Proposal for the construction of a Little Brown Bat (*Myotis Lucifugus*) BAC Library

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The importance of *Myotis lucifugus* to biomedical and biological research

Bats have acquired a number of evolutionary innovations in adapting to their particular niche. Microbats such as *Myotis lucifugus* have a number of interesting features including heavily modified limbs, the ability to echolocate, precise control over both thermoregulation and gestation, and an unexpectedly long life span. Many of the unique structures in bats have not arisen de novo, but are highly modified homologous structures found among other mammals. A good example is the bat forelimb. Despite the gross changes in morphology required for flight, the bat forelimb has maintained the basic pentadactyl skeletal elements in its wing. However, these bones have changed dramatically, having been elongated and reduced in girth, while tissue that is normally eliminated between the digits persists to form the different patagium (membranes forming the wings)(Adams 1992). The development of such marked evolutionary innovations is of great interest to our laboratory and many others. Understanding limb development in bats will be informative towards the basic mechanisms important in limb length determination and in the fate of interdigit tissues, both of which are key in human limb development and are often corrupted in limb malformations. One important aspect to an understanding of the mechanisms used in evolution is the differential regulation of genes held in common among mammals. An obvious but untested hypothesis is that elaboration of homologous structures in bats resulted from changes in regulatory elements for genes governing these structures. Studying these genetic changes will provide insight into basic processes during development

Large scale comparisons between human and mouse genomes reveal that 99% of the genes in the mouse have a human orthologous and 80% of these are 1:1 orthologs (Mouse Genome Sequencing Consortium, 2002). These data strongly suggest that spatial and temporal changes in the expression of existing genes is a primary mechanism in evolution. With the availability of the complete human and mouse genome sequences, comparative genomics have now become a powerful tool for determining conserved non-coding regions across species. These conserved regions are likely to be critical in gene regulation. While two species comparisons are valuable, the addition of a third and fourth species provides a much more robust determination of conserved elements. Equally important, modifications or changes that are conserved within a single group, but absent from others, should not be neglected. Such conserved outliers may correlate with phenotypic innovations unique to a particular group. The use of the bat genome as a tertiary comparator will be a great outlier to triangulate against the human and mouse genomes. Any changes observed in the *Myotis lucifugus* genome can be verified by consulting a second species of bat, *Rhinolophus ferrumequinum*. Finding these conserved non-coding regions exclusive to bats may allow us to identify unique functions. Bat exclusive sequences such as these would be strong candidates for effecting the changes in gene regulation required in the bats evolutionary path.

In addition to further understanding limb development, bats are a unique model for studying other structures and organs. The bat provides a novel system to investigate neuronal development, function and organization. The use of echolocation for hunting insects has required a number of morphological changes in both the larynx, nose, mouth and outer ears to send and receive auditory signals, and also major reorganizations to the inner ear and central nervous system to interpret and process various auditory calls (Smalling et al., 2003; Smotherman et al., 2003). Additionally, bats have an uncanny ability to thermoregulate and to couple this regulation with the control of fertilization and embryonic development (Barbour and Davis, 1969). Depending on food sources and ambient temperature, the female may delay fertilization, implantation, or arrest development in early stages (Hill and Smith, 1984; O'Farrell and Smith, 1984; Wilson and Gardner, 1980; Carter, 1970;). Determining how the bat controls its reproductive cycle will provide insight into the regulation of early mammalian development. Finally, the bat can be used as a model for the study of aging. The record for life expectancy has been thirty years for one *M lucifugus* banded in the wild (Keen and Hitchcock, 1980). This life span stands in great contrast to other mammals of its size which have a much shorter life expectancy, such as the lab mouse which under the most favorable conditions lives only up to three years. Further understanding of the genetic mechanisms underlying the evolution and development of unique bat structures will be of great benefit to both the biomedical and biological communities.

Uses to which the BAC library would be put, in addition to genomic sequencing.
The focus of our laboratory is to understand the genetic and molecular mechanisms responsible for the divergence of homologous structures between different species. To this end, we are using the mouse as a surrogate, in which to study the developmental and genetic programs of the bat. This will be done by targeting large pieces of bat DNA into mouse stem cells for the generation of mice with novel phenotypes. This method will provide a powerful tool in which previously inaccessible genetic programs, such as those of the bat, will be able to be manipulated with all of the established benefits of the mouse model system.

The sequence data that can be obtained from particular BAC clones is critical to creating a priority list of loci of interest. Mouse to bat and human to bat sequence comparisons will be instrumental; and conserved regions between these species will be significant and important. Even more interesting though, will be the comparison of two or more species of bats to determine modified conserved regions exclusive to bats. These regions will be a high priority for us in our experiments.

Currently, we are concentrating our efforts on the HoxD complex and its unique cis-distal regulatory element, the Global Control Region (GCR), located 200kb away from the complex (Spitz et al., 2003). Loss of function mutations within the HoxD gene cluster have demonstrated their critical role in limb development. Combined loss of function mutations in HoxD13, 12, and 11 exhibit a phenotype in the autopod or hand that has significantly shorter metacarpals and phalanges than wild type (Bruneau et al., 2001). These and other phenotypes associated with various mutations in the HoxD complex suggest a target that might be modified to execute evolutionary change. Of great interest

is the GCR, which as been shown to effect transcriptional control over the HoxD complex in the autopod. In the mouse, the complex spans over 45 kilobases in length and the importance of two highly conserved regions at either end of the GCR is suggested by comparisons with the genome of *Fugu rubripes*. While this element is also found juxtaposed next to the HoxD complex in *Fugu*, it is reduced in size to only 6kb. In the intervening expansion from *Fugu* to mouse, a number of sequences have arisen that are conserved amongst higher vertebrates (Spitz et al., 2003). Multi-species comparisons will further define regions of importance in the GCR.

We are using gene targeting methods to replace the mouse HoxD complex or its regulatory element with homologous bat sequences. Such a targeted swap can only be accomplished with the use of DNA constructs large enough to hold either the complex (≈ 120kb) or the GCR (≈45kb) on a single vector. A BAC library is most useful to this end in both a targeted and transgenic approach. Once this experiment is carried out, we can then use other candidate regions such as the HoxA complex in this assay to further understand the mechanisms responsible for differences in limb development between mouse and bat.

In addition to providing critical sequence data for particular loci and suitable vectors for our gene replacement experiments, the BAC library should also be of great use for the generation of transgenic mice harboring bat inserts. As well, the targeted sequencing of portions of this BAC library will provide a definitive tool for cladistic analyses to more accurately place bats within mammalia and to further understand the relationship between megabats and microbats.

The size of the research community that could potentially use the BAC library and the community's interest in and support for having a BAC library.
This BAC library will be an important resource for the greater developmental and evolutionary biology communities. We expect that once the efforts of our group and others (see below) are published, interest in bats as a model system will grow. Investigators such as Lee Niswander, John Rasweiler and Richard Behringer are working on approaches that complement our own (Cretekos et al., 2001). A number of investigators are interested in the neural innovations required for echolocation, in particular Walter Metzner, Albert Feng, these other research groups would benefit from access to a BAC library and in the future its sequence. Additionally this BAC library will be valuable to the large number of ecologists who study the role of bats in, and their evolutionary adaptations to, the environment (Ruedi and Castella, 2003; Kawai et al., 2002).

Whether the organism will be, or has been, proposed to NHGRI or another publicly funded agency for BAC-based genomic sequencing and the status of that request.
This proposal will not be sent to another funding agency during the review period.

Other genomic resources that are available that will complement this resource.
In working with *M lucifugus* we have developed a number of other resources to further characterize its genome. In collaboration with Robert Weiss at the Utah Genome Center,

we have created a whole genome shotgun library, of which 40,000 clones have been end sequenced (80,000 unique sequence reads). This has provided the unambiguous identification of over 3,000 landmarks within the *Myotis lucifugus* genome. In addition we have produced a phage library (≈ 12 kb inserts) that represent at least 4x coverage of the genome and which has been successfully screened for a number of genes. Based upon collection of a variety of different embryos representing various stages in development, tissue specific RNA pools have been established. Significant effort has gone into the derivation and detailed characterization of over 20 embryonic cell lines. Each fibroblast like cell line has been characterized with respect to growth conditions and rates, drug sensitivity, transfection efficiency and successive passage. These cell lines are extremely robust and well suited for many tissue culture based experiments. Early passage cell lines (P1) have been utilized in the construction of a high resolution G-banded karyotype for *Myotis lucifugus*. The resolution of this karyotype is sufficient to enable the production of chromosomal specific FISH paints based upon microdissection of G-banded chromosomes, an ongoing and successful effort. Finally, approximately 1,000 clones containing inserts representing the repetitive fraction of the bat genome are being sequenced and will provide a valuable tool for the masking of the existing 80,000 sequence reads, the analysis of repetitive sequence evolution and composition, as well as providing direct access to the Cot1 fraction of the bat genome.

The strain of the organism proposed and rationale for its selection.
We have chosen to study the bat *Myotis Lucifugus* for a number of reasons. First, its body size is similar to that of a mouse reducing potential problems with overgrowth in utero (Nowalk, 1994). Also, it is not endangered and is easily obtained domestically in large colonies in the eastern United States and in smaller colonies in the western United States (Nowalk, 1994). Already a large body of work exists on the physiology, ecology, biology and development of this species. Finally, our lab has developed a number of resources which will be useful to many investigators.

The size of the genome.
Work is currently underway to determine the size of the genome by flow sort analysis.

The availability of a source of DNA for construction of the BAC library (evidence of its quality for this purpose).
We have developed a number of collection sites in Utah, Pennsylvania and Wyoming from which we are currently collecting specimens. We now have DNA plugs from three females made from brain, kidney and spleen. These plugs are being evaluated for DNA content and quality. Additionally, embryos were taken from the females to derive primary cell culture lines than may be up to 50% isogenic with these DNA plugs. Previously, on two different occasions, we have collected bat specimens from which we have developed high molecular weight DNA of greater than120kb that was successfully ligated into a pBACe3.6 vector. Unfortunately, we do not have the resources to continue this project further.

Specifications for the library (e.g., library depth, BAC insert size) and supporting scientific rationale for these specifications. (Note: any request for an unusual vector for a particular application must be thoroughly discussed).
A standard library of at least 10X coverage with an average size of 120kb (100-200kb size range) would be sufficient in any standard BAC vector that is amenable to recombinogenic engineering (ET cloning) in *E. coli*. (Lee et al., 2001). 10X coverage and a 120kb average insert size is essential so that single clones can be found that contain an entire Hox complex or a large regulatory region. It is key to our applications that the actual back bone be selected with ET cloning in mind. This will facilitate the manipulations required for creating large targeting vectors in the range of 100 –200kb in size, where traditional restriction cloning in not feasible.

The time frame in which the library is needed.
All elements of our current experiments are in place except for the donating DNA from the bat. This library will allow us to proceed with a number of experiments and as stated above would represent an enormous resource for many other investigators.

Other support that is available or has been requested for the construction of the desired library.
Although resources to contribute to the development of a BAC library are limited, we are performing DNA collections and processing to the extent that is needed.

The need for an additional BAC library if one or more already exists.
First, mouse to bat and human to bat comparisons are useful but limited. Such comparisons allow for conserved regulatory sequences to be uncovered across large evolutionary distances. Sequences that are found in bats but not in mice or humans may represent novel regulatory regions or coding sequences exclusive to that group. Sequences exclusive to bats can only be uncovered by bat interspecies comparisons. This library will augment and be complementary to that of *Rhinolophus ferrumequinum* in uncovering these sequences (Thomas et al, 2002).

Second, with over 900 known species, bats represent one of the largest groups in mammalia and are second only to rodents in their diversity amongst mammals. This fact alone should be sufficiently compelling to strongly argue that two or more widely separate species of bats, such as *Myotis lucifugus*, and *Rhinolophus ferrumequinum*, should be sequenced in the very near future. A first step will be the development of BAC libraries for these two species.

Any other relevant information.

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Dear BAC Resource Steering Panel,

In response to your inquiries, regarding the evolutionary distance between *Rhinolophus ferrumequinum* and *Myotis lucifugus* and the significance of *M. lucifugus* to research, I present the following.

The large number of bat species (about 972) ranks second only to rodents amongst mammals with bat accounting for approximately 25% of all mammalian species. These facts alone are a major justification for the construction of a second BAC library from the Order Chiroptera. The Order Chiroptera is broken into two suborders, Megachiroptera, which are large old world fruit eating bats that do not echolocate and are grouped within the single family Pteropodidae; and Microchiroptera, which are small echolocating bats comprising at least 759 species and 16 families (4 superfamilies) distributed the world over (Jones et al. 2002, Simmons and Geisler 1998). Although *R. ferrumequinum* and *M. lucifugus* belong to the Suborder Microchiroptera the evolutionary distance between *Rhinolophus* and *Myotis* is rather large. In fact it has been suggested that Rhinolophus is more closely related to Pteropodidae (Megabats) than Vespertilionidae (the family of *Myotis*) (Teeling et al. 2000). The consensus is that they diverged during the Eocene, about 50 million years ago (M. Brock Fenton pers. com. 2003, Jones et al. 2002, Simmons and Geisler 1998). 50 million years far surpasses the Atlantic and Pacific oysters which are separated at most by 10 million years, are in the same genus, and for which BAC libraries are being constructed. Furthermore 50 million years is a significant separation when considering the mouse and human split is only 75 to 100 million years ago.

I would like to further address the scientific value of sequencing *Myotis lucifugus*. *M. lucifugus* is the predominant model for chiropteran biology and for the use of chiroptera in comparative biology and medical research. While *R. ferrumequinum* was chosen over the originally suggested *Myotis lucifugus* for accessibility, this has not been the case in practice. I have personally attempted to ascertain from UCLA (origin of the DNA for the *R. ferrumequinum* library) bat or bat DNA samples. They were not available since those at the UCLA facility are dedicated to neurobiological experiments and they are imported from China. *Myotis lucifugus* however is one of the most abundant native bat species on the North American continent thus negating import restrictions. I have personally collected specimens from three different states (Wyoming, Utah, Pennsylvania) with some colonies ranging up to 20,000 animals. It would seem that *M. lucifugus* is the more accessible bat.

Myotis lucifugus has been more extensively researched than any other bat inlcluding *R. ferrumequinum*. Therefore the current knowledge base and resources for *M. lucifugus* is substantially greater than that of *R. ferrumequinum* as shown by a survey of Entrez and Biosis, which gives more hits, for *Myotis* than for *Rhinolophus,* in some cases by a ratio of 3:1 (See table 1).

Table 1

Genus	Pub Med Hits	BIOSIS Hits	DNA Hits	Protein Hits
Rhinolophus	135	1062	430	92
Myotis	323	2538	473	395

Additionally, as noted in the original proposal, two bats are better than one for comparative genetics. While conserved sequences between bat, mouse or human will be of great interest, the fact that selection has maintained sequence integrity for the last 75 million years suggests that such sequences were not critical for effecting evolutionary change between these three species. Comparisons of two very distantly related bats however will reveal those sequences which are conserved between bats but not human nor mouse. These sequences are excellent candidates for having played a role in the evolution of novel chiropteran structures.

Our current work with *Myotis lucifugus* has involved a large number of molecular and genetic characterizations and the development of techniques that will propel this species into the forefront of chiropteran biology and provide a new developmental and genetic model. A BAC library and future sequencing of its genome will ensure the success of this model for critical research in limb and hindbrain evolution and development as well as thermoregulation, parturition and aging.

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