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PERSPECTIVE: EVOLUTIONARY DEVELOPMENTAL BIOLOGY AND THE PROBLEM OF VARIATION

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Abstract.—One of the oldest problems in evolutionary biology remains largely unsolved. Which mutations generate evolutionarily relevant phenotypic variation? What kinds of molecular changes do they entail? What are the phenotypic magnitudes, frequencies of origin, and pleiotropic effects of such mutations? How is the genome constructed to allow the observed abundance of phenotypic diversity? Historically, the neo-Darwinian synthesizers stressed the predominance of micromutations in evolution, whereas others noted the similarities between some dramatic mutations and evolutionary transitions to argue for macromutationism. Arguments on both sides have been biased by misconceptions of the developmental effects of mutations. For example, the traditional view that mutations of important developmental genes always have large pleiotropic effects can now be seen to be a conclusion drawn from observations of a small class of mutations with dramatic effects. It is possible that some mutations, for example, those in *cis*-regulatory DNA, have few or no pleiotropic effects and may be the predominant source of morphological evolution. In contrast, mutations causing dramatic phenotypic effects, although superficially similar to hypothesized evolutionary transitions, are unlikely to fairly represent the true path of evolution. Recent developmental studies of gene function provide a new way of conceptualizing and studying variation that contrasts with the traditional genetic view that was incorporated into neo-Darwinian theory and population genetics. This new approach in developmental biology is as important for micro-evolutionary studies as the actual results from recent evolutionary developmental studies. In particular, this approach will assist in the task of identifying the specific mutations generating phenotypic variation and elucidating how they alter gene function. These data will provide the current missing link between molecular and phenotypic variation in natural populations.

Key words.—*Cis*-regulation, development, evolution, evolutionarily relevant mutations, mutation, transcription, variation.

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> ''Only a thorough-going study of variation will lighten our darkness'' (Haldane 1932, p. 77).

Developmental evolutionary studies will play an increasingly important role in questions of microevolution. Recent developmental evolutionary studies have focused primarily on broad taxonomic comparisons. This macroevolutionary data has indicated, in broad strokes, that developmental systems have evolved largely by alterations in the regulation of a surprisingly conserved set of patterning genes (e.g., Akam et al. 1994; Carroll 1995; Gerhart and Kirschner 1997). However, the processes underlying these patterns of change and the identity of the individual mutations contributing to rearrangements in development remain largely unexplored. In contrast, an explosion of studies over the past several decades has illuminated patterns and processes of molecular evolution at the microevolutionary level. Little of this effort, however, has focused on the phenotypic consequences of molecular variation. This is unlikely due to a lack of interest, but primarily due to the difficulty, or at least the perceived difficulty, of the problem. I do not claim that this problem is easy to solve, but I will argue that a new perspective and a new set of tools, both borrowed from molecular developmental genetics, will ease the task.

The many questions associated with the occurrence and effects of the mutations that contribute to phenotypic evolution constitute a major challenge for contemporary evolutionary biology. What are the phenotypic and fitness effects of naturally occurring allelic variants? Is quantitative variation within populations due to the cumulative effects of many or few mutations? Is variation between species due to the fixation of precisely the same variants that commonly segregate within species or, instead, due to the fixation of rare variants of large effect? How is natural variation distributed across developmental networks and what is the influence of natural selection on the structure of this variation? What is the difference between the mutations that lead to

phenotypic change and the mutations, which can be detected in species hybrids, that alter gene function but do not result in obvious phenotypic change (Biddle 1932; Takano 1998)? Most of these questions are not novel, of course. For example, Haldane (1932), Goldschmidt (1940), and Dobzhansky (1941) were early champions of these problems, particularly the relationship between the genetics of population variation and species differences. Although grouping these questions emphasizes their unity as a set of problems, they can be answered at multiple levels of analysis by different methods, and various workers have addressed some of these problems using both classical and quantitative genetics (recent examples: Coyne 1983; Laurie-Ahlberg 1985; Shrimpton and Robertson 1988; Mackay and Langley 1990; Doebley and Stec 1991, 1993; Lai et al. 1994; Bradshaw et al. 1995; Fry et al. 1995; Long et al. 1995, 1996; Liu et al. 1996; Stam and Laurie 1996; True et al. 1997; Bradshaw et al. 1998; Wang et al. 1999). As new approaches and methodology have been developed, the level of analysis has moved from analysis of phenotypic variation to analysis of the genetic variance underlying phenotypic variance to the distribution of molecular variation itself. What is currently missing, and what developmental biology promises, is the opportunity to connect individual naturally occurring mutations to phenotypic variation.

I focus on what I call ''evolutionarily relevant mutations,'' mutations found at reasonable frequency in natural populations and those that differentiate species. These mutations may be different from the set of all possible mutations. It has been clear for some time that the rate of molecular evolution can be decoupled from the rate of phenotypic evolution (e.g., Meyer et al. 1990; Sturmbauer and Meyer 1992). This indicates, among other things, that we cannot extrapolate in a simple way from genotypic to phenotypic variation. The inverse approach, identifying the mutations responsible for phenotypic variation, is likely to be more fruitful. Much progress has been made in moving from phenotypic to genotypic variation using advances in methods for mapping quantitative trait loci (QTL) coupled with the use of molecular genetic markers (e.g., Liu et al. 1996; True et al. 1997). But this approach provides limited resolution, and the most feasible method for jumping to individual loci is still to make an educated guess, directed by knowledge of which genes map to the region of interest (e.g., Doebley and Stec 1991, 1993; Doebley et al. 1995, 1997; Long et al. 1995, 1996). A developmental approach will complement QTL mapping, but, perhaps more importantly, it will focus problems on individual developmental units. As I will discuss, this may provide important clues to identifying the individual mutations responsible for variation.

The distinction between evolutionarily relevant mutations and the class of all possible mutations is also helpful to emphasize that rare dramatic mutations found in nature and generated in the laboratory may unfairly represent the kinds of mutations that are allowed to persist in natural populations. The only way to determine the evolutionary relevance of such dramatic mutations is to focus on variation in natural populations.

This essay deals with three topics. First, I discuss how perceptions of mutations induced in the laboratory have clouded understanding of phenotypic evolution since at least the time of the New Synthesis. Second, I illustrate how one set of new data from molecular developmental genetics, the role and evolution of *cis*-regulatory DNA, promises to clarify some issues in microevolution. Third, I outline how adopting a developmental genetic perspective will ease the task of identifying and characterizing the individual mutations that contribute to phenotypic evolution within and between species.

This essay illustrates *one* potential direction of research at the interface of development and microevolution. I have focused on the group I know best, animals and particularly insects. However, findings from insects may not be representative of developmental evolution in other groups, particularly plants and fungi (e.g., Gottlieb 1984). I will also focus on one particular mode of developmental evolution, evolution of *cis*-transcriptional regulation, because these regions provide unique insights into microevolution that have not been widely noted. I ignore protein evolution and the evolution of novel proteins, not because I believe these are unimportant processes, but because they have been well reviewed by others (e.g., Gerhart and Kirschner 1997; Golding and Dean 1998; Kreitman and Cameron 1999; Patthy 1999). I also largely ignore natural selection. Some may feel that this is inadvisable because the selective consequences of such mutations must ultimately be addressed to understand the distribution of variants in nature. The goal of this essay is more modest, I focus on how individual mutations contribute to phenotypic variation to demonstrate one way that developmental genetics will contribute to microevolutionary studies.

Mutations in Evolutionary Thought

One of the oldest debates in evolutionary biology concerns the magnitude of the individual steps contributing to evolutionary change. In repeated attempts to settle this debate, biologists have compared variants observed in nature with mutations observed in the laboratory. However, depending largely on preconceptions generated from other evidence, observed mutations have been used to support both extreme micromutationism and extreme macromutationism as important modes of evolutionary change. Provine (1971) provides a detailed history of this problem from debates between Darwin and Thomas Huxley and Francis Galton up to 1932, after Fisher, Haldane, and Wright provided theoretical arguments supporting micromutationism. Several reviews detail developments since (Wright 1977; Lande 1981, 1983; Charlesworth et al. 1982; Coyne 1983; Charlesworth 1990; Orr and Coyne 1992; Orr 1998). Rather than redistill these reviews, I will instead illustrate how perceptions of mutations and their role in development have influenced the debate.

Micromutationism

Although it is possible to characterize Darwin's views as micromutationist, the modern conception of micromutationism derives largely from R. A. Fisher (1930; Orr and Coyne 1992; Orr 1998). Fisher's argument for the predominance of micromutations in evolution has three parts.

First, Fisher's model of adaptation (Fisher 1958, pp. 41–

44) assumes that most mutations are likely to be pleiotropic in their phenotypic effects, although he did not state this assumption so plainly. Fisher modeled adaptation as the change in fitness produced by a change in phenotype assuming the phenotype does not currently sit at the fitness optimum. In the simplest case, where the phenotype is represented in one dimension, a random change (either an increase or a decrease) will result in an improved phenotype in precisely half the cases. To make the model more realistic, Fisher assumed that the phenotype is multidimensional, and here I will use the two-dimensional case. Imagine that the phenotype currently sits on the edge of a circle; any position inside the circle represents an increased fitness and the center of the circle represents the optimal phenotype. A random change will then increase fitness less than half the time. For infinitesimally small changes, the probability of increased fitness approaches one-half, but for larger changes (in a random direction), the probability of increased fitness declines with increases in the size of the change (see Fisher 1958, fig. 3). The assumption of near-universal pleiotropy is buried within the assumption that changes in all directions are equally likely. Without pleiotropy, changes in all directions are not equally likely, and individual traits will only increase or decrease along one dimension. For each trait, the problem then reduces to the one-dimensional case, and the probability of increased fitness is again one-half, assuming that the change is not more than twice as large as the distance to the optimum.

The second part of Fisher's argument for the predominance of micromutations is his suggestion that organisms are rarely far from the optimum, so that, given his model of adaptation, large mutations would most often be deleterious, even without pleiotropy (Fisher 1958, pp. 44–45). If Fisher's model of adaptation is taken without any assumptions about the true rate of environmental change, however, the fitness consequences of a large change are dependent on the actual distance to the optimum. If the environment changes rapidly, large changes may be favored, particularly if pleiotropy is less pervasive than Fisher claims.

Finally, Fisher extrapolated from observations of large mutations in the laboratory to the probability that such changes would be fixed in natural populations: ''The case of large mutations . . . may first be considered. A considerable number of such mutations have now been observed, and these are, I believe, without exception, either definitely pathological (most often lethal) in their effects, or with high probability to be regarded as deleterious in the wild state'' (Fisher 1930, p. 44).

Of these three elements of Fisher's argument, the first is an assumption about development, the second is an assumption about the rate of change of environments, and the third is an assumption about the developmental and fitness consequences of mutations of large effect. Because this essay deals with the role of development in microevolution, I will focus on the first and third assumptions by discussing conceptions of the role of pleiotropy in evolution.

Darwin was probably the first to recognize that pleiotropy, what he called ''correlated growth,'' played an important role in evolution. Various authors, essentially following Fisher, have argued that mutations of large effect are unlikely to be selectively advantageous because they tend to have extensive deleterious pleiotropic effects (Wright 1941, 1963a,b, 1977, p. 463; Lande 1981, 1983; Charlesworth et al. 1982; Coyne and Lande 1985; Charlesworth 1990). Curiously, Dobzhansky's detailed examination of pleiotropy indicated that for the mutations he studied, mutations that have large direct effects have subtle pleiotropic effects (Dobzhansky 1927, 1930; Dobzhansky and Holz 1943). He wrote, ''a majority of the mutations . . . produce striking effect on a single character, and . . . their manifold effects, if any, involve changes which to our eyes appear trivial'' (Dobzhansky 1941, p. 32). However, more recently, authors have argued that mutations of large effect have extensive deleterious pleiotropic effects, which mitigate against a role for macromutations in evolution (Lande 1981, 1983; Charlesworth et al. 1982; Coyne and Lande 1985; Charlesworth 1990). The data supporting this claim are vague, and the claim has apparently persisted largely because it seems intuitive.

The intuitive nature of this idea may derive from the following types of descriptions of pleiotropy:

> Darwin himself was well aware of the correlation between different characters. Today we see the same phenomenon as the multiple effects of a single gene. Since the gene exists in every cell of the body, it may be expected to affect the organism as a whole, even if its most striking effect is on some particular organ or function. Thus the gene *Ch* in *Primula sinensis* incises the petals, doubles the number of sepals, breaks up the bracts, produces a more compact habit, increases the degree of crimping of the leaves when certain other genes are present, and so on. (Haldane 1932, p. 62–63)

This quotation illustrates two observations that we can now see are misconceptions of gene function. (I have chosen Haldane for his clarity of prose, not to single him out for abuse.) First, this quotation emphasizes the multiple effects of a single gene, although these are really effects of a particular *mutation* in a gene. This confusion of mutants and genes is common in the evolutionary literature and may derive from the early geneticists' view that single genes performed single functions, a reflection of the hegemony of the ''one gene–one enzyme'' theory.

The second misconception illustrated by Haldane's quotation is confusion between the pleiotropic *roles* of genes and the pleiotropic *effects* of mutations. Most or perhaps all gene products have pleiotropic roles in development, that is, they are required in multiple cell types for correct development. In contrast, individual mutations may or may not have pleiotropic effects. Mutations within protein-coding regions, particularly those that generate amorphic alleles, usually have pleiotropic effects (Thaker and Kenkel 1992; Miklos and Rubin 1996), but this observation primarily reveals the pleiotropic roles of genes in development. In contrast, I wish to emphasize that there may be a large class of evolutionarily relevant mutations that have minor or no pleiotropic effects. The distinction between pleiotropic roles and effects will be illustrated with an example later in the essay.

Macromutationism

The second view of the nature of evolutionarily relevant mutations argues that natural selection of small differences

is insufficient to account for the large differences between taxa (Bateson 1894; Goldschmidt 1940; deVries as discussed by Provine 1971).

In the laboratory, and occasionally in nature, mutations with large effects were observed that caused dramatic, but concerted, alterations to morphology. Two types of mutations, in particular, appeared to be attractive sources of evolutionarily relevant variation for macromutationism: mutations that caused apparent shifts in allometries and homeotic mutations, which transform organs in one step into the likeness of another (Bateson 1894). Combining these observations led to the hypothesis that large taxonomic differences are generated by extremely rare beneficial mutants of dramatic effect, so-called ''hopeful monsters'' (Goldschmidt 1940).

Despite an overwhelming rejection of Goldschmidt's specific model for the generation of hopeful monsters (see Dobzhansky 1941; Wright 1941, 1963a,b, Wright 1977, chs. 12, 13; Charlesworth 1990), the possibility remains that rare mutations causing dramatic rearrangements of development and morphology contribute to important evolutionary transitions. This is, in fact, a difficult hypothesis to reject because it relies on the rarity of the hypothesized mutations. The neo-Darwinian objection to this proposal has centered instead on the evidence that mutations of small effect can and do accumulate rapidly in natural populations combined with plausibility arguments for the generation of complexity by the accumulation of these small steps (e.g., Nilsson and Pelger 1994). However, neo-Darwinists have left open the possibility that mutations of relatively large effect may play a role in evolution (Dobzhansky 1941; Charlesworth 1990; Orr and Coyne 1992), particularly since the discovery that variation at individual loci can sometimes account for significant amounts of phenotypic variation (Orr and Coyne 1992). The debate has therefore evolved into the more realistic question of the distribution of phenotypic effects within and between species (Orr and Coyne 1992; Orr 1998).

Resolution

> Many of Goldschmidt's analogies between ''monstrous'' forms found in nature and large mutational steps observed in the laboratory . . . are valueless until we know that the natural forms have arisen at a single bound; they may well be merely phenotypically similar to the mutants, but be due to the accumulation of small gene-mutations. (Huxley 1942, p. 457)

As Julian Huxley emphasized, the appropriate data for resolution of this debate are functional studies of the individual mutations that segregate in natural populations and that differentiate species (see preface to Lewontin 1974; Laurie-Ahlberg 1985). By functional studies, I mean an understanding of the precise developmental and phenotypic consequences of alternative naturally occurring alleles. This level of understanding demands knowledge of the molecular action of genes and gene products.

The Importance of *cis*-Regulatory Logic

In an important sense, development is hard-wired into the genome (Arnone and Davidson 1997). To a large extent, development flows from the correct temporal and spatial transcription of genes, which requires the correct use of the instructions encoded in *cis*-regulatory DNA (Lawrence 1992; Latchman 1995). (Information encoded in *cis* can also exert post-transcriptional control, e.g., by influencing mRNA processing and translation. Although this information also originates in the genomic DNA and can be considered another form of *cis* regulation, I will focus on the control of transcription.)

The most striking generalizable result of recent studies of gene regulation is that the *cis*-regulatory DNA of many genes, and particularly those that are spatially regulated during development, is organized into independent modules (Travers 1993; Latchman 1995; Arnone and Davidson 1997). Individual modules direct or repress transcription in specific tissues at particular times in development and multiple modules for a single gene can display a remarkable amount of independence, even retaining their function when they are transposed to a new location in the genome. The modules themselves are constructed from multiple binding sites for individual transcription factors and often a single module contains multiple binding sites for the same transcription factor (Arnone and Davidson 1997). In the best studied case, the combined output of these modules resembles the action of a microprocessor (Yuh et al. 1998). These regulatory modules are often located near the transcription start site, but they are sometimes located up to tens of thousands of base pairs, either 3′ or 5′, from the transcription start site, or within introns (e.g., Duncan 1987; St. Johnston et al. 1990; Latchman 1995; Yin et al. 1997; DiLeone et al. 1998; Lehman et al. 1999; Tanaka et al. 1999).

It is not yet possible to inspect a piece of DNA and recognize *cis*-regulatory modules, in the way that the genetic code allows inference of open reading frames from raw sequence data. Sequence motifs of approximately six to 15 nucleotides mediate transcription-factor binding (Travers 1993), but these motifs are not always well conserved (Kassis et al. 1989; Kreitman and Ludwig 1996; Ludwig et al. 1998). In addition, blocks of sequence conservation within the *cis*-regulatory regions of genes do not always coincide with sequence motifs identified by protein/DNA binding assays (Kreitman and Ludwig 1996).

Transcription factors bind to their appropriate DNA sequences with low affinity and the binding affinity can be altered quantitatively by changes in the nucleotide sequence and by cooperative binding by two or more proteins (Travers 1993). From an evolutionary perspective, therefore, each of the nucleotides that forms part of the transcription factor recognition sequence is relevant to protein binding and mutations altering binding affinity may have quantitative or qualitative effects on gene transcription (Travers 1993).

The function of *cis*-regulatory modules can be conserved without conservation of sequences or conservation of specific transcription-factor binding sites. Evolutionary comparisons of regulatory regions have demonstrated dramatic sequence evolution, most often with changes in the distances between *cis*-regulatory modules and between individual transcription factor binding sites (Kassis et al. 1985; van Ooyen et al. 1985; Blackman and Meselson 1986; Bray and Hirsh 1986; Kassis et al. 1986, 1989; Minty and Kedes 1986; Henikoff

and Eghtedarzadeh 1987; Wilde and Akam 1987; Philippe et al. 1988; Treier et al. 1989; Maier et al. 1990; Langeland and Carroll 1993; Williams et al. 1994; Ludwig and Kreitman 1995; Kreitman and Ludwig 1996; Chiu et al. 1997; Hardison et al. 1997; Yin et al. 1997; Ludwig et al. 1998; Takahashi et al. 1999). In the few cases yet examined, divergent regulatory modules that contain small blocks of homology, which are in some cases known to act as transcription-factor binding sites, have largely retained their function, directing transcription in conserved spatial and temporal patterns (Bray and Hirsh 1986; Maier et al. 1990; Langeland and Carroll 1993; Yin et al. 1997; Ludwig et al. 1998). In one detailed study, Ludwig et al. (1998) demonstrated that some transcription-factor binding sites found in the *Drosophila melanogaster even-skipped* stripe-2 enhancer are recently derived in this clade. This was particularly surprising because one of these binding sites had previously been demonstrated to be functionally important in *D. melanogaster*; mutational removal of this site in *D. melanogaster* caused reduced levels of expression (Small et al. 1992). Nonetheless, when the enhancers from species without this binding site were transformed into *D. melanogaster*, the enhancers appeared to work much like the *D. melanogaster* enhancer. Ludwig et al. (2000) have recently presented an experiment that helps explain this apparent contradiction. They generated chimaeric enhancer elements that contained the first half of one species' element and the second half of a second species. These chimaeric elements did not precisely recover the expression pattern of the original elements, suggesting that compensatory mutations have arisen in the two halves of the enhancer (at least). As these authors stress, these results are consistent with a model of stabilizing selection on the entire enhancer module, with weak selection on individual binding sites allowing turnover of individual sites and compensatory mutations elsewhere within the enhancer.

Evolutionary Consequences

One evolutionary consequence of this revised understanding of gene function is that mutations within independent regulatory modules may have few or no pleiotropic effects. This contrasts with the extensive pleiotropic effects that may arise from mutations within protein-coding regions, which may affect protein function every time the protein is expressed. For example, amorphic mutations in the protein-coding region of the *decapentaplegic* locus of *D. melanogaster* cause embryonic lethality by disrupting many developmental processes. However, mutations in the regulatory regions of this gene can cause a variety of viable phenotypic effects. *held out* ($dpp^{d\text{-}ho}$), due to a 2.7-kb deletion 23 kb 3′ of the transcribed region, causes the wings to be held at right angles to the body, instead of in line with the body axis (Spencer et al. 1982; Blackman et al. 1987). This mutation also causes a reduction in the number of sensilla on the dorsal radius wing vein from 25 to eight (Bryant 1988). *short vein* (dpp^{s1}), a 0.9-kb deletion 3–9 kb upstream of the transcription start sites for four alternative transcripts, causes small gaps at the distal ends of two wing veins and the appearance of a small piece of extra venation (Segal and Gelbart 1985; St. Johnston et al. 1990). *blink* ($dpp^{d\text{-}blk}$), due to a 5.4-kb deletion 17 kb 3′ of the transcribed region, causes a reduction in the number of ommatidia in the compound eye from about 700 in a normal fly to between 100 and 200 (Blackman et al. 1987; Royet and Finkelstein 1997). These alleles demonstrate that mutations in the *cis*-regulatory DNA of one gene can have different, but highly specific, phenotypic consequences; in one case a dramatic reduction in eye size, in the others changes more reminiscent of variation within and between populations. If many or most mutations in *cis*-regulatory regions have few pleiotropic consequences, then dramatic changes in regulatory regions between distantly related taxa may have evolved either by the accumulation of a large number of much subtler changes in the *cis*-regulatory DNA or by a few large changes.

This example illustrates the distinction drawn earlier between pleiotropic genes and pleiotropic mutations. *dpp* acts at many points in development, and therefore has pleiotropic roles in development. Mutational removal of the protein affects all of these processes. In contrast, mutation of particular *cis*-regulatory regions may alter *dpp* function for only one or few developmental events, demonstrating that mutations in regulatory regions may sometimes have few or no pleiotropic consequences.

Another important implication of this model of gene function is that single genes involved in pattern formation may contain many times (in some cases, several orders of magnitude) more information than the traditional definition of a gene would imply. Although there are few estimates of the fine structure of *cis*-regulatory regions, the regulatory regions of several patterning genes have been reasonably well characterized and are reviewed by Arnone and Davidson (1997). One caveat they stress is that the number of characterized transcription-factor binding sites is almost certainly an underestimate of the true number. To estimate the amount of information encoded in *cis*-regulatory regions, I have estimated the number of DNA residues potentially involved in transcription factor binding for five *Drosophila* loci discussed in Arnone and Davidson (1997). For each of these five genes (*fushi tarazu*, *even-skipped*, *rhomboid*, *knirps*, and *Ultrabithorax*), only regulatory modules controlling part of the genes normal expression domain have been characterized, so these estimates are necessarily underestimates. On average, approximately 20 binding sites have been characterized for each gene. If we assume, conservatively, that each binding site involves five nucleotides, then regulation of each gene requires at the very least 100 nucleotides. Therefore, we can conservatively estimate that for genes involved in pattern formation, the number of nucleotides that may potentially be mutated to alter transcriptional regulation is, at a minimum, two orders of magnitude greater than the number of genes. Genes that are not themselves involved in pattern formation, or more specifically that act downstream of genes that are already discreetly patterned, may posses a simpler *cis*-regulatory organization (Arnone and Davidson 1997).

These observations provide a new interpretation of recent studies that have endeavored to map the genes contributing to quantitative variation. In many cases, these studies have found that individual loci account for a surprisingly large amount (sometimes greater than 25%) of the total variation (de Belle et al. 1989; Mackay and Langley 1990; Doebley

and Stec 1991, 1993; Bradshaw et al. 1995, 1998; Fry et al. 1995; Long et al. 1995; Liu et al. 1996; True et al. 1997). In none of these cases, though, has the large effect been attributed to a single mutation, and it remains possible that the observed effects are the product of multiple small-effect mutations. In the most detailed study yet, analysis of the regulatory control of *Adh* revealed that ''single'' alleles were actually due to the summation of mutations at several regulatory sites plus one amino-acid coding mutation, and mutations at different sites of the same allele sometimes caused opposite effects on *Adh* activity (Stam and Laurie 1996). An analysis of the molecular variation at the *scabrous* locus contributing to bristle number variation similarly suggested that the variation attributable to this locus was generated by multiple sites (Lai et al. 1994). The current best candidate for a large effect due to a single regulatory mutation is variation at the *teosinte branched1* gene of maize (Wang et al. 1999). Variation within a potentially small region of the *cis*-regulatory region appears to account for the difference in branching pattern between maize and teosinte and has apparently been fixed due to strong selection for the maize branch structure.

The clumping of variation at few loci is interesting for two reasons. First, this clumping may suggest that relatively few mutations of relatively large effect generate natural phenotypic variation. Some of the data reviewed above, however, suggest that a second model is more likely, that variation accumulates at relatively few loci because relatively few genes direct development of the trait. This interpretation is congruent with studies from developmental genetics. In particular, individual genes often play multiple, and sometimes different, roles in the development of single organs. Therefore, multiple mutations can accumulate within regulatory regions of individual loci to contribute to variation of specific traits.

It may seem that what I give to macromutationism with one hand, I take away with the other. The modular structure of *cis*-regulatory regions suggests that individual mutations causing dramatic regulatory changes, but with limited or no pleiotropic effects, can occur. However, the structure of the regulatory modules themselves, each with multiple transcription factor binding sites, implies that large regulatory changes can evolve by the accumulation of multiple mutations of small effect. Genetic mapping experiments alone cannot resolve this discrepancy. Resolution of this problem requires identification and functional study of the individual mutations contributing to phenotypic differences.

When considering developmental study of such mutations, it is important to put the actual magnitude of these ''large effects'' in perspective. In the study of *scabrous* mentioned above, this single locus was shown to account for approximately 10% of the total genetic variation for bristle number on the abdomen and sternopleura (Lai et al. 1994). However, bristle numbers varied, in the extremes, between about 10 and 20 bristles. Therefore this one locus can account for, at most, variation on the order of a single bristle out of about 15 bristles. The individual mutations within the *scabrous* locus presumably account for less than a single bristle of the total variation. Although such variation is likely to be of evolutionary relevance, the problem is, how do we study it? No developmental biologist interested in the preservation of her funding would study effects on the order of one bristle. The field of developmental biology has made progress precisely because it moved away from the study of subtle phenotypes to the study of null mutations, which can be used to reveal the complete role of genes in development. It is not yet clear how such subtle effects can be studied developmentally, although one approach may be to deconstruct a quantitative trait into developmental steps and study each of the steps in isolation. Each developmental step in the formation of a complex trait will, almost by definition, involve fewer genes (or at least fewer regulatory modules) and the ensuing simplicity may clarify the roles of individual genes.

In contrast, if even the most extreme mutations found in natural populations cause effects that are more subtle than can be reasonably studied developmentally, then the central question would seem to have been answered—these are not mutations of large phenotypic effect. Is the problem thereby solved? I believe that it is not. Even if ''genes'' of large effect actually harbor multiple mutations of much more subtle effect, we still want to understand the functional effects of such mutations, primarily because such mutations may be biased toward or away from certain parts of developmental networks. For example, one common theme in development is that diverse signals are integrated by cells that make binary decisions at particular times. Where in this process does variation tend to accumulate and why? Such questions may be answered by delving into the functional consequences of evolutionarily relevant mutations of subtle effect.

The Evidence

Most evidence for *cis*-regulatory evolution is inferred from qualitatively changed spatial patterns of expression (e.g., Dickinson 1980b; Dickinson et al. 1984; Cavener 1992; Schiff et al. 1992; Kelsh et al. 1994; Panganiban et al. 1994; Ross et al. 1994; Warren et al. 1994; Akam 1995; Averof and Akam 1995; Carroll 1995; Averof et al. 1996; Averof and Patel 1997; Grenier et al. 1997; Lowe and Wray 1997; Rogers et al. 1997; Abzhanov and Kaufman 1999). Several studies have provided experimental evidence supporting *cis*-regulatory evolution (Dickinson and Carson 1979; Dickinson 1980a; Rabinow and Dickinson 1981; Fischer and Maniatis 1986; Brennan et al. 1988; Schiff et al. 1992; Stam and Laurie 1996; Powell 1997, pp. 422–433; Stern 1998). In contrast, only a few studies have provided direct experimental evidence for the role of individual noncoding evolutionarily relevant mutations.

Cavener and coworkers were apparently the first to directly investigate, using P-element-mediated transformation, the evolution of individual enhancer elements responsible for transcription in particular tissues (Cavener 1992; Quine et al. 1993). They showed that a direct repeat of an AATTTAGACC element found upstream of the *D. melanogaster Glucose dehydrogenase* gene (*Gld*) is sufficient to drive transcription of a reporter gene in the pupal rectal papillae. *Drosophila melanogaster* expresses *Gld* in the pupal rectal papillae, whereas both *D. pseudoobscura* and *D. virilis*, which do not contain this DNA sequence motif, do not. This suggests that evolution of this repeat element is in fact respon-

sible for this altered expression pattern. But additional studies by Cavener and coworkers indicate that correlations between the presence or absence of functional enhancer elements and particular expression patterns must be interpreted with caution. Ross et al. (1994) searched for correlations between patterns of *Gld* expression and sequence changes in the enhancer region of *Gld* among species of the *melanogaster* subgroup. One promising result was that only *D. teissieri* lacks expression in the ejaculatory ducts and this species lacks all three TTAGA regulatory elements found within the *D. melanogaster* enhancer that had previously been shown to be capable of driving expression in the ejaculatory ducts (Quine et al. 1993). However, two other species, *D. erecta* and *D. yakuba* also lack these enhancer elements but express *Gld* in the ejaculatory ducts. There are many possible explanations for this lack of a strict correlation between presumptive enhancer elements and expression pattern, but I will focus on one. Quine et al. (1993) studied individual regulatory elements out of the context of the entire gene, in an attempt to isolate the individual elements driving tissue-specific expression. These assays revealed that specific elements were sufficient for driving tissue-specific expression, but not that they were the only elements necessary for proper expression. For evolutionary questions, a more relevant assay may be site-directed mutagenesis within the context of the entire gene.

Laurie and Stam (1994) demonstrated, using site-directed mutagenesis coupled with P-element-mediated transformation, that an intronic polymorphism caused a 1.5-fold difference in *Adh*-protein level in adult *D. melanogaster*. The polymorphism is due to replacement of a 29-bp sequence with an apparently unrelated 34-bp sequence that contains a repeated motif (TAATA) not present in the shorter sequence. It seems unlikely that this complex alteration arose in a single step, but no intermediates have been reported. Laurie and Stam (1994) further demonstrated that the altered protein levels were not due to altered transcription of total adult *Adh* mRNA. They therefore suggested that the polymorphism altered translational efficiency, perhaps because the polymorphism influences mRNA processing. It would be useful to directly test for altered translational efficiency, because it remains possible that the polymorphism actually influences transcription by altering tissue-specific regulation within adults. For example, the polymorphism may shift transcription between tissues that have intrinsically different translational properties.

Parsch et al. (1997) used phylogenetic comparisons to detect potential evolutionarily relevant mutations within noncoding regions of the *Adh* transcript. They were searching for compensatory interactions between sites of a single transcript that might influence gene regulation, for example, via mRNA secondary structure. They tested two potential sites by introducing the mutations found in related species singly and then together, to test for compensation, into a *D. melanogaster* sequence. One of the single mutations caused an approximately 15% decrease in *Adh* activity, and this drop in activity was compensated by the second mutation. The second mutation alone, however, had no effect. Both this and the previous example provide evidence for *cis* regulation of protein function at the posttranscriptional level, although the Parsch et al. study was biased to look for mutations altering posttranscriptional regulation.

Given the abundance of studies in molecular evolution, it is somewhat surprising that there is so little data on the evolution of noncoding DNA, let alone the functional consequences of such changes (cf. Hardison 1998; Ludwig et al. 1998; Singh et al. 1998). It is therefore premature to derive any strong inferences about the relative importance of *cis*-regulatory evolution versus changes in protein-coding regions (e.g., Choudhary and Laurie 1991; ffrench-Constant et al. 1998; Swanson and Vacquier 1998) to phenotypic evolution. However, the studies reviewed above demonstrate that such data are within reach, even for relatively small differences in function. It is also important to recognize that regulatory regions may sometimes evolve by dramatic rearrangements of DNA sequences. Two recent studies, which describe the origin of new chimeric genes, in one case by retrotransposition (Long and Langley 1993) and in a second by apparent tandem duplication followed by fusion (Nurminsky et al. 1998), indicate that regulatory regions can evolve by the most unlikely of routes. In the latter case, the gene acquired a new testes-specific promoter from a region that had previously coded for an exon. Gene regulation provides a potentially large diversity of molecular phenomena available for evolutionary modification, and many new kinds of *cis*-regulatory alterations probably await discovery.

A Developmental Genetic Approach to Microevolution

> During the period 1930–1950 *Drosophila* genetics began to move a little towards development. Still the emphasis was often on the mutant (rather than the wildtype). The standard approach of developmental genetics at that time was to make a detailed description of the mutant phenotype under different conditions and this was sometimes done without sparing a thought for the wildtype function of the gene. [For example], Adair Brasted chose the gene [engrailed] because 'the variability associated with the phenotypic effects of *engrailed* could be investigated experimentally, since each is sensitive to environmental changes.' This thought is followed by 26 taxing pages on the effect of body weight, temperature, crowding and intersexuality on the number of sex comb teeth (Lawrence 1992, p. 207).

The traditional genetic view of the phenotype invoked a developmental ''black box'' similar to the one that is still predominant in evolutionary biology. In genetics, detailed analysis of *mutants* was eventually replaced by the use of mutations to reveal the true function of genes (see above quotation). The primary techniques of modern developmental genetics currently include removal or misexpression of a gene product during development followed by examination of the effects, on the behavior of other molecules and on the resultant phenotype, in individual cells at particular times in development. This extreme reductionism to determining gene function represents a great intellectual leap and one that evolutionary biologists can use to similarly reveal the genetic basis of phenotypic variation.

To illustrate this approach, I review two studies of variation at the *Ultrabithorax* (*Ubx*) locus of *Drosophila*. *Ubx* is a member of the Hox cluster, a group of highly conserved genes that are differentially expressed along the anterior-posterior axis to provide spatial information allowing the development of diverse morphologies among trunk segments (Lewis 1978; Sánchez-Herrero et al. 1985; Kaufman et al. 1990). For example, the expression of *Ubx* protein in the dorsal appendage of the third thoracic segment causes these tissues to develop into a haltere, whereas the absence of *Ubx* from much of the second thoracic segment allows development of a wing. *Ubx* does not itself carry ''haltere-specific'' information, it simply marks this organ as different from the wing (Morata and Garcia-Bellido 1976; Morata and Kerridge 1981; Kerridge and Morata 1982). The haltere morphology is generated because *Ubx* binds to the *cis*-regulatory regions of a potentially large number of target genes that build the haltere (Weatherbee et al. 1998). Although Hox genes are expressed in broad domains along the trunk, recent evidence indicates that they are also regulated in complex spatiotemporal patterns within segments that are required for specifying particular aspects of segment morphology (Castelli-Gair and Akam 1995; Akam 1998; Castelli-Gair 1998; Stern 1998). The two studies reviewed below revealed variation within and between closely related species apparently altering this within segment regulation.

In the first study, Gibson and Hogness (1996) used knowledge of the normal role of *Ubx* in development to test the hypothesis that naturally occurring variation at the *Ubx* locus contributes to ether-induced *bithorax* phenocopies. When *D. melanogaster* eggs are exposed to ether for a short time, a certain proportion of individuals develop into adults with halteres enlarged and partially transformed into wings (Gloor 1947), a phenotype that is similar to that produced by *bithorax* mutations, which occur in the regulatory regions of the *Ubx* locus. Waddington (1956) demonstrated that this response had a genetic basis by successfully selecting on subsequent generations of individuals displaying the ether-induced *bithorax* phenocopy. Gibson and Hogness (1996) replicated Waddington's selection experiment and then demonstrated that a significant fraction of the genetic variation underlying the response to selection mapped to the *Ubx* locus. This study revealed that *Ubx* harbors intraspecific variation affecting its transcription under certain environmental circumstances. This study also revealed that ''canalization'' genes need not be distinct from the genes actually instructing the development of organs. That is, developmental genes harbor variation for their own canalization.

In the second study, I explored the developmental role of *Ubx* in contributing to a morphological difference between *Drosophila* species (Stern 1998). The study flowed first from an analysis of the function of *Ubx* in the normal patterning of the distribution of microtrichiae (often called trichomes) on the surface of the posterior femur of the second pair of legs of *D. melanogaster*. Using assays that removed and added *Ubx* protein to cells of the leg, I demonstrated that high levels of *Ubx* expression normally repress microtrichia development in this part of the leg during a short time window of pupal development. In addition, *Ubx* is expressed in a proximal-distal gradient in the second leg at this time of development, resulting in a small patch of naked cuticle in the proximal part of the leg. The next question was whether the *Ubx* locus harbors evolutionarily relevant variation for this trait. Using an interspecific complementation test, I demonstrated that some of the difference in naked cuticle distribution between *D. melanogaster* and *D. simulans* is due to a difference in *Ubx* function. Comparisons of the coding sequences revealed no changes in the presumptive amino-acid sequence of the *Ubx* protein between these species, suggesting that the difference resides in the *cis*-regulatory DNA.

The developmental approach to these studies provided several advantages. First, knowledge of normal Hox gene function immediately suggested Hox genes as likely candidates underlying variation in both the haltere and the second leg, because these are both organs that differ from their serial homologues for the morphological characters of interest. Second, manipulations of *Ubx* protein levels in the second study provided an understanding of how changes in *Ubx* regulation might alter the phenotype, which allowed a more precise interspecific test and suggested the appropriate time window to search for changes in *Ubx* function.

Both studies support the hypothesis that genes with conserved protein motifs can evolve by relatively minor changes in their *cis*-regulation. It is not yet clear whether similar changes accumulate over longer periods to generate the larger-scale differences observed between more distantly related taxa or whether these differences evolved by larger steps (e.g., Carroll 1995; Averof et al. 1996; Averof and Patel 1997). These studies also join a growing literature demonstrating that genes that are normally thought of as early-acting genes involved in the construction of the main features of the body plan also act throughout development, sometimes to pattern seemingly minor features. The key to understanding the ability of a single protein to carry out these diverse functions is to view these proteins as influencing relatively simple and specific decisions at particular times in development; in different locations and at different times, these proteins are used to make different kinds of decisions. The accumulation of all of a genes' functions can therefore appear dauntingly complex, when in fact it is the summation of a large number of much simpler events. And, of course, the organization of all these events resides in the *cis*-regulatory DNA.

To further illustrate this approach, I will outline a possible research program for dissecting one of the most challenging problems in the study of variation: the study of genetic effects of relatively small magnitude, such as the effects of quantitative trait loci on bristle number variation. Several studies have demonstrated that genetic variation in bristle number maps to or near loci that are known to be involved in bristle development (Mackay and Langley 1990; Lai et al. 1994; Long et al. 1995, 1996). The next problem, determining the actual mutations and how they alter gene function, appears daunting. Before beginning, however, there are several layers of complexity to the problem that should be revealed.

The determination of the presence or absence of a bristle at a particular location in the epithelium is the product of events occurring over an extended time in one cell combined with interactions between neighboring cells. Many of the genes involved in this process have been described in *Dro-*

sophila and the general outline of the process appears to be understood (Yan and Jan 1993; Simpson et al. 1999). First, a region of the epithelia is specified to be competent to produce bristles. The second step involves cell-cell communication to single out cells at a particular spacing that will become sensory mother cells. The third step involves a stereotypical series of cell divisions by the sensory mother cell that give rise to the multiple components of a single bristle. Defects at any of these stages will block bristle production and defects in the first two stages can increase or decrease the number of bristles in an epithelia.

One approach to dealing with this complexity is to focus the problem on particular developmental stages. This has been successfully accomplished in studies of hybrids between *D. melanogaster* and *D. simulans* that lack specific thoracic bristles. Takano (1998) examined developing hybrids with markers for different stages of bristle development and demonstrated that bristle loss is most likely due either to failure of sensory mother cells to maintain their status or to defects in the stereotypical cell divisions leading to bristle formation. The success of this study relied largely on the predictable loss of certain bristles, but in principle this approach could be applied to studies of quantitative variation. In conclusion, one possible way forward is to focus the question on the smallest number of developmental decision points.

How Mutants Can Mislead

There is a long and misleading history of comparing developmental mutations of large effect with inferred evolutionary transitions (Bateson 1894; Goldschmidt 1940; Lewis 1963; Whiting and Wheeler 1994). This point was first clearly stated by Raff and Kaufman (1983, p. 339). The misleading aspect of such comparisons is not that the mutations have large effects or are often amorphs (after all, both of these may, for all we currently know, contribute to evolutionary change), but that the effects of these mutations may provide inaccurate indications to the true evolutionary path. Such mutations are unlikely to represent evolutionary pathways in reverse, mainly because regulatory proteins acting early in organ specification probably regulate many target genes (Maynard Smith 1983, p. 19). It is easy to remove the regulation of all of these genes in one step by mutation, but essentially impossible to build up all of the required regulation in few steps. Therefore, it is misleading to extrapolate from mutational effects within a single species, particularly those that produce apparently atavistic changes, to likely evolutionary progressions (Coyne and Lande 1985). Arguments about evolutionary change derived from study of mutations producing atavistic or homeotic changes should always be treated with caution.

Ultrabithorax again provides a good example. Individuals that are heterozygous for an amorphic allele of the Hox gene *Ubx* carry slightly enlarged halteres, the dorsal ''balancer'' organs on the third thoracic segment that are evolutionarily derived from the hind wing. In addition, mutations in the regulatory regions of *Ubx* can make the halteres more wing-like by increasing their size and causing the appearance of extra bristles. The combination of some of these mutations in a single fly transforms this organ into a wing (Lewis 1963, 1978). This mutational series might be taken to imply that halteres evolved from wings by the fixation of mutations that caused *Ubx* expression in the hind wing of the ancestor of dipterans (Lewis 1963). However, *Ubx* is also expressed in the developing hind wings of Lepidoptera (Warren et al. 1994) and is required to promote the specific features of the butterfly hind wing (Weatherbee et al. 1999). Therefore, it is unlikely that the haltere evolved by changes in *Ubx* expression. Rather, halteres probably evolved from hind wings by the evolution of new *cis*-regulatory control regions recognized by *Ubx* protein in a panoply of target genes. This model is supported by the functional understanding of *Ubx* action discussed earlier; *Ubx* does not ''make'' halteres, it provides positional information along the anterior-posterior axis. That is, reconstruction of the probable path of haltere evolution required a detailed understanding of the ''normal'' role of *Ubx* in development. In fact, this functional understanding itself, combined with the observation that the fore and hind wings of most (or all) insects are different, suggests that the diversity of dorsal appendages in insects did not evolve by changes in regulation of *Ubx,* but rather by *cis*-regulatory changes of genes involved in wing patterning.

The second example involves the hypothesis that the Strepsiptera evolved their flight appendages, which are essentially the reverse of the Dipterans, with halteres on the second thoracic segment and wings on the third, via a dramatic set of homeotic mutations at the *Ubx* locus (Whiting and Wheeler 1994). A strict interpretation of this hypothesis requires at least three independent mutations, each of which causes large and, in the laboratory at least, deleterious morphological changes. One (*Contrabithorax*) causes expression of *Ubx* protein in the wings of Drosophila, whereas the others (*postbithorax* and *bithorax*) cause loss of *Ubx* protein in the haltere. It is easy to squabble over potential problems with this hypothesis, such as the fact that although *postbithorax* and *bithorax* alter appendage morphology they do not completely transform the flight muscles, so that mutant hind wings are nonfunctional (Egger et al. 1990). My main point is that this hypothesis argues from mutational effects, whereas an alternative hypothesis can be constructed based on our current understanding of *Ubx* function. In *Drosophila*, dorsal flight appendages respond to *Ubx* by differentiating a haltere, in Strepsiptera they may respond by differentiating a wing and the absence of *Ubx* may be the signal for differentiating a haltere. This morphological pattern could have evolved by minor alterations in the *cis*-regulatory regions of the genes responsible for building the dorsal appendages of Strepsiptera. This alternative hypothesis may be more congruent with our current understanding of development and microevolutionary theory, but that does not make it true. The test will come from the data.

RECAPITULATION

The research program I encourage is to approach the problem of variation as a multitude of many much smaller problems of individual variants. Development is surprisingly modular, not only at the level of *cis*-regulation that I have discussed (Gerhart and Kirschner 1997). I am encouraged by this modularity, because it implies that individual elements

can be studied in isolation to provide meaningful answers. I suspect that the biological reason that developmental genetics has succeeded is that development is composed of relatively independent modules and disturbance of one module need not disrupt development of the entire organism. Golding and Dean (1998) have similarly argued that the structural basis of adaptive protein evolution can be profitably studied largely because individual amino-acid substitutions tend to act independently of others. In fact, my emphasis on functional analysis of regulatory regions mirrors their encouragement to incorporate functional approaches into the study of protein evolution. Some may find this reductionism frustrating, because most ''traits'' of current evolutionary interest, such as body size, life span, and organ shape and size, are essentially composites of multiple developmental problems. However, if the question is, ''How does molecular variation generate variation in these traits,'' I would encourage the dissection of these composite traits into much smaller problems, preferably limited to a short time in development. It may currently be difficult to link the types of phenomena that are discovered by this approach to natural selection and this trade-off may prevail until we establish a more complete understanding of the relationship between genotypic and phenotypic variation. However, this link is required ultimately to provide a complete understanding of adaptive evolution.

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NOTE ADDED IN PROOF: We have recently reported that a relatively dramatic evolutionary change in larval morphology was caused by *cis*-regulatory evolution of a single gene. (Sucena, É., and D. L. Stern. 2000. Divergence of larval morphology between *Drosophila sechellia* and its sibling species caused by *cis*-regulatory evolution of *ovo*/*shaven-baby*. Proc. Natl. Acad. Sci. USA 97:4530–4534.)