Genetic Regulation of Mammalian Diversity

R.R. BEHRINGER,¹ J.J. RASWEILER IV,² C.-H. CHEN,¹ AND C.J. CRETEKOS³

¹Department of Genetics, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030; ²Department of Obstetrics and Gynecology, State University of New York Downstate Medical Center, Brooklyn, New York 11203; ³Department of Biological Sciences, Idaho State University, Pocatello, Idaho 83209

Correspondence: rrb@mdanderson.org

Mammals have evolved a variety of morphological adaptations that have allowed them to compete in their natural environments. The developmental genetic basis of this morphological diversity remains largely unknown. Bats are mammals that have the unique ability of powered flight. We have examined the molecular embryology of bats and investigated the developmental genetic basis for their highly derived limbs used for flight. Initially, we developed an embryo staging system for a model chiropteran, *Carollia perspicillata*, the short-tailed fruit bat that has subsequently been used for staging other bat species. Expression studies focusing on genes that regulate limb development indicate that there are similarities and differences between bats and mice. To determine the consequences of these expression differences, we have conducted an enhancer switch assay by gene targeting in mouse embryonic stem cells to create mice whose genes are regulated by bat sequences. Our studies indicate that *cis*-regulatory elements contribute to the morphological differences that have evolved among mammalian species.

The approximately 4800 extant species of the class Mammalia exhibit remarkable morphological and physiological diversity (Behringer et al. 2006). Most of these live on land, whereas others are born and live their entire lives swimming in aquatic environments or are capable of powered flight exploiting aerial niches. We are interested in the developmental genetic basis of the morphological and physiological variation observed among mammalian species.

Mammals are divided into three subclasses: the oviparous (egg-laying) monotremes, the viviparous (live-bearing) marsupials, and the eutherians. Most mammalian species are contained in the eutherian subclass, with a majority belonging to two orders: the Rodentia (~2000 species) and the Chiroptera or bats (~1200 species). We have focused our developmental studies on one of the bats, while also exploiting some of the numerous advantages of the laboratory mouse model.

The chiropterans have traditionally been divided into the megachiroptera and microchiroptera, i.e., simplistically, big bats and small bats. However, the biggest microchiropteran is larger than the smallest megachiropteran. Bats are widely dispersed throughout the world except at the poles. They feed on a wide variety of food sources, including pollen, nectar, fruits, other plant parts (flowers, leaves), insects, spiders, blood, and vertebrates. Many species have the ability of echolocation, using sound to “visualize” their environments and locate food. Among the mammals, bats have evolved the unique ability of powered flight that relies on highly modified limbs. This ability is distinct from other mammals such as flying squirrels (subfamily Petauristinae) and flying lemurs (order Dermoptera) that glide rather than actively sustain flight. The evolution of flight by bats allowed them to exploit a unique niche, the night sky.

The primary model we have chosen for most of our studies is a microchiropteran, *Carollia perspicillata* (the short-tailed fruit bat). *Carollia* possesses many useful attributes

as a model organism for studies of the Chiroptera. It is probably the most abundant mammal inhabiting forested areas in the lowland tropics of the New World and frequently establishes colonies in a variety of man-made structures, from which animals may be easily collected with hand nets. *Carollia* also readily adapts to captivity in a research setting. Indeed, it is feasible to accomplish this with no or negligible mortalities from the time of initial capture. In captivity, these animals are maintained in modest-sized cages that permit flight, but still facilitate efficient and noninjurious capture for experimental purposes. They are fed a fruit-based diet readily prepared from inexpensive canned and powdered components (Rasweiler et al. 2009). Because *Carollia* breeds very successfully in captivity with this husbandry program, self-sustaining colonies can be easily established. Finally, pregnancies can be conveniently timed from the first appearance of spermatozoa in daily vaginal smears. This has permitted the accumulation of substantial information on the timing of reproductive and developmental events in captive-maintained animals (Badwaik et al. 1997; Rasweiler et al. 2000; Cretekos et al. 2005). Using embryos derived from timed matings from our laboratory colony, we generated a standardized embryo staging system for *Carollia* (Cretekos et al. 2005), which has subsequently been modified for use with other species of bats (Giannini et al. 2006; Tokita 2006; Hockman et al. 2009; Nolte et al. 2009). These various factors have enabled our studies on the genetic basis of limb diversity among mammals.

LIMB DIVERSITY AND DEVELOPMENT BETWEEN MICE AND BATS

Comparisons of the forelimbs of adult mice and bats show significant differences in morphology (Fig. 1). The upper and lower arms of the mouse are approximately

FIGURE 1. Limb comparisons between mouse and bat. Diagram of the limb skeletal elements of the mouse and bat.

equal in length, whereas in the bat the lower arm is about twice as long as the upper arm. The mouse has digits that are separated due to programmed cell death of interdigital tissue during embryogenesis. In the bat, although the first digit of the forelimb is free of interdigital tissue, the four posterior digits (II–V) are connected by the wing membrane. In addition, digits II–V are highly elongated. In contrast, the relative lengths of the segments of the hindlimbs of the mouse and bat are similar. Another bat-specific feature includes a significant reduction of the ulna relative to that of the mouse. These morphological adaptations promote the quadrupedal and volant lifestyles of the mouse and bat, respectively.

Perhaps not surprisingly, the embryonic staging systems for all bat species examined so far are very similar in terms of the relative timing, pattern, and initial appearance of major anatomical features (Giannini et al. 2006; Tokita 2006; Hockman et al. 2009; Nolte et al. 2009). Species-spe-

cific differences in the shapes and sizes of some structures, such as craniofacial structures, limbs, and tail, appear after initial morphogenesis. In particular, limb outgrowth and early morphogenesis appear to be highly conserved among all bat species examined. Differences in wing proportions corresponding to different flight behaviors of the various species appear during the last third of development (Giannini et al. 2006; Tokita 2006; Hockman et al. 2009; Nolte et al. 2009). For example, digits II–V are spaced widely in the anteroposterior axis, with abundant wing membrane between them in *C. perspicillata* and *Pipistrellus abramus* (Cretokos et al. 2005; Tokita 2006), whereas digits II–IV are relatively closer together, with less interdigital tissue in *Molossus rufus* forelimbs, reflecting the relatively broad wing shape in the former species and the narrower wing shape of *M. rufus* (Nolte et al. 2009).

When limb morphogenesis is compared between bats and mice, a similar progression from early similarity to later differences is observed (Fig. 2). Limb buds form in both mice and bats at a similar stage of development and are morphologically similar in appearance during the early bud stage. Shortly after the formation of the forelimb handplate, the first overt differences appear, with the bat developing a proportionally larger handplate that is posteriorly expanded relative to the symmetric mouse handplate. During subsequent stages, programmed cell death occurs among all of the digits in the mouse, whereas only the interdigital tissue between digits I and II is lost in bat. The remaining interdigital tissue persists and later develops into the chiroptagium or “handwing” of the bat. Finally, during later stages of development, the bat forelimb shows a distinct proximal-to-distal bias in skeletal elongation, whereas the mouse limb skeleton elongates in a relatively uniform proximodistal pattern.

Formation of the axial skeleton begins during *Carollia* stage (CS) 14, with alcian-blue-positive cartilaginous condensations forming in the midline just ventral to the

FIGURE 2. Limb development in the bat and mouse. Whole-mount images of forelimbs from bat (A–F) and mouse (G–L). *Carollia* stages 14 (A), 15E (B), 15 (C), 16 (D), 16L (E), 19 (F); mouse stages 10 dpc (G), 11 dpc (H), 12 dpc (I), 13 dpc (J), 14 dpc (K), 16 dpc (L). Anterior is to the right. (Reprinted, with permission, from Cretokos et al. 2008.)

spinal cord (Fig. 3). During subsequent stages, the axial skeleton elaborates, with neural arches extending dorsally from the vertebral bodies by late-stage CS 15 and closing dorsally over the spinal cord by early-stage CS 17 and ribs extending ventrally beginning during CS 16. The first indication of the appendicular skeleton appears as an alcian-blue-positive condensation in the proximal forelimb during CS 14 (Fig. 3). During subsequent stages, the cartilaginous anlagen of each of the adult appendicular skeletal elements condense sequentially from proximal to distal (Hockman et al. 2008). In the forelimb, the scapula

and humerus precursors appear during late-stage CS 14, followed by radius and ulna during early-stage CS 15, carpals and metacarpals by late-stage CS 15, and phalanges during CS 16. During subsequent stages, the alcian-blue-staining cartilages are gradually replaced by ossified bone, again with a proximal-to-distal temporal bias, starting in the central region of each element and spreading with time toward the ends. Hindlimb skeletogenesis, consistent with the timing of limb bud formation, begins about one stage later than that of the forelimb, but catches up by CS 17 (Fig. 3).

Analysis of the cartilaginous skeleton of a stage-17 *Carollia* embryo reveals that the forming skeletal elements are relatively similar to the mouse, i.e., the upper and lower arm bones are of approximately equal length and each of the five digits are also approximately equal in length (Fig. 4A). However, by the time of birth, the forelimb skeletal elements have taken on the bat-specific morphological characters of the adult, including bone lengthening and ulna reduction (Fig. 4B). These findings suggest that limb form initially follows a general vertebrate pattern of development but, at later stages of gestation, species-specific characters emerge.

GENE EXPRESSION DURING BAT LIMB DEVELOPMENT

Many genes have been identified, predominantly in the chick and mouse systems, that are expressed in unique temporal and spatial patterns during vertebrate limb development. Their functions in limb patterning and morphogenesis have been determined for a subset of these genes. The expression of some of these genes have also been determined during bat limb development, including *Hoxd13* (Chen et al. 2005; Ray and Capecchi 2008), *Fgf8* (Weatherbee et al. 2006; Cretekos et al. 2007), *Bmp2*, *Bmp4*, *Bmp7*, *Msx2*, *Gremlin*, and *Spry2* (Weatherbee et al. 2006), phospho-Smad1/5/8 (Sears et al. 2006), *Prx1* (Cretekos et al. 2008), and *Shh* and *Ptc1* (Hockman et al. 2008). The general findings from these studies are that the expression patterns of orthologous genes in the mouse

FIGURE 3. Development of the cartilaginous skeleton of *Carollia perspicillata*. Whole-mount alcian blue staining of bat embryos from CS 14 through CS 20. The first column shows lateral views with anterior to the top and dorsal to the left. The second column shows high-magnification views of dissected forelimbs shown with anterior to the top and proximal to the left. The third column shows high-magnification views of dissected hindlimbs shown with anterior to the top and proximal to the left. Embryos are staged according to Cretekos et al. (2005) and indicated for each row. Views are not to scale.

FIGURE 4. Bat-specific characters develop late in gestation. *Carollia* forelimb skeletons at CS 17 (A) and newborn (B). (A) Alcian blue preparation, (B) alcian blue/alizarin red preparation. Digits are numbered I–V. (dpc) Days postcoitus, (H) humerus, (M) metacarpel, (P) phalange, (R) radius, (U) ulna. (B, Reprinted, with permission, from Chen et al. 2005 [© Wiley].)

and bat are generally similar, but many times, species-specific differences occur notably at later stages of embryogenesis. For example, *Hoxd13* is expressed in the posterior region of the developing mouse limb bud. Although *Hoxd13* expression in the bat forelimb is initially similar to that of the mouse, at later stages the anterior boundary of expression is shifted to a more posterior position relative to mouse *Hoxd13* (Chen et al. 2005). Likewise, *Fgf8* expression is detected in the apical ectodermal ridge (AER) of the forming mouse and bat limb buds. In the bat, the width of *Fgf8* AER expression is significantly greater than that of the mouse (Cretekos et al. 2007). These expression studies provided a foundation for ascribing the function of bat genes in the mouse.

PRX1

Prx1 (also called *MHox* or *Prrx1*) encodes a paired-class homeodomain transcription factor. The mouse locus produces two isoforms by alternative splicing, encoding proteins that differ only in the carboxy-terminal region. Alignment of the deduced amino acid sequences of *Carollia* PRX1 isoforms shows more than 99% identity with the orthologous isoforms of mouse PRX1 (Cretekos et al. 2008). Only two amino acid differences are found between bat and mouse PRX1 proteins, and these occur outside of the conserved homeodomain, PRX and OAR motifs, suggesting that the orthologs have identical biochemical activities.

Prx1 is expressed in a complex and dynamic pattern in the limb from the early undifferentiated bud stage through outgrowth and morphogenesis, suggesting roles in multiple steps of limb development (Fig. 5A) (Cserjesi et al. 1992; Martin et al. 1995). Expression of *Prx1* in the developing bat limb shows a striking up-regulation in the distal limb compared to mouse (Cretekos et al. 2008). Analysis of the mouse *Prx1* knockout (null) phenotype demon-

strates an essential role in the morphogenesis of limb and craniofacial tissues. The limbs of mice with *Prx1* function eliminated display shortened zeugopod (forearm and lower leg) elements and numerous autopod (hand and foot) element reductions and malformations (Fig. 5B) (Martin et al. 1995; ten Berge et al. 1998; Lu et al. 1999).

A region in which sequences capable of regulating expression in the limb reside, just upstream of the start of transcription, had been previously identified (Martin and Olson 2000). We identified the bat ortholog of the *Prx1* limb-specific enhancer by virtue of its conserved sequence and genomic position relative to mouse and human orthologs, and characterized the activity of this enhancer by combining it with a basal promoter and *lacZ* reporter in transgenic mice. The *Carollia Prx1* limb enhancer shares ~80% sequence identity with that of mouse and directs the expression of the reporter in a pattern similar to that of the orthologous mouse enhancer in the limbs of transgenic mice (Cretekos et al. 2008).

ENHANCER SWITCH AND DELETION BY GENE TARGETING

The challenge in studying the evolution of gene regulation is to distinguish between unselected genetic drift and significant functional divergence in noncoding *cis*-regulatory sequences (Cretekos et al. 2001). To address this challenge, we replaced the mouse *Prx1* limb enhancer with the bat limb enhancer within the endogenous locus by gene targeting in mouse embryonic stem (ES) cells and generated mice from these ES cells (*Prx1^{BatE}*) (Cretekos et al. 2008). We replaced the limb enhancer sequences without altering the coding sequence; thus, any change in *Prx* function in the *Prx1^{BatE}* mice is directly attributable to the bat regulatory sequences. Heterozygous and homozygous mice for the bat *Prx1* limb enhancer allele are viable, fertile, segregate in the expected Mendelian ratio, and show no readily apparent limb or craniofacial phenotypes. However, skeletal analysis of *Prx1^{BatE/BatE}* mice reveals that the forelimbs of these animals are about 6% longer than their wild-type littermates by the end of gestation, and this difference is statistically significant. Histological and molecular analyses indicate that bat enhancer-driven expression of *Prx1* results in accelerated endochondral bone growth in the mutant forelimbs, caused at least in part by increased proliferation of chondrocyte precursors at the ends of the long bones. Quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR) analysis of 17.5-day postcoitus (dpc) humerus from *Prx1^{BatE}* homozygotes showed ~1.7-fold higher levels of *Prx1* transcripts, suggesting that *Prx1^{BatE}* is a hypermorphic allele.

To test whether this *Prx1* limb enhancer is essential for limb-specific transcription, we also generated mice lacking it using a similar gene targeting strategy. To our surprise, mice homozygous for the *Prx1* enhancer deletion allele are viable, fertile, and do not exhibit the limb defects of *Prx1* null mutants. Q-RT-PCR analysis of 17.5-dpc humerus from enhancer deletion homozygotes showed no significant difference in *Prx1* RNA levels in comparison to controls. Thus, although the mouse limb enhancer is

FIGURE 5. *Prx1* expression and mutant forelimb phenotype in the mouse. (A) *Prx1* whole-mount in situ hybridization of 11.5-dpc mouse embryo. Expression is detected predominantly in the craniofacial region and limbs. (B,C) Alcian blue/alizarin red forelimb skeleton preparations from wild-type (B) and *Prx1^{-/-}* (C) newborn mice.

sufficient to direct limb-specific expression of heterologous genes, it is not required for limb transcription.

These results show that divergence of a single nonessential *cis*-regulatory region of one gene is by itself sufficient to elicit a discrete and measurable change in limb morphogenesis. They also suggest that regulatory redundancy may be part of a general mechanism for gain of novel expression patterns among conserved essential functions. In addition to *Prx1*, the mouse genome contains a *Prx2* gene, most likely a result of gene duplication. *Prx2* is also expressed in the mouse limb. *Prx2* homozygous mutant mice are viable with normal limbs (ten Berge et al. 1998). However, *Prx1;Prx2* double-mutant mice have severely shortened limbs, suggesting that *Prx1* and *Prx2* interact to regulate limb length (ten Berge et al. 1998). In our *Prx1^{BatE}* mutants, it is possible that PRX2 buffers the actions of bat-regulated PRX1 for limb development. These findings suggest that there are multiple levels of redundancy for buffering the effects of mutations on genes that regulate developmental processes.

CONCLUSIONS

The bat provides an excellent mammalian model system to explore the developmental genetics of animal diversity. The development of *C. perspicillata* as a model system has greatly facilitated research in the reproductive biology and embryology of bats. There are many morphological and physiological characteristics of developing bat embryos that show diversity relative to primary model organisms, such as the chick and mouse, beyond the limbs. For example, studying their craniofacial development would be a very exciting avenue for future research. Gene expression studies of developing bat embryos are still very limited. It would be useful to have a comprehensive analysis of gene expression in the developing limbs of bats. Such studies would likely generate candidate genes that are responsible for the generation of a wing. The reproductive biology of most bats, i.e., a relatively long gestation resulting in a single offspring and length to sexual maturity, makes the possibility of genetic studies, at best, unlikely. Thus, assaying the activity of bat sequences in the mouse with its tremendous genetic advantages can be a productive way to identify factors that lead to divergent morphologies among species.

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