Rescue of the embryonic lethal hematopoietic defect reveals a critical role for GATA-2 in urogenital development

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Mutations resulting in embryonic or early postnatal lethality could mask the activities of any gene in unrelated and temporally distinct developmental pathways. Targeted inactivation of the transcription factor GATA-2 gene leads to mid-gestational death as a consequence of hematopoietic failure. We show here that a 250 kbp GATA-2 yeast artificial chromosome (YAC) is expressed strongly in both the primitive and definitive hematopoietic compartments, while two smaller YACs are not. This largest YAC also rescues hematopoiesis *in vitro* and *in vivo*, thereby localizing the hematopoietic regulatory *cis* element(s) to between 100 and 150 kbp 5' to the GATA-2 structural gene. Introducing the YAC transgene into the GATA-2^{-/-} genetic background allows the embryos to complete gestation; however, newborn rescued pups quickly succumb to lethal hydro-ureteronephrosis, and display a complex array of genitourinary abnormalities. These findings reveal that GATA-2 plays equally vital roles in urogenital and hematopoietic development.

Keywords: embryonic rescue/GATA-2/hematopoiesis/urogenital defects

Introduction

The GATA family of zinc finger proteins plays pivotal roles in cellular proliferation and differentiation in numerous lineages. The founding member of the family, GATA-1 (Evans and Felsenfeld, 1989; Tsai *et al.*, 1989), is required *in vivo* for both erythropoiesis and megakaryopoiesis (Fujiwara *et al.*, 1996; McDevitt *et al.*, 1997; Takahashi *et al.*, 1997). GATA-2 and GATA-3 (Yamamoto *et al.*, 1990) have been shown to be crucial determinants of various developmental processes, including early hematopoiesis, neurogenesis and T lymphopoiesis (Kornhauser *et al.*, 1994; Tsai *et al.*, 1994; Pandolfi *et al.*, 1995; Ting *et al.*, 1996; Zheng and Flavell, 1997). GATA-4, -5 and -6, which comprise a structurally distinct subfamily, may play important roles in cardiac gene regulation (Kelley

et al., 1993; Heikinheimo *et al.*, 1994; Laverriere *et al.*, 1994; Grepin *et al.*, 1995; Gove *et al.*, 1997; Molkenstein *et al.*, 1997), and GATA-4 additionally appears to be involved centrally in ventral specification (Molkenstein *et al.*, 1997). Among these factors, GATA-2 has been implicated in cellular proliferative responses in numerous, often unrelated, developmental pathways (Dorfman *et al.*, 1992; Briegel *et al.*, 1993; Nagai *et al.*, 1994; Walmsley *et al.*, 1994; Brewer *et al.*, 1995; Ma *et al.*, 1997). Verification of the paramount importance of GATA-2 to embryogenesis was demonstrated by targeted ablation of the gene. Mutant embryos lacking GATA-2 fail to generate a sufficient number of primitive erythrocytes and die at mid-gestation, showing that GATA-2 plays a crucial role in the earliest phases of blood formation (Tsai *et al.*, 1994).

Since its inception more than a decade ago, gene targeting in the mouse has provided biologists with an exceptionally versatile tool with which to manipulate the mammalian genome (Smithies *et al.*, 1985; Thomas and Capecchi, 1987). Gene corrections, conversions, substitutions, temporal, spatial and conditional alterations and (most often) the generation of null mutations can now be accomplished in a rather straightforward manner, given the variety of specialized procedures that have been developed around the basic methodology. Despite its obvious success, the most frequently encountered secondary hurdle is that homozygous mutant embryos fail to survive gestation. While this establishes the functional consequences of gene loss as the first developmental block is encountered, it also conceals possible functions of the gene subsequent to this most proximal event. This complication, in turn, has hastened the development of practical surrogate tools to study gene function (e.g. by analysis of chimeras and hypomorphic alleles, and tissue-specific gene rescue or ablation). While powerful, these surrogate methods do not reveal mechanisms for transcriptional regulation within the locus, since they essentially focus only on the structural gene. To understand the details of the regulatory hierarchy that leads to tissue-specific gene expression, and to prove immediate epistatic relationships in genetic pathways, regulatory modules controlling the expression of a gene must be identified, dissected in molecular detail and tested for functionality *in vivo*.

Here we show that after modification by insertion of a *lacZ* reporter gene at the translational initiation site, a 250 kbp GATA-2-containing yeast artificial chromosome (YAC) recapitulates GATA-2 expression during murine embryogenesis. Two smaller YACs, ~200 or 120 kbp in size, respectively, fail to confer significant fetal liver (definitive hematopoietic) expression. We then show that the 250 kbp YAC can rescue the embryonic lethal defect in hematopoiesis that originally was attributed to targeted ablation of the GATA-2 gene. GATA-2 is expressed from the YAC at essentially wild-type levels, and normal

erythroid and myeloid lineage cells are generated *in vitro* and *in vivo*. Thus the hematopoietic regulatory controls for *GATA-2* lie within this YAC.

Complementation of the hematopoietic embryonic lethal defect due to gene targeting revealed a new deficiency in a tissue that had not been implicated as one requiring *GATA-2* function. The kidneys and ureters of *GATA-2*^{-/-} YAC compound mutant newborns are severely deformed, and neonates quickly succumb to fatal hydronephrosis. This abnormality was shown to result from a block in urine excretion due to an underlying mid-embryonic failure of the ureters to connect properly to the bladder. Other sex and excretory organs are also malformed in the YAC transgenic compound mutant mice, demonstrating that *GATA-2* plays a broad role in urogenital development. Since these urogenital phenotypes were revealed only after hematopoietic rescue (thus allowing the animals to survive gestation), these experiments suggest that analogous strategies may be valuable in revealing vital activities for other genes whose later developmental functions presently are obscured because of embryonic lethality.

Results

Isolation and characterization of *GATA-2* YACs

Four YAC clones containing the mouse *GATA-2* gene were identified originally from a YAC library (Research Genetics) by PCR. Extensive analysis of these YAC clones indicated that while all four contained the intact 14 kbp *GATA-2* structural gene, only one, YAC 22-5E7, was intact (Figure 1A; data not shown). YAC 22-5E7 is 620 kbp in size, and contains 520 and 80 kbp of 5' and 3' sequence flanking the *GATA-2* structural gene, respectively, and is oriented with the 5' end of the gene closest to the YAC right (non-centromeric) vector arm (Figure 1A). To facilitate its manipulation and microinjection, a series of YAC deletion mutants, containing varying amounts of 5' sequence, were generated using standard YAC deletion procedures (Heard *et al.*, 1994; Emanuel *et al.*, 1995; Figure 1B). YAC subclone d16, which is 250 kbp, contains large segments of 5'- and 3'-flanking sequence (150 and 80 kbp, respectively), while its overall size is still quite manageable for microinjection. Two smaller YACs, d27 and d18, which differ from d16 only in their 5' ends, are ~200 or 120 kbp in size, respectively (Figure 1B).

GATA-2 transcription in the hematopoietic system is recapitulated by a *GATA-2-lacZ* YAC reporter transgene

To define the transcriptional potential of these *GATA-2* YAC subclones, each was first tagged by inserting the bacterial *lacZ* gene into the *GATA-2* translation initiation site (Materials and methods; Lakshmanan *et al.*, 1998; Minegishi *et al.*, 1998). The *lacZ*-modified YACs (referred to below as d16Z, d27Z or d18Z) were isolated from pulsed-field gels, purified and then injected into fertilized CD1 ova to generate transgenic mice (Schedl *et al.*, 1993; Bungert *et al.*, 1995). Transgenic founders were identified initially by PCR using primer pairs specific for the YAC vector arms, as well as for the *lacZ* gene. Ten d16Z founders that were positive for all three PCR markers were identified, and seven of these transmitted the trans-

Fig. 1. Structures of the *GATA-2* YACs and PFGE mapping of the d16Z YAC transgenic lines. (A) The orientation and composition of the four isolated murine *GATA-2* YACs are depicted schematically. The top line represents the *NotI* restriction map of C57Bl/6 (B6) chromosome 6 in the vicinity of the *GATA-2* locus (cross-hatched), while the next four lines depict the deduced structures of the four isolated YAC recombinants, with their sizes indicated in parentheses. All four YACs contain an intact mouse *GATA-2* gene (black box). Clones 24-3F10 and 19-6B7 are chimeric, consisting of non-contiguous genomic DNA fragments (indicated by the open bar; data not shown). The YAC left and right vector arms are indicated by an open oval and an open rectangle, respectively. The size of each YAC recombinant is shown in parentheses. (B) B1 repeat recombinants of YAC 22-5E7 are illustrated diagrammatically. Yeast cells bearing YAC 22-5E7 were electroporated with a linearized B1 repeat recombinant plasmid (Heard *et al.*, 1994), and subclones bearing a new (*LYS2*) right vector arm (rectangle) contain different amounts of DNA deleted from the 5' end of the YAC. (C) Integrity of d16Z transgenic lines: PFGE mapping of four *GATA-2* YAC d16Z transgenic lines: d16Z5 (lanes 1), d16Z15 (lanes 2), d16Z31 (lanes 3) and d16Z43 (lanes 4; see Materials and methods). Probes used for each panel are indicated at the bottom of each panel. Transgenic lines Z5, Z15 and Z31 all contain at least one intact YAC transgene copy, while line Z43 does not. Three additional d16Z transgenic lines examined (Z1, Z8 and Z28) were also found to be fragmented using the same assays (data not shown).

gene through the germline. The integrity of the d16Z YACs in these seven lines was analyzed in F₂ or subsequent generation animals using PCR, pulse-field gel electrophoresis (PFGE) and YAC telomere analysis by Southern blotting of thymus DNA (Liu *et al.*, 1997). While several of the lines contained broken YACs, three contained at least one intact d16Z transgene copy (Figure 1C).

In the hematopoietic system, we noted that staining in the fetal liver of d16Z embryos became apparent at 10.5 days post-coitus (d.p.c.) and remained intense until

Fig. 2. YAC transgenes recapitulate the *in vivo* developmental expression of GATA-2. Embryos (11.5 d.p.c.) from transgenic lines d16Z5 (A), d27Z27 (B) and d18Z1 (C) were stained for β -galactosidase activity. All transgenic embryos displayed strong, identical staining patterns in the CNS (midbrain, hindbrain and spinal cord), the developing heart and the placenta (not shown). Only d16Z transgenic embryos displayed strong additional staining in the fetal liver (fl), which was conspicuously absent in the d27Z and d18Z transgenic lines. (D) Section of a 12.5 d.p.c. d16Z15 transgenic fetal liver stained for β -galactosidase activity. (E) Section of a wild-type 12.5 d.p.c. liver stained with a rat anti-GATA-2 monoclonal antibody RC1.1 (Materials and methods). (F) A 9.5 d.p.c. d16Z15 transgenic whole-mount embryo stained for *lacZ* activity. Note that the stained cells appear in a symmetrical pattern flanking both sides of the dorsal aorta. (G) Yolk sac of a 9.5 d.p.c. d16Z5 transgenic embryo. Distinct cells in the yolk sac were labeled, clearly identifiable as hematopoietic cells upon sectioning (H), which were not seen in d27Z or d18Z transgenic lines. (I) Bone marrow section from an adult d16Z15 mouse. (J) Spleen section of an adult d16Z15 transgenic mouse, displaying a strong punctate distribution of *lacZ*-positive cells in the red pulp.

~13.5 d.p.c., but faded gradually thereafter (Figure 2A; data not shown). Detailed examination of thin sections from 12.5 d.p.c. embryos showed that a similar number and kind of fetal liver cells were stained either by β -galactosidase in the d16Z YAC transgenic animals (Figure 2D) or by a GATA-2-specific monoclonal antibody in wild-type embryos (Figure 2E). At earlier stages (9.0–9.5 d.p.c.), robust *lacZ* staining was detected in two parallel cables of mesenchymal cells flanking the dorsal aorta of d16Z embryos (Figure 2F). This staining was strikingly reminiscent of the aorta, gonads and mesonephros (AGM) region that defines the earliest definitive hematopoietic stem cell compartment in the embryo (Muller *et al.*, 1994). Patchy staining was also detected in the visceral yolk sac at the same stage (Figure 2G), which upon detailed examination revealed exclusive labeling of primitive erythroid lineage cells (Figure 2H). The d16Z YAC is also expressed in the adult bone marrow as well as in a small fraction of hematopoietic cells in normal adult spleens (Figure 2I and J, respectively).

The two smaller GATA-2 YAC *lacZ* subclones (d27Z and d18Z; Figure 1B) were also examined in a similar fashion. Embryos from multiple transgenic lines bearing these two YACs exhibited staining patterns identical to the d16Z transgenic embryos at all developmental stages examined, except in one important respect. *lacZ* expression in hematopoietic tissues was virtually absent in the d27Z

and d18Z transgenic embryos (Figure 2A–C; data not shown).

In summary, a putative positive regulatory element(s) conferring high level expression to *GATA-2* in the primitive and definitive hematopoietic compartments resides somewhere between the 5' boundaries of the d27Z and d16Z YACs (which differ from one another by ~50 kbp; Figure 1B). Thus the hematopoietic regulatory element(s) is located between 100 and 150 kbp 5' to the *GATA-2* gene (Minegishi *et al.*, 1998). Since *GATA-2*^{−/−} embryos die of hematopoietic failure, YAC d16 appeared to represent the minimal locus that might be able to rescue this *GATA-2* deficiency.

GATA-2 transcription outside the hematopoietic system

We next determined whether the *lacZ* expression patterns in the intact transgenic lines coincided with normal *GATA-2* expression in non-hematopoietic cells. Detailed histological examination of the intact d16Z, d27Z and d18Z transgenic lines confirmed the coincidence of β -galactosidase staining and multiple sites of previously established *GATA-2* expression.

Numerous tissues in the d16Z transgenic embryos stained quite prominently: first, strong expression in the ectoplacental cone and parietal yolk sac was found as early as 8.5 d.p.c., and persisted throughout gestation,

coincident with normal GATA-2 expression in the placenta (Ng *et al.*, 1994; Ma *et al.*, 1997). A transient phase of expression is also detected in the endocardium of the developing heart between 9.5 and 12.5 d.p.c. (Figure 2A–C; data not shown), as well as in endothelial cells, another site of prominent GATA-2 expression (Dorfman *et al.*, 1992; data not shown). Finally, β -galactosidase activity conferred by the d16Z GATA-2 YAC is quite robust in the embryonic central nervous system (CNS), including the midbrain, hindbrain and spinal cord, between 10.5 and 14.5 d.p.c. (Figure 2A–C). Further studies showed that the CNS *lacZ* expression pattern precisely overlaps endogenous GATA-2 expression there (Kornhauser *et al.*, 1994; Y.Zhou, K.-C.Lim, K.Onodera and S.Takahashi, unpublished observations). We conclude that the elements conferring GATA-2 expression in the placenta, CNS and developing heart are all contained within the 250 kbp d16Z (Figure 2A), the 200 kbp d27Z (Figure 2B) and the 120 kbp d18Z transgenic YACs (Figure 2C).

GATA-2 YAC transgenes rescue the hematopoietic defect in *GATA-2*^{-/-} embryos

GATA-2-deficient embryos die at ~10.5 d.p.c. of embryogenesis, and current evidence suggests that this lethality is due to a failure in primitive erythroid proliferation (Tsai *et al.*, 1994). Since the d16Z transgene conferred robust expression of the GATA-2-directed reporter in embryonic tissues representing the sites and times of most abundant fetal blood formation, while two smaller YACs did not, we next asked whether or not the 250 kbp d16 YAC could complement the hematopoietic defect encountered in *GATA-2* homozygous mutant embryos. As the d16 YAC is structurally indistinguishable from the endogenous GATA-2 locus, except at the telomeric ends, we introduced a silent mutation into the YAC (by deleting a *Bam*HI restriction site present within intron 3, thereby generating subclone d16B) so that all three alleles (wild-type, germline gene-targeted and YAC) of *GATA-2* could be distinguished from one another.

Purified YAC d16B DNA was injected into fertilized ova according to standard protocols (Materials and methods). Ten founders were recovered, of which only four transmitted the d16B transgene. The integrity of the integrated YACs was characterized by PFGE Southern blotting, which showed that the established lines d16B15, d16B63 and d16B89 all bear at least one intact YAC transgene copy (Figure 3A). Each of these animals was then intercrossed to *GATA-2*^{+/-} mice to recover germline heterozygous mutant F₂ that carried the YAC transgene. These *GATA-2*^{+/-}::d16B YAC mice were intercrossed again with *GATA-2*^{+/-} animals, and the resultant litters were recovered at different embryonic times and genotyped to determine whether or not *GATA-2*^{-/-}::YAC d16B animals (abbreviated below as 'compound mutant' embryos or pups) were viable.

Since *GATA-2*^{-/-} mutant animals die of hematopoietic failure at 10.5 d.p.c., we first asked if the hematopoietic defect was rescued by the transgene. As shown in Figure 4A and D, 10.5 d.p.c. *GATA-2*^{-/-} embryos could be identified unequivocally by their pale yolk sac and vasculature containing very little blood. All of the compound mutant embryos, however, had a normal yolk sac which was well vascularized (Figure 4B and E) and indistinguish-

Fig. 3. YAC integrity and expression of GATA-2 in compound mutant transgenic mice. (A) Integrity of the *GATA-2* d16B YAC in four transgenic lines. Lines d16B15 (lanes 1), d16B63 (lanes 2) and d16B89 (lanes 3) bear intact transgene copies (the band detected in line d16B15 by the *GATA-2* 5' probe is >2 Mbp, not shown), while line d16B55 (lanes 4) is fragmented. (B) Southern blot of *Bam*HI-digested tail DNA isolated from individual pups in a single litter from an F₂ (*GATA-2*^{+/-}::d16B15 YAC) × *GATA-2*^{+/-} intercross. The expected *Bam*HI fragment sizes of the YAC (YAC; 5.6 kbp), *GATA-2* gene-targeted mutant (M; 4.6 kbp) and *GATA-2* wild-type (WT; 3.8 kbp) alleles are indicated on the left. Pups 10.1 and 10.4 are both of genotype *GATA-2*^{-/-}::d16B YAC. (C) RT-PCR analysis of *GATA-2* expression in 10.5 d.p.c. d16B15 YAC compound mutant embryos, using murine S16 mRNA levels as internal control. The genotypes and expression level (expressed as a percentage of wild-type; lane 5) were: lane 1, (-/-) 0%; lane 2, (-/-::YAC) 58%; lane 3, (-/-::YAC) 71%; lane 4, (+/-) 60%; lane 5, (+/+) 100%; lane 6, (+/-::YAC) 130%; lane 7, (+/+::YAC) 140%.

able from that of wild-type littermates (Figure 4C and F). Semi-quantitative RT-PCR analysis of total RNA recovered from compound mutant embryos at this stage showed that *GATA-2* is expressed at essentially wild-type levels (Figure 3C). Hence, the 250 kbp YAC d16B, containing 150 and 80 kbp of sequence flanking the structural gene, is capable of restoring *GATA-2* functions at this stage of embryonic development and reversing the effects of the null mutation.

Next, we asked if the compound mutant mice survived embryogenesis. When examined at P0, compound mutant pups were found amongst litters generated using all three intact d16 YAC transgenic lines for rescue (e.g. Figure 3B), and at the expected Mendelian frequency. These data show (as expected from the restoration of embryonic hematopoiesis) that the embryonic lethality observed in *GATA-2*^{-/-} embryos was indeed fully rescued by the d16B transgene integrated at any chromosomal position (Table I).

When the embryos and pups were examined in greater detail for hematopoietic phenotypes, we found that both

Fig. 4. Hematopoiesis is normal in compound mutant pups. At 10.5 d.p.c., the yolk sac of *GATA-2* null embryos (A) is paler than that of the compound mutant (B) or wild-type (C) embryos. The *GATA-2* homozygous mutant embryos reflect the same deficiency (D), which is not found in either the compound mutant (E) or wild-type (F) embryos. (G and H) Micrographs of liver tissue sections of *GATA-2*^{+/-}::YAC d16B or *GATA-2*^{-/-}::YAC d16B P0 pups, respectively, taken at the same magnification. Arrows indicate typical megakaryocytes surrounded by other nucleated and enucleated (erythroid) hematopoietic cells. (I and J) Tissue sections of wild-type or *GATA-2*^{-/-}::d16B compound mutant transgenic P0 pups, respectively, showing an approximately equal distribution of mast cells (arrows) near the surface of the skin.

Table I. The 250 kbp d16B YAC rescues the *GATA-2*^{-/-} embryonic lethal phenotype

YAC transgenic line	Without YAC			With YAC		
	+/+	+/-	-/-	+/+::YAC	+/-::YAC	-/-::YAC
d16B15 (12 litters)	22	41	0	9	25	16
d16B63 (9 litters)	12	24	0	10	23	10
d16B89 (2 litters)	3	10	0	0	5	2
Total	37 (0.33)	75 (0.67)	0	19 (0.19)	53 (0.53)	28 (0.28)

the number and types of hematopoietic colonies produced *in vitro* from 13.5 d.p.c. fetal livers of compound mutant embryos were within the range of wild-type and heterozygous *GATA-2* mutant controls (Figure 5). Peripheral blood smears taken from compound mutant pups were indistinguishable from those from wild-type littermates. Normal numbers of erythrocytes, megakaryocytes and leukocytes were detected in the bone marrow (data not shown) and liver (Figure 4H) of the newborn compound

mutant pups. Finally, a normal number and distribution of mast cells was found in the skin of these animals (Figure 4J) in comparison with their wild-type littermate controls (Figure 4G and I, respectively). These data show that the d16B YAC transgene contains sequences sufficient for the rescue of myeloerythroid cell lineages in *GATA-2*^{-/-} animals, and further that this rescue leads to the survival of the *GATA-2*-null mutant mice through full-term gestation. These data therefore support the contention

Fig. 5. *In vitro* fetal liver hematopoietic progenitors in compound mutant mice. Two standard intercross matings were conducted between males (genotype $GATA-2^{+/-}::d16B$ YAC) bearing either the d16B15 or d16B63 YAC transgenes and $GATA-2^{+/-}$ females. Fetal livers were recovered from embryos at 13.5 d.p.c., dispersed and plated in methocel cultures containing IL-3, IL-6, SCF and Epo (see Materials and methods). Colonies were scored 7 and 10 days after seeding. The total number of fetal livers recovered, genotyped and assayed were: (+/+) = 4; (+/-) = 8; (-/-) = 0; (+/+):YAC = 6; (+/-):YAC = 3; (-/-):YAC = 2.

that the original embryonic lethal mutant defect was indeed hematopoietic failure (Tsai *et al.*, 1994).

Rescued compound mutant pups develop a fatal urogenital disorder

As described above, compound mutant pups were born in the expected Mendelian ratio (Table I) while no homozygous $GATA-2$ mutant animals survived gestation if they did not additionally carry the transgene. However, none of the compound mutant pups survived to weaning. Further examination revealed that compound mutant neonates rarely flourished beyond the first few days after birth, and they succumbed quickly to wasting.

Necropsies of the compound mutant pups revealed a single consistent abnormality: $GATA-2^{-/-}::YAC$ newborns rescued with any of the three d16B transgenes developed hydronephrosis, a condition characterized by fluid-filled dilation of the kidneys and ureters. At mild to moderate manifestation, the kidneys were enlarged with a single, fluid-filled cavity, while at its most severe extreme, the kidneys were riddled with multiple large ancillary cysts causing the morphology to be completely disrupted (Figure 6A and B). Both ureters were extremely bloated, resembling a clinical condition in humans called megaureter (Figure 6A and H).

Multiple embryonic stages were examined in the hope of determining the etiology of this defect. At 14.5 d.p.c., the metanephros of the compound mutant embryos appeared normal, with regular numbers of S- and comma-shaped bodies, indicating that glomerulogenesis *per se* was unaffected in the compound mutant embryos; the ureters also appear to be normal at this stage (Figure 6E). By 17.5 d.p.c., huge cysts were found in the kidneys of the compound mutant embryos (Figure 6F). Glomeruli and tubules were still relatively normal in appearance, although the lumen of Bowman's capsules and the tubules appear to be dilated (not shown). Therefore, nephrogenesis overall appeared to be relatively normal at 14.5 d.p.c. in the compound mutant embryos.

The phenotype detected first in the kidney and ureters

appeared to originate at 14.5–17.5 d.p.c., which coincides with the onset of urine excretion. This suggested that the hydronephrosis in the compound mutant pups was associated with a distal urinary obstruction. We therefore determined the position of the obstruction by dye injection. When dye was injected into the renal pelvis of wild-type pups, it flowed freely through the ureter into the urinary bladder (Figure 6G). In contrast, when compound mutant pups were treated in a similar fashion, the dye passed into the ureter but was trapped, demonstrating that the ureter-bladder junction was blocked (Figure 6H).

Further histological analysis of the compound mutant embryos and pups revealed multiple excretory and sex organ developmental defects later during embryogenesis. In compound mutant males, the conspicuously enlarged ureters entirely failed to connect to the bladder (Figure 7B). They were, instead, either joined aberrantly to the vas deferens or terminated blindly before impinging on any organ. The testis and epididymis of the compound mutant males appeared normal, but the seminal vesicles were often hypoplastic. Furthermore, the seminal vesicles and vas deferens were fused aberrantly into a single, common structure. In compound mutant females, the ureters were unconnected to other genitourinary organs, and terminated dorsally to the urinary bladder (data not shown). Compound mutant females also displayed abnormally developed uteri and vaginas, and several ducts appeared to be developmentally arrested at earlier stages than represented by their chronological age (Figure 7D). In addition, ~50% of the compound mutant animals, regardless of genetic sex, had a grossly distended rectum (Figure 7B).

Since the vas deferens and seminal vesicles are developmental descendants of the Wolffian (mesonephric) duct, while the uterus and vagina are derived from the Müllerian (paramesonephric) duct, we examined the Wolffian and Müllerian ducts in 14.5 d.p.c. embryos in greater detail (Figure 7E and F). In wild-type embryos, both Wolffian and Müllerian ducts were evident in both males and females (e.g. Figure 7E), and the ureter, which initially buds from the wall of the Wolffian duct, was in direct contact with the urogenital sinus (not shown). In contrast, compound mutant littermates displayed agenesis of the distal Müllerian ducts, and the ureter was not in contact with the urogenital sinus, but instead remained close to the Wolffian duct (Figure 7F). More caudally, there was evidence for continuity only of the ureters and Wolffian ducts.

Finally, we examined $GATA-2$ expression in the early embryonic urogenital system. At 11.5 d.p.c., $GATA-2$ is expressed strongly in the ureteric bud, the ureters and the mesonephric (future Wolffian) ducts, while the mesenchyme surrounding the bud (the metanephric blastema) and that surrounding the ureters do not express $GATA-2$ (Figure 8A). The strongest $GATA-2$ expression at 12.5 d.p.c. is detected in the ureteric bud branches in the metanephros, destined to become the collecting tubules of the definitive kidney (Figure 8C). The Wolffian ducts intensely express $GATA-2$ (Figure 8A, B and D), whereas the paramesonephric (Müllerian) ducts appear to be completely devoid of $GATA-2$ (Figure 8B). $GATA-2$ is expressed in the mesenchyme of the 12.5 d.p.c. urogenital ridge at moderate levels (not shown), and at low levels in

Fig. 6. *GATA-2*^{-/-}::d16B YAC compound mutant mice develop hydronephrosis. (A) The kidneys and ureters from compound mutant (cm) P0 neonates (right) are enlarged dramatically in comparison with those of wild-type (wt) littermates (left). (B) An extremely dilated kidney from another compound mutant pup. Kidney sections of wild-type (C and D) or *GATA-2*^{-/-}::YAC compound mutant (E and F) transgenic embryos at either 14.5 (C and E) or 17.5 d.p.c. (D and F). By 17.5 d.p.c., compound mutant kidneys have developed prominent central cysts (F). Injection of trypan blue into the renal pelvis of either wild-type (G) or compound mutant (H) neonates. Note that the colored dye does not enter the bladder (b) of the compound mutant animal, but rather accumulates in the kidneys (ki) and ureters (u).

the wall of the urogenital sinus in direct contact with the mesonephric ducts (Figure 8D).

In contrast, sectioning of comparable 11.5 and 12.5 d.p.c. 16Z YAC transgenic embryos revealed that there was no β -galactosidase staining in any part of the urogenital system in any of these lines (Figure 8E–H). Although intense blue staining was readily detectable in other tissues on the same slides in the fetal liver, spinal cord and dorsal aorta (e.g. Figures 2D and 8F are separate images from the same tissue section), β -galactosidase was never detected in the developing genitourinary tract. Since all the *lacZ* YAC transgenic lines exhibited the same expression pattern in the caudal regions of these embryos, the data show that the element(s) directing *GATA-2* expression in the developing urogenital system lies beyond the boundaries of these YACs.

Based on these observations, we conclude that a developmental defect in compound mutant animals leads to numerous urogenital abnormalities. This failure leads

to obstructed urine flow, which causes the severe bilateral hydronephrosis (Figure 6A and H) and postnatal lethality. We therefore conclude that in the absence of *GATA-2*, numerous other urogenital aberrations, affecting multiple genitourinary organ systems, occur, and that an underlying defect in ureter/bladder morphogenesis is the most likely cause of post-embryonic lethality observed in the compound mutant animals. All of the reported phenotypes were observed consistently in compound mutant animals derived from all three *GATA-2* YAC transgenic lines, but were not observed in wild-type littermates carrying the YACs. Therefore, the urogenital defects must be due specifically to a lack of *GATA-2* function during early genitourinary development, and cannot be due to any transgene-specific gain of function, nor to loss of important functions because of disruption by transgene integration. Thus the rescuing d16B *GATA-2* YAC lacks a sequence(s) that is required for normal *GATA-2* expression at early, and obviously critical, steps in urogenital morphogenesis.

Fig. 7. GATA-2 deficiency leads to aberrant organogenesis in the male and female reproductive tracts. Cross-sections of the lower trunk of wild-type (A and C) or compound mutant (B and D) male (A and B) or female (C and D) neonatal pups. (E and F) Cross-sections of 14.5 d.p.c. wild-type or compound mutant embryos, respectively, at the lower lumbar level; dorsal is up in both panels. At 14.5 d.p.c., the ureters (u) and Müllerian duct (md) are distinct from the Wolffian duct (wd) in the wild-type embryo (E), but the ureters are joined to the Wolffian duct caudally, and the Müllerian ducts are missing in the compound mutant embryo (F). In the compound mutant male shown here (B), the left ureter ends blindly while the right ureter connects to the vas deferens (vd). Therefore, the vas deferens, the seminal vesicles (sv) and epididymis (ep) are all dilated on the right side of the embryo, while they appear superficially normal on the left side in this section. The rectum (r) of compound mutants is often greatly enlarged, and the wall was stretched into a very thin layer with almost no supporting musculature. In the compound mutant female (D), both the uterus (not shown) and vagina (v) are severely affected; other aberrant structures are also observed (*). Other abbreviations: h, hindgut; b, bladder; ur, urethra; t, testis.

In summary, these data show that the loss of GATA-2 expression in compound mutant neonates leads to organ-specific pathology. Furthermore, the data demonstrate directly that at least one key regulatory element controlling GATA-2 expression during urogenital development is located beyond the boundaries described by the d16B

YAC transgene (i.e. either >150 kbp 5', or >80 kbp 3', to the GATA-2 gene). Finally, the data prove that GATA-2 is indispensable for both normal hematopoiesis and urogenital morphogenesis, a surprising conclusion since GATA-2 had not been implicated previously in ureter, bladder or sex organ development.

Fig. 8. GATA-2 is expressed in the nascent urogenital system. Transverse sections through the lower lumbar region of 11.5 (A) or 12.5 d.p.c. (B, C and D) wild-type embryos, stained with the RC1.1 anti-GATA-2 monoclonal antibody (Materials and methods). Comparable tissue sections of 11.5 (E) or 12.5 d.p.c. (F, G and H) d16Z15 embryos stained for β -galactosidase activity. Abbreviations: u, ureter; wd, Wolffian (mesonephric) duct; ub, ureteric bud and branches; mb, metanephric blastema; us, urogenital sinus; md, Müllerian (paramesonephric) duct.

Discussion

These experiments illustrate three important features concerned with genetic manipulation of embryogenesis. First, that YAC transgenes, when examined in combination with well-characterized mutations, can be used to complement embryonic lethal phenotypes. Secondly, and most importantly, they show that rescuing transgenes can reveal novel gene functions at different developmental stages, and in different tissues, that previously were obscured by the original mutation. Additionally, these experiments provide direct evidence that transcriptional control elements can be located extremely far from structural genes.

Gene rescue

Despite the original expectations for employing YAC approaches to gene rescue (Capecchi, 1993), it has been difficult to implement routinely. The complications probably arise from both conceptual and empirical concerns: for example, from analysis of the limited data set presented here, randomly isolated individual YACs appear to contain a substantial number of non-contiguous chromosome segments. This realization, in turn, demands that each YAC recombinant must be analyzed thoroughly before initiating experiments. Secondly, as is already broadly appreciated, there are significant difficulties involved in YAC DNA preparation and manipulation relative to smaller DNAs. Our modified YAC DNA purification protocols consistently yield intact DNA preparations, which significantly improves transgenic efficiency (Materials and methods).

Finally, even when these initial obstacles are surmounted, the routine recovery and transmission to progeny of intact YAC transgenes larger than 300 kbp is, in our experience, not easily quantified. Despite these more or less vexing complications, even a cursory survey of the literature demonstrates that these large transgenes can be of inestimable value in the rescue of mutant activities. α 1 procollagen, HGPRT, tyrosinase, *clock* and CFTR have all been rescued using transgenic YACs or bacterial artificial chromosomes (BACs) (Jacobvits *et al.*, 1993; Strauss *et al.*, 1993; Montoliu *et al.*, 1996; Antoch *et al.*, 1997; Manson *et al.*, 1997). In several of these instances, investigators resorted to the use of these large DNAs simply because the structural genes themselves were large or their limits unknown, and thus YAC or BAC cloning represented the most effective means of generating fully coding transgenes.

The *mGATA-2* structural gene is ~14 kbp in size (Minegishi *et al.*, 1998). The 250 kbp *mGATA-2* YAC, that we have shown here to be capable of rescuing embryonic and fetal liver hematopoiesis, is ~20 times the size of the structural gene. While this transgene does contain the appropriate patterning elements for GATA-2 expression in, e.g., the placenta, fetal liver, heart and brain, it is clearly not sufficient to evoke GATA-2 transcriptional response in all tissues where it normally is required. Thus the element(s) that regulates GATA-2 expression in multiple tissues within the urogenital system must, deductively, lie very far (>150 kbp 5', or >80 kbp 3') from the gene. While previous mapping and genetic studies have implicated transcriptional control elements that lie

vast distances from their target genes (e.g. Bedell *et al.*, 1995), none have been demonstrated previously by rescue, as has the hematopoietic element defined here.

This strategy for gene rescue initially was based on the expression of a *GATA-2* YAC used as a reporter gene. We first showed that one of four isolated YACs harbored contiguous DNA sequence representing only the *GATA-2* locus, while at least two others are chimeric DNA molecules. We then reduced the size of the intact *GATA-2* YAC to facilitate its manipulation. *GATA-2* YAC subclone d16 was first marked by insertion of the bacterial *lacZ* gene. We found that this *GATA-2-lacZ* YAC reporter transgene was expressed strongly in fetal liver hematopoietic cells, while two smaller YACs were not. Given the intense fetal liver staining, we therefore assumed that YAC d16 might be able to complement the defect in hematopoiesis which was compromised by the inactivating *GATA-2* germline-targeted mutation (Tsai *et al.*, 1994).

***GATA-2* function in hematopoiesis**

Intercrosses between YAC transgenic and *GATA-2* gene-targeted mutant animals showed that the transgene was indeed able to overcome the embryonic lethality caused by the original mutation. The data, taken together, show that the transgenic *GATA-2* YAC gave rise to normal numbers of myeloerythroid lineage-definitive progenitor cells, that mature hematopoietic cells are anatomically disposed in a normal pattern and abundance, and that the *GATA-2* mRNA levels generated from the transgene are comparable in abundance with the levels in wild-type mice. The genetic, biochemical and histological data thus show that this 250 kbp transgene rescues the original defect in embryonic blood maturation, thereby allowing the embryos to complete gestation, and provides additional evidence supporting the hypothesis that the lethality of the *GATA-2*-targeted mutation was indeed due to defective hematopoiesis.

During the course of these studies, we also noted that at the 9.5 d.p.c. embryonic stage, the *GATA-2-lacZ* YAC-labeled cells corresponded closely both temporally and spatially to the AGM, the mesenchyme that gives rise to long-term reconstituting hematopoietic stem cells (Muller *et al.*, 1994; Medvinsky and Dzierzak, 1996; Miles *et al.*, 1997). Since analysis of RAG-2 chimeras showed that *GATA-2* is required for definitive hematopoiesis (Tsai *et al.*, 1994), the labeling pattern of the YAC is consistent with the notion that *GATA-2* may be among the transcription factors regulating differentiation and/or proliferation in hematopoietic stem cells (Hu *et al.*, 1997).

***GATA-2* function in urogenital development**

The rescued *GATA-2*^{-/-}::d16B YAC compound mutant transgenic animals did not survive for long after birth. When the newborn compound mutant pups were examined in detail, they were found to display a spectrum of urogenital phenotypes in both sexes, all of which indicated a single etiological origin: the kidneys and ureters were badly misshapen, displaying varying degrees of hydronephrosis. Thus, when the *GATA-2* YAC was combined with the original germline null mutation, the union revealed a previously masked lethal phenotype in tissues that require *GATA-2* for proper function at a later time in development. These later embryonic defects arise

in tissues not implicated previously as being among those requiring *GATA-2* activity. Thus, these experiments show that *GATA-2*, in addition to its paramount function(s) in hematopoiesis, is also vital for normal urogenital development.

What is the nature of the defect in *GATA-2* expression that gives rise to such a broad spectrum of urogenital phenotypes? The most likely possibilities are either that cell-autonomous functions for *GATA-2* are required in each of the affected tissues in the developing urogenital system of normal mice, or that *GATA-2* directs the expression of cell signaling ligands and/or receptors which then induce the appropriate tissue remodeling and differentiation responses in those tissues. The first possibility predicts that *GATA-2* would be expressed in all of the affected developing ducts and organs (which it is not; Figure 8), while the second possibility suggests that *GATA-2* activity need only be expressed in a subset of the affected tissues, and only at specific times during embryogenesis. The latter alternative also predicts that the inducing molecules required for appropriate tissue remodeling would be under the direct or indirect regulatory influence of *GATA-2*. Immunohistochemical analysis of the initial stages in genitourinary development shows that *GATA-2* is widely, but not ubiquitously, expressed there. Thus we conclude that *GATA-2* probably regulates both cell-autonomous and non-autonomous functions during genitourinary morphogenesis, and the identity of the ligands and/or receptors controlled by *GATA-2* await discovery. Interestingly, similar phenotypes have been observed in distal *hox* gene mutant animals, suggesting that *GATA-2* may perhaps lie in the same regulatory pathway as those genes (*hox10-13*) during urogenital morphogenesis (Warot *et al.*, 1997).

It has been estimated that developmental abnormalities of the genitourinary system affect 10% of the human population (Ashcraft, 1990). Such defects account for one-third of all congenital malformations, and are responsible for 40% of renal diseases. Many of these anomalies are known to have a genetic etiology. More than a dozen inherited diseases involving chromosomal rearrangements or single-gene mutations are known to cause hydronephrosis, hydroureter or cystic kidney diseases. The evidence presented here demonstrates that *GATA-2* dysfunction can lead to all three abnormalities *in vivo*, suggesting that *GATA-2* may be involved intimately in some of the analogous human conditions. The data also demonstrate that *GATA-2* is not only required for very early morphological steps in urogenital development, but further suggest (from its expression pattern in the developing kidney; data not shown) that it may participate in multiple steps in elaboration of the genitourinary tract. We currently are attempting to test this hypothesis by isolation and molecular dissection of the element(s) required for *GATA-2* expression in the developing urogenital tract.

Materials and methods

YAC isolation

YACs bearing the *GATA-2* gene were isolated by PCR screening of a murine YAC DNA library from Research Genetics (Birmingham, AL) using primers derived from the *mGATA-2* 3' untranslated region (5'S:

5'...CACGGAGAGTGTGTTGTTGGAGAGC...3' and 3' AS: 5'...CCCC-TTTCAGAGAATGTTTCCTTCC...3'), yielding a 431 bp product. The four recovered YAC recombinants were called 19-6B7, 22-5E7, 23-7D2 and 24-3F10, and each bears the intact *GATA-2* structural gene (data not shown). Their sizes were determined to be 780, 620, 550 and 450 kbp, respectively, by PFGE.

YAC characterization

Miniprep yeast DNA recovered from each YAC was digested with *EcoRI*, *HindIII*, *BamHI* and *SacI*, and probed with *GATA-2* cDNA. All four clones were found to contain the intact *GATA-2* gene (data not shown). Each yeast clone was embedded in agarose plugs (Birren and Lai, 1994), digested with *NotI* and then subjected to PFGE and Southern blotting with gene-specific as well as left and right YAC vector arm probes (Figure 1C). The *NotI* restriction endonuclease recognition site within exon IS (Minegishi *et al.*, 1998) of the locus was used to determine the orientation and position of the *GATA-2* gene within each of the YACs. Clone 23-7D2 was determined to contain too little 5'-flanking DNA to be of further use. When the YAC DNA was compared directly with mouse genomic DNA, we found that 22-5E7 was the only one that contained a genomic *NotI* site (located 230 kbp 5' to the *NotI* site in the gene) as C57Bl/6 strain genomic DNA (from which the YAC library originally was prepared). Thus 22-5E7 was the only clone used in subsequent studies. The position of the *GATA-2* gene and the size of each of the four original *GATA-2* YACs are shown in Figure 1A. The original data are available upon request.

B1 deletion mutagenesis

B1 deletion mutagenesis on the 620 kbp YAC 22-5E7 was done essentially as described (Emanuel *et al.*, 1995; Lakshmanan *et al.*, 1998). Since the non-centromeric right vector arm (bearing the *URA3* marker) of clone 22-5E7 is located 5' to the *GATA-2* gene, the mutagenesis generated a series of smaller YACs, with a new (*LYS2*) yeast selectable marker, bearing from 400 to <50 kbp of DNA 5' to the *GATA-2* structural gene (Figure 1B).

Gene replacement

Since the d16, d27 and d18 YAC subclones bear the *LYS2* selectable marker, *lacZ* was targeted into the gene using homologous recombination in yeast to select both for, and against, a *URA3* marker borne in the *lacZ* targeting plasmid (Guthrie and Fink, 1991). The linearized plasmid first was transformed into yeast bearing the YACs, and the correct integration and subsequent excision events (Winston *et al.*, 1983; Becker and Guarente, 1991; Bungert *et al.*, 1995; Liu *et al.*, 1997) were verified by Southern blotting (data not shown).

Purification/microinjection of YAC DNA

A procedure originally modified from Schedl *et al.* (1993) was used to purify YAC DNA for microinjection. Yeast DNA embedded in agarose plugs was fractionated on a 0.8% low-melt agarose gel under appropriate PFGE conditions. A slice was cut off from both sides and the middle of the gel and stained with ethidium bromide to visualize the DNA. The position of the YAC was marked, and the corresponding region on the unstained part of the gel was isolated. The gel pieces were then equilibrated in 20 ml of injection buffer (10 mM Tris, 0.1 mM EDTA and 100 mM NaCl) on ice, changing the buffer every 30 min three times. The gel slices were then blotted dry to remove as much residual buffer as possible. The slices were then transferred into 1.5 ml microcentrifuge tubes and incubated at 68°C for 10 min to melt the gel completely. The tube was then transferred to 42°C and incubated for 10 min. Agarase (1 U/100 µl) was added to the 400–500 µl sample, gently swirled, then incubated for 2 h and finally dialyzed against 40 ml of injection buffer at 4°C overnight. The samples were diluted 3- to 5-fold in injection buffer immediately prior to microinjection.

YAC transgene integrity

Thymus cells from animals bearing the d16B or d16Z YAC transgenes were embedded in low melting point agarose (10⁷ cells/ml) and digested with proteinase K to release DNA. The plugs were then digested with *NotI*, electrophoresed on pulsed-field gels, transferred to nylon and hybridized with YAC vector arm or gene probes. Since there is only a single *NotI* site in YAC d16 (Figure 1B), a transgene was deemed intact if the right arm probe hybridized to the same band as the 5' gene probe and was >150 kbp, and if the left vector arm probe hybridized to the same band as the 3' gene probe and was >80 kbp. The actual sizes of the bands in independent transgenic lines vary, depending on the

transgene integration site (and thus on the distance to the next randomly encountered *NotI* site in the genome).

In vitro colony assays

Individual fetal livers and yolk sacs were isolated from 13.5 d.p.c. conceptuses from *GATA-2*^{+/+} × *GATA-2*^{+/-}:YAC d16B matings; the yolk sacs were used for genotyping by Southern blotting (e.g. Figure 3B). Single cell suspensions from individual fetal livers (1.5 × 10⁴ cells) were seeded in duplicate in methocel supplemented with erythropoietin (Epo), interleukin-3 (IL-3), IL-6 and stem cell factor (SCF) (Stem Cell Technologies Inc., Vancouver, BC). Colonies (>30 cells) were scored as mixed (CFU-GEMM), myeloid (CFU-G, CFU-M and CFU-GM) or erythroid (BFU-E) at day 7 and day 10 of culture.

RT-PCR

Total RNA from 10.5 d.p.c. embryos was isolated using an Isogen RNA preparation kit (Nippon Gene, Japan) and quantified. Reverse transcriptase (RT) reactions were carried out as described (Foley and Engel, 1992) using 1 µg of total RNA from each embryo sample. One µl of the RT reaction (20 µl total volume) was diluted into 10 µl of H₂O: half was used for the *GATA-2* PCR analysis and half for murine *S16* PCR amplification as an internal control. The *GATA-2* primers used were: 5S 5'...ACTATGGCAGCAGTCTCTTCCATC...3' (from exon 3) and 3' AS 5'AAGGTGGTGGTGTGTCGTCTGAC...3' (from exon 5), and the size of the cDNA amplification product is 301 bp. The *mS16* primers were described previously (Leonard *et al.*, 1993), and yield a 190 bp product.

Immunohistochemistry

Embryos were harvested at 11.5 or 12.5 d.p.c. and fixed in phosphate-buffered saline (PBS) containing 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 for 30 min at 4°C. They were then equilibrated in 20% sucrose for 4–6 h prior to embedding in OCT. For antibody staining, 15 µm sections were blocked with 5% goat serum and 0.25% bovine serum albumin (BSA) in PBS. Purified rat anti-chicken *GATA-2* monoclonal antibody, RC1.1 (1:100; unpublished), which we determined to be cross-reactive with murine *GATA-2*, was then added and incubated overnight at 4°C. The next day, after treatment with hydrogen peroxide, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat secondary antibody (1:200) at room temperature for 2 h. Color development was carried out using diaminobenzidine (DAB) as chromogen.

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