BAC transgenic analysis reveals enhancers sufficient for *Hoxa13* and neighborhood gene expression in mouse embryonic distal limbs and genital bud

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SUMMARY We previously demonstrated that a ∼ 1 Mb domain of genes upstream of and including *Hoxa13* is co-expressed in the developing mouse limbs and genitalia. A highly conserved non-coding sequence, mmA13CNS, was shown to be insufficient in transgenic mice to direct precise *Hoxa13*-like expression in the limb buds or genital bud, although some LacZ expression from the transgene was reproducibly found in these tissues. In this report, we used β-*globin* minimal promoter LacZ recombinant BAC transgenes encompassing mmA13CNS to identify a single critical region involved in mouse *Hoxa13*-like embryonic genital bud expression. By analyzing the expression patterns of these overlapping BAC clones in transgenic mice, we show that at least two sequences remote to the *HoxA* cluster are required collectively to drive *Hoxa13*-like expression in developing distal limbs. Given that the paralogous posterior *HoxD* and neighboring genes have been shown to be under the influence of long-range distal limb and genital bud enhancers, we hypothesize that both long-range enhancers have one ancestral origin, which diverged in both sequence and function after the *HoxA/D* cluster duplication.

INTRODUCTION

Hox genes encode transcription factors that are conserved among all vertebrates and are essential for appropriate axial and appendicular embryonic patterning (McGinnis and Krumlauf 1992; Veraksa et al. 2000). Over the course of vertebrate evolution, a single ancestral Hox cluster underwent individual gene, and entire cluster, duplications leading to 39 Hox genes organized into four clusters (*HoxA–HoxD*) characteristic of all mammals (Kappen et al. 1989; Ruddle et al. 1994; Prince 2002). During embryonic axial patterning the Hox genes are colinearly expressed, both spatially and temporally (Duboule and Morata 1994). For appendicular patterning, the posterior *HoxA* and *HoxD* genes are re-expressed in the presumptive appendicular tissues. Specifically, the *Abd-B* derived genes in the posterior *HoxA* and *HoxD* clusters are necessary for limb and urogenital development (Fromental-Ramain et al. 1996; Mortlock and Innis 1997; Warot et al. 1997; Zakany et al. 1997). Mutations in human *HOXA13* are associated with dominantly inherited hand-foot-genital syndrome (HFGS; OMIM #140,000) (Mortlock and Innis 1997; Goodman et al. 2000) which is characterized by malformations of the distal limbs as well as urogenital structures. Similar malformations were also

observed in the spontaneous mouse mutant, *Hypodactyly* (Mortlock et al. 1996), and in engineered *Hoxa13* null mouse models (Fromental-Ramain et al. 1996; Stadler et al. 2001).

The function of posterior *HoxA* and *HoxD* genes in developing appendicular structures is believed to have been acquired secondarily to the primary, ancestral role of all four clusters in axial patterning (Herault et al. 1998; Spitz et al. 2001). Regulatory sequences responsible for the expression of the *HoxD* genes in the developing limb buds and genital bud have been mapped to locations remote from the cluster (Spitz et al. 2001; Spitz et al. 2003; Gonzalez et al. 2007), and we have hypothesized that the *HoxA* cluster is under a similar mode of regulation (Lehoczky et al. 2004). In previous work, we showed that a set of genes upstream of *Hoxa13*, (*Evx1*, *Hibadh*, *Tax1bp1*, and *Jazf1*), are expressed in *Hoxa13*-like distal limb and genital bud domains between E10.5 and E13.5 (Lehoczky et al. 2004). We hypothesized that a broadly acting enhancer is responsible for the shared spatial and temporal gene expression patterns. In addition, we hypothesized that the enhancer exerts function over a finite domain because genes immediately proximal and distal to the neighborhood (*Hoxa11* and *Crebpa*, respectively) are not expressed similarly.

A similar global enhancer has been described for the *HoxD* locus (Spitz et al. 2003; Gonzalez et al. 2007). At this locus,

Evx2 and *Lnp* are expressed in the distal limb and genital bud in a spatial and temporal pattern common to the posterior *HoxD* genes. Given that the four mammalian Hox clusters are derived from a single ancestral cluster, we hypothesized that a single regulatory innovation occurred in the *HoxA/D* lineage before cluster duplication. This regulatory mechanism was retained by both the *HoxA* and *HoxD* clusters, driving posterior *HoxA/D* gene expression in the developing appendicular and urogenital structures. Results of comparative sequence analysis supported our hypothesis, as five paralogously conserved (*HoxA* locus vs. *HoxD* locus) non-coding DNA sequences (putative regulatory elements) are shared between the *HoxA/D* upstream regions (paraCNS1-5) (Lehoczky et al. 2004). No similar sequence conservation was found between *HoxA/D* and the *HoxB* or *HoxC* genomic regions. This is consistent with absence of posterior *HoxB/C* expression in the *HoxA/D* expression domains of distal limbs and genital bud (though *Hoxc13* is expressed in the nail beds of the distal limbs (Godwin and Capecchi 1998; Godwin et al. 1998)). On the *HoxA* chromosome, paraCNS4 and 5 map to a conserved 2.25 kb element (mmA13CNS) and they also correspond to the "distal limb enhancer" critical region on the *HoxD* chromosome (Spitz et al. 2003). We tested the ability of mmA13CNS to direct LacZ expression in the developing limbs and urogenital system and found that mmA13CNS was insufficient for appropriate posterior *HoxD*-like/*Hoxa13*-like distal limb or genital bud expression. Although inappropriate LacZ expression was reproducibly found in those tissues, in multiple lines, we hypothesized that mmA13CNS may function in *Hoxa13* tissue-specific expression, but additional surrounding sequences were necessary for appropriate expression.

In this report, we created transgenic mice with BACs encompassing mmA13CNS with β-*globin* LacZ reporters in *cis* to examine our hypothesis and to begin to identify broad enhancer domains within which to focus future attention. By deduction, expression from overlapping BAC clones allowed us to demonstrate the involvement of a single critical region for *Hoxa13*-like embryonic genital bud expression. By contrast, the same BACs illustrate the existence of at least two separate loci remote to the *HoxA* cluster involved in *Hoxa13*-like distal limb expression from E10.5 to E13.5.

MATERIALS AND METHODS

LacZ expression from mmA13CNS mouse transgenic lines generated with β-*globin*-LacZ (data not shown) corroborated the expression found from the *Hsp68*-LacZ lines (Lehoczky et al. 2004). Both constructs were insufficient to drive appropriate *Hoxa13*-like distal limb or genital bud expression at any embryonic stage. We concluded that the two minimal promoters were comparable for the purposes of our experiments and proceeded to use the β-*globin* minimal promoter LacZ construct for all future experiments.

BAC modification
BAC clones from the *HoxA* locus RPCI-23-347D13 and RPCI-23-420L19 (BACPAC Resources) were identified by end sequences AZ032610, AZ032607, AZ068097, and AZ068091 (www.tigr.org). Original clones were both fingerprint (*Not*I and *Eco*RI) and PCR verified to assure identity and structure. The minimal promoter LacZ reporter cassette, pFNeoH1H2βglob-LacZ, was constructed to target the *sacB* gene in the pBACe3.6 backbone in either BAC. The neo cassette flanked by FRT sites cloned into the *Sma*I site of pBKS was obtained (gift of J. D. Engel) and modified to insert the β-*globin* minimal promoter LacZ reporter (kindly provided by R. Krumlauf) into the *Sal*I site.

BAC homology arms H1 and H2 were individually PCR amplified with restriction site tailed primers (H1_*Pme*I TTGTTTA AACGTTACGACTGCACTTCTGG, H1_*Pac*I.R TTTTAATTA ACGAATTGAGGCACTTGGT, H2_*Xho*I.F AACTCGAGCG ATATTTACATGCTTGGTT, and H2_*Kpn*I.R AAGGTACCCA TGTAGCTTGTGATAACCA) and directionally cloned into the targeting construct. The linear fragment for recombineering was generated from the resultant clone by insert excision with a *Pme*I/*Kpn*I double digest followed by gel purification. Targeting of the linear fragment into the BAC vector was done as previously reported (Yu et al. 2000; Lee et al. 2001; Khandekar et al. 2004) with the following exceptions. EL250 (kindly provided by N. A. Jenkins and N. G. Copeland) heat induced, electrocompetent cells (100 ml culture) were prepared when the density of the culture reached an A600 of 0.2. The culture was centrifuged 15 min at 10,000 rpm at 4°C and the resultant pellet was resuspended in 10 ml of ice cold 10% glycerol. This was repeated four times and the final pellet was resuspended in 100–200 μl of 10% glycerol. One hundred nanograms of the linear targeting construct was electroporated into 25 μl of freshly prepared, heat-induced cells. Clones that were chloramphenicol- and kanamycin-resistant were verified by PCR for the presence of the targeting event, and were used for subsequent arabinose induction of flp recombinase to excise the neo cassette. Resultant clones that were chloramphenicol-resistant and kanamycin-sensitive were both fingerprint and PCR verified to assure that the intended recombinant clones were obtained.

Transgenic mice and LacZ embryo staining
BAC transgene preparation was performed by standard alkaline lysis protocols followed by sepharose-CL-4B column purification as previously described (Yang et al. 1997). Undigested BAC transgene DNA was microinjected into (C57BL/6 × SJL) F2 fertilized eggs at ∼ 1 ng/μl (University of Michigan TAMC). All DNA samples extracted from yolk sacs or tail snips were genotyped for the BAC insert/BAC vector SP6 junction as well as the β-*globin*/LacZ junction. The preliminary 347D13-LacZ transgene microinjection was used for an E12.5 founder embryo harvest. This injection yielded five transgenic of 80 total embryos, two of which had LacZ expression. Additional 347D13-LacZ microinjections were performed to obtain permanent lines. Eight transgenic founders were obtained from a total of 331 pups. Three founder lines were used for timed embryo analysis (three founders did not

pass the transgene through the germline and two founder lines did not express the transgene). A single 420L19-LacZ transgene microinjection was performed and yielded 10 transgenic animals out of 108 total offspring. Seven founder lines were used for timed embryo analysis (two founders did not transmit the transgene and one founder line did not express the transgene). All timed matings and LacZ embryo staining were carried out as previously described (Lehoczky et al. 2004).

BAC transgene contribution analysis

Because the BAC clones were derived from the C57Bl/6 mouse strain, the transgenic lines were crossed onto a polymorphic strain to assess, by SNP analysis, what portion(s) of BAC transgene had integrated into the genome. Four 347D13-LacZ and seven 420L19-LacZ transgenic lines were individually crossed onto the DBA2/J strain, which is polymorphic relative to C57Bl/6J throughout and upstream of the *HoxA* locus. In all cases, F1 litters were genotyped for the presence of the transgene and these positive animals were obligate DBA2/J heterozygotes. Transgene+/−; DBA+/− animals were subsequently backcrossed onto the DBA2/J strain and N1 litters were once again screened for the presence of the appropriate transgene. Transgenic DNA samples were then genotyped for the C57/SJL/DBA polymorphic SNPs, RS3023069 and RS13478756, positioned adjacent to the endogenous region from which the BAC clones were derived. Resultant DNA samples that were transgenic and DBA congenic for the upstream *HoxA* region were used to PCR amplify across SNPs throughout the region of the BAC transgene. SNPs RS29851258, RS29857058, RS30707421, RS13478755, RS29863749, and RS30170317 were used for the 347D13-LacZ lines and only the latter three were used for the 420L19-LacZ lines (supplementary Fig. S3). Lines 250 and 268 were excluded from this analysis due to repeated unsuccessful attempts to generate transgenic DBA+/+ animals. PCR products were directly sequenced (University of Michigan Sequencing Core), and resultant chromatograms were visually inspected for the DBA SNP only (no transgene contribution), or DBA/C57 SNP (transgene contribution).

Transgene copy number analysis was performed with phenol/chloroform prepared spleen DNA from N1 or N2 C57Bl/6J backcross animals. Southern blots were prepared by standard methods. In brief, 10 µg of transgenic or C57Bl/6J wild-type genomic DNA was digested with *Eco*RI to completion. Before electrophoresis, *Eco*RI digested pBACe3.6 vector was spiked in fixed amounts into the wild-type digests to serve as transgene copy number controls. An *Eco*RI/*Kpn*I pBACe3.6 fragment was used for random hexamer primed labeling with ^{32}P-dATP. Blot hybridization and washing was performed by standard methods. Transgene copy number was determined by relative densitometry using the publicly available ImageJ software (NIH).

RESULTS

In this report, we compare transgenic expression to the endogenous expression patterns of *Hoxa13* in the limbs and genital bud, yet we believe our transgenic expression data

reflects the entire *Hoxa13* neighborhood of genes (*Evx1*, *Hibadh*, *Tax1bp1*, and *Jazf1*) (Lehoczky et al. 2004). We use *Hoxa13* expression as the representative gene for comparison because of its established necessity in limb and genital development. Endogenous expression of *Hoxa13* for stages E10.5–E13.5 is presented in supplementary Fig. S1.

BAC 347D13-LacZ is sufficient for proper *Hoxa13*-like expression

To address the hypothesis that additional sequences in the vicinity of mmA13CNS are needed for appropriate *Hoxa13*-like distal limb expression, we obtained a ~ 220 kb mouse BAC clone, RPCI-23-347D13, which overlaps mmA13CNS by a wide margin (~ 73 kb on one side and ~ 145 kb on the other) skewed toward the *HoxA* cluster (Fig. 1). We used homologous recombination to place the β-*globin*-LacZ reporter construct into the BAC vector backbone (Yu et al. 2000; Lee et al. 2001; Khandekar et al. 2004) and used the resultant recombinant BAC, 347D13-LacZ, for transgenic microinjection. E12.5 founder embryos were harvested and three of five transgenic founders had no LacZ expression in any tissue and were thus excluded from the analysis. The two remaining embryos both had LacZ expression in the central nervous system, limbs, and genital bud. The expression pattern found in the hindbrain and neural tube of both embryonic founders was consistent with the original mmA13CNS transgene expression (Lehoczky et al. 2004). We used this central nervous system *Evx1*-like (not *Hoxa13*) pattern of expression as a positive control for expression from the injected transgene.

The expressing founder embryos showed *Hoxa13*-like distal limb and genital bud LacZ expression. Autopod expression in the fore- and hindlimbs of founder 69 at E13 was virtually identical to the expected *Hoxa13* expression domain (Fig. 1, B, C, F and G and supplementary Fig. S1) (Haack and Gruss 1993; Mortlock et al. 1996; Innis et al. 2004; Lehoczky et al. 2004; Williams et al. 2005). Expression in the genital bud was posteriorly restricted (discussed in later section; data not shown, similar to Fig. 4E′). The limb expression in founder 18 was restricted to the posterior autopod (discussed in later section; shown in supplementary Fig. S2), yet genital bud staining was appropriate when compared with endogenous expression of *Hoxa13* (Fig. 1D and H) (Stadler et al. 2001; Lehoczky et al. 2004; Scott et al. 2005). Collectively, these embryos demonstrate that 347D13-LacZ is sufficient for E12.5 *Hoxa13*-like expression in the distal limb and genital bud.

To further interrogate the ability of 347D13-LacZ to direct *Hoxa13*-like expression at additional developmental stages, we repeated the transgene injection and generated three permanent lines (8, 710, and 733). Lines 8 and 733, while exhibiting the expected central nervous system LacZ staining
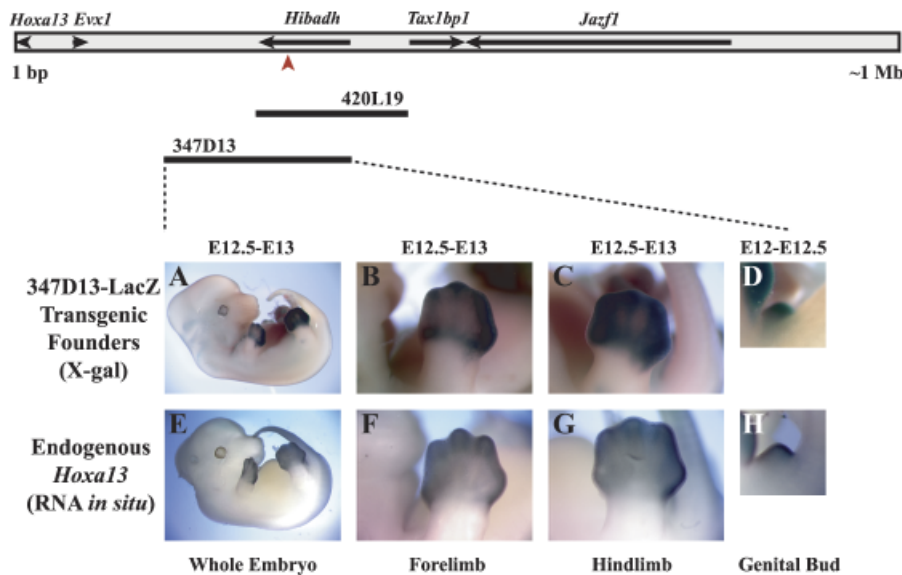
Fig. 1. (Top) To-scale representation of the 1 Mb region of mouse chromosome 6 upstream of the *HoxA* cluster that is under the influence of a long-range distal limb and genital bud enhancer module. Base 1 is the first nucleotide adjacent to the *Hoxa11* promoter and base 1041528 is the nucleotide before the start of the 5′ UTR of *Creb5*. Red arrowhead denotes the position of mmA13CNS, found within intron 4 of *Hibadh*. 347D13 and 420L19 are the two BAC clones used in this report. (Bottom) (A–C) X-gal stained E12.5–E13.0 347D13-LacZ founder embryo 69 and (D) E12.0–E12.5 347D13-LacZ founder embryo 18. LacZ expression was detected in the distal autopod and genital bud in a *Hoxa13*-like pattern (E–H). Central nervous system LacZ expression was also found in both transgenic embryos in an *Evx1/Evx2*-like pattern, albeit at a weaker level in founder embryo 69 (A).

pattern, had no additional LacZ activity at any stage (data not shown). In contrast, line 710 had strong distal limb and genital bud LacZ expression in addition to the expected central nervous system staining between stages E10.5 and E13.5 (Fig. 2, A, D, H, L, P, and T). A description of stage-specific LacZ expression in this line follows and the data is summarized in Table 1.

At E10.5, line 710 expresses the 347D13-LacZ transgene in the distal forelimb and the ventral, distal trunk mesenchyme surrounding the hindlimbs, urogenital tissues, and tail. Forelimb expression is largely similar to the endogenous E10.5 RNA expression domain of *Hoxa13*, with appropriate anterior/posterior boundaries and distal placement (Fig. 2B arrowhead). Therefore, 347D13-LacZ is sufficient for E10.5 *Hoxa13*-like distal forelimb expression. The LacZ expression pattern found in the trunk is not consistent with the known E10.5 expression of *Hoxa13* or any of the genes in the *Hoxa13* expression neighborhood (*Evx1*, *Hibadh*, *Tax1bp1*, and *Jazf1*). However, expression does encompass the expected genital bud domain of *Hoxa13*, so in addition to its additional activity, 347D13-LacZ is sufficient for *Hoxa13*-like genital bud expression (Fig. 2C).

The sufficiency of 347D13-LacZ to drive *Hoxa13*-like distal forelimb expression is maintained through E11.5 where the LacZ expression remains restricted to the distal boundary and extends to appropriate *Hoxa13*-like anterior/posterior boundaries of the autopod (Fig. 2E). The broad trunk staining from the previous stage becomes strikingly restricted to the mesenchyme of the developing genital bud at E11.5 as is expected for the endogenous expression of *Hoxa13*. Thus, the transgene is sufficient for E11.5 *Hoxa13*-like forelimb and genital bud expression. The hindlimb expression in this line is not sufficient for complete *Hoxa13*-like limb expression (Fig. 2F). While the proximal/distal boundaries are appropriate, the anterior/posterior expression boundaries are posteriorized.

For stages E12.0–E12.5 in line 710, 347D13-LacZ is sufficient for *Hoxa13*-like expression in the developing genital bud (Fig. 2, K and O). This corroborates the results obtained from founder 18 (Fig. 1D). In contrast, the limb staining is not consistent between line 710 and either founder embryo (69 or 18) (Fig. 2, I, J, M, and N). Instead, this expression, while appropriate at the distal boundary, extends beyond the expected posterior and proximal boundaries. In addition, in comparison to endogenous *Hoxa13*, the transgenic LacZ expression domain does not completely extend into the anterior autopod. Variation in expression patterns among founders and lines is common in such experiments and can be due to complexity in the BAC transgene insert DNA configuration and/or genomic site effects.

At E13.0–E13.5 347D13-LacZ in line 710 remains sufficient for *Hoxa13*-like genital bud expression (Fig. 2, S and W).
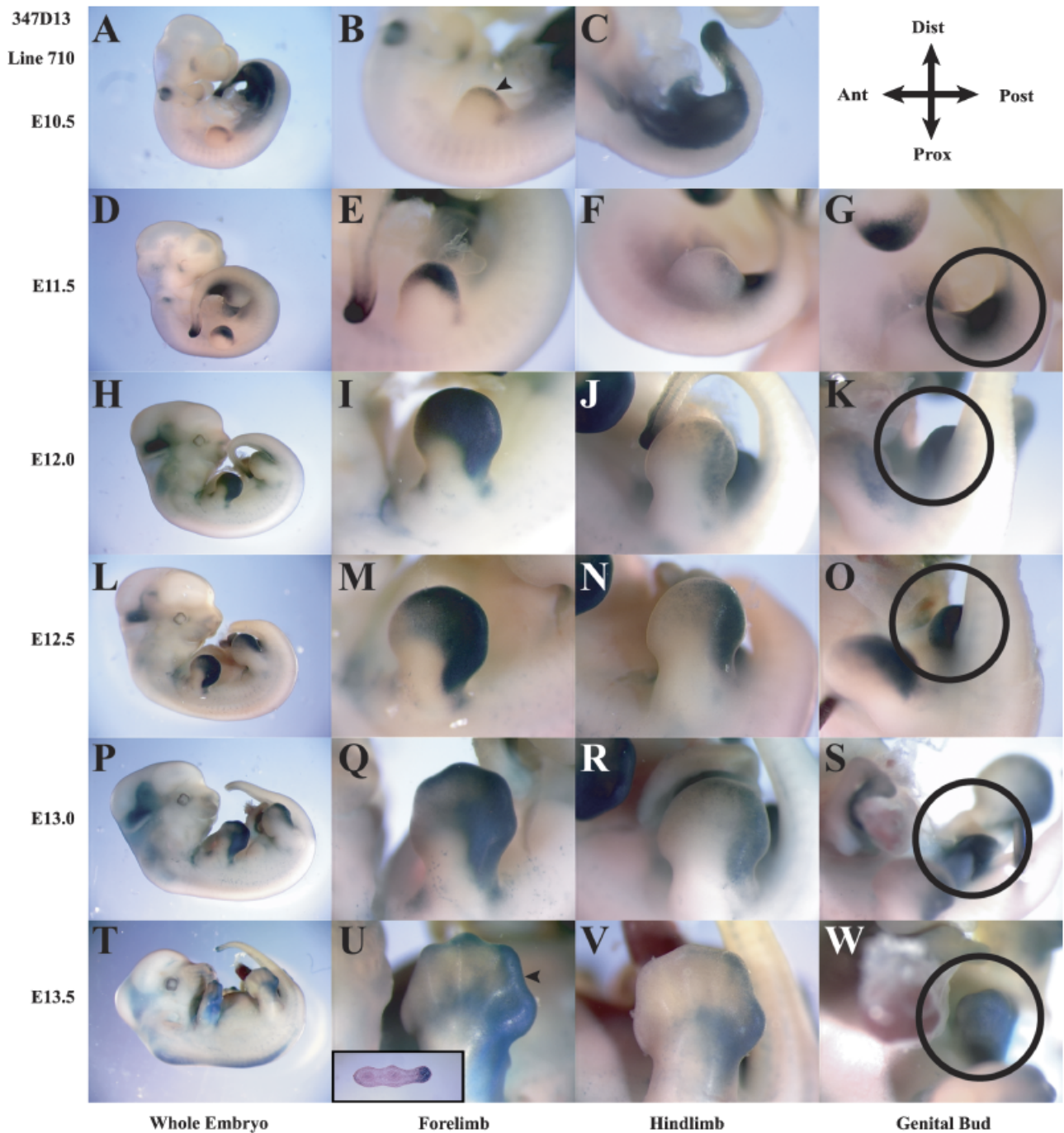
Fig. 2. Timed embryo analysis of LacZ expression in a 347D13-LacZ transgenic mouse line 710. This transgene insertion drives distal limb and genital bud LacZ expression similar, but not identical, to the expression pattern of *Hoxa13*. All embryos were stained with X-gal and photographed with distal positioned to the top and anterior positioned to the left. Embryos were analyzed at multiple stages: (A–C) E10.5, (D–G) E11.5, (H-K) E12.0, (L–0) E12.5, (P–S) E13.0, and (T–W) E13.5. B, E, I, M, Q, and U, are the forelimbs, photographed at a higher magnification, of embryos in A, D, H, L, R, and T, respectively. Black arrowhead in panel B shows distal autopod expression domain. (U inset) Eosin counter-stained cross-section of X-gal stained forelimb at level of black arrowhead with dorsal to the top and ventral to the bottom. C, F, J, N, R, and V are the stained hindlimbs and G, K, O, S, and W are the stained genital buds (circled).

Table 1. Summary of LacZ expression from BAC transgenic lines

		Distal limbs				Genital bud			
	CNS	E10.5	E11.5	E12.5	E13.5	E10.5	E11.5	E12.5	E13.5
347D13-LacZ									
8	Y	N	N	N	N	N	N	N	N
18	Y	?	P	?	?	?	Y	?	?
69	Y	?	?	Y	?	?	?	P	?
710	Y	Y	Y	P	P	Y	Y	Y	Y
733	Y	N	N	N	N	N	N	N	N
420L19-LacZ									
215	Y	N	P	P	P	Y	Y	P	P
222	N	N	N	N	P	N	N	N	N
237	Y	N	P	P	P	Y	P	P	P
250	Y	N	P	P	P	Y	P	P	P
267	Y	N	P	P	P	Y	P	P	P
268	Y	N	N	P	P	N	N	N	N
273	Y	N	N	N	N	N	N	N	N

Y, appropriate expression; P, partial expression; N, no expression; ?, not done.

The expression remains concentrated in the genital bud mesenchyme and has appropriate anterior/posterior boundaries. However, at this stage line 710 does not exhibit complete distal fore- or hindlimb *Hoxa13*-like transgene expression (Fig. 2, Q, R, U and V). As was seen with the previous stage, limb staining extends to the distal tip of the autopod but does not reach the expected anterior boundary, and reporter expression extends beyond the expected posterior and proximal boundaries. In addition, at this stage there is minor regression of reporter activity in the anterior/central autopod. It is unclear whether this regression is similar to that of endogenous HOXA13 protein, but a similar regression of reporter expression was observed in the autopods of *Hoxa13*-GFP mice at similar stages (Stadler et al. 2001). Despite the skewed expression domains on the other axes, the expression on the dorsal/ventral axis is appropriate (Fig. 2U inset), as was true with the earlier stages (data not shown). Thus, line 710 demonstrates appropriate early expression of the transgene reporter, however, later expression domains are skewed in the autopod along the anterior/posterior and proximal/distal axes. Yet, complete *Hoxa13*-like expression of this transgene at E12.5–E13.0 in founder 69 suggests sufficiency at this stage for appropriate proximal/distal, anterior/posterior and dorsal/ventral expression.

BAC 420L19-LacZ is partially sufficient for *Hoxa13*-like expression

In order to better define sequences responsible for directing *Hoxa13*-like expression, we selected another BAC clone,

RPCI-23-420L19 that overlaps with mmA13CNS by a wide margin (~ 38 kb to one side and ~ 139 kb to the other), but is skewed further away from the *HoxA* cluster and overlapping RPCI-23-347D13 by 114 kb (Fig. 1). We modified this BAC to contain the β-*globin*-LacZ minimal reporter in *cis* and used the resultant construct, 420L19-LacZ, to create transgenic mice. Seven expressing transgenic lines (215, 222, 237, 250, 267, 268, and 273) were used for timed embryo analysis. Embryos from all seven lines were collected between stages E10.5 and E13.5 and analyzed for LacZ expression. Combined results from all lines revealed this transgene was capable of expressing LacZ in the distal limbs and the genital bud, as well as the expected domains of the central nervous system (Fig. 3, A, C, G, K, O, and S). As previously mentioned, central nervous system LacZ activity was used to gauge if our transgenic lines (either 347D13-LacZ or 420L19-LacZ) were expressing the incorporated transgene. While this served as a suitable positive control, we still observed inter-line differences in timing/onset, levels, and domains of LacZ expression in the developing genital bud and limbs (Fig. 4 and supplementary Fig. S2). While there was inter-line variation, line 215 was representative of the expression timing and distribution of all 420L19-LacZ lines, and thus we present it here as an example of the enhancer capabilities of 420L19-LacZ. We conclude that 420L19-LacZ is sufficient for *Hoxa13*-like genital bud expression at E10.5 and E11.5, yet insufficient for this complete expression from E12.5 to E13.5. We also conclude that this transgene is insufficient for complete *Hoxa13*-like distal limb expression at any stage. Detailed stage-specific LacZ expression follows and all results are summarized in Table 1.

At E10.5, 420L19-LacZ embryos have no discernible forelimb LacZ expression (Fig. 3A). As was seen with 347D13-LacZ, these embryos have a broad region of distal/ventral trunk LacZ expression, encompassing both the presumptive hindlimbs and the urogenital tissues but not the tail (Fig. 3B). This expression, while not identical to the expected *Hoxa13* genital bud expression, does encompass the expected *Hoxa13* expression domain. We conclude that 420L19-LacZ is sufficient to direct expression in the genital mesenchyme and insufficient for forelimb expression at E10.5.

At E11.5, consistent with 347D13-LacZ, 420L19-LacZ reporter expression in the caudal/ventral mesenchyme becomes excluded from the surrounding tissues and is solely expressed in the genital bud (Fig. 3F). This property was observed for both transgenes and may represent a true capability of the enhancer module that is used or modified in the endogenous locus in a promoter-specific manner. The expression in the genital bud appears identical to the expected endogenous pattern of *Hoxa13* RNA, thus we conclude that 420L19-LacZ is sufficient for *Hoxa13*-like genital bud expression at E11.5. In contrast, this transgene is insufficient for appropriate *Hoxa13*-like distal limb expression. LacZ expression is found in both fore- and hindlimbs (Fig. 3, D and E), yet the domain
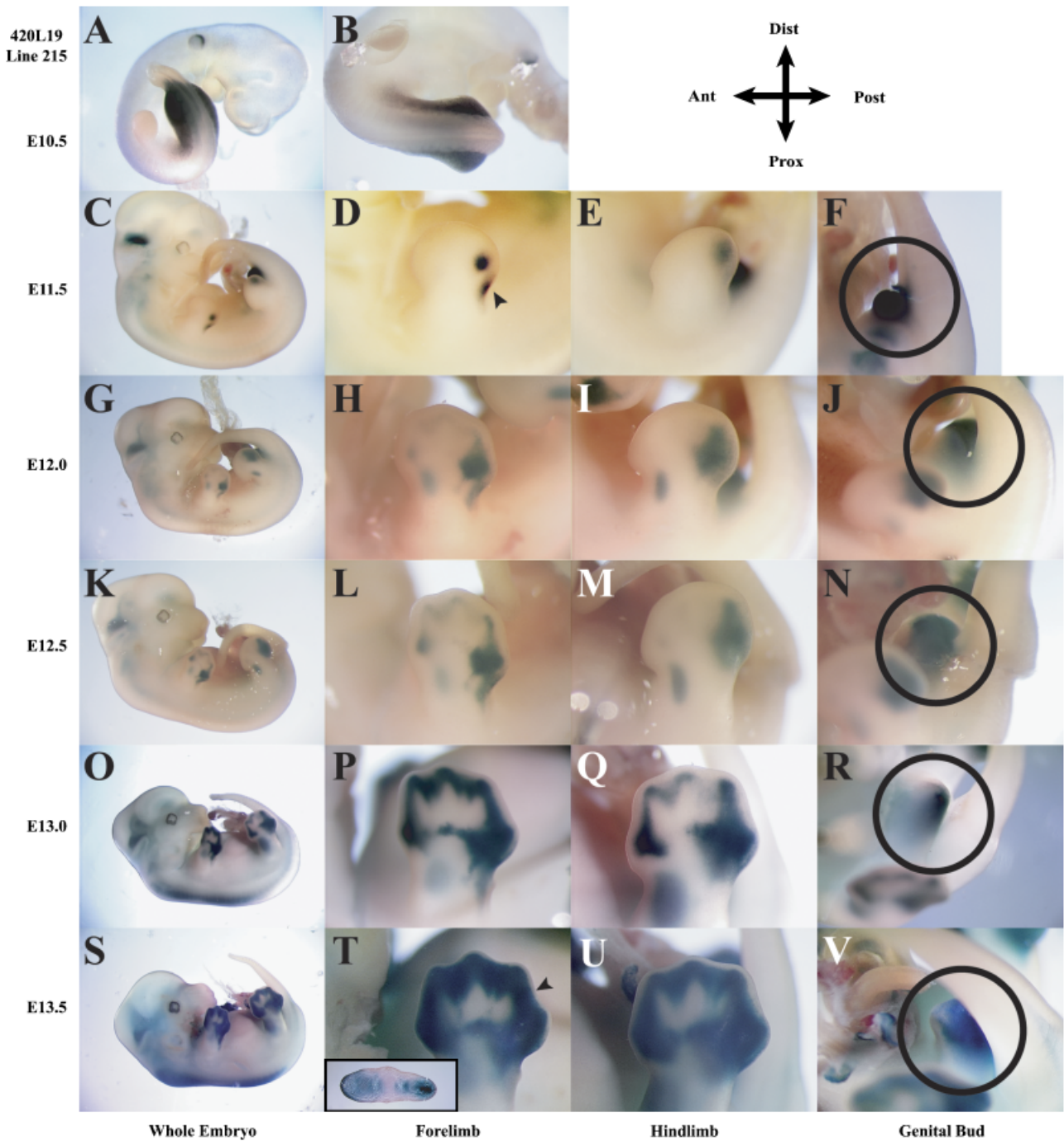
Fig. 3. Timed embryo analysis of LacZ expression in a 420L19-LacZ transgenic mouse line 215. This transgene is capable of expressing LacZ in a peripheral subdomain of the expected *Hoxa13* expression pattern in the autopod and genital bud. All embryos were stained with X-gal and photographed with distal positioned to the top and anterior positioned to the left. Embryos were analyzed at multiple stages: (A and B) E10.5, (C–F) E11.5, (G–J) E12.0, (K–N) E12.5, (O–R) E13.0, and (S–V) E13.5. A, D, H, L, P, and T are the forelimbs, photographed at a higher magnification, of embryos in A, C, G, K, O, and S, respectively. (T inset) Eosin counter-stained cross-section of X-gal stained forelimb at level of black arrowhead with dorsal to the top and ventral to the bottom. B, E, I, M, Q, and U are the stained hindlimbs and F, J, N, R, and V are the stained genital buds (circled) at these stages. Note there is no expression in forelimbs at stage E10.5.
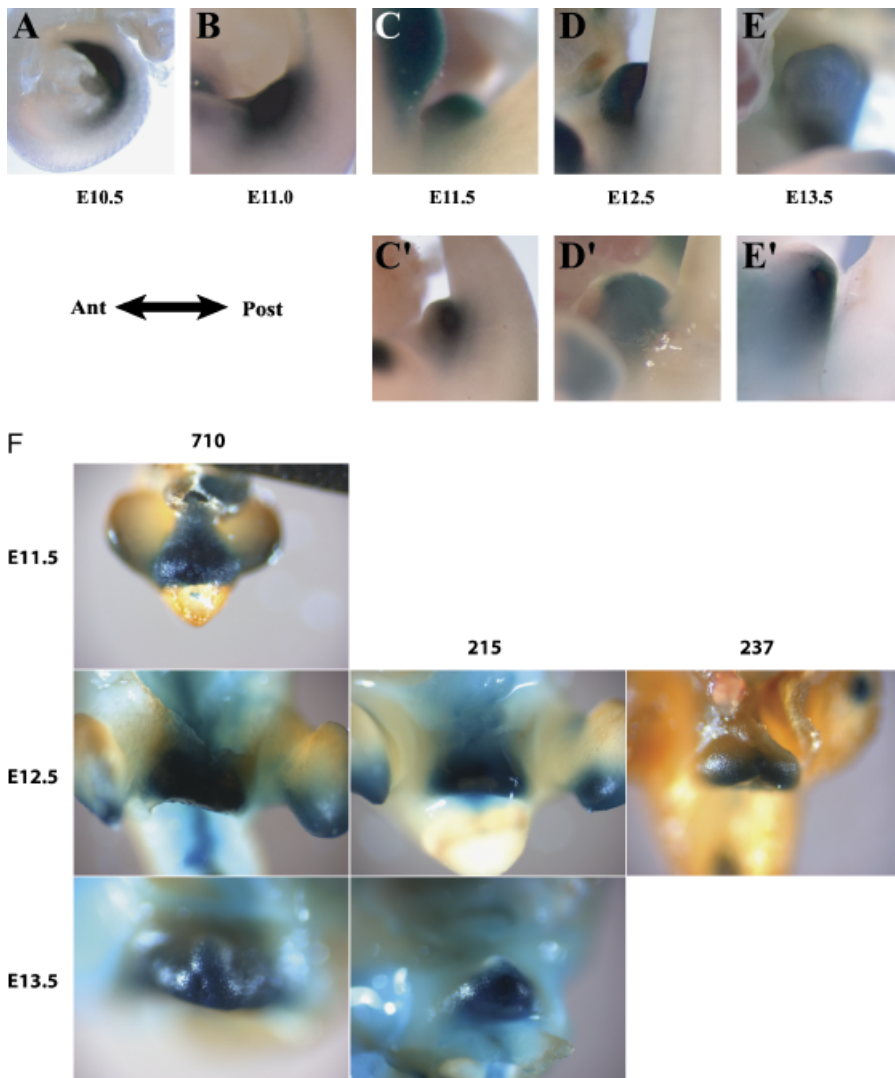
Fig. 4. Representative E10.5–E13.5 genital bud LacZ expression common to both lines 347D13-LacZ and 420L19-LacZ. Embryos are pictured with anterior to the left. When any genital bud expression was present at E10.5–E11.5 in embryos from either transgene it was consistently expressed in the same domains (A and B). Between stages E11.5–E13.5, both BAC transgenes were observed to have expression in the genital bud in two different ways: (C, D, and E) complete *Hoxa13*-like genital bud expression or (C′, D′, and E′) posteriorized *Hoxa13*-like genital bud expression. (F) Shows ventral views of genital buds of line 710 (BAC 347D13) and lines 215 and 237 (BAC 420L19). Tails were removed from all embryos presented in (F); hindlimbs were removed from the E13.5 embryos. Anterior is up.

is posteriorly restricted. In addition to the incorrect anterior/posterior boundaries, the proximal/distal boundaries are also misplaced. The 420L19-LacZ expression domain extends past the appropriate proximal boundary (arrowhead Fig. 3D), and does not extend to the distal-most region of the autopod. This transgenic expression domain represents only a small posterior subdomain of the expected *Hoxa13* expression pattern, thus we conclude that sequences within 420L19-LacZ are insufficient for *Hoxa13*-like E11.5 autopod expression.

Between stages E12.0 and E12.5, 420L19-LacZ no longer directs *Hoxa13*-like genital bud expression. In the transgenic embryos, LacZ expression is only seen in the posterior half of the tubercle (Fig. 3, J and N), whereas the endogenous expression of *Hoxa13* is found throughout the entire genital bud at this stage. In addition, we conclude that this transgene is insufficient for *Hoxa13*-like distal limb expression between E12.0 and E12.5. At E12.0, anterior and distal *Hoxa13*-like

LacZ domains begin to be expressed in addition to a broadening of the posterior expression domain from the previous stage (Fig. 3, H, I, L, and M). This LacZ expression is in conflict with many aspects of the endogenous *Hoxa13* expression pattern. While there is expression in both the anterior and posterior portions of the autopod, there is absence of LacZ expression throughout the central autopod. In addition, the proximal/distal boundaries are inappropriate. LacZ expression never reaches the distal tip of the autopod, leaving a thin strip of non-expressing mesenchyme, and LacZ expression extends too far proximally.

At stages E13.0 and E13.5, the conclusions drawn at E12.5 were corroborated. 420L19-LacZ is insufficient for complete *Hoxa13*-like genital bud expression (Fig. 3, R and V). The LacZ staining, while partially appropriate, is posteriorly restricted and only represents half of the endogenous *Hoxa13* expression pattern. In addition, the distal limb LacZ expres-

sion only represents a portion of the stage-appropriate *Hoxa13* expression (Fig. 3, P, Q, T, and U). While the expression does extend to the correct anterior/posterior and dorsal/ventral boundaries (as is true at all stages, Fig. 3T inset), expression throughout the central autopod is absent. In addition, the proximal/distal boundaries are still skewed such that the LacZ expression domain does not extend to the distal-most tip of the autopod, and the proximal boundary extends past the expected boundary into the zeugopod. Because of the ability of this transgene to recapitulate some, but not all, endogenous *Hoxa13* expression domains of the autopod, we conclude that 420L19-LacZ is insufficient for *Hoxa13*-like distal limb expression at any stage. We also conclude that 420L19-LacZ is sufficient for *Hoxa13*-like genital bud expression at E10.5 and E11.5, yet insufficient for this complete expression from E12.5 to E13.5.

Transgene integrity

Analyzing all of the 347D13-LacZ/420L19-LacZ lines collectively (supplementary Fig. S2) enabled us to tease out different aspects of the transgenic expression, namely: timing of expression onset, posterior and anterior expression domains, distal and proximal expression boundaries, and central autopod expression domain. To address whether the differences found between our BAC transgenes and between lines of the same transgene were due to technical issues arising from the transgene injection/insertion or to subtleties in the function of the enhancer(s), we pursued experiments to determine BAC copy number and to validate the integrity of the transgenes. By Southern hybridization, we quantified the number of integrated transgenes in fully constituted, N1 transgenic animals. We found that among our transgenic lines, there is no correlation between transgene copy number and level of LacZ expression (supplementary Table S1).

Because BAC transgene copy number was not playing a significant role in the inter-line expression variation, we turned to analyzing the integrity of the incorporated transgene by polymorphic SNP analysis (see 'Materials and methods'). Results from this analysis for 347D13-LacZ transgenic lines correlated well with the LacZ expression. Lines 8 and 733 had LacZ expression in the central nervous system and no additional tissues. Marker analysis showed that in both of these cases the incorporated BAC was fragmented into at least two (line 8) or three pieces (line 733) and large portions of the transgene missing (supplementary Fig. S3). These lines were excluded from the analysis.Line 710 contained the entire BAC as assessed by markers A–D and F–I (supplementary Fig. S3). This analysis could not be done for the two founder embryos. Because circular BAC clones were used as the substrate for microinjection, random BAC breakage must have occurred before integration of the transgene into the genome. Our analysis did not determine the structure of the transgene

integration and does not rule out a microdeletion between any two of the retained markers upon transgene breakage. Thus, the anterior and central autopod expression loss observed late in this line cannot be readily explained by content of transgenic material.

Differences in BAC integrity were also found for the 420L19-LacZ lines. Lines 215, 237, and 267 all had the full complement of BAC markers E–H and J (supplementary Fig. S3). The finding that these three lines largely reflect the same transgenic sequences is consistent with the similar LacZ expression patterns in these embryos. We did not detect any major deletions and we conclude that the lack of central or distal autopod expression, or late onset of the expression are consistent with true insufficiency of 420L19-LacZ to perform these enhancer functions and that the regulatory elements responsible for these domains must lie outside of this clone. Line 273 also contains all of the tested markers, yet no limb or genital bud staining was found for this line. We hypothesize that potential limb and genital bud expression capabilities in this transgene are likely being silenced by the site of integration or the overall integrated structure is not conducive to expression. Line 222 was only positive for markers E and J. The absence of markers F and G indicate a probable deletion of mmA13CNS in this transgene, likely explaining the absence of central nervous system staining in this line.

DISCUSSION

Genital bud enhancer(s)

Our data demonstrate that 347D13-LacZ is sufficient for *Hoxa13*-like genital bud expression at stages E10.5–E13.5. 420L19-LacZ is sufficient for *Hoxa13*-like genital bud expression at stages E10.5–E11.5 (Fig. 3). This indicates that the region of intersection of the two overlapping transgenes is sufficient for E10.5–E11.5 *Hoxa13*-like genital bud expression (Fig. 4). While the genital bud expression from E12.5–E13.5 in the 420L19-LacZ lines was consistently posteriorized, variability was found for the E11.5 expression domain which shows the potential of the transgene to drive two types of expression. Line 215 was sufficient for entire E11.5 genital bud expression, whereas all of the remaining expressing lines had a posteriorized expression domain even in the context of the same transgenic sequence content (similar to Fig. 4, C vs. C′). The ability of the same transgene to be expressed in either a complete or incomplete manner suggests the presence of an intact regulatory element whose function is perhaps context dependent. This is further demonstrated by the results from 347D13-LacZ. This transgene was shown to be sufficient for genital bud expression at all stages (line 710 and founder embryo 18), yet founder embryo 69 had a posteriorized genital bud expression domain (similar to Fig. 4E′). We hypothesize based on this data that a regulatory element(s) sufficient

for *Hoxa13*-like genital bud expression is contained within the ∼ 114 kb fragment in common between BACs 347D13 and 420L19, yet our data does not exclude redundant enhancer contribution from outside the 114 kb domain. We also hypothesize that the enhancer(s) within the BACs is sensitive to genomic context and is capable of being expressed in either a *Hoxa13*-like domain or in a restricted posterior domain between E11.5 and E13.5.

Distal limb enhancer(s)

We propose a model to combine the expression data we have obtained from our 347D13-LacZ and 420L19-LacZ transgenic embryos that requires enhancers influencing the onset of expression, proximal/distal and anterior/posterior boundaries, and tissue expression subdomains. We considered the commonalities in *Hoxa13*-related limb expression between the two transgenes, because the enhancer(s) directing that expression should be localized to the overlapping BAC sequences. Generally speaking, both BACs are capable of directing distal autopod expression between E11.5 and E13.5. This expression is *Hoxa13*-like in the anterior/posterior domains, the dorsal/ventral domains, as well as at the proximal boundary. We hypothesize that an enhancer(s) within this ∼ 114 kb fragment is responsible for E11.5 expression onset and related expression domains and boundaries.

Our data suggests that at least one additional enhancer is located within the ∼ 107 kb fragment of 347D13 that does not overlap with 420L19, though we have not determined whether it functions autonomously or cooperatively with the enhancer(s) in the adjacent ∼ 114 kb fragment. We showed that the 347D13-LacZ transgene was expressed in the autopod at stage E10.5 and it was expressed in the central and distal-most boundary of the autopod. This stage of expression onset and these tissue sub-domains were never observed in any of seven 420L19-LacZ lines, thus our model proposes the existence of an enhancer(s) in this ∼ 107 kb region responsible for this expression. Effectively, the remote enhancers for *Hoxa13*-like limb expression fall within two separate, yet still broad regions. A *Hoxa13* regulatory network contingent upon multiple remote enhancers is supported by the recent finding of a similar, multi-component system upstream of the *HoxD* locus (Gonzalez et al. 2007).

Evolution of embryonic distal limb and genital bud expression domains

We recognize the experiments in this report are testing the sufficiency of putative regulatory elements that are comparable to, but not proven to be biologically involved in, *Hoxa13* gene expression. Our prior work showed that *Hoxa13*, *Evx1*, *Hibadh*, *Tax1bp1*, and *Jazf1* all have distal autopod and genital bud expression from E10.5 through E13.5 (Lehoczky et al. 2004), and we have no direct evidence that the sequences we are mapping are not merely local regulatory elements specific to one of these genes. Yet, we interpret our LacZ reporter data as expression from regional enhancers rather than gene-specific enhancers primarily due to our original *HoxA/D* paralogous sequence comparison that led us to focus on this region (ParaCNS4+5).

The similarities in expression and function of the posterior *HoxA/D* genes, combined with their common genomic origin, are most parsimonious with the existence of a limb/genital, or more simply a distal appendage, regulatory module before the *HoxA/D* cluster duplication. Under this model, the remote regulation that exists for the extant posterior *HoxA* and posterior *HoxD* genes resulted from divergence over the course of vertebrate evolution. Presumably, introduction and/ or degradation of genes upstream of the *HoxA* and *HoxD* clusters led to the extremely different upstream genomic neighborhoods (Lehoczky et al. 2004), yet the enhancers required for distal limb and genital bud expression were retained. The distinct upstream regions inevitably placed different evolutionary constraints on these pre-existing regulatory sequences. On the *HoxD* chromosome, the distal limb enhancers were recently mapped to two discrete, remote loci: GCR and Prox (Gonzalez et al. 2007). GCR exhibits sequence conservation to the *HoxA* upstream region corresponding to mmA13CNS (Lehoczky et al. 2004), but there is no paralogous Prox element in the *HoxA* locus detectable by standard sequence conservation. Absence of conservation between the Prox *HoxD* region and any sequence at the *HoxA* locus does not preclude the existence of a functionally comparable enhancer element at the *HoxA* locus. One explanation could be that Prox and a functionally paralogous enhancer at the *HoxA* locus have diverged since the *HoxA/D* cluster duplication thus escaping the sequence conservation thresholds.

GCR and Prox enhancers are proposed to interact with two genes (*Evx2* and *Lnp*) in addition to the posterior *HoxD* genes. Similarly, on the *HoxA* chromosome our data supports the existence of at least two discrete enhancers functioning in long-range regulation. Yet distinct from the *HoxD* regulatory organization, which has remote enhancers positioned within a gene desert, the *HoxA* limb/genital bud remote enhancers are within a gene neighborhood. The remote *HoxA* enhancers that we infer from our data appear to interact, bidirectionally, with four other genes (*Evx1*, *Hibadh*, *Tax1bp1*, and *Jazf1*) in addition to *Hoxa13*. Future work should identify the smallest functional sequence elements within these broad, remote upstream *HoxA* domains, and compare their function(s) with GCR and Prox via endogenous deletion of the elements in mice.

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SUPPLEMENTARY MATERIALS

The following material is available for this article online:

Fig. S1. Endogenous *Hoxa13* expression is revealed in mouse embryos using RNA in situ hybridization from E10.5 through E13.5 in the developing limbs and genital bud.

Fig. S2. X-gal stained forelimbs from all 420L19-LacZ (top) and 347D13-LacZ (bottom) expressing, transgenic lines or founders (f). Autopods are positioned distal to the top and anterior to the left. Interline variability for 420L19-LacZ is shown for stages E11.5 through E13.5 as no line had any forelimb expression at E10.5. Line 710 (347D13-LacZ) shows LacZ expression domains when compared to founder embryos 69 and 18 at stages E11.5–E12.5. None = we observed no expression in forelimb; NA = not available.

Fig. S3. BAC transgene integrity analysis. Transgenic DBA+/+animals were genotyped at the indicated markers excluding E and J for the 347D13-LacZ lines and markers E-H, and J for 420L19-LacZ lines. Markers are: A = 347D13-T7 insert/vector junction; B = RS29851258; C = RS29857058; D = RS30707421; E = 420L19-T7 insert/vector junction; F = RS13478755; G = RS29863749; H = RS30170317; I = 347D13-SP6 insert/vector junction; and J = 420L19-SP6 insert/vector junction. Arrowhead represents location of mmA13CNS. Solid dots indicated the transgenic insert is positive for that marker and X's depict absence of the marker. Lines 250 and 268 were not included in this analysis due to multiple unsuccessful attempts to generate transgenic DBA+/+animals.

Table S1. BAC copy number of transgenic lines.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1525-142X.2008.00253.x (This link will take you to the article abstract).

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