Defining the Functional Boundaries of the Gata2 Locus by Rescue with a Linked Bacterial Artificial Chromosome Transgene

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Transcription factor GATA-2 is vital for both hematopoietic progenitor cell function and urogenital patterning. Transgenic mapping studies have shown that the hematopoietic and urogenital enhancers are located hundreds of kbp 5' and 3' to the Gata2 structural gene, and both are vital for embryonic development. Because the size of mammalian genes, including all of their associated regulatory elements, can exceed a megabase, transgenic complementation in mice has, in specific instances, proven to be a formidable hurdle. After incorporating the Gata2 structural gene as well as the distant hematopoietic and urogenital enhancers into a single, contiguous piece of DNA by fusing two bacterial artificial chromosomes (BACs) into one, we formally tested the hypothesis that the functional boundaries of this locus are contained within this contiguous genomic span. We show that two independent lines of transgenic mice bearing a multicyclic 413-kbp-linked Gata2 BAC transgene (bearing sequences from −187 to +226 kbp of the locus) are able to fully rescue Gata2 null mutant embryonic lethality and that the rescued animals behave and reproduce normally. Surprisingly, the linked BAC confers expression in the ureteric epithelium, whereas sequences within any of the overlapping parental BACs and a yeast artificial chromosome that were originally tested do not, and thus these experiments also define a novel synthetic enhancer activity that has not been previously described. These genetic complementation studies define the required outer limits of the Gata2 locus and formally demonstrate that enhancers lying beyond those boundaries are not necessary for Gata2-regulated viability or fecundity.

Transcription factor GATA-2 is exemplary of a vital developmental factor whose regulation reveals many of the intrica-

cies of the controlling apparatus that is required for proper temporal and tissue-specific expression of a gene in many different tissues and organs that are critical for mammalian development. The evolutionarily conserved C4 zinc finger GATA transcription factors play demonstrably crucial roles in embryogenesis. GATA-2, originally cloned from a chicken cDNA library (1), was shown to be essential for the proliferation and/or differentiation of early hematopoietic progenitors, as Gata2 null mutant mice die around mid-gestation from a block in primitive hematopoiesis (2). Further examination of Gata2 gain-of-function mice and in vitro differentiation of Gata2 null mutant ES cells underscored the fundamental conclusions from the initial loss of function experiments, and showed that GATA-2 plays a pivotal role in the proliferation of very early hematopoietic progenitors (3–5).

We showed several years ago that Gata2 null mutant embryonic lethality could be rescued by complementation with a 247-kbp transgenic yeast artificial chromosome (YAC)3 bearing sequences from −174 to +73 kbp (revised endpoints relative to the Gata2 translational initiation site); the YAC contains all of the regulatory information required to rescue Gata2 function in primitive and definitive hematopoiesis (6). We subsequently found that Gata2 hematopoietic regulatory activity is conferred by at least two distinct cooperative cis elements, one lying about 170 kbp 5' to the gene and another lying somewhere between 40 and 100 kbp 5'4. However, compound mutant mice bearing this YAC, but lacking endogenous Gata2, expired perinatally of kidney failure and uretero-vesicular obstruction, because the YAC-rescued Gata2 null mutant mice failed to develop a patent connection between the ureters and the bladder, causing the mice to develop cystic, non-functional kidneys and megareters (6). Because the rescuing YAC was not expressed in the urogenital system, while endogenous GATA-2 is prominently expressed there, we speculated that the development of hydroureters and cystic kidneys was due to the absence of tissue-specific enhancers that were responsible for directing proper Gata2-mediated patterning of the developing urogenital system in the YAC transgene.

Several years ago, we devised a BAC transgenic founder mapping strategy (BAC-traps) that ultimately revealed the positions

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2 The online version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

3 The abbreviations used are: YAC, yeast artificial chromosome; BAC, bacterial artificial chromosome; GFP, green fluorescent protein; Q-PCR, quantitative PCR.

4 N. Suwabe, R. Shimizu, T. Moriguchi, and J. D. Engel, unpublished observations.
of the regulatory elements that control Gata2 expression in the developing urogenital system (7). One cis element, responsible for Gata2 activation in the ureteric epithelium, was not specifically localized, but was predicted to lie very far, either upstream or downstream, from the structural gene. However, two enhancers that were finely localized are responsible for mesenchymal expression: one (UG2, located +113 kbp 3’ to the translational initiation site) controls Gata2 activation in the rostral metanephric mesenchyme, whereas a second (UG4, centered at +75 kbp) is responsible for Gata2 activation in the caudal periureteric mesenchyme and urogenital sinus.

We have now regionally or precisely localized about a dozen individual cis elements that are required for Gata2 expression, including two hematopoietic (6), two central nervous system (8), one endothelial (9), and three urogenital enhancers (7), as well as the two Gata2 promoters (10); these individual elements are scattered over 400 kbp 5′ and 3′ to (as well as within) the Gata2 structural gene. However, dozens of other elements must deductively exist, because Gata2 is involved in many other quite diverse organotypic developmental events (e.g. for the generation of placental trophoblasts, fat, and pituitary subtype specification (11–13)), and indeed, our initial BAC-trap surveys demonstrated that other Gata2 enhancers lie even further away (e.g. a whisker follicle enhancer that maps to between +560 and +787 kbp 3’ to Gata2 (7)).

To determine whether the Gata2 urogenital enhancers that were identified in the initial BAC-trap survey defined the required functional domains of gene activity that are responsible for proper ureter/bladder morphogenesis, in this study we created a transgene that would recapitulate the expression of Gata2 in both the hematopoietic as well as the urogenital compartments. Although YACs have been generated that contain genomic inserts of the size required, numerous technical problems with YACs have also come to light, including chimerism (6, 14) and innate replicative instability (15). BACs, while surmounting many of the problems encountered with YACs, posed a different problem, because the average genomic DNA insert size in BACs is only ~200 kbp (16), which is insufficient in this case to span the interval between the most distant Gata2 hematopoietic (~170 kbp) and urogenital (~113 kbp) enhancers.

To circumvent the size limitation of BACs, we devised a new general strategy to precisely link two BACs together to generate a single recombinant that contained a very large but stable genomic DNA fragment. Generation of mice bearing this BAC were predicted to create a transgene spanning the entire interval between the distant enhancer elements that were either known (hematopoietic) or suspected (urogenital) of being required for Gata2 function. Following breeding between Gata2 BAC transgenic mice and Gata2 germ line null mutant mice, we were able to formally test whether complementation of both the hematopoietic and urogenital functions would fully rescue Gata2 null mutants. Alternatively, complementation with the linked BAC might only partially rescue, and thereby reveal novel Gata2 mutant phenotypes due to as yet undiscovered enhancers that lie beyond the boundaries of the linked BAC.

The present experiments revealed that rescue of the Gata2 null mutant phenotype with a 413-kbp linked BAC was complete using either of two independent rescuing BAC transgenic lines. Both lines allowed animals to survive to adulthood, and the rescued mice exhibited no obvious behavioral or reproductive anomalies. Therefore, we can place outer boundaries on the size of the Gata2 locus, including all sequences that are required to fulfill its most vital developmental functions. These data suggest that documented enhancers that lie outside these boundaries are either unnecessary for vital GATA-2-mediated physiological functions in some tissues where that factor is expressed, or that those functions are fully complemented by other molecules.

**EXPERIMENTAL PROCEDURES**

**BACs**—BACs spanning the mouse Gata2 locus were identified (16), purified (17), and modified (7) as previously described. **Construction of Specialized Recombineering Plasmids**—lox514Neo: Two loxP14 sites (18) with convenient cloning sites were created. lox514nx oligonucleotides (supplementary Table S1) were annealed and digested with NotI and XbaI and then cloned into the Eagl and SpeI sites of the pFrtNeo plasmid. The resulting plasmid was digested with PstI and EcoRI and ligated to annealed lox514 pm oligonucleotides (supplementary Table S1), which had been digested with NsiI and MfeI. The resulting plasmid, plox514Neo, was verified by sequencing and then used as a template for the generation of the BAC targeting constructs. pFrtAmp: to generate a plasmid containing the Frt-flanked ampicillin gene, we used Famp oligonucleotides (supplementary Table S1) containing Frt sites, sequences complementary to the pUC19 AmpRgene and convenient cloning sites. Using these primers, the AmpR gene was amplified from pUC19 by PCR, gel-purified, and digested with BamH1 and EcoRI. This fragment was cloned into the BamH1/EcoRI sites of the pIRES2-eGFP vector (Clontech). The fragment was sequenced to verify correct generation of the flanking Frt sites. Tn5FlpeTet: a plasmid that constitutively expresses Flp recombinase was generated in a manner similar to that described by Liu et al. (19).

**Generation of Targeting Constructs by PCR**—To generate the bacterial targeting constructs, synthetic oligonucleotides were used as outlined in previous experiments (20). Targeting fragments were generated by PCR with ExTa (Takara), purified from agarose using GeneCleanII (Q-bioengineeringe), and digested with DpnI to remove (methylated) template DNA. 100–200 ng of targeting fragment was used in each experiment. To generate single lox sites, the same primers (supplementary Table S1) were used to amplify a targeting fragment containing tandem lox sites. After insertion of this fragment into the BAC by homologous recombination, induction of cre recombinase resulted in deletion of the intervening selection marker, leaving a single loxP site incorporated at the point of selectable marker insertion.

**Cre Recombinase Induction**—The EL350 strain was used for cre recombinase induction, as previously described (7). Because the frequency of recombination between loxP and lox511 or loxP514 sites is finite, varying the duration of cre induction was used to optimize recovery of the desired homologous recombination product.

**Functional Boundaries of the Gata2 Locus**
Modification of Parental BACs 115E9 and 81F7—To generate the 5′ BAC, homologous recombination was used to insert a loxpP514 site at 13 kbp upstream of the Gata2 translational initiation site. Simultaneously, this insertion was designed to delete a loxP site in the pBACe3.6 vector backbone by directing the 3′ homologous recombination to position 4862 of the vector sequence (21). The primers (−13loxP514(f) and bacdellox(r); supplemental Table S1) and the template plasmid ploxP514Neo were used for PCR to generate a targeting fragment, which was then used to modify the 115E9 BAC by homologous recombination. The neomycin gene that was used as the positive selection marker was removed by cre induction, leaving a single loxP514 site at −13 kbp, and at the same time deleting the loxP site in the vector backbone. The resulting BAC was further modified to include an Frt-flanked PGK-directed neomycin resistance gene for positive selection. BacNeo primers (supplemental Table S1) were used to generate a targeting fragment by PCR using the pFrtNeo plasmid as template. The resulting fragment was then inserted into the 115E9lox BAC by homologous recombination.

To generate the 3′ BAC, a loxP514 site was again inserted 13 kbp upstream of the Gata2 translational start site, at the same position that was modified in BAC 115E9. The −13loxP514 forward and reverse primers (supplemental Table S1) and the ploxP514 plasmid were used to generate a fragment that was subsequently inserted into the 81F7 BAC by homologous recombination. The neomycin gene was again removed by cre induction, leaving a single loxP514 site at the −13 kbp position. This BAC was further modified to insert an Frt-flanked ampicillin resistance gene to allow for selection of products that had undergone intermolecular recombination. This was achieved by using Famp2977 and Famp4233 primers (supplemental Table S1) together with the FrtAmp plasmid to generate a targeting fragment that was then inserted into 81F7lox BAC by homologous recombination, and in doing so, inserted the FrtAmp cassette between positions 2977 and 4233 of the pBACe3.6 vector backbone (21).

cre-mediated BAC Recombination—To join the two modified BACs, Escherichia coli EL350 bearing the 115E9loxNeo BAC was grown in overnight culture. After diluting the culture 1:10 and allowing it to grow for 10 min, arabinose was added to 1 mg/ml before growing the culture for an additional 2 h. The cells were made competent for transformation (7) and then electroporated with the 81F7loxAmp BAC; recombinants were selected on Cam/Kan/Amp plates. Homologous recombination was verified by HindIII fingerprinting, as well as by P1-SceI digestion and pulsed field gel electrophoresis under the conditions: 6 V/cm, linear ramp switch time (10–50 s) 24 h at 14 °C (Fig. 1C).

Verification of Homologous Recombination—To verify recovery of the correct recombination products by Southern blot analyses, three probes from different regions of the recombined BAC were employed. A 5′ Nco/Spe probe fragment corresponding to −173.9 kbp of the Gata2 locus, which was predicted to yield a restriction fragment of 3.9 kbp only from the parental 115E9 BAC and the recombinants, was hybridized to HindIII-digested BAC DNAs (Fig. 1E). To generate a −13 kbp probe, a 600-bp fragment was generated by PCR from the 81F7 BAC using the G2–13 primers (supplemental Table S1). Using this probe and digesting the BACs with Pml and PvuI was predicted to yield a 5-kbp restriction fragment in the case of the 115E9 parental BAC, or a 10-kbp fragment after appropriate recombination at the loxP514 site (Fig. 1F). For the 3′ probe, a 900-bp fragment corresponding to a position +200 kbp (relative to the Gata2 translational initiation site) was generated by PCR from the 81F7 BAC using G2 + 200 primers (supplemental Table S1). Digestion with HindIII followed by hybridization with this probe was predicted to yield a 7-kbp band from the 81F7 parental BAC as well as the homologous recombinants but not hybridize to the 115E9 BAC (Fig. 1G).

Flp-mediated Removal of the Neomycin Selection Cassette—Because the neomycin resistance gene carries a eukaryotic promoter, it was removed from the linked BAC to avoid possible promoter interference effects. To accomplish this, the Tn5FlpeTet plasmid was transformed into bacteria carrying the linked BAC (R, Fig. 1B), which were then grown on plates containing Cam/Kan/Amp/Tet. The Tn5FlpeTet plasmid constitutively expresses Flp recombinase, which catalyzed excision of the Neo gene. To subsequently eliminate this plasmid from the bacteria bearing the linked BAC, we used nickel ion-based counterselection, because bacteria that are tetracycline-resistant are sensitive to nickel death (22). We reasoned that growth in L-broth containing NiSO4 would select for the rare bacteria that had lost the Tn5FlpeTet plasmid during cell division. Cam/Kan/Amp/Tet colonies were grown in 5 ml of L-broth plus Cam for 3 h, at which time NiSO4 was added to 5 mM. The cultures were grown until turbid and then streaked onto Tet/Cam, Kan/Cam/Amp, and Cam plates. Cultures that showed slower growth on Tet/Cam and Kan/Cam/Amp plates versus Cam plates were replica-spotted onto Cam and Kan/Cam plates to verify loss of neomycin resistance. Miniprep DNA from the resulting CamR/Kan5 clones was verified by HindIII restriction digest, pulsed field gel electrophoresis, and Southern blot analyses as described above.

Transgenic and Knock-out Mice—BACs were purified by double banding on CsCl/EtBr gradients and then the purified supercoiled DNA was microinjected into oocytes as previously described (7). The Gata2 El1-KI mice have been previously described (23).

Immunohistochemistry—Embryos were harvested and prepared as previously described (7). Purified rat anti-chicken GATA-2 monoclonal antibody, RC1.1 (1:10) (6), which is cross-reactive with murine GATA-2, was added along with an anti-green fluorescent protein (anti-GFP) antibody (Molecular Probes, A11122, 1:2000) to the embryo sections, which were then incubated overnight at 4 °C. The next day the RC1.1 antibody was detected using a Cy3-conjugated goat anti-rat secondary antibody (Zymed Laboratories Inc.), whereas the anti-GFP antibody was detected using Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Both secondary antibodies were incubated at 1:200 for 1 h. Finally the sections were treated with 4′,6-diamidino-2-phenylindole and ProLong Gold antifade reagent (Invitrogen). Images were recorded electronically and merged as previously described (7).
RESULTS

Linking BACs—In this strategy, we first identified two overlapping BACs, which together encompass the complete structural gene as well as the regulatory domains required for Gata2 hematopoietic and urogenital expression (6, 7), from BAC library RPMI-23. The selected parental BACs, 115E9 (P1) and 81F7 (P2), bear genomic sequences from −187 to −10 kbp and from −34 to +226 kbp of the Gata2 locus, respectively (Fig. 1, A and B). To fuse the two BACs precisely in register, we employed cre-mediated homologous recombination to exploit the documented differential recombination efficiency between homologous versus heterologous loxP sequences (24). A single loxP514 site (yellow arrow, Fig. 1B), which is distinct from the loxP511 sites (green arrows, Fig. 1B) in the pBACe3.6 vector backbone (21), was inserted at −13 kbp (relative to the Gata2 start codon) of the Gata2 locus in both BACs, P1 and P2. Next, Frt-flanked antibiotic resistance markers (neomycin or ampicillin) were introduced at the 5′ end of P1 or at the 3′ end of P2, respectively, to facilitate drug selection of intermolecular recombinants. The electroporation of P1 into P2-harboring bacteria, in conjunction with cre recombinase induction in the E. coli EL350 strain, led to simultaneous loxP514/514 and loxP511/511 homologous recombination in trans (Fig. 1B, gray bars) and the insertion of Gata2 genomic DNA from P1 into P2, thus generating the 413 kbp (from −187 to +226 kbp of the Gata2 locus) recombinant BAC (R, Fig. 1B).

After Flp-mediated deletion of the Frt-flanked positive selection markers from this recombinant, the structural integrity of the resultant linked BAC (referred to hereafter as G2BAC) was confirmed by pulsed field gel electrophoresis (Fig. 1C), restriction digest fingerprinting (Fig. 1D), and Southern blotting analyses using three radiolabeled probes dispersed throughout the Gata2 locus (Fig. 1B, the positions of the probes are indicated as E, F, and G). Hence, we successfully generated a 413-kbp Gata2-linked BAC using cre-mediated intermolecular recombination.

Linked BAC Transgenic Mice—Due to fragmentation problems encountered when very large linear or nicked circular BAC DNAs are used for microinjection,6 we injected supercoiled DNA into fertilized mouse ova. Founder animals were genotyped using PCR primers (supplemental Table S1) located at the junction between the BAC vector and the most 5′ end of the Gata2 genomic DNA insert at −187 kbp, at the loxP514 insertion site situated at −13 kbp of the Gata2 locus, and in the chloramphenicol acetyltransferase gene. Fourteen founders were verified to be transgenic by Southern blot analyses of tail DNA (data not shown). Of these, at least two appeared to contain complete transgenes that were transmitted stably to progeny for multiple

6 M. Khandekar, and J. D. Engel, unpublished observations.
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FIGURE 2. Gata2-linked BAC copy number as determined by Q-PCR. Tail DNA was recovered from animals in which the transgenes in two different established lines (called 2.2 and 2.12) were stably transmitted to offspring for more than four generations. PCR primers were developed using Primer Express, which would detect unique sequences approximately every 50 kbp along the length of the linked BAC, as well as at positions outside the boundaries of the BAC (as a diploid copy number control; supplemental Table S1). The normalization strategy for each genomic DNA sample involved first standardizing the quantified PCR product from Gata2-specific primer pairs to that of the β-actin gene, and then normalizing the values of transgenic mice to those of wild-type controls.

To simultaneously quantify the BAC copy number and roughly assess the integrity of the transgenes, we employed a quantitative PCR (Q-PCR) strategy. After breeding the lines for several generations to ensure that no further segregation of unlinked transgenes occurred, DNA was prepared from the tails of several transgenic littermates of each line. Q-PCR was performed on these DNA samples in triplicate using Sybr green (Applied Biosystems). PCR primer pairs were designed (supplemental Table S1) to amplify Gata2 genomic sequences at ~50-kbp intervals throughout the length of the linked G2BAC. Gata2 genomic sequences lying immediately beyond the boundaries of the G2BAC (at approximate positions corresponding to ~230 and ~270 kbp) as well as the β-actin gene were used as diploid copy number controls to ensure the validity of the assay. As shown in Fig. 2, transgenic line 2.2 appears to have incorporated three stably integrated transgene copies (three transgenic plus two endogenous = 5 copies), whereas line 2.12 appears to bear two. Given the similarity of the quantified results reflected by PCR primer pairs spread across the entire locus, we assume that at least one of the integrated BAC transgenes is intact in each line, but we performed no additional experiments (e.g. pulsed field Southern mapping) to test this hypothesis.

Rescue of the Gata2 Null Mutation with a Linked BAC Transgene—A Gata2 null mutant (EII-KI) mouse in which the green fluorescent protein (GFP) was integrated at the translational start site in Gata2 exon II has been previously characterized (23). GFP expression was detected in all of the anticipated organs and tissues (23). We mated the two independent linked BAC transgenic (TgG2BAC) lines (2.2 and 2.12) to Gata2 germ line mutant heterozygotes to generate Gata2+/−:TgG2BAC compound mutant animals; none of the compound mutant offspring exhibited abnormal behavior. The transgenic heterozygotes were backcrossed again to Gata2 heterozygotes to generate Gata2 homozygous null mutant transgenic mice, which were genotyped by Q-PCR (Fig. 3A). The quantitative methodology was validated by breeding an F2 Gata2−/−: TgG2BAC (presumptive rescued null mutant) parent to wild-type females: all progeny contained one germ line mutant Gata2 allele (Fig. 3C), thus demonstrating unambiguously that this parent was indeed of Gata2−/−:TgG2BAC genotype.

Because a Gata2 ureteric epithelial enhancer had not been previously identified (7), we speculated that rescue of the Gata2 null mutants with the linked BAC might lead to full complementation of the hematopoietic phenotype, as we previously observed with a smaller transgenic YAC (6). However, an additional expectation was that the BAC-rescued mice might also exhibit perinatal or postnatal urogenital deficiencies, given that a ureteric epithelial enhancer was not detected among the BACs surveyed in the original BAC-trap screen, and was therefore expected to lie somewhere outside the boundaries described by the linked BAC transgene (7).

After crossing the two independent TgG2BAC lines into the Gata2 null mutant background, we were able to draw several conclusions. First, all genotypes were recovered at the anticipated Mendelian frequency from Gata2+/− × Gata2−/−: TgG2BAC intercrosses (actual: 60:127:23; expected: 52:105:26, for Gata2−/− (both with and without the G2BAC), Gata2+/− (again, both with and without the G2BAC), and Gata2+/− (with the G2BAC only)) genotypes (supplemental Table S2), respectively, when performed with either of the G2BAC transgenic lines. Second, BAC-complemented null mutant mice suffer from neither hematopoietic nor urogenital defects; neither do they exhibit any adult urological deficiency. Furthermore, Gata2−/−: TgG2BAC mice exhibit normal life expectancy, body weights, and litter sizes (of expected Mendelian frequencies) compared with wild-type controls.

Given the lack of expression of any of these BAC and YAC transgenes in the ureteric epithelium (7), the observation that adult animals were generally normal was somewhat surprising, and therefore we collected, sectioned, and stained e14.5 embryos to specifically assess the early development of the ureter and the expression of the linked BAC transgenes. Hematoxylin and eosin staining showed that the overall morphology of the genitourinary system is unaltered in the Gata2−/−: TgG2BAC embryos (Fig. 4, A and E). GFP expression (from the Gata2 knock-in allele) localized to the same cells in BAC-comple-
formed on genomic DNA samples prepared from the tails of sibling pups (Gata2) from a linked BAC transgene allele (TgEII-KI) sequences together encompass sequences represented by both the examined in the "BAC-trap" revised genome sequence, and an overlapping BAC (333I12, compartments in the expressed in both the urogenital epithelial and mesenchymal

However, much to our surprise, GATA-2 protein was lacZ-mice might be rescued by the linked BAC possibly via non-cell

mates that map to positions 60 to 268 kbp of the Gata2 locus) was also previously examined in the "BAC-trap" lacZ reporter assay. These two sequences together encompass sequences represented by both the 5′ BAC (115E9, −187/−10 kbp of the Gata2 locus) and the 3′ BAC (81F7, −34/+226 kbp) that we examined in this study, and in neither of the former reporter transgenic studies were we able to

detect GATA-2 activity reflected as reporter staining in the ureteric epithelium. Thus the present data indicate that the linked BAC is able to confer tissue specificity for epithelial expression that is not observed in the BACs or YAC when they are analyzed individually (7).

**DISCUSSION**

GATA-2 is expressed in multiple tissues during vertebrate development and has been shown in several instances to be required for their differentiation. However, there are at least an equal number of examples of tissues in which this transcription factor is robustly expressed but also for which its in vivo requirement has not yet been completely elucidated. Because it is well established that Gata2 null mutants expire from hematopoietic failure (2) and that Gata2 YAC-rescued null mutants, after surmounting the lethal hematopoietic block, expire from urogenital patterning failures (6), it seemed reasonable to ask: if both of these embryonic deficiencies were complemented by a transgene bearing activities that would correct both deficiencies, would the animals survive? The alternative, which might have been expected in this case, is that the animals would probably not survive to reproductive age, but expire of a second perinatal lethal urogenital


dmented Gata2+/−:TgG2BAC compound mutant animals as in heterozygous mutant (Gata2+/−) controls (Fig. 4, B and F). However, much to our surprise, GATA-2 protein was expressed in both the urogenital epithelial and mesenchymal compartments in the G2BAC-rescued transgenic mice (Fig. 4, C and G). Although we anticipated that these compound mutant mice might be rescued by the linked BAC possibly via non-cell autonomous inductive effects (perhaps as a consequence of the mesenchyme inducing ureteric epithelial differentiation), on the basis of previous data we did not expect to observe GATA-2 expression in the epithelium (7). The previously characterized lacZ-tagged YAC transgene (called d16, Fig. 1A (6)) has boundaries that map to positions −174 to +73 kbp according to the revised genome sequence, and an overlapping BAC (333I12, from +60 to +268 kbp of the Gata2 locus) was also previously examined in the "BAC-trap" lacZ reporter assay. These two sequences together encompass sequences represented by both the 5′ BAC (115E9, −187/−10 kbp of the Gata2 locus) and the 3′ BAC (81F7, −34/+226 kbp) that we examined in this study, and in neither of the former reporter transgenic studies were we able to

failure (due to the anticipated absence from the transgene of any urogenital epithelia-complementing activity), or even from a deficiency in some new tissue that was regulated by an enhancer located outside the boundaries circumscribed by the linked BAC.

Through the analyses of two independent transgenic lines, complementation of the germ line Gata2 null mutation by the 413-kbp linked BAC appears to be complete: Gata2+/−:TgG2BAC mice are born in the anticipated Mendelian ratio, they display no obvious physiological deficiencies, and they parent offspring without apparent difficulty. The size and general health of all viable progeny derived from Gata2+/−×TgG2BAC intercross is indistinguishable from that of wild-type mice (data not shown, supplemental Table S2).

One major unexpected consequence resulting from the BAC linking was the observation that fusion of the two parental BACs led to Gata2 expression in the urogenital epithelium, whereas all of our previous studies examining the expression of lacZ-tagged BACs and YACs that fully overlapped this domain

**FIGURE 3.** BAC-complemented Gata2+/− mice are born in a Mendelian distribution. A, Q-PCR was performed on genomic DNA samples prepared from the tails of sibling pups (S1–S10) from a presumptive Gata2+/−:TgG2BAC×Gata2+/−:TgG2BAC intercross (primer sequences for the EII-KI allele are shown in supplemental Table S1). Q-LacZ Q-PCR was used as a diploid copy number control. The strategy was designed to detect the EII-KI (Gata2+−) allele in all GFP+ mice (supplemental Table S1), and therefore wild-type mice have a ratio (Gata2+/−:lacZ) of 0, EII-KI heterozygotes (e.g. both parents) have a ratio of 1, and EII-KI homozygotes (Gata2 null mutant mice) have a ratio of 2. B and C, representative semi-quantitative PCR results depicting detection of the linked BAC transgene allele (B, using BAC5′jx primers, supplemental Table S1) or the EII-KI (−) or wild-type (+) alleles (C, referred to as GFP or wt alleles, supplemental Table S1) from a presumptive Gata2+/−:TgG2BAC×Gata2+/−:TgG2BAC intercross. Note that every sibling (S1–S10) from this mating carries a (−) germ line allele, whereas only seven of nine sibs harbor a BAC allele, proving that the genotype of the original transgenic parent (P2) is Gata2+/−:TgG2BAC. Note that the + allele can be contributed either through the germ line or from a BAC-derived Gata2 gene.
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Gata2
t-\textasciitilde

Gata2-\textasciitilde::TgG2BAC

FIGURE 4. The G2BAC transgene recapitulates mesenchymal and epithelial Gata2 urogenital expression. Transverse cryosections of e14.5 Gata2-\textasciitilde\textsuperscript{+/-} (A–D) and Gata2-\textasciitilde::TgG2BAC (E–H) embryos were subjected to co-immunostaining using anti-GFP (B and F) and anti-GATA-2 (C and G) antibodies, prior to hematoxylin and eosin histological staining (A and E). B and F, GFP immunofluorescence reflects the endogenous GATA-2 expression pattern in the urogenital mesenchymal and epithelial cells of Gata2-\textasciitilde\textsuperscript{+/-} and Gata2-\textasciitilde::TgG2BAC embryos. C and G, GATA-2 protein was immunologically detected in the urogenital mesenchymal and epithelial compartments of the Gata2-\textasciitilde::TgG2BAC embryo (C). In the Gata2-\textasciitilde::TgG2BAC embryo, anti-GATA-2 immunoreactivity was present not only in the urogenital mesenchyme, but also in the ureteric epithelium (G). D and H, merged images of anti-GFP and anti-GATA-2 immunofluorescence. e, ureteric epithelium; m, urogenital mesenchyme.

gave no indication that a reporter gene could be expressed in that specific tissue (6–8). The current genetic and histological data support the notion that urogenital epithelial expression is functional only when the two BACs are joined in cis (1). This novel concept of a delocalized synthetic enhancer has no precedent insofar as we are aware. Recent studies have also described the existence of domain boundaries, which is established by an array of cis elements (including insulators, boundary elements) that serve to delimit an active transcriptional state in a given region of the genome (25–28). Thus a possible scenario is that there is a single Gata2 ureteric epithelial enhancer, which requires the full complement of domain boundaries present only in the linked BAC to activate GATA-2 expression in the ureteric epithelium. Further investigation into the mechanism by which the ureteric epithelial enhancer becomes functional promises to reveal novel and fundamental insights into transcriptional regulation.

We conclude from the data shown here that the urogenital deficiency detected in the YAC-rescued Gata2-\textasciitilde\textsuperscript{-/-} mice is indeed the cause of their perinatal lethality (6). We also conclude that Gata2 enhancers that have been identified lying outside the −187/+226 kbp boundaries (7) encompassed by the G2BAC are unnecessary for viability or healthy reproductive status. The possibility that these even more distant enhancers control functions for which GATA-2 activity is redundant seems likely, but this hypothesis remains to be tested.

Finally, recombineering-based mutagenesis strategies to link overlapping BACs via homologous recombination have been previously described (29–31), although none of the earlier reports attempted transgenic rescue with the manipulated DNA. Here, we employed a somewhat different approach by utilizing cre-mediated homologous recombination to exploit the documented differential recombination efficiency between homologous versus heterologous loxP sequences to create a fully rescuing BAC transgene. This could provide the scientific community with a powerful genetic tool that could be used to quickly and efficiently mutate specific sequences in vivo in any rescuing transgene. Interrogation of genomic locus activity using these straightforward recombineering strategies could provide a much more rapid way of assessing the effects of any mutation when compared with the lengthy process involved in the generation of germ line mutants by ES cell targeting, especially if one wishes to examine (e.g.) dozens of different mutations in the same genetic locus. Thus this BAC complementation strategy may offer several advantages over the creation of conditional germ line mutants for animals in which stable null mutations already exist.

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