

QTL analysis and genome-wide mutagenesis in mice: Complementary genetic approaches to the dissection of complex traits.

John K. Belknap^{1,2,3,5}, Robert Hitzemann^{1,2,3}, John C. Crabbe^{1,2,3}, Tamara J. Phillips^{1,2,3}, Kari J. Buck^{2,3} and Robert W. Williams⁴.

¹Research Service (R&D5), Veterans Affairs Medical Center, Portland, OR 97201

²Portland Alcohol Research Center

³Department of Behavioral Neuroscience, Oregon Health Sciences University

⁴Center for Neuroscience, Department of Anatomy and Neurobiology,
University of Tennessee, Memphis, TN 38163

⁵To whom correspondence should be addressed at Research Service (R&D5), VA Medical Center, Portland, OR 97201

Phone: 503-273-5317; FAX 503-721-7839; email: belknajo@ohsu.edu.

Abstract:

Quantitative genetics and QTL mapping have undergone a revolution in the last decade. Progress in the next decade promises to be at least as rapid, and strategies for fine mapping QTLs and identifying underlying genes will be radically revised. In this commentary we address several key issues: first, we revisit a perennial challenge—how to identify individual genes and allelic variants underlying QTLs. We compare current practice and procedures in QTL analysis with novel methods and resources that are just now being introduced. We argue that there is no one standard of proof for showing QTL=gene; rather, evidence from several sources must be carefully assembled until there is only one reasonable conclusion. Second, we compare QTL analysis with whole genome mutagenesis in mice and point out some of the strengths and weakness of both of these phenotype-driven methods. Finally, we explore the advantages and disadvantages of naturally occurring vs mutagen-induced polymorphisms. We argue that these two complementary genetic methods have much to offer in efforts to highlight genes and pathways most likely to influence the susceptibility and progression of common diseases in human populations.

Key words: quantitative trait loci (QTL), ENU, mutagenesis, complex traits, gene mapping.

QTLs that are critical in behavior and neurological disease are being mapped at a rapid pace in mouse and human populations (Belknap et al., 1997; Crabbe et al., 1999; Burmeister, 2000; Tecott and Wehner, 2001; Phillips et al., in preparation). Numbers of significant QTLs for behavioral and CNS traits in mice have increased more than tenfold in the last 5 years—from 3 in 1995 to over 40 by 2000. This issue of *Behavior Genetics* covers a cross-section of this exciting work. We hope to shed light on two questions that are important in such a rapidly evolving field: where do we want to be in the next few years, and how can we get there? This involves three key issues. One issue concerns the methods and prospects for identification of single genes associated with well mapped QTLs. The second issue is the relative merits of QTL versus whole genome mutagenesis approaches. Finally, we discuss the pros and cons of genetic variation induced by mutagens compared to that found in existing mouse strains and lines. We explore these issues in the context of looking at the problems that beset both QTL and genome-wide mutagenesis screens of complex traits.

A provocative essay in a recent issue of *Nature Genetics* reviewed the obvious challenges associated with gene identification (Nadeau and Frankel, 2000) and made the case that mutagenesis of the entire genome provides an alternative, more rapid, and more certain route around an apparent “roadblock” in QTL analysis. We agree with facets of this review, but we disagree with the gloomy assessment of the current status and immediate prospects of QTL gene identification. In this commentary, we revisit this issue and present QTL analysis and mutagenesis as complementary (rather than competing) methods that will both need to be used to screen an entire genome for subsets of genes that influence specific traits. These two genetic strategies share numerous scientific and clinical objectives (Lander and Schork, 1994; Takahashi 1994), but there are key differences between methods, realms of application, scientific goals, and the areas of expertise of practitioners who work in these fields. We also consider new opportunities associated with the influx of sequence data, comprehensive CNS gene expression data, and high-resolution mapping resources. Finally, we note some of the problems we see in whole-genome ENU mutagenesis screens as applied to genetically complex traits such as behavior. Our conclusion is that QTL analysis will be a lively and crucial partner in functional genomics in the foreseeable future as will genome-wide mutagenesis screens. Without losing sight of genuine challenges, even

pessimists should be encouraged by the continued hybridization of quantitative and molecular genetics.

Standard operating procedures: QTL analysis and genome-wide mutagenesis in mice

A complex trait is a phenotype resulting from multilocus determination coupled with multiple environmental influences. The first step in QTL analysis is to select a cluster of closely associated complex traits that reflect a single well-defined biological problem. Biological questions and specific phenotypes drive the entire program. The operational difficulties of measuring important traits usually do not dissuade investigators, as shown by the employment of elaborate behavioral paradigms, immunohistochemical procedures, and quantitative electron microscopy in the last few years to map QTLs. Investigators are often specialists with highly focused interests in the genetic basis of the very particular traits under study. They also usually share strengths in statistical and quantitative genetic analysis. QTL studies are usually carried out in a single laboratory or as part of small group projects.

In a typical QTL analysis of a complex trait, a mapping population is generated by crossing two highly differentiated progenitor strains or lines of mice. Most often, several hundred F₂ or backcross progeny are tested and genotyped genome-wide. Sometimes, recombinant inbred strains are used as a preliminary screen coupled with other mapping populations. The initial goal is to dissect existing continuous or quantitative genetic variation into its component loci (QTLs), and to map them to broad chromosomal regions. This is only the first step; the aim of this effort is to rapidly return to the biology to gain a better understanding of underlying molecular and cellular control of the target trait as well as at the systems and organismic levels—initially in mice, but ultimately, in human populations (Williams, 2000). The most effective route to this goal is to identify genes unambiguously associated with QTLs. These genes then become key entry points from which to explore the network of genes, proteins, pathways, systems and environments important in the determination of a complex trait. Strong homologies between genes in mice and humans usually guarantee overlapping or even near identical biological function. Furthermore, polymorphic genes in mice may also be polymorphic in humans; at least functionally important allelic variation may be discovered in the same pathways if not the same genes.

The first step in a typical genome-wide mutagenesis screen is to select a set of traits that can be scored rapidly and with high throughput in a thousand or more mice. The choice of traits is governed as much by economy and throughput as by biological interest. For this reason, directly and readily observable abnormalities, such as dysmorphology or abnormal motor movements, figure prominently in the range of traits chosen for study. Investigators who lead this type of research are usually molecular geneticists with special competence in gene structure and cloning (See reviews by Brown and Nolan, 1998; Noveroske et al., 2000; Nolan et al., 1997, 2000; Hrabe de Angelis et al., 2000; Schimenti and Bucan, 1998; Wells and Brown, 2000). ENU screens of adult mice are massive and very expensive undertakings that are now often coordinated at a national level (e.g. Hrabe de Angelis et al., 2000; Nolan et al., 2000).

In a typical whole genome mutagenesis screen, inbred male mice are treated with a strong alkylating agent, ethylnitrosourea (ENU), to induce several hundred germline mutations per mouse. They are crossed to wild type females of the same strain, and large cohorts of offspring are run through a gauntlet of tests to identify the individual outlier or extreme-scoring mice most likely to bear a large-effect Mendelian mutation. These outlier mice, usually defined as those that are >3 SD units from the mean, are each progeny-tested to determine whether their abnormality segregates bimodally in their offspring with the expected 1:1 or 3:1 Mendelian ratios. Those that pass this test (a minority of all extreme-scoring mice) are

subsequently mapped at moderate resolution (10-20 cM) in a cross to a different inbred strain using genome-wide markers and methods similar to QTL mapping studies.

To fine-map each mutation to a 1 cM interval (95% confidence interval) typically requires a cross of about 500 progeny (Wells and Brown, 2000). At this level of precision, brute-force sequencing can be effective in identifying the ENU-induced mutation since there are expected to be about 750 mutations throughout the genome, or about one every 2 cM (Schimenti and Bucan, 1998). These induced mutations, because of their large-effect Mendelian patterns of inheritance and fewer polymorphisms to sort through, currently have an important advantage over QTLs in ease of gene identification.

Isolating Genes underlying QTLs

What are the prospects for gene identification for QTLs? At present, it is difficult to establish connections between continuous phenotypic variants and the associated set of mapped gene variants (Darvasi, 1998). Nadeau and Frankel describe this task as a “long and bumpy road.” However, it is worth pointing out that QTL studies that were almost inconceivable a decade ago are now routine. As we discuss below, many new developments are on the horizon that will fundamentally alter how we identify genes underlying QTLs. To modify the metaphor, that “long and bumpy road” is better seen as a high-speed highway under construction.

Current and evolving developments will greatly simplify fine mapping and gene identification. One key factor is, of course, complete genome sequences for the most commonly-used inbred progenitor strains. Provisional coverage is already available for 129/SvJ, DBA/2J, and A/J from Celera Genomics (see www.pecorporation.com/press/prccorp101200.html), and C57BL/6J is now being sequenced as part of an NIH-supported effort (see www.ncbi.nlm.nih.gov/genome/seq/Mmprogress.shtml). As a result, generating comprehensive lists of functional polymorphisms between these four major strains will soon be routine for any part of the genome. Among crosses between any pair of these strains, the source of all QTLs will be known. A parallel situation will also exist at the mRNA and protein levels; the development of array-based methods will reveal numerous strain differences, the source of many QTLs. In a few years, QTL studies will begin with complete lists of differences in gene expression and protein levels in several commonly-used progenitor strains of mice.

This is already beginning to happen. For example it is now possible to generate a list of differences in the expression level of 7169 genes in the hippocampus between strains C57BL/6J and 129 (Sandberg et al., 2000; <ftp://ftp.gnf.org/pub/papers/brainstrain/>). Identifying hippocampal-dependent QTLs from crosses between these two strains may be more like “cruising in a Cadillac” than a hard road trip. In contrast to the enormous benefit of these developments for QTLs, the benefit for ENU mutants will be less because much of this work at the sequence, transcript and protein levels must be done anew with each newly-created mutation of interest.

Steps from QTL to gene. Surprisingly, the actual steps involved in moving from QTLs to genes have received only cursory attention. The following outline and flowchart (Figure 1) summarizes our views of the likely steps. We assume that a QTL has been refined to intervals of 1 cM (95% confidence interval) that will contain an average of about 50 genes. [This is based on an estimated 75,000 genes distributed over 1450 cM, a worst case scenario since recent estimates suggest only 30,000 to 40,000 genes.] The 1-cM criterion is not unreasonable since several behavioral QTLs have now been mapped with high LOD scores and impressive precision (Crabbe et al., 1999; Demarest et al., 2001; Talbot et al., 1998; Fehr et al., in press). We assume that the cells and tissue types related to the phenotype are

known or strongly suspected. This will almost always be the case for morphometric traits (Le Roy, 2001, Williams et al., 2001, both in this issue), but for higher-order behavioral traits, inferences will be provisional at early stages of analysis.

Genes Expressed in the Tissue of Interest. Only a fraction of the genes within the QTL interval will be expressed in the tissue of interest at some point in the life of the mouse. Microarray technology can easily address this problem for specific subsets of genes. At most, about half of the genes in an interval will be expressed in brain (Sandberg et al., 2000), and consequently, only this half need be considered for further testing for most behavioral traits. Carrying out expression analysis at an early stage is based on the assumption that the sequence information for multiple inbred strains will be of variable accuracy for the next several years; thus, some sequencing will still be necessary. Therefore, a reduction in the number of candidate genes by expression studies should save time. However, once accurate sequences are readily available, expression and proteomic studies would more efficiently become part of the end game.

Polymorphic Genes - Open Reading Frames (ORFs). From the completed genomic sequence for the primary inbred strains of interest (e.g. C57BL/6J and DBA/2J), it will be possible to screen and map the polymorphisms within the ORFs *in silico*. Nonconservative amino acid substitutions between progenitor strains in ORFs are relatively uncommon (10-20%) (Buck and Finn, 2000; Fehr et al., in press) and we assume that about 50% of these polymorphisms will have functional significance. *With this estimate, the number of genes with functional polymorphisms in the ORFs is reduced to about 3-5 genes per 1 cM that will merit serious consideration.* In the short term, the function of some (if not most) of these genes will not be well known, but we can often make strong inferences from homology with other better-characterized genes. Strategies for determining which (if any) of these polymorphisms may be associated with the QTL are described below.

Expression Array Approaches A useful approach would be to look for expression differences among the strains and treatment groups defining the phenotype; this could be combined with the methods discussed above, being mindful that important differences in expression may have occurred earlier in development. There are several problems with expression array analysis that deserve comment. First, the method is only semiquantitative and any apparently significant result will require confirmation with a quantitative technique, e.g., quantitative RT-PCR. Second, differences in RNA expression do not always lead to differences in protein content; thus, evidence that protein levels have actually changed must be provided, e.g., quantitative Western analysis. Third, small but functionally relevant differences in gene expression may not be detected. And fourth, not all relevant genetic sequences are currently available for analysis. Fortunately, these problems are likely to be relatively short-lived and should disappear with advances in expression technology and proteomics.

Emerging data indicate that only a small percentage of genes will show detectable differences in expression between pairs of inbred strains. Sandberg et al. (2000) found that about 1% of genes varied in expression level by more than two-fold between the C57BL/6 and 129 strains across several brain regions. Therefore, we would expect an average of less than one gene showing detectable differential expression between a pair of inbred strains within a 1 cM interval. While expression studies cannot by themselves rule out candidates, they can certainly provide important support for particular candidates.

Narrowing the List of Potential Candidate Genes One could argue that although the "gene list" for the QTL interval can be substantially reduced based on the above considerations, to be efficient we need to reduce the number to one or two candidates. In some cases, there will be an obvious candidate that is plausibly associated with the

phenotype, such as the cluster of GABA-A subunit genes in the region of a QTL affecting both alcohol and pentobarbital withdrawal severity (Buck et al., 1997, 1999; Buck and Finn, 2000). To date, this opportunistic approach has led to successes in moving from QTL to gene. However, in general we assume that the function of the remaining candidates will either be unknown or only partially known.

Can the list of candidates be further reduced? One practical method is to use much higher resolution community-based mapping resources capable of sub-cM precision. For example, chromosome substitution strains (Nadeau et al., 2000) can be rapidly converted into interval-specific congenic strains for high resolution mapping (Darvasi, 1998; Williams, 1999). Heterogenous stock, advanced intercrosses and recombinant progeny testing are additional effective methods that can attain 0.5 cM precision or better (e.g., Talbot et al., 1998; Mott et al., 2000; Darvasi, 1998; Lyons et al., 2000; Fehr et al., in press; Demarest et al., this volume). Recombinant inbred strains could be easily extended for high resolution mapping. A set of 100 BXD RI strains would permanently archive about 6000 recombination events and this would often be sufficient to confine a QTL to a 0.25 cM interval using RIST (Darvasi, 1998) and other methods. Such precision mapping coupled with the above considerations will often narrow the list of plausible candidates to just one gene.

The End Game - QTL to Gene. We believe that existing technology in combination with technology that will soon be acquired will soon lead to one or two very strong candidates without relying on knowledge of gene function. The question that now arises is what will be acceptable as proof that a particular gene underlies a QTL. Nadeau and Frankel (2000) claim that an allele “swap” should be the formal proof of identity, or the “gold standard” (our words). Certainly, if the allele swap produced the expected phenotypic changes, this would be a powerful argument. However, in simpler systems such as bacteria, allele swapping has not always produced the expected phenotypic results because of genetic background (epistatic) effects (e.g., Malke et al., 2000). Thus, it would appear that we need to consider additional means of “proof.” We propose the scheme depicted in Figure 1 as a reasonable strategy for determining whether a gene is responsible for a phenotypic difference. The following are some likely strategies.

One, the process described above for reducing the number of candidates to one can provide compelling evidence.

Two, physiological/pharmacological approaches can be used for genes where function is known, e.g. will specific inhibitors of the gene product produce the expected phenotype?

Three, transgenic (overexpressing, underexpressing, null) mutants should be useful especially for genes of unknown function, despite the well known limitations of this approach. Some of these limitations can be overcome through tissue-specific inducible mutants (Tecott and Wehner, in press).

Four, antisense and related techniques can be used to knockdown genes transiently; this approach is particularly well suited to “brain” phenotypes, where one may wish to knockdown gene function in only a specific region or nucleus. Viral transfer strategies can be used to produce the opposite effect--targeted gene overexpression.

Overall, we would argue that there is no single proof of identity, or “gold standard”, for proving that a gene underlies a particular QTL. Rather, *proof will rely on the careful assembly of evidence from several sources that leads to only one reasonable conclusion.* Finally, it is important to note that given the current and expected advances in genomics, proof that QTL = gene can occur largely independently of any knowledge of gene function, the most difficult scenario. However, in the shorter term, knowledge of gene function *vis a vis* the phenotype will no doubt be an important contributor to gene identification successes.

Natural vs Mutagen-Induced Genetic Variation

What advantages and disadvantages do we incur when we choose to induce genetic variation rather than rely on naturally occurring variants? The advantages are great. Rendering presently monomorphic loci into polymorphic ones is a marvelous capability. This opens a whole range of genes for investigation that cannot be studied by QTL or other nonmutagenic approaches. The prospects are especially exciting for the study of genes that direct early development since they normally cause little variation.

Working with loci that have large rather than small effects is another important advantage. While naturally occurring or “spontaneous” mutations have led to many valuable genetic disease models, the ones that have most often led to gene identification have been principally large effect mutations showing single locus or Mendelian inheritance. Recent studies with neurological and development mutants such as *vibrator* (Hamilton et al., 1997) are prime examples. ENU mutagenesis seeks to amplify on this successful approach by systematically extending the range of large effect mutations available for study. Largely for these reasons, two of us are committed to carrying out mouse ENU studies in our laboratories. While we support the growing interest in applying genome-wide mutagenesis to complex traits, this approach should not be considered uncritically, especially for behavioral traits. Therefore we ask—are there disadvantages to mutagen-induced variation? Yes—and in the next several paragraphs we describe seven drawbacks that should be considered in designing such studies.

Some of the drawbacks stem from errors in the process that must be used to detect and recover valuable mutations. There are three steps involved in this process, (1) the phenotypic screen to detect individual outlier mice, (2) the progeny test to determine whether the outlier phenotype is due to single locus inheritance, and (3) genome-wide chromosomal mapping to provide a further check on single locus inheritance as well as to begin gene identification efforts. For complex traits showing considerable background variation, there are Type I (false positive) and II (false negative) errors to consider at each of the three steps that rarely matter with simple traits showing little background variation. We argue below that such errors can greatly diminish the usefulness of genome-wide mutagenesis in the study of complex traits, especially behaviors, which are often the most complex of all.

First, for some traits, it may be very difficult to identify which animals bear a valuable mutation. The favorite example of a successful mouse mutagenesis experiment is the isolation of the *clock* gene by Takahashi and his group (Vitaterna et al., 1994). The success of this experiment depended at least in part on the extremely small variability in circadian rhythm photoperiod in the background inbred strain. Thus, a single outlier mutant mouse could easily be detected against a nearly uniform phenotypic background. For complex traits, the existence of many environmental influences causing mice to vary phenotypically will make this task much more difficult (Tarantino et al., 2000). This problem arises because the detection of a valuable mutant hinges critically on the phenotype of a *single* outlier mouse relative to the background phenotypic variation. We expect that as environmental variation increases, fewer outliers will be apparent against an increasingly variable phenotypic background. This will have the effect of reducing the outlier rate, or percent of outliers, and thus the yield of recovered mutations. Moreover, the risk of false-positive outliers, i.e., those *not* due to a large-effect single locus mutation, increases as the background variance increases, making the recovery of valuable mutations more difficult.

Increased environmental variance is often associated with reduced reliability or repeatability of measurement (Falconer and MacKay, 1996). This problem is evident when a mouse, appearing to be an outlier when first tested in a phenotypic screen, may not be an outlier when tested a second time on the same assay. This has been reported for some

behaviors (Nolan et al., 2000) and may reflect the regression toward the mean expected when reliability is less than perfect (Falconer and MacKay, 1996). Retesting of each mouse may be needed to insure that an outlier mouse truly is an outlier, or in other words, to reduce false positive errors in the phenotypic screen. For many behaviors, retesting is not feasible because only the first test is valid (e.g., learning or anxiety), or because the first test alters the outcome of later tests, so the phenotype is no longer the same. False positive outliers at this stage are troublesome because they lead to progeny testing with little hope of passing the progeny test. This reduces the percent of all outliers ultimately shown to be valuable mutations. The difficulty and cost of recovering each valuable mutation are also increased.

Similar problems arise at the progeny-testing step. To pass the progeny test, the offspring phenotypes must be distributed bimodally as expected from 1:1 or 3:1 Mendelian ratios of wild type to mutant genotypes. To generate a bimodal rather than a unimodal distribution, a single mutant locus would have to account for two-thirds or more of the phenotypic variance (Belknap et al., 1993). Put in other words, the variance due to the mutant locus would have to be at least double that of the background variance for detection to occur. [Unfortunately, because the animals cannot be genotyped to differentiate mutant from nonmutant genotypes, the trait distribution is our only means for separating the two genotypes.] As the background variance increases, the probability diminishes that a single mutant locus will meet this criterion in a progeny test. This has the undesirable effect of decreasing the percent of extreme-scoring mice passing the progeny test by increasing the frequency of false-negative errors, which are valuable mutants that are not detected. Alternatively, one could abandon the bimodal distribution requirement and simply require that the progeny score differently than the background strain by a less stringent criterion. But this would increase the rate of false positives, which would then undergo expensive chromosome mapping studies with little hope of recovering a valuable mutant. Either way, the difficulty and cost of recovering each valuable mutation will be substantially increased as a function of the magnitude of the background variance.

Second, the successful screening and mapping of a mutant is just the beginning of the process of determining whether it has any utility to increase our understanding of pathways important for a complex trait. Much effort must often be expended to answer the basic question—what is this mutant good for? Let's assume in carrying out a screen for learning ability, we find a mutant that exhibits almost no learning of a given task. Considerable effort could be devoted to mapping and characterizing this mutant, only to find that its performance is due to reasons unrelated to learning—a sensory or effector deficit may be the cause. Another example is a mutation that seriously impairs vision—it will likely be detected and recovered on a screen for anxiety since most assays for this trait presume normal vision (e.g., Cook et al., 2000). For lack of a better word, we call these trivial (for a given trait) mutations because they are unlikely to shed light on the fundamental processes involved in either learning or anxiety. [Of course, a trivial mutation for one trait may serendipitously prove to be valuable for another trait.]

The more complex the trait genetically, the more genes (and pathways) will be involved across several organ systems. Since many if not most of these pathways will be trivial to an understanding of the trait, it can be difficult to sort out which mutants are trivial and which are not. Because trivial mutations will pass the progeny test as readily as nontrivial ones, they will undergo expensive mapping efforts with little hope of being particularly valuable. This implies that greater genetic complexity can be expected to lead to a reduced recovery rate of *nontrivial* or valuable mutations, as well as increase the cost and difficulty of recovering each valuable mutation. [There are undoubtedly trivial QTLs as well, but this is much less of a problem compared to induced mutants.]

Third, mutagen treatment induces an average of several hundred mutations in every mouse. For genetically very complex traits, defined as those with large numbers (potentially many thousands) of mutable trait-relevant genes, phenotypic screens will likely select individual outlier mice with several trait-relevant mutations (*polymutations*), not just one. If so, then the effects of the individual polymutations will be much smaller than expected, thus compromising one of the advantages of this method, the production of large-effect Mendelian mutations. The more complex the trait genetically, the more often trait-relevant polymutations will predominate among the outlier mice. These mice are unlikely to pass the progeny test because *each* polymutation is unlikely to account for two-thirds or more of the trait variance required for detection. To make matters worse, the effects of the smaller polymutations will add to the background variation, making a bimodal distribution due to the largest of them even less likely. This has the effect of reducing the percent of outlier mice passing the progeny test, and increasing the cost and difficulty in recovering each mutant. We now have another reason for believing that increased genetic complexity will be associated with a diminished recovery rate of valuable mutants. Moreover, since mutant gene mapping requires crosses between different inbred strains, large-effect mutations are essential if they are to be discriminable from the QTLs also segregating in the mapping population.

One important implication of the last three points noted above is that there may be subsets of complex traits, particularly behaviors, with outlier rates and recovery rates so low that the mutagenesis approach is only of marginal utility. In this situation, one could adopt a brute-force strategy and progeny-test much larger numbers of mice to increase the probability of recovering some nontrivial mutations. To do this implies that we must either relax our standards for what qualifies as a positive result for the first (outlier detection) or second (progeny testing) steps (which increases false-positives and further reduces recovery rates), or we must expand the total size and scale of the screen well beyond that needed for simple traits such as kinked tails and circling movements. Either way, the cost and effort will be greatly increased.

For the reasons given above, recovery of each valuable mutant for a complex trait is likely to be much more difficult and expensive than for a simple trait. To be sure, complex traits offer more targets for mutagenesis compared to simple traits, and thus the potential number of valuable mutants is greater *per* trait, but the increased difficulty and cost in recovering each mutation will take its toll on the usefulness of this method. This conclusion runs counter to that implied by some proponents of genome-wide mutagenesis (e.g., Nadeau and Frankel, 2000). Unfortunately, for those of us interested in behavior, it does not appear that ENU is going to save us from the complexity of our preferred phenotypes. At present, the analysis of complex behavioral traits by induced mutagenesis is too new to allow an adequate empirical test of our concerns, but preliminary results thus far are consistent with our expectations (e.g., Sayah et al., 2000; Nolan et al., 1997).

Fourth, there are strong biases in favor of mutation detection in some genes over others, and this reduces the proportion of all trait-relevant genes likely to be recovered in mouse ENU screens. Mutated genes most likely to be detected are those that have large effects on the phenotype resulting from base-pair substitutions at any one of hundreds of sites within the gene. A good example is a gene where point mutations at many sites all lead to premature stop codons; thus, this gene will likely emerge often in a screen while genes without this property may go undetected. [Multiple detections of the same gene are already apparent in ongoing mouse ENU studies, which can be useful if multiple allelic series are created, but this does not help the detection bias problem.] Trait-relevant genes unlikely to emerge are those with considerable phenotypic effects, but not enough to induce an outlier mouse no matter where the site of the mutation. For such genes, even null or constitutive

mutants won't be enough to lead to their detection and recovery. This detection bias will likely be greatest for genetically complex traits compared to simpler ones because of the higher frequency of trait-relevant genes whose mutated effects on the phenotype are too small to allow their recovery. Thus, the claim that all trait-relevant genes are potentially recoverable is highly questionable for complex traits. In addition, mutants showing recessive inheritance (the majority) are much less efficiently detected than those showing dominance, another major source of detection bias.

Strong bias also applies to the range of all behaviorally important phenotypes amenable to study by genome-wide mutagenesis. Phenotypic screens of mutagenized mice require large numbers of animals, much more than a typical QTL study. This introduces a bias for practical reasons in favor of traits that require little time or effort to phenotype each mouse, and do not affect the outcome of subsequent tests of other phenotypes carried out on the same mice. This is one reason why dysmorphological traits predominate in major mouse ENU screens since they can be detected by simple observation, and among behaviors, abnormal locomotor activity is a favorite phenotype.

The mutant detection and recovery bias against traits with large amounts of background variation has already been mentioned. Complex traits that show floor or ceiling effects (common with behaviors) do not work well because of the truncated distribution, making the identification of outliers almost impossible; however, these traits often work well for QTL studies. Also, traits that require sacrificing the animal to measure them, such as neurochemical or neuroanatomical measures, can be studied easily by QTL methods using replicated, isogenic genotypes inherent in recombinant inbred strains, congenics, recombinant congenics or chromosome substitution strains (consomics), but do not lend themselves well to mutagenesis screens for outlier genotypes that are neither isogenic nor replicated when the phenotypic screen is performed. This disadvantage means that outlier mice must serve as breeders or as sperm or ova donors prior to sacrifice for phenotyping. Therefore, detecting such outliers in the first place will require sperm or ova freezing for *all* of the several thousand mice in the screen prior to phenotyping, followed by *in vitro* fertilization/implantation procedures to propagate the outlier genotypes. These burdensome requirements make such traits undesirable if not unworkable for mutagenesis screens.

Fifth, phenotype-driven mutagenesis screens, for practical reasons, are designed to detect only those mutations with the largest effects on a given trait. These are precisely the ones most likely to cause developmental compensation on a scale seen in some targeted mutagenesis (knockout) mice (Gerlai, 1996; Bilbo and Nelson, 2001; Crawley, 2000). Indeed, the majority of recovered mutants from mouse ENU screens are null mutants (Neveroske et al., 2000). This can introduce a troubling confound, for we will always be unsure of the degree to which a particular phenotype we observe in a mutant strain is due to an induced mutation or to other *nonmutated* genes whose expression has changed to compensate for the mutation effect. In addition, perturbation of pathways by a mutation may be so great as to cause a cascade of secondary effects not seen in normal mice, many of them in pathways far removed from the site of the mutation. In such cases, the question becomes—to what degree does a particular observed “phenotype” of a mutant reflect aberrant secondary effects? Strong secondary effects, particularly during development, can obscure the normal function of affected pathways and greatly complicate inferences about the cause of an observed phenotype because of their largely unknown nature and because there can be so many of them. In addition, such effects often disrupt the normal interplay among genes (epistasis), an increasingly important focus for complex trait studies.

Secondary effects pose other problems as well. Among those mutants that are viable, many show reduced health and vigor that may *nonspecifically* confound a phenotypic assay

when a “sickly” mutant genotype is compared to a more vigorous wild type (normal) genotype. When differences are found (e.g., the mutants may be less active), we may incorrectly attribute this to a specific pathway of a known mutation when the true explanation lies with unknown secondary effects serving to impair health or vigor. This problem is likely to be of greatest concern in the study of behavior.

Sixth, “shotgun” mutagenesis is inherently indiscriminate. For every mutation that is detected and mapped in the offspring of each outlier mouse, hundreds more exist unknown to the experimenter. While one could eliminate most extraneous mutations by repeated backcrossing leading to congenic strains, this is rarely considered in the mouse genome-wide mutagenesis literature.

Seventh, most genes that are monomorphic are so for a reason. For many if not most such loci, natural selection has eliminated any functional polymorphisms induced by spontaneous mutations over evolutionary time. Rendering these polymorphic by chemical mutagenesis often results in a loss of fitness or even lethality. Among those that are viable, their health may be compromised. These concerns cause many mutant stocks to be difficult to maintain and propagate, which increases cost and reduces their utility as disease models.

For the reasons given above, the impressive successes of mutagenesis screens in dissecting simple traits in simple organisms are likely to be much more difficult to attain with complex traits (behavior) in genetically complex organisms (mice). Moreover, the price tag in mice is very high, especially for complex traits, raising issues of cost-effectiveness compared to other approaches. Consider also the demands for housing potentially thousands of new mutant mouse stocks when many animal facilities are already full to capacity with knockouts and transgenic mice.

Finally, natural genetic variation is of interest in its own right in many biological disciplines, particularly from evolutionary, ecological, and population genetic perspectives. For example, if we wish to understand the genetic architecture of a trait in an evolutionary context, naturally occurring variation is much more likely to provide insightful clues. There are also immediate biomedical reasons for interest in the large polymorphic subset of genes for breeding (e.g., selective breeding) of better animal models of disease states in humans. The demonstrated usefulness of selection lines for the study of hypertension and alcohol withdrawal severity are prime examples (Phillips et al., in preparation). Existing variation found in laboratory stocks of mice and rats are the basis of hundreds of valuable disease models, and new ones will no doubt continue to be discovered or developed.

More than Just Gene Identification

While much of our discussion has focused on gene identification, it is important to note that this is not the *sine qua non* of the QTL approach. Standard practice is to isolate mapped QTLs into congenic strains, which, when compared to the background strain, allow the study of the functional effects of the QTL at any desired level of analysis from the molecular to the organismic. This allows assessment of the effects of a given QTL on multiple traits (pleiotropy), interactions with other loci (epistasis) and with environments. This effort to understand QTL effects in a broader genomic, organismic and environmental context can be quite productive without knowing the specific gene(s) involved. [Of course, this effort will be more powerful if the gene has been identified.] Moreover, such studies will undoubtedly provide important functional clues as to gene identity. A similar approach could be used for induced mutations as well, but this is rarely mentioned in the mutagenesis literature.

Epistasis is becoming an increasingly important focus of QTL studies recently, an important new development (e.g., see Hood et al., this volume). ENU studies do not lend themselves to the study of epistasis as readily because of secondary effects which compound

the difficulty of determining which interactions are important to the *normal* organism. The same situation exists with regard to gene-environment interactions and correlations, another important consideration for gaining valuable insights from mouse disease models.

Finally, we note that the QTL approach over the past five years has led to the identification of scores of highly probable candidate genes for many useful mouse and rat disease models. Most of these would likely not have been implicated without QTL screens. Thus, QTL studies often provide an important and powerful hypothesis *generating* function in contrast to hypothesis *testing*. ENU screens also offer this capability.

Summary

QTL analysis and genome-wide mutagenesis will continue to contribute greatly to functional genomics in the next decade. Neither approach is an optimal solution to understanding genetic modulation of complex traits in mammals. The more we learn about even Mendelian mutations, the more we appreciate that there are relatively few genuinely simple traits. Epistasis, genetic background, parental effects, imprinting, and innumerable gene-environment interactions intrude into originally simple stories. No matter what the technique, the ride is likely to be “long and bumpy” when challenges are faced squarely and realistically. The general aim is to decipher the coordinated actions of many genes and even highly reduced systems will involve dozens of molecules and dozens of potential exogenous modulators. In our view, we will need all possible complementary approaches in functional genomics because the strengths of one will often offset the weaknesses of another. Given the rapid progress in technology and reagents that has occurred over the past decade, we are encouraged that the means to solve, sidestep, or mitigate these problems will be developed.

Consider the technology available for QTL work only a decade ago. In 1990, full genome searches were restricted to RFLPs, a cumbersome and expensive method of genotyping, and software to implement interval mapping and appropriate Type I error control were not yet available. The first successful genome-wide search for QTLs in a mouse disease model did not appear until one year later (Rise et al., 1991). Today, full genome QTL searches using microsatellite markers are routine, and hundreds of QTLs have been reported for many valuable phenotypes (Moore and Nagle, 2000; Phillips et al., in preparation). Readily available software has greatly increased both the power and accuracy of genome-wide searches (Manly and Olson, 1999). Higher resolution mapping to 1 cM is now straightforward (Darvasi, 1998), and sub-cM mapping is beginning to emerge. [These QTL advances have already greatly enhanced the mapping step of ENU projects.] The availability of full genome sequence data for four of the most commonly-used inbred strains is almost at hand. Because of technological advances, both recent and near future, we are confident that the problems that presently hinder progress will serve as the instigation for success in the years to come for both the QTL and mutagenesis approaches.

Acknowledgements

Work cited as originating in our laboratories was supported by grants AA10760, AA06243, DA10913, DA05228 and four Department of Veterans Affairs Merit Review programs.

References:

Belknap, J.K., Metten, P.A., Helms, M.L., O'Toole, L.A., Angeli-Gade, S., Crabbe, J.C. and Phillips, T.J. (1993). Quantitative Trait Loci (QTL) applications to substances of abuse: Physical dependence studies with nitrous oxide and ethanol. *Behav Genet* **23**:211-220.

- Belknap, J.K., Dubay, C., Crabbe, J.C. and Buck, K.J. (1997). Mapping quantitative trait loci for behavioral traits in the mouse. In K. Blum and E.P. Noble (eds) *Handbook of Psychiatric Genetics*, CRC Press, Boca Raton, FL, pp 435-453.
- Bilbo, S.D. and Nelson, R.J. (2001). Behavioral phenotyping of transgenic and knockout animals: A cautionary tale. *Lab Animal* 30:24-29.
- Brown, SD and Nolan, P. (1998). Mouse mutagenesis--systematic studies of mammalian gene function. *Hum Molec Genet* 7:1627-33.
- Buck, K.J., Metten, P., Belknap, J.K. and Crabbe, J.C. (1997). Quantitative trait loci involved in genetic predisposition to acute alcohol withdrawal in mice., *J Neurosci* 17:3946-3955.
- Buck, K.J., Belknap, J.K., Wenger, C., Merrill, C. and Crabbe, J.C. (1999). Quantitative trait loci involved in genetic predisposition to acute pentobarbital withdrawal in mice. *Mamm Genome* 10:431-437.
- Buck, K.J. and Finn, D. (In press). Genetic factors in addiction: QTL mapping and candidate gene studies implicate GABAergic genes in alcohol and barbiturate withdrawal in mice. *Addiction* 96:xxx-xxxx.
- Burmeister, M. (1999). Basic concepts in the study of diseases with complex genetics. *Biol Psychiat* 45:522-532.
- Cook M.N., Williams R.W. and Flaherty L. (In press). Anxiety-related behaviors in the elevated zero maze are affected by genetic factors and retinal degeneration. *Behav Neurosci* xx:xxxx.
- Crabbe, J.C., Phillips, T.J., Buck, K., Cunningham, C. and Belknap, J.K. (1999). Identifying genes for alcohol and drug sensitivity: Recent progress and future directions. *Trends Neurosci* 22:173-179.
- Crawley, J. N. (2000) What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice. Wiley-Liss, NY, NY.
- Darvasi, A. (1998) Experimental strategies for the genetic dissection of complex traits in animal models. *Nature Genet* 18:19-24.
- Demarest, C., Koyner, J., McCaughran J., Cipp, L. and Hitzemann R. (In press). Further characterization and high resolution mapping of ethanol-induced motor activity. *Behav Genet* XX:XXX-XXX.
- Falconer, D.S. and MacKay, T. (1996). *Introduction to Quantitative Genetics*, Longman, London.
- Fehr, C., Belknap, J.K., Crabbe, J.C. and Buck, K.J. (In press) High resolution mapping of an alcohol withdrawal locus *Alcw2* to the *D4Mit80-Mpdz* region on mouse chromosome 4.

- Hamilton B.A., Smith D.J., Mueller K.L., et al. (1997). The *vibrator* mutation causes neurodegeneration via reduced expression of P1TP alpha: positional complementation cloning and extragenic suppression. *Neuron* **18**:711-22.
- Hrabe de Angelis, M. et al. (2000). Genome-wide large-scale production of mutant mice by ENU mutagenesis. *Nature Genet* **25**:444-447.
- Lander, E.S. and Schork, N.J. (1994). Genetic dissection of complex traits. *Science* **265**:2037-2048.
- Le Roy, I. (In press). Possible causal link between cerebellar patterns of foliation and hindlimb coordination in laboratory mice: a quantitative trait loci analysis. *Behav Genet* **XX**: XXX-XXX.
- Lyons, P.A., et al. (2000). Congenic mapping of the Type 1 diabetes locus, *Idd3*, to a 780-kb region of mouse chromosome 3: Identification of a candidate segment of ancestral DNA by haplotype mapping. *Genome Res* **10**:446-453.
- Manly, K.F. and Olson, J.M. (1999). Overview of QTL mapping software and introduction to Map Manager QT. *Mamm Genome* **10**:327-334.
- Malke, H., Steiner, K., Gase, K. and Frank, C. (2000). Expression and regulation of the streptokinase gene. *Methods* **21**:111-24.
- Moore, K.J. and Nagle, D.L. (2000). Complex trait analysis in the mouse: the strengths, the limitations and the promise yet to come. *Ann Rev Genet* **34**:653-686.
- Mott, R., Talbot, C.J., Turri, M.G., Collins, A.C. and Flint, J. (2000). A method for fine mapping quantitative trait loci in outbred animal stocks. *Proc Natl Acad Sci* **97**:12649-12654.
- Nadeau, J.H., Singer, J.B., Matin, A. and Lander, E.S. (2000). Analysing complex genetic traits with chromosome substitution strains. *Nature Genet* **24**:221-225.
- Nadeau, J.H. and Frankel, W.N. (2000). The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. *Nature Genet* **25**:381-384.
- Noveroske, J.K., Weber, J.S and Justice, M.J. (2000). The mutagenic action of N-ethyl-N-nitrosourea in the mouse. *Mamm Genome* **11**:478-483.
- Nolan, P.M., Kapfhamer, D. and Bucan, M. (1997). Random mutagenesis screen for dominant behavioral mutations in mice. *Methods* **13**:379-396.
- Nolan, P.M., et al. (2000). A systematic genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nature Genet* **25**:440-443.
- Rise, M. L., Frankel, W. N., Coffin, J. M. and Seyfried, T. N. (1991). Genes for epilepsy mapped in the mouse. *Science* **253**:669-673.
- Sandberg, R., et al. (2000). Regional and strain-specific gene expression mapping in the adult mouse brain. *Proc Natl Acad Sci* **97**:11038-43.

- Sayah, D.M., Khan, A.H., Gasperoni, T.L. and Smith, D.L. (2000). A genetic screen for novel behavioral mutations in mice. *Mol Psychiat* **5**:369-377.
- Schimenti, J. and Bucan, M. (1998). Functional genomics in the mouse: Phenotype-based mutagenesis screens. *Genome Res* **8**:698-710.
- Takahashi J.S, Pinto L.H, Vitaterna M.H. (1994). Forward and reverse genetic approaches to behavior in the mouse. *Science* **264**:1724-1733.
- Talbot, C.J., Nicod, A., Cherny, S.S., Fulker, D.W. Collins, A.C. and Flint, J. (1998). High-resolution mapping of a quantitative trait loci in outbred mice. *Nature Genet* **21**:305-308.
- Tarantino, L.M., Gould, T.J., Druhan, J.P. and Bucan, M. (2000). Behavior and mutagenesis screens: the importance of baseline analysis of inbred strains. *Mamm Genome* **11**:555-564.
- Tecott, L.H. and Wehner, J.M. (In press). Mouse molecular genetic technologies: promise for psychiatric research. *Arch Gen Psychiat*, **58**:XXX-XXX.
- Vitaterna, M.H., King, D.P., Chang, A-M, et al. (1994). Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science* **264**:719-725.
- Wells, C. and Brown, S.D.M. (2000). Genomics meets genetics: towards a mutant map of the mouse. *Mamm Genome* **11**:472-477.
- Williams, R.W. (1999). A targeted screen to detect recessive mutations that have quantitative effects. *Mamm Genome* **10**:734-738.
- Williams, R.W. (2000). Mapping genes that modulate brain development: a quantitative study. www.nervenet.org/main/papers.html.
- Williams, R.W., Airey D.C., Kulkarni A., Zhou G, and Lu, L. (in press). Genetic dissection of the olfactory bulbs of mice: QTLs on chromosomes 4, 6, 11, and 17 modulate bulb size. *Behav Genet* **XX**:XXX-XXX.