis of 1 kb of sequence upstream of FLJ10761 exon 1 with TRANSFAC revealed a number of potentially interesting transcription factor binding sites including MTF-1 (metal element protein-1), which is active in liver, kidney, and testes (just as FLJ10761), and ANF-2, which is reported to be liver specific (36). The most interesting finding was the identification of a fully consensus binding site for LF-A1 ~85 bp upstream of the transcription start site. This site is present in several liver-specific genes including α1-antitrypsin, pyruvate kinase, and apolipoproteins A1, B1, and A4 (23). Additional studies will be needed to clarify the importance of this site in liver-specific expression of FLJ10761.

There is scant information on the functional relevance of FLJ10761. The Swiss-Prot database lists it as a hypothetical protein potentially encoding a choline- or ethanolamine-kinase (EKI2). EKI proteins normally catalyze the first step in phosphatidylethanolamine biosynthesis, an abundant phospholipid in cell membranes. It is evolutionarily conserved with homologs in mouse, zebrafish, worms, and yeast. Knockdown of EKI homologs in worms using RNA interference does not result in any observed phenotype (from WormBase database, http://www.wormbase.org/).

On the contrary, substantial information continues to emerge on the function of KISS1. KISS1 is a metastasis suppressor gene which inhibits metastasis in a number of experimental model systems (reviewed in Ref. 12). A COOH-terminal peptide derived from KISS1, termed metasin, released into the circulation from the placenta during pregnancy, is thought to mediate the biological effects of KISS1 (13). Recently, a G-protein-coupled receptor has been identified as the receptor for metasin (32). This pathway may play a role in the gonadotropic axis, as loss of function mutations of the metasin receptor can cause hypogonadotropic hypogonadism (5). A similar phenotype was observed in knockout mice lacking the receptor (24).

KISS1 is abundantly expressed in the placenta and has been reported in brain (18). In PAC160 mice, KISS1 was abundantly expressed in lung with lower levels in brain and kidney. Although its expression in brain is consistent with other reports, its expression in kidney and lung cannot be easily reconciled. Moreover, KISS1 expression in the placenta of PAC160 transgenic fetuses was also substantially lower than anticipated. It is possible that since the pattern of KISS1 expression in PAC160 mice was similar to the expression ofREN, the KISS1 promoter may have been influenced by sequences regulating REN. Recall, placental KISS1 was detected in lines of PAC160 mice containing multiple copies of the transgene. Along similar lines, a second transcriptional enhancer termed the chorionic enhancer (CE in Fig. 1) has been reported ~5 kb upstream of human REN (9). This enhancer is capable of strongly trans-activating the human REN promoter in primary chorionic cells, but less strongly in renin-expressing cells derived from the kidney and lung. It becomes tempting to speculate that the chorionic enhancer may have dual actions as a REN and KISS1 enhancer. As above, we are directly testing this in transgenic mice containing a modified PAC160 transgene lacking the chorionic enhancer (PAC160ΔCE).

In conclusion, transgenic mice containing large chromosomal segments can be excellent tools with which to study the coordinate expression and regulation of closely linked genes in an experimental context where the human expression pattern is retained. Mutational studies provide further opportunities to identify regulatory elements that act in a gene-specific or locus-specific manner.

ACKNOWLEDGMENTS
DNA sequencing was performed at the University of Iowa DNA Core Facility. We thank Dr. Kenneth W. Gross for comments on the manuscript. A. W. Cowley, Jr., served as the review editor for this manuscript submitted by Editor C. D. Sigmund.

GRANTS
The work described herein was funded by National Institutes of Health Grants HL-58048, HL-61446, and HL-55006. We gratefully acknowledge the generous support of the Roy J. Carver Trust.

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