Integrative biology and the developing limb bud[1]

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SUMMARY The identification or selective construction of mutations within genes has allowed researchers to explore the downstream effects of gene disruption. Although these approaches have been successful, a limitation in our assessment of the consequences of conditional changes, and thereby our understanding of roles or function of genes, limits the degree to which we examine the effects of our manipulations. It is also clear that linear associations are incorrect models for describing development, and newer methods now give us an opportunity to practice an integrative biology. In our attempts to explore the consequences of *Hoxa13* disruption in mice and humans, it has become clear that a better understanding of the consequences of gene alteration may be achievable by taking a broader approach with a long-term view. Fundamental questions regarding Hox gene function in vertebrates, in-cluding those related to the number of target genes; the degree of overlap of target gene regulation among paralogs; the magnitude of modulation exerted; and the identity of genes that are activated versus repressed need to be explored if a more thorough mechanistic understanding is to be achieved. To begin to address these questions, we undertook a comprehensive analysis of the expression of genes within developing limb buds of mice, and here we present some of our preliminary results. Our efforts will further (1) the exploration of the broader genetic relationships of expressed genes, (2) the determination of parallels or variations in target usage for a given gene in different tissues and between different organisms, (3) the evaluation of limb patterning mechanisms in other animal model systems, and (4) the exploration of gene expression hierarchies regulated by HOX proteins in developmental systems.

INTRODUCTION

Limb development models are very useful for dissecting molecular constituents that determine morphology (Cohn and Tickle 1996; Johnson and Tabin 1997; Innis and Mortlock 1998; Tickle 2000). Our laboratory uses the mouse limb model system to explore the contribution of Hox genes to growth and patterning. The expression of Hox genes in both the developing limbs and the genitalia and malformations in those structures in selected Hox mutant suggests that both structures require the growth and patterning determination conferred by Hox genes. Even though the context of Hox expression is different, it is likely that at a certain level there is a parallelism, or commonality, in the mode of Hox function in those tissues that remains to be determined. Here we summarize our efforts to explore the limb and genitourinary phenotypes of *Hoxa13* mutant mice and, as a prelude to future experiments into Hox function, present our preliminary work to document the complexity of gene expression in serially homologous structures, namely, the forelimb and hindlimb in developing mice.

Hoxa13 mutant mouse limb phenotype

Two mutants of *Hoxa13*, Hypodactyly (*Hoxa13^{Hd}*) and engineered *Hoxa13-/-* mice, were examined (Fromental-Ramain et al. 1996; Mortlock et al. 1996). *Hypodactyly* (*Hoxa13^{Hd}*) is a spontaneous semidominant mutation that arose in 1969. Heterozygous mutants exhibit hypoplasia of the first digit in all paws of variable severity and almost always have distal phalangeal shortening. The mutant phenotype is easier to identify in the hindlimbs of young animals. In skeletal preparations, there are alterations in maturation of specific skeletal elements (Mortlock et al. 1996; Post and Innis 1999a). In particular, the middle phalanx is usually delayed in ossification and may be smaller.

Homozygous mutants are more severely affected in terms of limb development and usually die in utero at around E14.5-E17.5 (Post and Innis, unpublished data). Homozygous mutants have a single digit on each paw. The *Hoxa13^{Hd}* mutation was identified through a combination of positional cloning and candidate gene mutation search (Mortlock et al. 1996). A 50-bp deletion in the first exon 25 bp downstream of the initiator methionine was identified, yet a stable mRNA is made in mutant limb buds. The phenotype of this mutant was noted to be more severe than was expected compared with those of engineered null mutations for other Hox genes expressed in the mouse limb (Small and Potter 1993; Davis and Capecchi 1994; Davis et al.

1995). Two engineered null *Hoxa13* genes created by gene deletion and by insertion of a neomycin resistance gene into the homeobox resulted in mice with similar phenotypes but with considerably milder effects compared with *Hoxa13^{Hd}* (Fromental-Ramain et al. 1996). Not all heterozygous mutants can be identified grossly due to very mild shortening of the first digit. Homozygous mutants also died in utero; however, the limbs developed four shortened digits, which stood in contrast to the single digits that developed in the *Hoxa13^{Hd}* mutants.

Both *Hoxa13* mutants also exhibit genitourinary malformations in regions of gene expression (Kondo et al. 1997; Warot et al. 1997; Post and Innis 1999). Rare male or female survivors are infertile due to hypoplasia of the penian bone or to cervical/vaginal hypoplasia, respectively (Post and Innis 1999). The gene is expressed in the developing Mullerian ducts, and homozygous *Hoxa13^{Hd}* females have an anterior transformation of cervical tissue to uterine-like stromal characteristics and severe hypoplasia of the cervix and vagina (Post and Innis 1999). In surviving homozygous mutant males, the endochondral portion of the penian bone is hypoplastic. These physical defects may explain the inability of homozygous mutants to reproduce. Heterozygous *Hoxa13^{Hd}* males show alterations in prostate branching (Podlasek et al. 1999). *Hoxa13-/-* mice also fail to live past E16.5 (Warot et al. 1997).

The phenotypic differences between the *Hoxa13-/-* and *Hoxa13^{Hd}* mice were found to be unrelated to genetic background (Post and Innis 1999) and therefore were hypothesized to be intrinsic to the mutations. Support for this came from *Hoxa13-/Hoxa13^{Hd}* compound heterozygotes that exhibited an intermediate phenotype (Post and Innis 1999). Also, development of single digits on the paws of homozygous *Hoxa13^{Hd}* mice may result, in part, from cell death in regions of the developing autopod outside of the condensing mesenchyme that will give rise to the digit (Post and Innis 1999; Robertson et al. 1996). This is associated with development of a very loosely associated apical ectodermal ridge (AER) over those regions undergoing cell death and prolonged persistence of an enlarged AER over the single digit (Robertson et al. 1996). In addition, cells from *Hoxa13^{Hd}* mutant limb buds have reduced adhesiveness and fail to form nodules in vitro, implying a role for HOXA13 in activation of expression of cellular adhesion molecules needed for mesenchymal condensation. Additional support for this role was found in misexpression experiments with chicken *Hoxa13* (Yokouchi et al. 1995). In summary, HOXA13 may be involved in proliferation of mesenchyme, alteration of cell adhesiveness, and AER survival/regression. For each, the underlying mechanisms are unknown largely because the identity and nature of the regulation of target genes is unknown.

The *Hoxa13^{Hd}* allele was known to synthesize a stable mutant mRNA, and based on the sequence of the message it was hypothesized that a protein could be synthesized that would be 300 amino acids long and would be devoid of a homeodomain (Mortlock et al. 1996). Importantly, this protein would have 25 amino acids of the authentic HOXA13 protein followed by 275 amino acids of lysine-arginine rich novel sequence as a result of the frameshift. A polyclonal antibody capable of recognizing the amino-terminal 25 amino acids was found to identify not only wild-type protein in limb buds, but also the expected shorter HOXA13^{Hd} mutant protein in heterozygotes and in homozygotes (Post et al. 2000). No wild-type protein could be identified in homozygous mutants. Therefore, the *Hoxa13^{Hd}* mutation eliminates wild-type protein but creates a new protein that was found to be stable in limb buds.

To explore the hypothesis that the HOXA13^{Hd} protein was interfering with limb bud growth in its domain of expression, the HOXA13^{Hd} mutant protein was expressed in limb buds of wild-type transgenic mice (Post et al. 2000). This was shown to lead to limb truncation in 3 of 15 founder animals, a finding consistent with the variable expression of transgenic mice and with expression of the mutant protein in a wild-type HOXA13-proficient genetic background. Such data should engender reconsideration in the interpretation of phenotypes for presumed "null" alleles. Further work to explore the nature of the negative effect of the mutant protein is ongoing.

HOXA13 mutant phenotype in humans

The *Hoxa13^{Hd}* mutant mouse phenotype was instrumental in predicting human malformation phenotypes that might be due to mutation of the human *HOXA13* gene. This prediction was based on the expectation that similarity in phenotype would result from mutations in the same gene given the highly conserved primary structure of HOX proteins in vertebrates.

In the hand-foot-genital syndrome (HGFS), the hands and feet exhibit hypoplasia of the first digit (Innis 1997; Mortlock and Innis 1997). The fifth fingers of most individuals with this disorder are curved inward (clinodactyly), and this occurs from hypoplasia of the middle phalanx of the fifth finger. A homeodomain nonsense mutation leading to truncation of the last 20 amino acids of the protein was identified in the original family described with this disorder. Since then, additional families with other mutations have also been reported, including alanine expansion coded for in the first exon (Goodman et al. 2000). In addition, a large deletion of this region of the chromosome, including part of the HOXA cluster, has been described in a patient with hand and foot anomalies consistent with HFGS, suggesting that the other mutations so far described in human HOXA13 are likely to be null alleles.

Although the limb phenotype appears to be the most consistent, there is some variability in the genitourinary malformations. For example, some males exhibit hypospadias, whereas others do not. Similarly, in females there is variable incomplete Mullerian fusion and/or abnormal reflux. Thus, the phenotype in humans for heterozygous mutation in this gene is similar to that in mice; however, humans seem to have more serious, yet variable, problems with the genitourinary system.

Growth of a particular segment depends on HOX dosage, yet how do they work?

The phenotype of Hox gene mutations created by knockout technology has allowed us to learn that HOX proteins expressed within overlapping domains cooperate in the limbs to promote growth (Capecchi 1996; Davis and Capecchi 1996; Rijli and Chambon 1997). This helps explain the hypoplastic phenotype associated with most double mutants and suggests that one of the main functions of HOX proteins is to promote proliferation in their domains of expression. However, how HOX proteins function in terms of target gene selection (Liang and Biggin 1998; Guss et al. 2001), how HOX dosage output is realized at the transcriptional level (Davis and Capecchi 1996; Rijli and Chambon 1997; Zakany et al. 1997; Veraksa et al. 2000), how specificity is realized (Hayashi and Scott 1990; Veraksa et al. 2000), and the functional redundancy between paralogs (Greer et al. 2000) are only beginning to be understood.

In terms of HOX protein target genes, it is clear that identification of downstream targets will be difficult using only sequence information provided by the Genome Projects. In addition, between-species variation in the targets used by a Hox gene is expected (Kenyon 1994; Carroll 1995; Graba et al. 1997; Liang and Biggin 1998; Mannervik 1999; Veraksa et al. 2000). Therefore, information is needed not only about targets, but also for the principles governing relationships between HOX proteins and their targets.

Drawing relationships such as these is confined to the participants that are known, that is, to those genes whose expression is controlled by HOX proteins. There is incomplete knowledge of the genetic cascades or epistatic relationships that exist for Hox genes and their upstream and downstream regulators and effectors. Since to date neither all potential genes involved are known nor are the functions of many genes that have been identified as targets, it is impossible to discuss such complex relationships. Attempts to assess complex relationships cannot be built on purely theoretical constructs; thus identification of the genuine genetic factors and their properties must be elucidated if we choose to move beyond the current linear working models.

Some work elucidating the complexity of relationships is available. Recent knowledge in *Drosophila* suggests that (Guss et al. 2001) HOX proteins have numerous targets and homeobox proteins cooperate with cell signaling molecules on promoters, thereby potentially linking growth signaling with transcriptional activity of HOX proteins. Other work in *Drosophila* supports the conclusion that homeodomain gene products control the expression of most genes in the genome (Liang and Biggin 1998). However, whether this is true for vertebrates with duplicated Hox clusters, allowing potentially for diversification of some functions, is unknown. In addition, little is known of how gene expression differences are altered and which gene expression differences are critical for the cellular, and ultimately phenotypic, effects observed in vertebrate Hox mutants. Furthermore, how mutant HOX proteins act and what their effect is at the molecular level is unclear. Other phenomena, such as posterior prevalence, which is the dominant effect of one HOX protein over other HOX proteins in more anterior regions of expression, are not understood at the molecular level (Duboule and Morata 1994; Capovilla and Botas 1998). Acquisition of such knowledge would be aided by experimental approaches that capture a broader view of transcriptional changes in cells expressing HOX proteins.

In summary, positional cloning, gene knockout, and misexpression experiments for *Hoxa13* and other Hox genes have revealed important roles for it and other Hox proteins in proliferation and control of differentiation. However, the question is how these proteins do it and what changes in gene expression accompany the loss of Hox expression during normal differentiation. If more progress is to be made in this area, identification and evaluation of expression of the target genes that are affected by loss- or gain-of-function mutations will be needed.

Methods for comprehensive assessment of gene expression

Several newer methods seeking to gain information about the expression of thousands of genes simultaneously include cDNA microarrays (Schena et al. 1995, 1996; Shalon et al. 1996; DeRisi et al. 1996, 1997), photolithographic oligonucleotide chips (Pease et al. 1994; Lockhart et al. 1996; Wodicka et al. 1997), and serial analysis of gene expression (SAGE) (Velculescu et al. 1995, 1997; Zhang et al.

1997). The cDNA microarray and photolithographic methodologies rely on hybridization to a glass slide or oligonucleotide chip on which are arranged thousands of immobilized probes. These methods are currently limited by the need for expensive machines to manufacture slides or oligonucleotide chips with selected probes and reproducible characteristics, the need for efficient hybridization with a complex prelabeled cDNA mixture, and the need to use sophisticated expensive scanning devices coupled with software-based data acquisition and interpretation. These latter two methods have not yet been widely used, and both require prior knowledge of sequence and of the genes that are involved in a conditional change in the experimental system of interest.

SAGE is a comprehensive, quantitative, and relatively inexpensive method in which through a series of standard enzymatic manipulations, small representative segments of individual cDNA molecules, called tags, are concatenated, cloned, and sequenced. From one concatenated clone as many as 40–60 distinct tags can be determined in one sequencing reaction. Sequencing hundreds of independent concatenated clones provides direct information about frequency and identity of thousands of mRNA molecules. Two advantages of SAGE are that it allows accurate quantitative interpretations of variations in mRNA abundance and, because the SAGE method does not presuppose what differences in gene expression may be present, it facilitates the discovery of new genes and genetic pathways as well as assessments of changes in pattern involving multiple genes. It is important to point out that for SAGE experiments we derive a snapshot, not an ongoing readout. For this reason, many investigators use microarray data and time course experiments.

Statistical evaluation of gene expression changes is critical to appropriate interpretation of results (Audic and Claverie 1997; Margulies and Innis 2000; Margulies et al. 2001a,b; Margulies 2001). Genetic relationships do not rely on single gene–gene effects; therefore, mathematical models capable of assessing the relationships between multiple varying components that rely on known data must be developed. As a result of these factors, for some time our abilities to measure changes will greatly exceed our abilities to process or interpret the biological meaning of such changes.

Use of SAGE to assess gene expression in developing limb buds

Other than dissecting the HOX protein functions, one of the applications that would be significantly aided by a comprehensive view of gene expression would be in elucidating the distinctions between forelimbs and hindlimbs. Specifically, what makes these serially homologous structures distinct from one another?

The developing tetrapod limb is an excellent model system for exploring the molecular basis of homology and evolutionary diversity within a substructure of the whole organism (Carroll 1995; Shubin et al. 1997; Capdevila and Izpisua-Belmonte 2000; Ruvinsky and Gibson-Brown 2000; Tickle 2000). This model system has long been the subject of study by evolutionary biologists, paleontologists, and molecular biologists. Nearly 150 years ago, Charles Darwin documented the fascinating similarity observed between the limbs of different species. But it is more than the homology between different species that makes this an ideal system for implementing novel integrative approaches to the study of complexity in development. Even before the publication of Darwin's monumental

new theories of evolution, Richard Owen noted the similarity between forelimbs and hindlimbs of the same species (Owen 1849).

The relationship between forelimbs and hindlimbs represents a special kind of homology. Owen believed that these kinds of homologous repeated structures within the same body plan should be considered a separate category of homology and thus coined the term "serial homology." Although Owen considered serial homologous structures to be constructed on the same rational plan, a more modern theory originates from Darwin (1859), who considered these homologies to indicate common evolutionary descent. Darwin's concepts are, in fact, the backbone of current genetic theories that attempt to explain the evolution of tetrapod appendages.

All jawed vertebrates (gnathostomes) share two sets of paired appendages at specific locations along the primary body axis (Carroll 1988). Tetrapod forelimbs and hindlimbs are believed to have evolved from the pectoral and pelvic fins of an ancestral fish (Coates 1994; Hinchliffe 1994). Positioning of the limbs may have arisen by a co-option of the "Hox code" that had originally evolved in the splanchnic mesoderm to regulate rostral-caudal patterning of the digestive tract (reviewed by Ruvinsky and Gibson-Brown 2000). Thus, both classical and modern concepts of homology can be used to explain the similarities observed between forelimbs and hindlimbs as well as their evolutionary origins. However, there are very distinct morphological and functional differences between these two serially homologous structures that require more specific terminology and cannot be entirely explained with the concepts of homology. Novel more comprehensive experimental strategies are required to identify the molecular mechanisms that underlie these observed differences.

Significant progress has been made toward understanding the molecular signals that regulate initiation and maintenance of limb bud outgrowth, establishment of the anterior-posterior and dorsal-ventral axes, and patterning the overall morphology of the limb (Gilbert 1997; Johnson and Tabin 1997). Until recently, however, little was known about the genetic mechanisms for determining the ultimate identity of a limb structure, that is, whether it will become a forelimb or a hindlimb. Both limbs share the same molecular mechanisms for axis specification and growth regulation, yet a subset of signals unique to each limb structure regulating their differential morphology was suspected.

The earliest molecular signals that play a role in the specification of limb-type likely originate from the Hox code along the primary body axis very early in morphogenesis. Evidence for this comes from studies by Cohn et al. (1995) whereby the placement of fibroblast growth factor-soaked beads into the flank of chick embryos was found to induce ectopic forelimbs or hindlimbs, depending on the position of the soaked bead along the flank. In other studies, alterations in the anterior-posterior Hox code resulted in a corresponding shift of positional information (Charite et al. 1998) and initiation of limb bud outgrowth ectopically along the embryonic axis (Rancourt et al. 1995; vandenAkker et al. 1999, 2001).

Interestingly, the first two pieces of evidence for the existence of "selector genes" in determining limb identity came from studies on the evolution of T-box genes and studies to identify novel transcription factors regulating the development of the pituitary gland. T-box genes encode a family of transcription factors that have regions of homology to the DNA-binding domain of the Brachyury T locus product (Chapman et al. 1996). Four of these genes, *Tbx2–*

Tbx5, are represented as two distinct unlinked gene pairs that likely arose from a series of chromosomal duplication events (Agulnik et al. 1996). These four T-box genes were all found to be expressed in selected domains of the developing embryo, suggesting specific roles during embryonic development. Interestingly, *Tbx4* and *Tbx5* were found to have mutually exclusive expression patterns in the developing limbs such that *Tbx4* is expressed in the hindlimb and *Tbx5* is expressed in the forelimb (Gibson-Brown et al. 1996; Isaac et al. 1998; Ohuchi et al. 1998).

Two separate groups working to identify novel factors regulating pituitary development identified *Pitx1*. First, *Pitx1* was identified as a transacting factor that bound to a critical *cis*-acting sequence in the PomC promoter (Lamonerie et al. 1996). Second, a yeast two-hybrid screen was used to identify a novel protein (the *Pitx1* gene product) that interacted with the transactivation domain of the pituitary-specific POU domain protein, Pit-1 (Szeto et al. 1996). The gene encoding this novel protein had also been simultaneously identified from a yeast one-hybrid screen for factors that interact with the mitochondrial transcription termination site (Shang et al. 1997a,b). This gene, originally referred to as *Bft*, *P-Otx*, or *Ptx1* and now officially named *Pitx1*, not only has specific expression in the developing pituitary gland but also exhibits expression in the caudal regions of the embryo that include the hindlimbs and not the forelimbs. *Pitx1* has been found to be part of a homologous gene family that now includes *Pitx2* and *Pitx3* (Gage et al. 1999). However, *Pitx1* is the only member of this family whose gene expression is specific to a particular limb type.

A number of functional studies have shown that these three transcription factors, *Tbx4*, *Tbx5*, and *Pitx1*, play a critical role in determining the ultimate identity of the developing limb. Experiments in chick have shown that *Pitx1* and *Tbx4* can exert a transformation of limb type when misexpressed in the developing wing. Similarly, *Tbx5* misexpression in the developing leg results in the growth of a wing-like morphology (Logan and Tabin 1999; Rodriguez-Esteban et al. 1999; Takeuchi et al. 1999).

Misexpression of *Tbx5* in the chick hindlimb was also shown to suppress expression of *Tbx4*. Misexpression of *Pitx1* in prospective forelimbs was able to induce expression of *Tbx4*, *Hoxc10*, and *Hoxc11* but had no effect on *Tbx5* expression (Logan and Tabin 1999). Similarly, *Tbx4* induced *Hoxc9*, *Hoxc10*, and *Hoxc11* and suppressed *Hoxd9* (Rodriguez-Esteban et al. 1999; Takeuchi et al. 1999). Therefore, these transcription factors mediate their limb transforming properties, in part by regulating each other, as well as specific downstream target genes (Fig. 1).

Supporting the misexpression studies in chick, engineered mice lacking *Pitx1* develop hindlimbs with reduced *Tbx4* gene expression and skeletal and muscle features more characteristic of forelimbs (Lanctot et al. 1999; Szeto et al. 1999). This observation illustrates the importance of *Pitx1* in *Tbx4* regulation and the necessity of *Pitx1* for correct hindlimb morphogenesis. Furthermore, appendage-specific expression of these genes has also been observed in the developing embryos of *Xenopus* and *Danio rerio* (Tamura et al. 1999; Takabatake et al. 2000), indicating that the genetic determinants of tetrapod limb identity have ancient origins and that differences in final limb morphologies are likely to be related to target gene selection (Weatherbee and Carroll 1999).

A number of observations support the hypothesis that additional regulators exist in the limb identity genetic pathway. First, *Pitx1*,
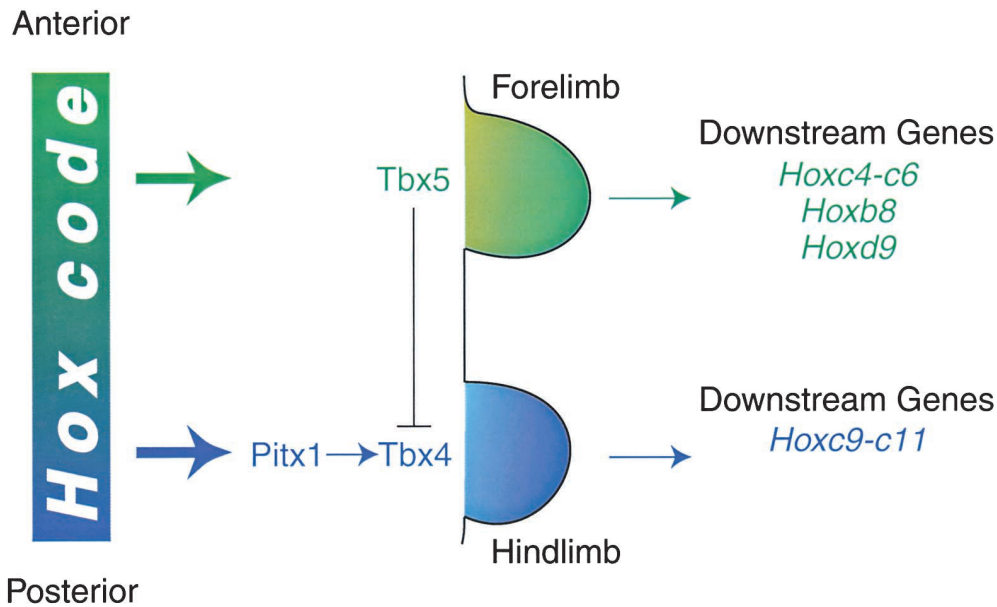
Fig. 1. Genetic pathways that determine limb identity. The Hox code in the lateral plate mesoderm specifies the expression of *Tbx5* or *Pitx1* and *Tbx4* in the prospective forelimb and hindlimb buds, respectively (reviewed in Ruvinsky and Gibson-Brown 2000). *Tbx5* down-regulates *Tbx4* and *Pitx1* is capable of inducing expression of *Tbx4*.

Tbx4, and *Tbx5* are transcription factors; however, the identity of their downstream target genes and the upstream regulators that restrict expression to specific limbs are not known (Ruvinsky and Gibson-Brown 2000). Second, limb-type transformations that occur in *Pitx1*, *Tbx4*, or *Tbx5* misexpression experiments are incomplete. These incomplete transformations also occur in mice with loss of *Pitx1* expression in the hindlimb. Although these results may simply reflect experimental limitations in the timing, domain, or level of expression, they highlight the need for further work. Third, even though *Pitx1* is capable of inducing expression of *Tbx4*, *Pitx1*-/- mice express low levels of *Tbx4*, suggesting the existence of alternative regulatory pathways. Finally, *TBX5* mutations cause Holt-Oram syndrome in humans, resulting in upper limb and cardiac malformations, both regions of *TBX5* expression in development (Basson et al. 1997; Li et al. 1997). The existence of numerous other inherited human malformation syndromes that predominantly involve either the upper or lower limbs suggests the potential for numerous unidentified genes with differential limb expression. For these reasons, it is likely that additional regulators of limb identity, yet to be cloned or identified as such, have differential gene expression profiles between developing forelimbs and hindlimbs.

A broad, comprehensive, and integrative approach for investigating limb identity

A shift in experimental design is required to more comprehensively explore the molecular mechanisms driving morphological diversity. We must incorporate the complexity that is inherent in biological systems into the design of our experiments rather than to simply ignore this phenomenon. Synergistic advances in scientific discovery are possible by developing and applying methods that integrate knowledge gained from the reductionist experiments of the previous millennium with the novel experiments of today and tomorrow.

With this in mind, one of our goals has been to develop a rational experimental plan to prospectively identify novel regulators of limb identity on the basis of differential gene expression. An ideal experimental system should maintain the complex spatial and temporal morphogenetic networks of these limb substructures, such as the zone of polarizing activity and the AER. These complex three-dimensional cellular and molecular interactions, vital for proper functionality of the different morphogenetic regions in the developing limb, can only be maintained by the use of an in vivo model system. Furthermore, the method to assay gene expression should be sensitive, quantitative, comprehensive, and unbiased; should have the ability to identify novel genes; and should integrate current knowledge with that gained from these experiments. One approach that meets these criteria uses SAGE (Velculescu et al. 1995) on tissue collected from intact developing limb buds.

We examined limb-specific gene expression by generating and analyzing comprehensive SAGE gene expression profiles from intact developing E11.5 mouse forelimbs and hindlimbs. Using this experimental design, we integrated the results of these experiments with current knowledge and identified novel genes.

RESULTS

SAGE tag-to-gene matching

We generated SAGE libraries from carefully staged forelimbs and hindlimbs at E11.5 and sequenced approximately 68,000 tags from each population. We found 36,300 unique SAGE tags. The proportion of SAGE tags matching genes was highly skewed toward the abundant tags (Fig. 2). We found that 86% of the unique tags in the highest abundance class matched a gene, whereas only 3.2% of all tags in the lowest abundance class matched a gene. Overall, 33.1% of the unique SAGE tags matched genes or expressed sequence tags (ESTs) (for details of the tag-to-gene mapping and tag libraries, please see Margulies et al. 2001b; Margulies 2001). Excluding the lowest abundance class of tags observed once,
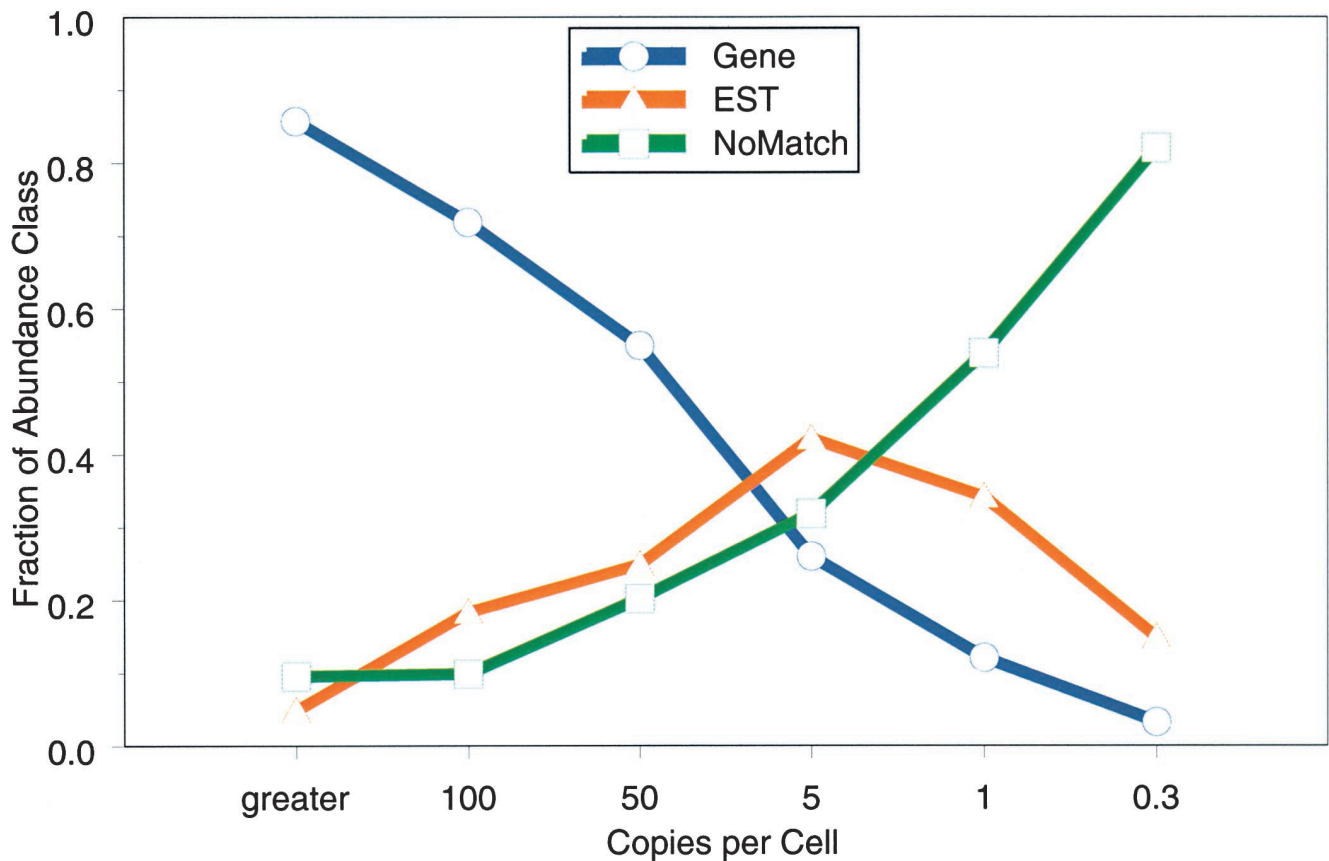
Fig. 2. Proportion of unique tags representing genes, expressed sequence tags (ESTs), or with no match, sorted by abundance class. Copies per cell was calculated assuming, on average, 500,000 total transcripts in a cell (Velculescu et al. 1995). Fraction of abundance class refers to the proportion of unique SAGE tags in a particular abundance class representing genes, ESTs or with no match.

this proportion increased to 58%. Sixty-six percent of the unique tags were observed only once and correspond to genes expressed, on average, at a level less than 0.3 copies per cell (Fig. 3).

This highly complex abundance class comprised only 17.6% of all sequenced SAGE tags (24,046 of 136,856 tags). Unique SAGE tags continued to accumulate at a rate of 16% toward the end of our sequencing effort, indicating that we have likely sampled over 85% of the unique transcripts present in the developing limbs. This observation was consistent with an analysis of 3.5 million SAGE tags from 19 different human tissues (Velculescu et al. 1999). Interestingly, 45% of the transcription factors we identified (113 of 251) are expressed in the lowest abundance classes of 1 copy per cell or less.

Fold-differences analysis

Figure 4 shows a histogram of fold-differences in gene expression between forelimbs and hindlimbs. The three tags with the greatest fold-difference in the hindlimb represent *Pitx1* and *Tbx4*, genes previously known to have hindlimb-specific expression patterns. Because the tag frequencies of other genes

previously identified as differentially expressed (*Tbx5* and several Hox genes) were low and in this fold-difference analysis tag counts of 0 were treated as 1, they appeared to be only 2-fold different. One of the limitations of using fold-difference as a measure of differential expression is that at low abundance levels, it is impossible to distinguish between genes that are not expressed ("true" zero) and those that are at such low levels they simply have not been observed in the SAGE sample (a "nonzero" element). Therefore, the genes in this low range are all potential candidates for novel regulators of limb identity.

By comparing the forelimb and hindlimb SAGE libraries, we were able to detect the differential expression of the three currently known major regulators of limb identity: *Pitx1*, *Tbx4*, and *Tbx5* (Table 1). Furthermore, we also detected the differential expression of several Hox genes previously identified by whole-mount in situ hybridization experiments as being differentially expressed in chick and mouse. Because *Tbx5* and many of the Hox genes were expressed at low levels in our SAGE libraries, their differential expression was not statistically significant at this level of sequencing. Nevertheless, the appropriate detection of previously known dif-
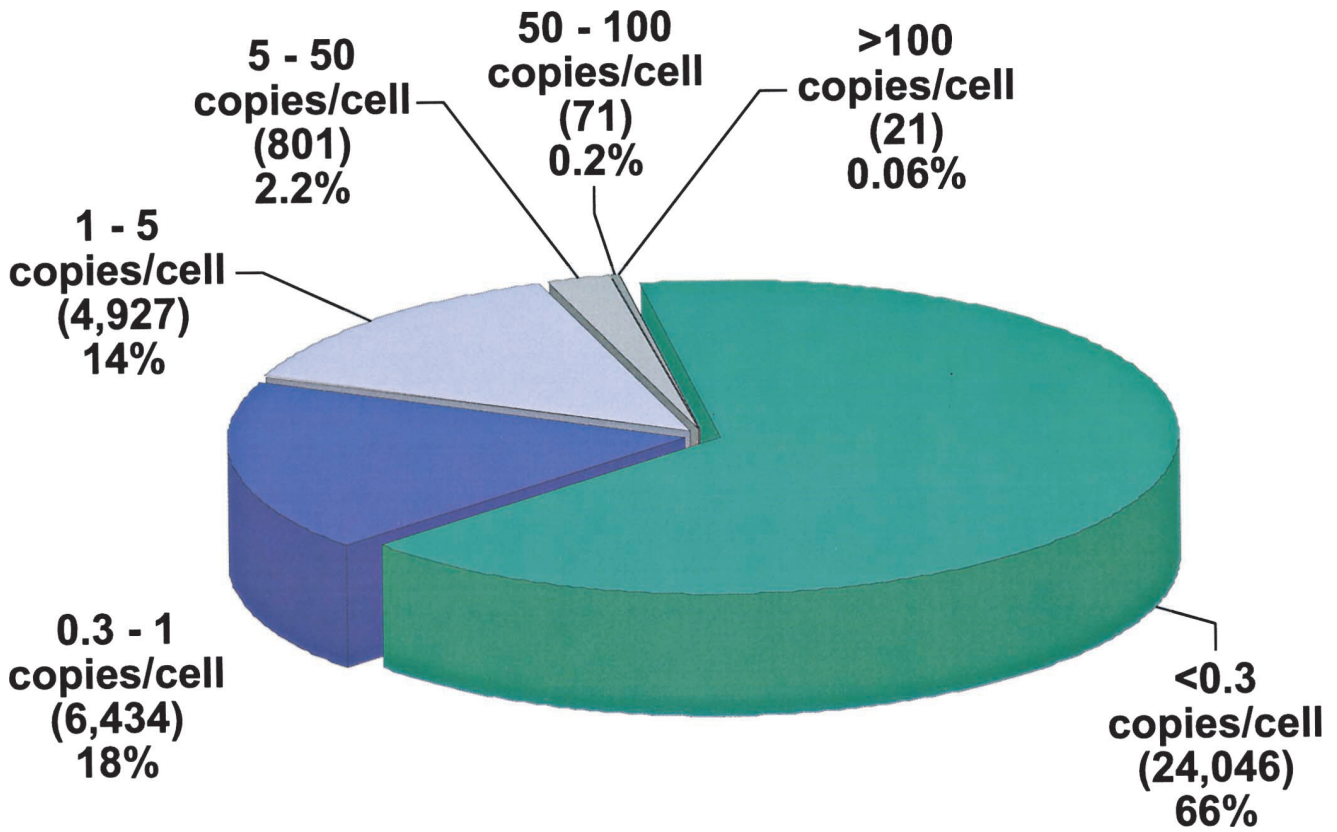
Fig. 3. Distribution of unique tags separated by abundance class. Copies per cell were calculated as in Fig. 2. Numbers in parentheses indicate the amount of unique transcripts in a given abundance class. The percentages indicate the fraction of the total unique tags represented by a given abundance class. Note that the sliver of the pie representing the highest two abundance classes are too thin to be drawn to scale and have been omitted from this representation.

ferentially expressed genes has validated this approach to detect novel in vivo differences. Further details of limb-specific gene expression can be accessed in Margulies et al. (2001b).

DISCUSSION

Pitx1 was the most abundant limb-specific transcription factor in our combined limb SAGE libraries and the most differentially expressed SAGE tag between our SAGE libraries. We did not expect the other known differentially expressed genes to be in the low abundance class. Nevertheless, the fact that known differentially expressed genes (*Tbx5* and limb-specific Hox genes) are in this low abundance class supports our hypothesis that other SAGE tags at this expression level are valid candidates for novel regulators of limb identity and morphology.

Interpretation of a SAGE tag count of 0

One of the difficulties in making comparisons between "digital" gene expression profiles is how to treat a discrete SAGE tag count of 0. In the context of our SAGE experiments, this number can take on two different meanings. The first mean-

ing is that the transcript is actually not present in the mRNA population. The second meaning challenges the stochastic properties of an event that has not yet been observed. Should 0 take on the value of 1, 0.1, 0.00001 . . . ? The dilemma of how to treat a SAGE tag count of 0 is exemplified in a fold-difference analysis. In our fold-difference analysis, we treated tag counts of 0 as 1. However, is a tag that is 2 and 0, 2-fold different, 200-fold different, or not different at all? A SAGE analysis cannot differentiate between the different interpretations of 0. Therefore, these genes must be verified with a secondary screening method that can differentiate between the different meanings of 0, such as a Northern analysis, reverse transcriptase polymerase chain reaction, or whole-mount in situ hybridization.

Mechanistic future studies

Our forelimb and hindlimb SAGE libraries were very similar (even though there is an overall statistical difference between their distributions); 93.4% of the genes were less than or equal to 2-fold different and 99.8% of the SAGE tags were not statistically different at a significance level of 1%. How-
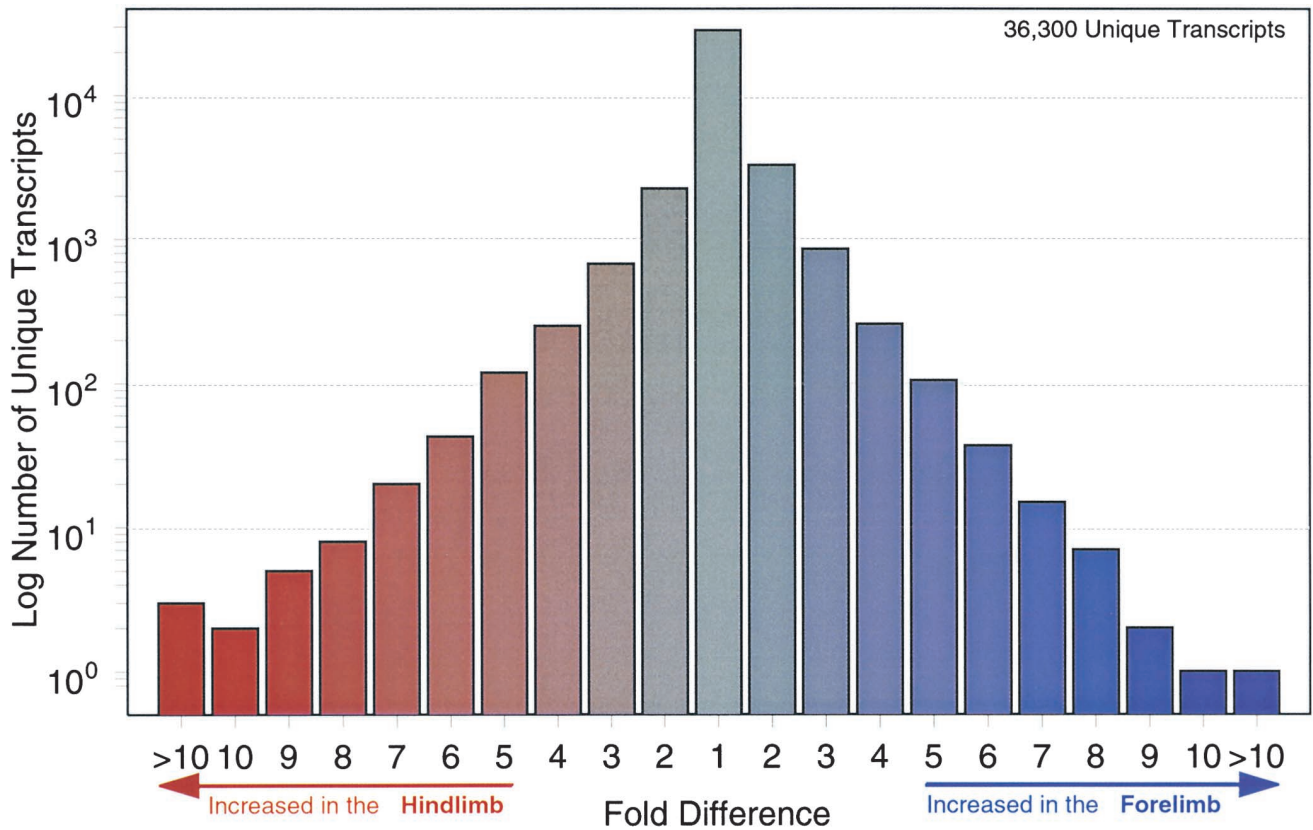
Fig. 4. Histogram of fold-differences in gene expression between forelimbs and hindlimbs. Note that the *y* axis is in log scale. Each category on the *x* axis contains values greater than or equal to the number. Red columns represent tags predominantly expressed in the hindlimb, and blue columns represent tags predominantly expressed in the forelimb. 0 tag counts were assigned the value of "1" to avoid division by zero. Over 93% of the unique transcripts are ≤2-fold different.

ever, statistical tests and fold-difference calculations cannot accurately assess the low abundance transcripts. In these cases, a virtual subtraction approach may be better suited. Resources would be better spent verifying this candidate subset of differentially expressed genes with other methods rather than to sequence an additional 1 million SAGE tags to determine which tags will become statistically significant.

Pitx1, *Tbx4*, and *Tbx5* are expressed throughout the entire limb structure during a time that starts before and continues after the specific stage of limb development assayed here. Nevertheless, this experiment may not detect potential upstream regulators in the *Pitx1-Tbx* hierarchy that are no longer expressed at this stage of development. This system may also have difficulties detecting genes expressed in a particular subset of limb cells such that their representation in the entire population is too low to be observed at this depth of sequencing. With the improvement of methods to perform SAGE on 1000-fold less RNA (Datson et al. 1999; Peters et al. 1999; Virlon et al. 1999), it will be possible to investigate gene expression at earlier time points of limb development, potentially identifying genes upstream of the *Pitx1-Tbx* hierarchy.

Functional testing of candidates

The current SAGE experiment analyzed gene expression at a very specific stage in limb development. However, *Pitx1*, *Tbx4*, and *Tbx5* are all expressed throughout a very broad range of development that includes the assayed time point. In fact, *Pitx1* is expressed in the flank before limb bud outgrowth occurs. Determining the temporal expression pattern for any novel differentially expressed gene would provide information on its potential role in limb identity. It would also be beneficial to determine if translational regulation correlates with transcriptional regulation of the candidate gene by raising antibodies to the gene product if none are currently available and performing immunolocalization experiments.

Confirmed gene expression differences should also be further evaluated as to their relationship in the *Pitx1-Tbx* genetic hierarchy. The expression pattern of a candidate gene can be compared between normal and *Pitx1-/-* embryos (Lanctot et al. 1999; Szeto et al. 1999). If the candidate gene normally has hindlimb-specific gene expression, down-regulation of the candidate gene in *Pitx1-/-* embryos would sug-

Table 1. Tag frequencies for genes known to have differential expression between forelimbs and hindlimbs

Gene	SAGE Tag	Tag Count	
		Forelimb	Hindlimb
Pitx1	TACGTCTATT	0	26
Tbx4[1]	TCGCCGGGCG	0	9
Tbx5[1]	TTCCCCGATT	3	0
Hoxc9	TACGGCTCGC	0	2
Hoxc10	TAGCTTCCTT	0	4
	CAAAGTTGAG	0	5
Hoxc11	TGCGTGAGTG	0	1

[1]These SAGE tags were identified by reverse transcriptase polymerase chain reaction cloning and sequencing of cDNA 3′ ends from limb mRNA.

gest that *Pitx1* regulates this gene. If the candidate gene normally has forelimb-specific expression, up-regulation in the hindlimbs of *Pitx1*-/- mice would be consistent with the phenotypic transformations of the hindlimb into a forelimb.

Misexpression experiments in chick would also provide valuable functional information about the candidate gene. The effects of limb morphology and gene expression can be determined by misexpressing the candidate gene in the opposite limb type, similar to the experiments performed with *Pitx1*, *Tbx4*, and *Tbx5* (Logan and Tabin 1999; Rodriguez-Esteban et al. 1999; Takeuchi et al. 1999). This would identify any roles this candidate gene may have as a limb identity selector gene and/or regulator of other forelimb or hindlimb specific genes. Furthermore, the *Pitx1-Tbx* misexpression experiments can be repeated, this time assaying for the candidate gene expression.

Additional studies would be valuable in determining the functional roles of such genes. For example, a yeast two-hybrid study could be used to identify other proteins that interact with and mediate the effects of the candidate gene product. Additionally, expression of the candidate gene in a cell culture system could be used to identify potential downstream target genes by assaying gene expression with microarrays or even SAGE. For this type of experiment, it would be valuable to use a cell line that closely recapitulates the developing limb, such as primary limb bud cells. If this candidate gene appears to play a major role in specification of limb type, generating mice with a targeted deletion of the gene would provide a valuable resource for functional characterization and determination of this gene's relationship to *Pitx1*, *Tbx4*, and *Tbx5*. One interesting experiment would be to analyze the phenotype of mice with deletions of both the candidate gene and *Pitx1*.

Integration with other biological information

Rather than looking at individual differences between multiple SAGE libraries, it will be more powerful to look at the trends for groups of genes, sorted by a number of biological features such as function, classification, or intracellular location. Recently, a method was developed (Bouton and Pevsner 2000) that attempts to link together all publicly available biological information about a particular gene and place this information in a searchable database called DRAGON (Database Referencing of Array Genes On-line). This database is publicly available (http://pevsnerlab.kennedykrieger.org/dragon.htm) and has numerous features for viewing, linking, and analyzing large-scale gene expression data. The ability to perform this type of analysis with SAGE data relies on the ability to match a SAGE tag to a known gene. Because DRAGON keys all biological information by UniGene cluster, the UniGene clusters identified with the ehm-tag-mapping method can be used to integrate SAGE tag information with the publicly available biological information in the DRAGON database.

SAGE at an earlier time point

It is now possible to perform SAGE on as little as 1 μg of total RNA (Datson et al. 1999; Ye et al. 2000). This makes it feasible to perform the SAGE experiments presented in this thesis at earlier time points in limb development. Because presumptive limb mesenchyme retains the ability to generate a limb when it is transplanted to a new location on the flank (Saunders and Reuss 1974), a SAGE experiment could be performed on flank tissue before limb bud outgrowth has occurred to identify potential additional "identity" regulators. Assaying multiple time points of gene expression from the same tissue will be a valuable resource for identifying patterns of gene expression similarities and differences between the serially homologous structures of the forelimb and hindlimb. Furthermore, this type of analysis could provide valuable information about the regulation and mechanisms of morphological variation throughout evolution.

Human malformation syndromes

There are numerous inherited limb malformation syndromes, with or without additional organ system involvement, for which the genetic basis has not been identified. A novel approach for identifying candidate genes can be envisioned by using a set of genes expressed in the limb, the rapidly expanding mouse genomic sequence data, and methods to map expressed genes to physical chromosomal locations (Caron et al. 2001). Further refinement of a candidate gene set could come from the ability to rapidly compare additional independently generated SAGE libraries (representing an expanding variety of tissue sources) with the phenotypic knowledge of a particular syndrome.

Finding correlations between the tissue distribution of specific genes and affected organ systems, combined with linkage data and chromosomal mapping information, will be a powerful integrated approach for the study of inherited diseases. Understanding the complex regulation that results in

the divergence of a single structure within the same organism may provide new insights into the evolutionary complexity observed between species (Capdevila and Izpisua-Belmonte 2000, Ruvinsky and Gibson-Brown 2000). In summary, research into the invariant properties of biological systems has provided a valuable framework for the next phase of scientific exploration: the examination of how variations in the relationships of expressed genotypic information are translated into phenotypic change.

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