Long human CHGA flanking chromosome 14 sequence required for optimal BAC transgenic “rescue” of disease phenotypes in the mouse Chga knockout

Sucheta M. Vaingankar,1 Ying Li,1 Angelo Corti,5 Nilima Biswas,1 Jiaur Gayen,1 Daniel T. O’Connor,1,2,3,4 and Sushil K. Mahata1,4
Departments of 1Medicine and 2Pharmacology, and 3Center for Human Genetics and Genomics, University of California at San Diego, 4Veterans Affairs San Diego Healthcare System, La Jolla, California; and 5Department of Oncology, San Raffaele Scientific Institute, Milan, Italy

Submitted 15 May 2009; accepted in final form 14 December 2009

Vaiingankar SM, Li Y, Corti A, Biswas N, Gayen J, O’Connor DT, Mahata SK. Long human CHGA flanking chromosome 14 sequence required for optimal BAC transgenic “rescue” of disease phenotypes in the mouse Chga knockout. *Physiol Genomics* 41: 91–101, 2010. First published December 15, 2009; doi:10.1152/physiolgenomics.00086.2009.—Chromogranin A (CHGA) plays a catalytic role in formation of catecholamine storage vesicles and also serves as precursor to the peptide fragment catestatin, a catecholamine secretory inhibitor whose expression is diminished in the hypertensive individuals. We previously reported the hypertensive, hyperadrenergic phenotype of Chga−/− knockout (KO) mice and rescue by the human ortholog. In the present study, we compare two humanized CHGA mouse models. Into the Chga null background, by bacterial artificial chromosome transgenesis human CHGA transgene has been introduced. Both lines have the complete ~12 kbp CHGA gene integrated stably in the genome but have substantial differences in CHGA expression, as well as consequent sympathochromaffin biochemistry and physiology. A mouse model with longer-insert HumCHGA31 displays integration encompassing not only CHGA but also long human flanking sequences. This is in contrast to mouse model HumChga19 with limited flanking human sequence co-integrated. As a consequence, HumCHGA19 mice have normal though diminished pattern of spatial expression of CHGA, and 14-fold lower circulating CHGA, with failure to rescue KO phenotypes to normalcy. In the longer-insert HumCHGA31 mice, catecholamine secretion, exaggerated responses to environmental stress, and hypertension were all alleviated. Promoter regions of the transgenes in both HumCHGA19 and HumCHGA31 display minimal CpG methylation, weighing against differential “position effects” of integration, and thus suggesting that lack of cis elements required for optimal CHGA expression occurs in HumCHGA19 mice. Such “humanized” CHGA mouse models may be useful in probing the physiological consequences of variation in CHGA expression found in humans, with consequences for susceptibility to hypertension and cardiovascular disease.

blood pressure; catecholamine; stress; hypertension; chromogranin A; bacterial artificial chromosome

CHROMOGRAHIN A (CHGA) is present with catecholamines in their storage vesicles/chromaffin granules and co-released by exocytosis in response to sympathoadrenal stimulation (37). It is a proprotein that is processed into several biologically active peptides, including the dysglycemic hormone pancreaticatin, vasodepressor vasostatin, and catecholamine release-inhibitory peptide catestatin (12, 17, 20, 23, 35). The catestatin fragment of CHGA seems to be processed inefficiently in hypertensives, leading to lower levels in plasma (25). As a nicotinic cholinergic antagonist, catestatin inhibits secretion from dense core granules, thereby attenuating levels of circulating catecholamines (20, 21). It also augments the release of the vasodilator histamine from mast cells, thereby enhancing its hypotensive action (14, 16).

*Chga−/−* mice develop hypertension, along with disordered catecholamine storage and release, as evidenced by a decrease in number and size of chromaffin granules coupled with elevated catecholamine levels in the plasma (18). A central role for catestatin deficiency in the elevated blood pressure (BP) is supported by the “rescue” of BP elevation by exogenous catestatin (18). Here we report the effects of introducing human CHGA transgenes into knockout (KO) mice. The two “humanized” CHGA models we describe in this study differ in length of inserted chromosome 14 sequence containing CHGA. The Tg(CHGA:RPC11-862G15)31Smv (HumCHGA31) mice have a single copy/haploid genome of an entire ~211 kbp bacterial artificial chromosome (BAC, RPC11-862G15) insert stably integrated into the genome, while mouse model Tg(CHGA:RPC11-862G15)19Smv (HumCHGA19) integrated only ~72 kbp of the BAC RPC11-862G15.

The ability of these mice to rescue KO phenotypes varies dramatically. Long flanking stretches 5′ and 3′ of CHGA appear to be essential for the optimal CHGA expression and consequent rescue of the KO phenotypes. Such mouse models, with sufficient vs. insufficient levels of CHGA, may effectively mimic variability in CHGA expression in the human population (5, 6, 27, 33) and offer tools to study the mechanism of action of CHGA/catestatin and its role in the pathophysiology of developing hypertension.

METHODS

Generating BAC transgenic humanized CHGA mice. Mice were utilized in the study according to a protocol approved by the Animal Subjects Committee of the University of California at San Diego, and research was conducted in accordance with institutional guidelines. RP11-862G15 BAC human genomic clone, obtained from Children’s Hospital Oakland Research Institute, has a ~211 kbp insert that spans the ~12 kbp CHGA locus (human chromosome 14: 92,459,198–92,471,393) and was used to generate transgenic and humanized mice as described earlier (18). The founder mice Tg19 (with shorter integration) and Tg31 (with longer integration) were used to establish transgenic lines homozygous for the transgene: Tg19 (Tg(CHGA19+/+; Chga+/+) or Tg31 (Tg(CHGA31+/+; Chga+/+) and subsequently backcrossed with Chga−/− (C57BL6 × 129/SvJ background) mice to...
generate humanized transgenic mice $\text{TgCHGA}^{+/+};\text{Chga}^{+/−}$ with the human CHGA transgene and lacking mouse Chga alleles. Once longers HumCHGATg31 and shorter HumCHGATg19 lines were derived, PCR analysis using five primer sets to amplify the 12 kbp CHGA gene as well as seven primer sets for the ~44 kbp upstream (92,415,173–92,459,199) region and 10 sets for the downstream ~156 kbp region (92,471,394–92,626,041 bp) of the BAC insert, with respect to the ~12 kbp human CHGA boundaries established the limits of insertion of the RP11-862G15.

**Mouse breeding.** The ability of the transgenes in Tg19 and Tg31 to rescue the reported partial prenatal lethality of mice with genotype $\text{Chga}^{+/−}$ was determined by two types of mating designs for each of the transgenes. Using the PCR method described earlier (18) pups derived from the matings were genotyped for presence or absence of the human transgene and the mouse Chga allele(s). The PCR assay also provides information on the copy number of the mouse alleles ($\text{[0 copy in KO, 1 copy in heterozygote (Het), 2 copies in wild type (WT)]}$; however, the assay was not informative regarding copy number of human CHGA alleles. In the first type of mating, $\text{CHGA}^{+/+}\text{;Chga}^{+/−}$ was crossed with KO mice ($\text{CHGA}^{+/−}\text{;Chga}^{+/−}$). CHGA refers to transgene $\text{Tg19 or Tg31}$. The expected ratios and genotypes of the offspring are 1:1:1:1 for $\text{CHGA}^{+/+}$: $\text{CHGA}^{−/−}$: $\text{Chga}^{+/+}$: $\text{Chga}^{−/−}$ (Het) : $\text{CHGA}^{−/−}$: $\text{Chga}^{−/−}$. Of 38 offspring, the observed ratio was 13:10:9:6.

The second type of mating was between mice heterozygous for both human and mouse alleles. $\text{CHGA}^{−/−}$: $\text{Chga}^{−/−}$ crossed with $\text{CHGA}^{+/−}$: $\text{Chga}^{+/−}$ . There are nine expected genotypes, however, the PCR distinguishes offspring into six groups ($\text{CHGA}^{+/+}$: $\text{Chga}^{+/−}$ and $\text{CHGA}^{+/−}$: $\text{Chga}^{+/−}$): ($\text{CHGA}^{+/+}$: $\text{Chga}^{−/−}$ and $\text{CHGA}^{+/−}$: $\text{Chga}^{−/−}$): $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{+/−}$ : $\text{Chga}^{+/−}$ : $\text{CHGA}^{+/−}$ : $\text{Chga}^{+/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{+/−}$ : $\text{CHGA}^{+/−}$ : $\text{Chga}^{+/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{+/−}$. The second cross $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{+/−}$ : $\text{CHGA}^{−/−}$ : $\text{Chga}^{−/−}$, in the instance of mice with the transgene Tg19, produced offspring in the genotypic ratio (described above) 9:6:26:2:19:3 (total 65 pups). For transgene Tg31, of a total of 74 offspring the observed ratio was of 14:12:34:2:9:3. Any mice with the transgene Tg19, produced offspring in the genotypic ratio.

**DNA analyses:** slot and Southern blot. Genomic DNA was prepared from tails of founder or offspring mice using a Puregene DNA purification kit (Gentra Systems). The Minifold II apparatus (Schleicher & Schuell, Keene, NH) was used for slot blot analysis. Genomic DNA digests with Xmn1 (8-base recognition restriction enzyme not sensitive to methylation) were used for Southern analysis. Probe specific for human CHGA was used for both blots.

**Real-time PCR, immunoblot, ELISA, and confocal microscopy.** The animals were humanely killed, and the tissues isolated from the dissected animal; particular brain regions were obtained by excision with a razor blade. The tissues were homogenized in TRIZOL reagent (Invitrogen, Carlsbad, CA) and processed according to manufacturer’s instruction.

For RNA analyses, total RNA was then DNase digested and further purified using RNeasy mini-kit (Qiagen, Valencia, CA). The first-strand cDNA synthesis was carried out using 500 ng of total RNA as template, with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The primers employed were specific for human CHGA (NM_001275) (NM_001275): CHGATg/H11001/5’-GCCAGACCGCTGGGTCGAG-3’ and R 5’-TTCTGCTTCTACCTGCTTAGAG-3’; for BX674904: F 5’-AGGAGGTGTCGTCGTCATC-3’ and R 5’-TGTGCAGAGGCGGCTATTTT-3’; for AK093301: F 5’-GAATCTGACCTGAAGGTTCAC-3’; and R 5’-TTAGGGG- AGGAGGAGGATGTAGTAG-3’; and for BCD420841: F 5’-AGACGCTTGCCTCTCATTC-3’ and R 5’-CCGGTGTACCCCATTCATC-3’ were employed. WT tissues were used as control.

For immunoblots, adrenal glands were homogenized, the protein concentration was determined using Bio-Rad assay reagent (Hercules), and 5 μg of total protein was loaded per well. The primary polyclonal antibody was generated against human cathepsin domain of CHGA using the vector Strategic Biosolutions. Sandwich ELISAs between monoclonal anti-chromogranin A (MAB B4E11) antibody and rabbit polyclonal anti-human CHGA/cathepsin antisera have previously been described in detail (7). Plasma samples of each animal were assayed in duplicate.

To analyze the microarray of adrenal glands, they were fixed overnight in 3.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and then transferred to 30% sucrose in 1x PBS for 48 h. The gland was fixed in OCT compound (Tissue-Tek) and stored at −70°C until sectioning. Sections 10–15 μm thick were cut using Leica CM3050S. Once the medullary region of the gland was well defined by light microscopy, the subsequent sections were stained for indirect immunofluorescence. The primary antibody used is rabbit anti-recombinant human CHGA (gift from Dr. L. Taupenot). The secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and TO-PRO iodine (2 μM in DMSO) used to stain nuclei were obtained from Molecular Probes (Eugene, OR). Samples were analyzed using a Zeiss Axiosvert 100M laser-scanning microscope.

**Measurements of BP and plasma catecholamines in mice.** BP of mice was measured using BP-2000 Blood Pressure Analysis System (Visitech Systems, Cary, NC). The instrument employs a noninvasive tail-cuff method. Mice were placed in individual rodent restraint holders on a preheated specimen platform at 38°C. BP values were recorded 10 times in rapid succession, and the results were averaged. This process was repeated three or four times daily between 10:00 and 12:00 AM, for 5 consecutive days. Over the first week the readings were not collected, so as to allow the mice to adapt. Over the following week, the data were analyzed. During measurements, data were continuously stored in a notebook computer running the BP Analysis software package (Visitech) via a PCMCIA data acquisition card. Averaged readings of systolic BP (SBP) having standard deviation of <10 mmHg were accepted for further analysis.

Catecholamines were measured in plasma drawn from mice euthanized by deep anesthesia in isoflurane (Baxter) chambers followed by cervical dislocation. High performance liquid chromatography coupled to an electrochemical detector (Waters 600E Multisolvent Delivery system and Waters 2465 Electrochemical Detector) was used. Separation was performed on an Atlantis dC18 column (2.1 × 150 mm, 3 μm) from Waters. The mobile phase used was a mixture composed of phosphate-citrate buffer (2 mM NaH2PO4, 268 μM Na2EDTA, 50 mM sodium citrate, 10 mM diethylamine hydrochloride, pH 3.1 adjusted using phosphoric acid, and 2.2% N,N-dimethylacetamide) and acetonitrile at 95:5 (vol/vol). A flow rate of 0.25 ml/min was used with isocratic mobile phase. The electrode potential was set at +0.6 V. The data were analyzed using Empower software from Waters, and CA levels normalized according to the recovery of an internal 3,4-dihydroxybenzylamine (DHBA) standard. To 0.3 ml of plasma sample, 1 ng of DHBA and 0.125 mM sodium meta-bisulfite were added. Following the addition of 12 mg of alumina (aluminum oxide, activity grade: Super I, type WA-4, Sigma-Aldrich), the pH of the plasma was raised to pH 8.6 by adding Tris · HCl buffer, pH 8.9. After a 30 min incubation, the sample was centrifuged (5,000 rpm, 5 min), the supernatant was discarded, and the beads were washed with water. The catecholamines were eluted with 80 μl of 0.1 N HCl supplemented with 100 mM sodium meta-bisulfite, and injected for analysis.
Immobilization stress. This experimental protocol had prior approval by the Animal Care and Use Committee of our university, following humane National Institutes of Health (NIH) guidelines. Gentle and safe immobilization stress of mice was carried out using the Universal Restrainer apparatus (Braintree Scientific, MA). Prior to start of the immobilization stress, the BP of the mice was measured as described above. For a period of 21 days, the 4-mo-old mice were immobilized daily for 2 h between 8:00 and 10:00 AM. The BP of the stressed mice was monitored twice weekly. The stressed mice were given a 2 h recovery period prior to BP measurement.

Human CHGA promoter CpG methylation assay. CpG methylation was assayed using Pyrosequencing after bisulfite treatment and PCR amplification at EpigenDX (Worcester, MA). Genomic DNA was prepared from adrenal gland (does express CHGA) and liver (does not express CHGA) tissues of HumCHGATg31 and HumCHGATg19 mice. It was subjected to bisulfite conversion, and an amplicon of 156 bp, upstream to ATG and including the TATA box, was PCR amplified from the region of human CHGA promoter in the transgene. Pyrosequencing analyzed five of the CpG sites in the amplicon for DNA methylation. In-house EpigenDx control human transgene. Pyrosequencing analyzed five of the CpG sites in the amplicon for DNA methylation. In-house EpigenDx control human transgene. Pyrosequencing analyzed five of the CpG sites in the amplicon for DNA methylation.

RESULTS

Humanized CHGA mouse models generated by BAC transgenes. The human BAC clone RP11862G15 ~211 kbp insert (Fig. 1A) was used to microinject the F2 embryos of the hybrid strain CB6 (F1 hybrid between BalbC and C57BL6 mice). Of 58 microinjected, implanted embryos that were born, six had integration of the CHGA BAC insert. However, only founders Tg19 and Tg31 gave rise to stable transgene inheritance over several generations and were characterized further. Founders Tg19 and Tg31 differed in the lengths of human flanking sequence that was co-integrated along with the entire CHGA. Figure 1B shows a schematic representation of these differences. The integrity of the RP11862G15 BAC inserts (posttransgenesis) was determined by PCR amplification of the BAC insert region from mouse genomic DNA preparations. By a series of PCR amplicons, the limits of insertion of the BAC in the transgenic lines were determined. Tg19 contains ~72 kbp of stably integrated RP11-862G15 insert, including in addition to the ~12 kbp CHGA gene, ~41 kbp upstream (5’) and ~19 kb downstream (3’) of the gene. Tg31, on the other hand has a near-complete 210,868 bp of RP11-862G15 insert.

To determine the copy number of the transgene CHGA integrated in the genome of the founders, slot blot analysis was employed (Fig. 2A). A densitometric scan revealed that only one copy of the transgene is integrated in the genomes of the founder mice (Fig. 2B). Qualitative Southern blot probing also confirmed the presence of a single copy of transgene (Fig. 2C). The F1 pups obtained by a cross between the founder and inbred strain CB6F1 mice are hemizygous for the CHGA transgene and have two of the mouse Chga alleles. In these mice we probed expression patterns of CHGA by RT-PCR (Fig. 2D). In neuroendocrine tissues the human allele is expressed. No expression of the allele is detected in liver and spleen, indicative of the expected pattern of fidelity of expression specific to neuroendocrine tissues in both Tg31 and Tg19 mice. The expression of CHGA in the transgenic mice clearly indicates elevated expression in Tg31 compared with Tg19. All the data values lie left of the line of identity with the highest levels of expression in Tg31 adrenal gland.

**Fig. 1.** Structures of the integrated human chromogranin A (CHGA) transgenes. A: schematic depicting the ~220 kbp BAC (bacterial artificial chromosome) construct used to create “humanized” CHGA mice. The insert comprises the ~211 kbp chromosome 14 sequence encompassing the entire 12.1 kbp human CHGA gene, as well as ~44 kbp upstream and ~155 kbp downstream sequence. B: integrated sequence in the 2 founders Tg(CHGA:RPC11-862G15)19Smv; Chga +/- (“Tg19”) and Tg(CHGA:RPC11-862G15)31Smv; Chga +/- (“Tg31”) is shown. Dotted lines indicate the extent of flanking sequence incorporated in each transgene. The founder Tg31 has almost the entire BAC insert integrated stably into the mouse genome. In founder Tg19 with limited flanking sequences, ~41 kbp upstream and ~19 kbp downstream are integrated along with the entire CHGA gene.

**Structure of the human CHGA transgenes**

**A**

Insert: human BAC clone RP11862G15 (spanning the CHGA locus)

**B**

“Humanized” CHGA mouse: Insertion boundaries

<table>
<thead>
<tr>
<th>FOUNDERS</th>
<th>Hum Tg19 (shorter)</th>
<th>Hum Tg31 (longer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(CHGA:RPC11-862G15)19Smv</td>
<td>Tg(CHGA:RPC11-862G15)31Smv</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NcoI (210.66)</th>
<th>EcoRI (0.01)</th>
<th>NcoI (210.88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NcoI (201.63)</td>
<td>EcoRI (0.01)</td>
<td>NcoI (210.88)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NcoI (70.39)</th>
<th>NcoI (210.66)</th>
<th>NcoI (210.88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NcoI (201.63)</td>
<td>EcoRI (0.01)</td>
<td>NcoI (210.88)</td>
</tr>
</tbody>
</table>

| CHGA-BAC Insert: 210.868 kbp Vector: 8.791 kbp |
|-----------------|-----------------|
| 210.868 | 160.645 | 154.649 |
| 160.645 | 154.649 | 148.649 |

<table>
<thead>
<tr>
<th>UPSTREAM SEQUENCE</th>
<th>CHGA</th>
<th>DOWNSTREAM SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(CHGA:RPC11-862G15)19Smv</td>
<td>Tg(CHGA:RPC11-862G15)31Smv</td>
<td></td>
</tr>
</tbody>
</table>
Expression of chromogranin A in humanized CHGA mice.

The fully humanized CHGA mice lack both copies of the mouse Chga allele but have two copies of the human CHGA transgene. Once both HumCHGA19 and HumCHGA31 lines were generated, we compared the lines for expression of chromogranin A mRNA and protein (Figs. 3 and 4). Human CHGA is expressed in several neuroendocrine tissues (data not shown), including the adrenal gland and brainstem, and is detected in circulating plasma in both lines. Comparison of the HumCHGA31 and HumCHGA19 adrenal extracts to WT and KO revealed more CHGA expression in HumCHGA31 (Fig. 3A). A stronger signal (>3-fold) is seen in case of HumCHGA31 than WT adrenal extracts, since the immunoblotting uses polyclonal anti-human catestatin antibody (Fig. 3B). The HumCHGA19 and KO adrenal extracts did not give a signal. Figure 3C shows mRNA expression of CHGA in the HumCHGA31 and HumCHGA19 strains. HumCHGA19 has significantly lower CHGA expression, ~23-fold lower in adrenal and ~7-fold lower in the brain stem, compared with HumCHGA31. The ELISA employed is based on capturing antibody B4E11 and detecting polyclonal antibody raised against full length CHGA (Fig. 3D). Only the human epitope is detected: it does not cross-react with either WT or KO mouse plasma (7). The ELISA results show that HumCHGA31 has ~14-fold higher circulating levels of CHGA in the plasma compared with HumCHGA19. Immunohistochemistry of adrenal glands of HumCHGA31 and HumCHGA19 (Fig. 4) also supported the evidence by immunoblot and RT-PCR analysis, in that expression is significantly higher in HumCHGA31. Also notable was the fact that CHGA staining was restricted to the medulla of the adrenal gland in the humanized mice. It appears therefore that the ~198 kbp flanking sequence in HumCHGA31 mice is evidence that SBP is rescued to normalcy in both WT (++) and humanized mice. Thus, cell type (i.e., chromaffin)-specific expression seemed to occur for both mouse and human CHGA.

Ability of the humanized CHGA insert to rescue the Chga null phenotype. Both the transgenes rescued prenatal lethality observed in KO mice. Thus in case of Tg19 transgenic mice, the combined expected ratio of humanized (CHGA+/− : Chga−/− and CHGA+/− :Chga−/−) mice to rest of the offspring is 17:68 compared with the observed ratio of 8:77 ($\chi^2 = 3.0$ and $P = 0.083$). Similar analysis of Tg31 offspring displays a ratio of 22:90 and the expected ratio of 24:88. The $\chi^2$ (0.027) analysis indicates that the observed ratio is not significantly deviant ($P = 0.87$) from the expected ratios.

The dramatic phenotypic differences of the Chga KO mice compared with WT mice include elevated BP as well as plasma catecholamine levels (18). In this study we analyzed these two disease traits in both of the humanized CHGA lines. Shown in Fig. 5A is evidence that SBP is rescued to normalcy in HumCHGA31 mice, with final values comparable to WT. HumCHGA19, with lower levels of CHGA, was unable to...
completely rescue the SBP to normalcy. Indeed, while ANOVA across five strains [WT (+/→), KO (−/−), Het (−/+), HumCHGA31, and HumCHGA19] yielded significant effects of strain on both SBP (Fig. 5 A, \( P < 0.0001 \)) and DBP (Fig. 5 B, \( P < 0.0001 \)); post hoc Bonferroni-corrected comparisons were not significant for WT vs. HumCHGA31, KO vs. HumCHGA19, HumCHGA19 vs. Het, or Het vs. KO.

Circulating catecholamines parallel the BP changes in these mouse strains. One-way ANOVA analyses across the four strains for NE (\( P = <0.0001 \)) and E (\( P = 0.0015 \)) are significant (Fig. 6). The plasma levels of norepinephrine (NE) and epinephrine (E) are significantly higher in KO compared with WT mice (Fig. 6, A and B). There is no significant difference in either the NE or E levels between HumCHGA31 and WT mice (Fig. 6, C and D). On the other hand, both of these groups have significantly lower levels of NE and E compared with HumCHGA19. These findings are congruent with the observation that HumCHGA19 display elevated BP in contrast with WT and HumCHGA31 mice.

Thus we conclude that HumCHGA31 reverts to the phenotype of the WT mice and is thereby effectively rescuing Chga−/− disease phenotypes. By contrast, replacement in the KO by two copies of the Hum Tg19 transgene does not completely rescue either BP or catecholamine phenotypes. The difference in the two humanized lines resides in the length of the flanking sequence that gets inserted along with the entire CHGA locus. This difference results in lower levels of CHGA in HumCHGA19 compared with HumCHGA31; the insufficient levels of CHGA, in turn, result in failure to rescue the KO phenotype to normalcy in HumCHGA19.

Differential response to stress by HumCHGA31 (Tg31, longer) vs. HumCHGA19 (Tg19, shorter) mice. Both lines of human CHGA replacement mice (HumCHGA19 and HumCHGA31) were subjected to chronic immobilization stress, with BP responses measured, as well as in a set of age- and sex-matched mice not subjected to immobilization stress. Older mice (4 mo old) were used in this experiment, compared with 2-mo-old mice in Figs. 5 and 6. At the end of the stress period, SBPs of WT and Chga−/− mice were comparably elevated (Fig. 7 A); thus it appeared that both the −/− and +/+ mice had reached an upper limit or “ceiling” for ability to raise BP. Once again, transgene Tg31 mice had lower basal SBPs than Tg19 mice, though at the end of stress SBP values were similar in HumCHGA31 and HumCHGA19 mice (Fig. 7 B), further suggesting an SBP ceiling to which all mice ascend during stress.

Location and expression of CHGA flanking genes. The transgene Tg31 represents the entire insert of the BAC clone RP11862G15 (Fig. 8 A). In addition to the complete CHGA gene, the BAC insert spans AK093301, BC042084, BX647940, and BC127782. Their transcripts (usually as expressed sequence tags) have been detected, but their functions are unknown. In the shorter Tg19 only AK093301 and BC042084 are additional transcripts of HumCHGA19 by RT-PCR in all tissues tested. An exception is the absence of expression of AK093301, the other three transcripts are detected in HumCHGA31 by RT-PCR in all tissues tested. An exception is the absence of expression of AK093301.
with consequent silencing. In contrast (and as expected), endogenous mouse LINE-1 methylation was highly methylated in adrenal genomic DNA from both the HumCHGA19 line (at 89.7 ± 1.8%) and the HumCHGA31 line (89.9 ± 1.0%), confirming that this assay can discover hypermethylation in the same animals.

**DISCUSSION**

BAC transgenic mice are currently indispensable to address the in vivo role of the human CHGA gene. Aligning the 10,059 bp of the murine gene (ENSMUSG00000021194) located on chromosome 10 with the 12,194 bp of human CHGA (ENSG00000100604) on chromosome 14 using the MacVector 7.2 software reveals that only ~4,713 residues are conserved. Given sequence divergences, it is unlikely that these orthologous genes are absolutely identical in expression. Unlike conventional transgenics, BAC constructs integrated into the mouse genome are quite large. Therefore the entire panoply of cis-regulatory elements, including long-range elements required for faithful expression of the transgene, are co-integrated. The extensive flanking sequences may also “buffer” the transgene from “position effects.” It has been observed in Drosophila, yeast, and plants that when genes are located in close proximity to the heterochromatin region, their expression is unstable (30, 31). In mouse embryonic stem cells, transgenes located near the telomeres are silenced (26). Unlike conventional transgenics, the possibility also exists that essential mouse sequences at the site of the transgene integration are disrupted. HumCHGATg31 displayed ~14-fold higher circulating levels of CHGA compared with HumCHGATg19 (Fig. 3D), in conjunction with more extensive co-integration of flanking sequence (Fig. 1B). Enhanced CHGA expression likely results in superior rescue of the Chga (−/−) disease phenotypes of elevated BP and catecholamines to normalcy by the longer HumCHGATg31 transgene. These differences in expression of HumCHGATg31 and HumCHGATg19 likely result from a “positive” consequence of retained essential cis-acting sequences for human CHGA expression by longer HumCHGATg31. The difference in expression is unlikely to be a “negative” consequence of position effects (36) arising from inadequate control.
“insulation” by incorporation of the shorter HumCHGATg19 into surrounding mouse heterochromatin, since the proximal CHGA promoters in both the transgenic lines display low methylation levels (Fig. 8D), consistent with active promoters. While methylation is known to be a lasting influence on the heterochromatin organization of a gene influencing in turn its expression, it appears here that the integration locus of the human CHGA transgene in both lines (HumCHGATg31 and HumCHGATg19) does not result in promoter methylation differences likely to alter gene expression (10, 13). Lack of adequate insulation of the transgene does not appear to be the cause for silencing of either HumCHGATg19 or HumCHGATg31.

While the region differentially integrated in HumCHGATg19 vs. HumCHGATg31 does contain several regions of conserved sequence across species (Fig. 8A), our experiments cannot resolve the relative contributions of positive cis-acting enhancer in HumCHGATg31.

Longer (Tg31) vs. shorter (Tg19) flanking sequences. Both HumCHGATg31 and HumCHGATg19 represent lines that span the entire 12.1 kbp CHGA gene. While HumCHGATg31 mice have ~44 kbp upstream and ~155 kbp downstream human chromosome 14 sequences, HumCHGATg19 mice have ~41 kbp upstream but only ~19 kbp downstream sequences. Additional expressed genes (AK093301, BC042084, BX647940, and BC127782) in the longer transgene HumCHGATg31 may influence the final phenotypic differences between the two lines. Thus far, the human CHGA transgenic lines do not appear to have detrimental developmental effects. The two humanized mouse models for CHGA (HumCHGATg31 and HumCHGATg19) were analyzed for rescue of Chga KO phenotypes. Phenotypes of Chga-KO (~/-) mice already suggest that Chga regulates SBP, catecholamine secretion, and perhaps catecholamine storage vesicle biogenesis (18). In both HumCHGA replacement lines, the spatial expression pattern is comparable to WT mouse Chga as previously reported (18, 19) and restricted to

Fig. 5. Preferential “rescue” of elevated blood pressure to normalcy by the longer human CHGA transgene Tg31. Higher systolic blood pressure (SBP) and diastolic blood pressure (DBP) are observed in KO, HumCHGATg19, and heterozygote (mouse Chga +/-, Het) mice, indicating that lower levels of CHGA expression in HumCHGATg19 or Het (+/-) fail to completely rescue the KO (~/-) BP to normalcy. HumCHGATg31 mice in contrast have BP indistinguishable from WT (Chga+/-). The age of the mice was 10-12 wk. WT, n = 8; KO, n = 12; HumCHGATg31, n = 21; HumCHGATg19, n = 30; heterozygotes (Het) n = 17. A: SBP. Overall (1-way ANOVA across all groups), the SBP difference was significant at P < 0.0001. Bonferroni-corrected P values are shown for individual pair-wise comparisons. B: DBP. Overall (1-way ANOVA across all groups), the SBP difference was significant at P < 0.0001. Bonferroni-corrected P values are shown for individual pair-wise comparisons.

Fig. 6. Rescue of catecholamine levels to normalcy by the longer human CHGA transgene Tg31. Plasma catecholamines were measured in 10-12 wk old mice, n = 8/group. A and B: comparison of norepinephrine and epinephrine levels in WT and KO mice. C and D: humanized mouse catecholamines compared with WT. HumCHGATg19 mice have elevated norepinephrine and epinephrine in the plasma compared with WT and HumCHGATg31 mice. HumCHGATg31 mice with 2 copies of the human CHGA gene effectively compensate for loss of Chga alleles and rescue the catecholamine levels to normalcy.
neuroendocrine tissues (Figs. 2D and 4, B, D, and E). However, only the longer HumCHGA31 transgene completely rescued the KO phenotypes to normalcy: mice with HumCHGATg31 regulate BP, catecholamines and response to stress, in a manner comparable to WT (+/+). Mice heterozygous (+/−) for Chga deletion have been characterized earlier (18), and their BP values resemble those of the HumCHGA19 and KO mice; the three groups display similar BP elevations (Fig. 5, A and B) with catecholamine excess (Fig. 6, C and D).

Thus the most pertinent gene expression difference between the two humanized CHGA lines seems to be in amount (rather than the distribution) of expression. Why should this quantitative difference come to be? The shorter HumCHGA19 and longer HumCHGA31 transgenes are likely integrated at different sites in the mouse genome, but each seems to exist as a single copy per haploid genome (Fig. 2, A–C). While the region differentially integrated in Tg31 vs. Tg19 does contain several regions of conserved sequence across species (Fig. 8), our experiments cannot resolve the relative contributions of positive cis-acting enhancer in HumCHGA31.

CHGA level significantly influences blood pressure in humans (24, 32, 33). Hypertensive patients differ from the normotensives in their levels of circulating CHGA, with elevated levels seen in the former. These humanized mouse models with elevated and stressed conditions. HumCHGA19 mice have higher SBP at the start of the experiment at age 4 mo, and chronic stress does not further elevate SBP.

Transgene rescue of mouse gene function by BAC-encoded transgenes has been reported for several loci. Some examples of murine BAC transgene rescue include that of neural tube defect by BAC transgene Ltap (15) mouse circadian Clock gene (2), pcd mouse defect by Nnal bearing BAC, and tattered mouse phenotype by BAC transgene Ebp (22).

Human ortholog transgenes have also been demonstrated to rescue deficient function in mice. This approach differentiates differences in regulation of mouse and man genes as has been shown with Mc1r BAC transgenesis in Mc1r-deficient mice (11). The mouse α-thalassemia phenotype was rescued by BAC containing human α-thalassemic gene (1), Krabbe disease rescue by BAC transgenesis employed BAC with GALC (9), and Abca1−/− deficient mice were compensated by ABCA1 BAC transgene (8). In each of these cases the inset integrated into the transgenic mouse contained the gene and its native flanking chromosomal sequences. The length of the flanking sequence varied. The transgene BRCA1 rescued embryonic lethality of Brca1 1700T mutant mice (4). Similarly, the role of BRCA2 in gametogenesis was delineated using a BAC transgene to rescue the Brca2 +/− phenotype (29), ABCA1 gene was sufficient for rescue of Abca1−/− phenotype (8), and finally, an extremely large 413-kbp Gata2 BAC transgene fully rescued Gata2 null mutant embryonic lethality (3). In each of these rescue scenarios the mouse of human gene required a unique length of flanking native sequence to rescue the disease or mutant phenotype by BAC transgenesis.

Caveats and alternative approaches. We were able to characterize the insertion boundaries of two large CHGA BAC inserts, ranging from ~72 kbp (shorter Tg19) to ~211 kbp (longer Tg31); these two flanking inserts, coupled with careful biochemical and physiological phenotyping, allowed us to draw inferences about required boundaries for optimal expression and phenotype rescue. One limitation of this study is the random (initially uncontrollable) site and extent of BAC transgene integration, and the subsequent development of only two transgenic lines in great detail. The precise site of integration of a BAC transgene cannot be well controlled, nor can the extent of human flanking sequences. Therefore, this study features one transgenic CHGA line with higher and the other with lower levels of CHGA expression. Random integration of the TgCHGA19 vs. TgCHGA31 into different sites in the mouse genome might result in interruption of an essential mouse gene in one case but not the other. Although the TgCHGA19 and TgCHGA31 strains displayed no readily detectable differences in morphology or breeding, we cannot exclude the possibility of such disruption at one or the other integration site. A refinement of the BAC transgenic procedure known as the “co-placement” strategy (34) allows placement of two different haplotypic variants of a gene within the same human BAC into a single chromosomal site within the mouse genome. However, even this approach cannot ensure that the entire BAC is...
Fig. 8. HumCHGA19 and HumCHGA31 transgenes: expression and location. A: human CHGA. Local genomic region in the BAC insert. The image was created at http://genome.ucsc.edu using the assembly February 2009 human reference sequence (GRCh37). Displayed are local genes, regions of sequence conservation across vertebrate species, and repeat regions. The limits of the BAC clone RP11862G15 contained in the integrated transgene of HumCHGA19 and HumCHGA31 are depicted in gray at the bottom. B: genes contained in the BAC clone RP11862G15. The BAC clone RP11862G15 spans human chromosome 14 region 93,345,420–93,556,350. Therefore, ITPK1 gene is incomplete and 5' end of the gene (~26 kb) is deleted in RP11862G15. Four coding genes along with CHGA are co-inserted in line HumCHGA31. AK024887 is a noncoding RNA. C: expression of flanking genes co-inserted with CHGA. Differential expression of CHGA flanking genes is observed in the two strains. In adrenal gland, brain (total), testis, lung, and spleen, human CHGA mRNA was detected by RT-PCR in both HumCHGA19 and HumCHGA31 strains. D: methylation status of the human CHGA promoter in transgene. Both transgenic lines HumCHGA31 and HumCHGA19 were analyzed for the degree of methylation in the first 5 CpG sites within the proximal promoter of the transgene in adrenal DNA by Pyrosequencing analysis. CHGA promoter of both the transgenes display low methylation status, consistent with expression of the genes. As a genome-wide control, mouse LINE-1 sequence methylation was also assayed, and expectedly displayed higher methylation, consistent with “silencing.”
integrated into the mouse genome, and the integration site cannot be controlled.

Conclusions and Perspectives

Human BAC transgenes, to complement loss of mouse gene function, are a feasible approach to complement loss of mouse gene function, and to humanize the target locus. Such “humanization” should permit more specific studies of the function of the human CHGA gene in vivo, under circumstances where studies in intact humans are inappropriate or impossible. Generation of humanized mouse models may also serve the purpose of differentiating between regulation of human and mouse genes. Finally, the results of replacement by two humanized lines reinforce the impression that effective expression of CHGA is important for optimal catecholamine release, BP and the response to environmental stress. Finally, since genetic variation at the human CHGA locus results in variability of CHGA expression as well as susceptibility to hypertension (5, 6, 27), these models may represent a useful approach to understanding the coupling between the CHGA gene and disease risk.

GRANTS

This work was supported by NIH Grants DK-069613 (to S. M. Vaingankar, R01 DA-011311 (to S. K. Mahata), DK-60702 (to D. T. O’Connor), and P01 HL-58120 (to S. K. Mahata and D. T. O’Connor) and by the Department of Veterans Affairs (S. K. Mahata and D. T. O’Connor).

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES


