Published in final edited form as:

Evol Anthropol. 2015 July 8; 24(4): 130–136. doi:10.1002/evan.21454.

How are we made?:

Even well-controlled experiments show the complexity of our traits

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The fact of evolution seems as well established as anything in science. However, there are many questions for which we don't yet have clear answers. That's fortunate because it allows us to have careers in evolutionary sciences, during which we can try to understand the origin and genetic basis of traits that interest us. From what is currently understood, the development and evolution of physical and even behavioral traits are genetic at their core. We should be able to design studies to explore what that basis is.

An important criterion for success in genetics, as in any other science, is the ability to frame a well-posed question on which to base one's research. A well-posed question should enable focused research to yield a unique, clear answer. That's particularly difficult for questions about the evolution of complex traits because we have only fragmentary physical evidence from the past; fossils that happened to be preserved, though how representative they are we can't really say, and ancient DNA from a small number of sources. Consequently, our understanding of how traits evolved necessarily rests on historical narrative rather than on direct observation of mechanism or process. We must find indirect ways to use contemporary material to pose questions about how traits are produced and evolve. Fortunately, the same evolutionary process that generated the complex traits in our ancestors also provides connections among present-day organisms and thus suggests clever strategies we can take.

GETTING AHEAD OF WELL-POSED QUESTIONS

Upright posture and thumbs have long interested anthropologists, but we often like to think headfirst about ourselves. Much of what makes us different from other species, and perhaps more vain, has to do with our heads. However, understanding the genetic basis of the head, or its individual traits, has been difficult. Many genes that ostensibly are important to the evolution of the head have been identified because, when mutated, they can cause serious craniofacial disorders. Many or most mutational variation in those genes seems to be so serious that the embryo doesn't survive development. In that sense, more fine-tuned adaptive evolution doesn't seem to work by purging the truly pathologic genes, but more

NOTES

I welcome comments on this column: kenweiss@psu.edu. I co-author a blog on relevant topics at EcoDevoEvo.blogspot.com. We thank John Fleagle for critically reading this manuscript. The Box reports collaborative work done with co-investigators James Cheverud and Jeffrey Rogers, whose contact information is, respectively, cheverud@luc.edu and jr13@bcm.edu; also see <http://hominid.psu.edu>. This column is written with the assistance of funds provided to Penn State Evan Pugh professors. Contact information for the major investigators: Jim Cheverud: jcheverud@luc.edu, Jeff Rogers: jr13@bcm.edu, Ken Weiss: kenweiss@psu.edu, Joan Richtsmeier: jta10@psu.edu, Anne Buchanan: abw1@psu.edu.

typically by gradual “tinkering” within the viable range. Heads and other traits have mainly evolved through the gradual process of chance circumstances and natural selection leading to genetic variants that produced minor, essentially normal, variation in traits. Small advantages and good luck within that variation proliferated over time, yielding what we are today. To understand this, we need research models to identify relevant genetic contributions to normal craniofacial variation.

One simple, direct approach seems obvious: Take tissues from an embryonic head and see what genes are expressed as the head develops. The idea is to identify and document the genetic mechanism responsible. The technology is readily available, but a problem is quickly apparent. Thousands of genes are expressed in developing head tissues, but many of those are not involved in development *per se*, as in the shaping of a particular trait; instead, they are involved in basic cellular, neural, and skeletal physiology. Since most genes serve many different functions, how can the genes and gene interactions most critical to development be identified in the complex mix of relevant developmental subsets?

One approach to understanding genetic mechanisms is to use genetic variation to find the relevant genes. Like all anatomical structures, the head is a 3D object. Among closely related species, homologous landmarks that presumably reflect important function can be identified on the skull, as shown in Figure 1. The 3D coordinates of these locations can be digitized and used directly in analysis; alternatively, linear distances among the landmarks can be estimated and used.¹

There have been many large studies of the genetic basis of such isolated metric traits as body weight or stature. It seems that it should be possible to ask well-posed questions about these studies, in which the entire genome is typed in each member of a chosen sample of individuals to find sites in the genome in which variation is statistically associated with variation in the trait measured in the individuals. This process “maps” the association of variation in the measured trait to variation in the genome, assigning a causal effect to the genomic site. Some such studies have been done specifically to identify genetic associations with facial variation in humans.³ Usually, many different genome sites are implicated. Depending on the way data are acquired, facial dimensions can include soft tissues like skin and muscle, as well as the underlying bone, and hence can be affected by various life-style effects, as well as genes. Facial shape and superficial appearance are relevant to things like mating success, but the underlying skull has historically been the target for genetic research on the head. The skull is a hard structure, the shape of which is affected by life events, not just genes. The head has been of traditional and comparative value because it is relatively well preserved in burials and in the fossil record. 3D data on skull shape can be obtained in various ways, optimally by CT-scanning. However, obtaining adequately large samples, even of living individuals, is also costly and difficult.

Fortunately, there is an alternative to doing studies directly on humans. This method takes advantage of the evolutionary connections that make it possible for one species to serve as a practical surrogate for another. Homologous traits in other animals are not identical to those in humans or other primates, but countless precedents suggest that the basic genetic

mechanisms responsible for their development will be similar and, if we are properly circumspect, can at least provide testable leads that are relevant to humans.

The laboratory mouse is an obvious choice. Many researchers have shown correspondence in mouse skull phenotypes resulting from mutations in genes that, when present in humans, cause disease, thus revealing conservation of the basic developmental genetic program that underlies mammalian skull morphology and validating the use of mouse models in the analysis of primate skull phenotypes such as models of craniofacial disorders in humans.² Controlled breeding of laboratory mice is an attractive approach for many reasons: There is an extensive research infrastructure to build on, measurements can be taken accurately, large samples can be studied at affordable cost, and their genotypes can be controlled, so that otherwise confounding environmental variation is minimal. Generation times are short and any sex or stage of pre- or postnatal growth and development can be studied.

The mouse, which enables us to ask what should be well-posed questions about the genetic basis of craniofacial variation, is a research mainstay that is being adopted by many anthropologists. Inbred strains with a wide range of traits are commercially available. These have been produced by maintaining a small colony formed by mating only close relatives, such as siblings, for many generations, perhaps after or while using artificial selection, which allows mating only of individuals having some trait of interest, such as obesity.⁴ The resulting animals then have the chosen trait. It is important to note that the resulting response to selection depends on the variation that is present in any given data set. The response to selection may also involve traits that were not of particular interest or were not selected for, and may or may not be relevant to what was selected “for” in natural adaptations. This colony-generating process fixes variants across the genome that are compatible with survival under these breeding conditions. A subset of those genetic variants will have specifically affected the selected trait of interest. However, the mice, having been inbred, no longer vary at either relevant or irrelevant sites. So how can we find the genes having the variants that made the selected trait?

One way is to take mouse strains that have been produced by artificial selection so that they differ in the trait you’re interested in. Often such strains are already commercially available. One’s first thought might be simply to sequence the genomes of a mouse from each of the differing strains, then compare them to see where they differ. Unfortunately, there will likely be millions of such differences. You may be lucky and find variants in candidate genes for which there is already some evidence of involvement, such as previously identified mutations that cause human disease, or having known function that suggests possible involvement in the development or function of the trait of interest. But the function of most genes is incompletely understood, complex, or as yet unknown. If you count only on what you already know, what have you learned?

Alternatively, one can obtain more detailed information by using a controlled cross between strains that differ in your trait of interest. Many such studies have been done. An illustrative example based on work we and collaborators have undertaken is described in Box 1. From studies of this type, we can hope to find what we’re looking for. But in important ways,

which make complete evolutionary sense, what is found raises questions about what we think we should be looking for in the first place.

CRANIOFACIAL MAPPING IN THE MOUSE: WHAT DOES IT SHOW?

Several independent studies have mapped craniofacial variation in laboratory mice. They identified parts of the mouse genome that are statistically associated with various distance measures such as those shown in Figure 1. These associations may reflect elements in the implicated regions that causally affect individual metric traits or modules — sets of correlated traits represented by distance measures — in the mouse head. Once a genome region has been identified, investigators can look at the annotated mouse genome sequence to see what known functional units are within the region in an attempt to identify genes they believe might be responsible for the measured size or shape variation in their mice. Often, the suspected genes are identified as candidates because some previous evidence, often human disease, has shown an association with craniofacial disorders or osteogenesis or some other fundamental cellular process that seemingly is fundamental to skull growth.

For example, Klingenberg and colleagues⁵ mapped variation in mouse mandible size, shape, and symmetry, in an earlier offspring generation of the same cross as that described in Box 1. They found that variation in each measured trait was affected by multiple genome regions, each of which was long enough to contain tens or even hundreds of potentially causal genes or regulatory sites. In a recent study of similar design, Maga and coworkers⁶ mapped landmarks and craniofacial linear craniofacial distances in 433 animals from a backcross of two inbred mouse strains. They reported finding seven genomic regions affecting skull size and 30 affecting skull shape. Though the individual effect sizes of these identified regions were very small, Maga and coworkers noted 16 “high-priority” genes that they consider to be candidates responsible for these effects, primarily choosing genes previously identified to be associated with craniofacial anomalies.

Pallares and colleagues⁷ did a genome-wide association study of craniofacial variation in a kind of natural experiment, using wild mice captured from a natural hybrid zone between two subspecies, in which there would be much more genetic variation than in a cross between two inbred strains. They identified various relevant sites across the genome that affected craniofacial measures, but together these sites explained only 13% of variation in skull shape and 7% of variation in mandible shape. Their candidate genome regions basically did not overlap with those of other studies, a general problem that, as the authors point out, reveals the complexity of the genetic architecture of face shape.

Our own study (Box 1) took a similar approach between inbred mouse strains. However, in our “hit” genome regions there were only a few genes previously known to be associated with craniofacial disease. At the same time, there were many genes within the mapped intervals having documented tissue expression in Genepaint (genepaint.org) or other published sources, which make them at least potentially important in craniofacial development. But from these data alone, we had no way of claiming a single candidate or even a small number of these potential candidates as being more likely than any other to affect variation of the trait within the interval. This isn’t really a surprise. Genes can

function in head development in ways that are so important that mutations would likely be lethal, so they cannot vary substantially in normally developing individuals. Hence, there can be a functionally relevant gene involved in development of the head in the mice in our study, but it will not be responsible for significant variation in the skull in our particular sample. Further, one or more genes in an interval can vary in ways that have only small but nonpathological, effects. A mapping exercise alone cannot resolve such issues. Since the whole mapped genome region is statistically associated with the trait's variation as a unit, without extensive additional testing one cannot specify which one or more of these candidate genes within the region is associated with the trait's variation. This, along with the likelihood that trait variation results from the combination of many different genetic effects, points to fundamental problems in understanding the genetic basis of how traits are made and how morphology is constructed.

EVOLUTION PUTS IT ALL TOGETHER, BUT HOW?

So identifying the genetic basis of individual craniofacial measures, spot by individual spot along the genome, is a challenge. An obvious question relates to the way multiple, potentially even competing, functions such as defense, thinking, posture, chewing, and so on have evolved, as they must have, to work within the single compatible structure of the head. That is, what is the nature of morphological integration in the skull?

Morphological integration, a concept originally introduced by Olson and Miller (1958)¹⁸ shows up empirically as co-variation among developmentally, functionally, and/or evolutionarily related measures; that is, they may vary together. Several cranial measures may have coevolved and hence grown together to form a morphological module within the skull. If you think of the head's many functions from an evolutionary point of view, a modular arrangement is a necessity. But even if distinct areas like the cranium and face do constitute modular units, the overall pattern has to be modular in other ways, based on known histological criteria. The different functional units must themselves be compatible with a single skull. Different elements of the human skull are derived from disparate primary cell layers of the early embryo; that is, some skull bones are derived from neural crest cells, while others derive from paraxial mesoderm, which ultimately join together to form a skull as a single structure. Different bones of the skull also mineralize by two types of ossification (intramembranous and endochondral), and yet, regardless of their mode of ossification, the bones all change together to accommodate the growing brain and other soft-tissue structures, and eventually fuse as a unified skull. The development of the skull is the result of a complex, nested, overlapping, and/or serial modularity.

An obvious issue is that standard metric distances traverse different developing structures, or modules, such as different cranial vault plates. An example is shown by the arrows in Figure 1. One could limit analyses to distances that don't cross more than one functional region, but all of the structures must evolve cooperatively to fit with each other in a viable way. Thus, one would still have the challenge of connecting the developmental dots. It's not clear what level and sort of correlation among dimensions we should expect and whether or how these relationships change throughout ontogeny and over evolutionary time as different adaptations are acquired.

In fact, it's also unclear to what extent we should expect such correlations to be due to the same gene(s) having evolved to regulate the growth of both structures or to different genes in which variation was separately and adaptively molded to be consistent. There are examples of both kinds of orchestration among closely related species. This is a rather fundamental question about the way evolution generally works. The head seems to be a good thing to consider in this light, given the many studies of its variation and genetics that have been done. But if the question seems simple, the problem isn't. Every trait varies. Correlations might simply reflect aspects of sampling or chance variation, or even morphological correlations specific to a particular time in ontogeny that have no particular unitary functional evolutionary basis.

Methods for empirically searching for such correlations have been developed.⁸⁻¹¹ The idea is that if single genes are pleiotropic in this context — that is, they affect multiple traits — then variation in the trait should be correlated with the variation at the gene. Unfortunately, these are statistical approaches to data; there is no formal theory for the expected amount or nature of morphological integration we should expect.¹² The evidence consists largely of empirical correlations among measures and comparison of observed variation to expected patterns. Statistical associations that have been studied to date generally do not clearly show that morphological correlations are functional. They could just reflect sampling and measurement choices or the genomic variation that happened to be present in a given study design. Our own study found individual genome regions associated with multiple traits, but a given variant among the many variants within such a region might affect only one of the traits in which variation is associated with that genome region as a whole. One can't tell from this sort of study alone. A study of an earlier generation of the Lg/Sm (described in Box 1) cross asked whether development of the mouse mandible was genetically modular in the sense of being developmentally isolated from other cranial structures.¹³ It was found that some genome sites associated with all measured distances to some extent, suggesting that modularity is not due to distinct subsets of genes.¹³

There's no reason to be surprised or disappointed at these uncertainties. Even an individual metric distance usually reflects several different evolutionarily relevant functional constraints. For example, facial length accommodates teeth for diet and defense, reflects the orientation and extent of visual and olfactory systems, the location of eyes for direction detection, the appearance for mate choice, and more. But those functions are affected by posture and neural connections, which relate to other parts of the skull, as well as mode of locomotion and consumption (predation, herbivory, etc). In addition, of course, variation in each region is affected by many different genes. The different parts of a trait have to be compatible, changes in one adjusting to changes in the other during development (in mammals, for example, the jaws adjust for suckling and then gradually for either carnivory or herbivory) and evolution. Finally, there is no one way that is theoretically "correct" relative to development and evolution even to choose or analyze measures in 3-D structures such as the head. Any claims of methods or data as the most appropriate assume prior knowledge of what one seeks to learn.

HOW CONSERVED IS THE GENOMIC BASIS OF A CONSERVED TRAIT?

Carefully controlled experiments cannot capture all the genetic effects on craniofacial development, even in the mouse. They can however, show us what happens to vary in the available data we choose to look at. What has been found provides some clear messages that are wholly consistent with mapping studies on other plant and animal traits and the extensive mapping approach used to study human disease-associated traits.

Hundreds of genes have been reported to have pathologic effects on craniofacial shape, but these are only a fraction of the genes that are expressed in specific tissues in the developing head. At least 80% of all known genes are expressed in the brain, according to one report.¹⁴ We estimate, from the searches we have done (Box 1), that about 95% of all genes are expressed somewhere in the head even at the single embryonic stage that has been most systematically characterized (genepaint.org). Expression in a relevant tissue, however, does not imply that the gene or its variation is relevant to any particular metric trait, such as in a particular mouse cross or species comparison; rather, expression in a relevant tissue implies only that the gene is *a priori* possibly relevant. Demonstrating specific causation when a plethora of potential candidates exist is more than trivially difficult. Even if a specific variant can be demonstrated as contributing to a trait, that does not mean it acts alone. A major limitation is that the sample size of any mapping study determines the strength of effect below which statistically significant evidence cannot be found. Also, a mapping study is a search for relevant variation, and cannot identify functionally important genes that don't vary in the particular study material. In addition, because mutations are always arising, doing so differently in each population, the idea of a true replication study is at least somewhat problematic.

The latter points are relevant to understanding evolution. A variant that is too rare for us to detect statistically in mapping studies or that has individual effects that are too weak will be comparably difficult for natural selection to screen, so it's unlikely to have strong adaptive value for the population in which it occurs. Some genes contributing to the developmental mechanism may be so vital that they simply can't vary among viable individuals. And, just as only some genes will vary in any particular study sample, only some will vary in any local population or between species. Variation, not replication, is the fuel of evolution, enabling natural selection and chance to work on different variants in different times and places. By the same token, variants likely affect traits at different ages, so that mapping results may depend on the age of subjects chosen to study. A highly adaptive trait may stay stable while its underlying genetic basis varies over time, among individuals, and between populations.¹⁵

The DNA sequences of candidate genes identified in studies of craniofacial dysmorphogenesis, like most clearly functional genome regions, are conserved among and within species, in part because they serve similar functions and are important. Most were not found by mapping studies but, instead, were found because they are fundamental to development or because when mutated they cause viable but diseased or maladaptive phenotypes such as dwarfism or blindness. From an evolutionary point of view, what is conserved may be the core mechanisms of development (for processes that govern

symmetry, gastrulation, patterning of body axes) or the association of changing shapes as organisms change in scale, but not the particular variants that account for the details of a given measure in a given individual or sample.

MAKING US WHAT WE ARE

The upshot is that asking which genes make a structure like the head may not be asking a well-posed evolutionary or developmental question. A sensible reply is that no one would ask such a general question, but would only consider individual dimensions or parts of the head that one suspected made functional, structural, developmental or evolutionary sense as units, such as in determining the degree of prognathism or the orientation of the orbits. But each trait is affected by other traits and may serve several functions that evolved at separate times. Although we have lists of traits that define vertebrates, or traits associated with *Hominins*, each one, in and of itself, might have been inconsequential to the success of these groups. Instead, it is the acquisition of a coordinated set of characters fundamental to evolving behavioral and social repertoires that are critical to evolution. From this perspective, is not clear just what we should want to know about individual traits at the gene level.

In sum, studies like those we have described have consistently shown that even in well-controlled experiments, the dimensions of skull shape vary due to the effects of many genes. Different studies even in the same species find different results. Different but related species, such as dogs, mice, humans, and other primates, may share basic genetic developmental mechanisms, but their patterns of variation may not be identical among study samples or populations. One has to expect that aspects even of the mechanisms themselves will have evolved to differ among species. If your question is about the genetic basis of such traits, you need to be aware of these complexities. There is no rule for how complex traits develop or evolve, except that each instance will be different. The nature of genetic causal variation is such that careful study designs addressing well-posed questions are required. Unless genetics itself is your interest, for many purposes, one can try to understand the trait and its evolution without worrying about the genes that make it or account for its variation. After all, the trait, and not its genes, is what evolution cares about.

Heads are specific structures made of a composite of coordinated anatomical features, but the issues of how we are made are general. What we've said applies to other physical and physiological traits, and to behavior, where defining and measuring appropriate traits with appropriate precision is always a challenge. In fact, mapping studies of almost any imaginable type of trait have clearly shown that genes in which a mutation by itself causes serious disease are easy to find but represent only a fraction of the causal landscape across the normal and even the disease range. What that suggests is that the reputation of evolution as being a highly prescriptive process at the gene level may be the opposite of the deeper truth that there are many more paths to success than to failure.

ACKNOWLEDGMENTS

This project was supported by the NSF Anthropology Hominid program, grants BCS 0725227, 0725068, 0725031, as well as 0522112, 0523305, 0523637 and the National Institutes of Health 5R01-DE018500, R01DE018500-S1, R01DE022988, P01HD078233, which is gratefully acknowledged.

REFERENCES

1. Richtsmeier J, DeLeon V, Lele S. The promise of geometric morphometrics. *Yearbk Phys Anthropol.* 2002; 45:63–91.
2. Richtsmeier J, Baxter L, Reeves R. Parallels of craniofacial maldevelopment in Down syndrome and Ts65Dn mice. *Dev Dyn.* 2000; 217:137–145. [PubMed: 10706138]
3. Claes P, Liberton DK, Daniels K, et al. Modeling 3D facial shape from DNA. *PLoS Genet.* 2014; 10:e1004224. [PubMed: 24651127]
4. Silver, LM. *Mouse genetics: concepts and applications.* Oxford University Press; Oxford: 1995.
5. Klingenberg C, Leamy L, Routman E, et al. Genetic architecture of mandible shape in mice: effects of quantitative trait loci analyzed by geometric morphometrics. *Genetics.* 2001; 157:785–802. [PubMed: 11156997]
6. Maga A, Navarro N, Cunningham M, et al. Quantitative trait loci affecting the 3D skull shape and size in mouse and prioritization of candidate genes *in-silico*. *Frontiers Physiol.* 2015; 6 Article 92.
7. Pallares L, Harr B, Turner L, et al. Use of a natural hybrid zone for genome-wide association mapping of craniofacial traits in the house mouse. *Mol Ecol.* 2014; 23:5756–5770. [PubMed: 25319559]
8. Cheverud J. Quantitative genetics and developmental constraints on evolution by selection. *J Theor Biol.* 1984; 110:155–171. [PubMed: 6492829]
9. Falconer, D.; MacKay, T. *Introduction to quantitative genetics.* 4th ed. Benjamin Cummings; San Francisco: 1996.
10. Cheverud J. Developmental integration and the evolution of pleiotropy. *Am Zool.* 1996; 36:44–50.
11. Lande R. The genetic covariance between characters is maintained by pleiotropic mutations. *Genetics.* 1980; 94:203–215. [PubMed: 17248993]
12. Linde-Medina M, Diogo R. Do correlation patterns reflect the role of development in morphological evolution? *Evol Biol.* 2014; 41:494–502.
13. Klingenberg CP, Leamy LJ, Cheverud J. Integration and modularity of quantitative trait locus effects on geometric shape in the mouse mandible. *Genetics.* 2004; 166:1009–1921.
14. Lein ES, Hawrylycz MJ, Ao N, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature.* 2007; 445:168–176. [PubMed: 17151600]
15. Weiss KM, Fullerton SM. Phenogenetic drift and the evolution of genotype-phenotype relationships. *Theor Popul Biol.* 2000; 57:187–195. [PubMed: 10828213]
16. Cheverud J, Routman E, Duarte F, et al. Quantitative trait loci for murine growth. *Genetics.* 1996; 142:1305–1319. [PubMed: 8846907]
17. Roseman C, Kenny-Hunt J, Cheverud J. Phenotype integration without modularity: testing hypotheses about the distribution of pleiotropic quantitative trait loci in a continuous space. *Evol Biol.* 2009; 36:282–291.
18. Olson, EC.; Miller, RL. *Morphological Integration.* University of Chicago Press; Chicago: 1958.

Box 1**An Example of a Study of Craniofacial Genetics**

Here we describe a mapping study of craniofacial variation in a highly controlled mouse experiment. The study was done through collaboration of the research groups of Jim Cheverud, Jeff Rogers, and ourselves. We took advantage of two strains of mice that had been inbred over many years to have different body size. Large (Lg) and Small (Sm) mice had been independently made from unrelated founding colonies that were selected over several generations for having large or small adult body size. Each strain was then maintained by inbreeding for many subsequent generations. Lg and Sm mice are commercially available.

The study strategy was to take advantage of what is called an advanced intercross (Fig. 1). Years ago the Cheverud lab crossed 10 Lg and 10 Sm mice.¹⁶ In subsequent generations, the mice have been bred only with each other, in a colony size that reached and was maintained at about 1,000 animals. Our study used F34 mice; that is, they were from the 34th offspring generation. In each generation, recombination between parental chromosomes occurs, which mixes regions originally from either the Lg or Sm ancestors. Each mouse's chromosomes are therefore a patchwork of segments ultimately derived from one or the other ancestral strain. Approximately 2,800 "marker" sites spread across the mouse genome (except for the X chromosome) were identified in which the fixed allele in Lg mice differed from that in Sm mice. These marker alleles were genotyped in each F34 mouse, which means that in each mouse, the genotype at any marker site identified the founder strain from which that part of that mouse's genome originated.

Micro CT (μ CT) images were acquired of the skulls of about 1,200 F34 mice by the Center for Quantitative X-Ray Imaging at Penn State (<http://www.cqi.psu.edu>). 3D coordinates of craniofacial landmarks like those in Figure 1 were recorded for each mouse. These landmarks were used to estimate linear distances between landmarks. Then, for each of 15 cranial linear distances and each marker, craniofacial effects were mapped by identifying correlations between the craniofacial dimensions and marker alleles. That means that some sequence difference(s) in the marker's chromosome region between what has been inherited from the Lg and Sm ancestors is statistically associated with variation in the given distance measure. The spacing between the markers meant that typical mapped candidate regions were several megabases wide. The regions together included a total of 2,384 different genes.

We were interested in identifying candidate genes in each mapped region without assuming that genes previously associated with craniofacial anomalies were the most likely causal sites within a region. The mapped chromosomal segments were long enough to include typically tens and sometimes hundreds of genes, not including potentially regulatory or other nonprotein-coding sites, most of which are not yet identified; however, one region included no protein coding genes at all. For each gene in each mapped region, multiple gene expression and gene function databases were searched to see if the gene was known to be expressed in the developing head or involved in

development of the head, or whether variation in the gene was known to have craniofacial effects.

There were 76 chromosomal regions in the genome to which at least one of the tested craniofacial distances mapped with statistical significance. Within these regions were found approximately 10% of all known mouse genes and 28% of the entire mouse genome (other than the X chromosome). We evaluated potential candidate genes by assessing what was known about their function and whether they were expressed in tissue locations in the developing head that were relevant to the distance that implicated the region. Of those genes for which tissue-specific expression data were available, 95% were expressed in the mouse head, even at just one stage in development (mouse embryonic day 14.5, about 2/3 of full gestational length, and a standard stage for embryonic study because most structures or tissues or their precursors are identifiable, as recorded in genepaint.org). Using these criteria, we identified 103 plausible candidate genes within the mapped regions. Sixteen of the 76 statistically significant mapped genome regions were associated with, and so presumably affected by, more than one distance measure in these mice. However, these multiple measures were not patterned in a way that reflects obvious aspects of correlated function.

Although some genes that have been identified by other studies of craniofacial variation (see main text) were included in the areas mapped in our study, most were not. Even so, given the multiple possible genes within each of the mapped areas, we could not conclude that these known candidates in those regions were specifically responsible for the trait or that they were the only responsible sites within a given mapped area. Thus, had we just searched for previously known candidates in a region, we would have found them and might then have closed our search and concluded that these few genes were responsible. However, that would have been entirely unwarranted, if for no other reason than that it is known that genome regions commonly contain multiple genes jointly involved in a given trait. Moreover, we would be setting out to discover something we already knew, but with no more conclusive evidence than when we began. Finally, in an earlier generation of this same intercross (F10), metric dimensions of the mandible were mapped,¹⁷ but with little overlap between those chromosomal regions and what we found in the F34 crania. The mandible develops independently, but one would expect it to provide additional or at least confirmatory information regarding the genetic basis for morphological integration of the skull. One would expect at least some similar sets of genes to control the construction of both the maxilla and mandible, since these two bones require proper occlusion for survival.

Figure 1.

Study design of craniofacial mapping in a 34th-generation intercross between differing mouse strains. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 1.

Examples of corresponding craniofacial landmarks and distances in human and mouse skulls, with some of the bony structures shown. Only 2-dimensional sites and distances can be shown in a figure. Arrows show an example of a distance that traverses more than one clearly functional area. Adapted from Richtsmeier, Baxter, and Reeves (2000).² [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]