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Chromosome substitution strains: gene discovery functional analysis and systems studies

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Abstract

Laboratory mice are valuable in biomedical research in part because of the extraordinary diversity of genetic resources that are available for studies of complex genetic traits and as models for human biology and disease. Chromosome substitution strains (CSSs) are important in this resource portfolio because of their demonstrated use for gene discovery, genetic and epigenetic studies, functional characterizations, and systems analysis. CSSs are made by replacing a single chromosome in a host strain with the corresponding chromosome from a donor strain. A complete CSS panel involves a total of 22 engineered inbred strains, one for each of the 19 autosomes, one each for the X and Y chromosomes, and one for mitochondria. A genome survey simply involves comparing each phenotype for each of the CSSs with the phenotypes of the host strain. The CSS panels that are available for laboratory mice have been used to dissect a remarkable variety of phenotypes and to characterize an impressive array of disease models. These surveys have revealed considerable phenotypic diversity even among closely related progenitor strains, evidence for strong epistasis and for heritable epigenetic changes. Perhaps most importantly, and presumably because of their unique genetic constitution, CSSs, and congenic strains derived from them, the genetic variants underlying quantitative trait loci (QTLs) are readily identified and functionally characterized. Together these studies show that CSSs are important resource for laboratory mice.

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Introduction

Research progress with many biological problems and disease models depends on integrating genetic and epigenetic approaches, developmental and physiological studies, and network and systems analyses. These genetic and epigenetic studies focus on the identity of genetic variants, dynamic changes in epigenetic features, their interactions with each other, and their dependencies on environmental factors. Molecular, developmental, and physiological studies focus on the function of genes and regulatory DNA elements and the ways in which phenotypes are coordinated across cell and organ systems, with time, and in response to perturbations. Integration of this information into networks of gene, protein, and functional interactions provides complementary insights about the molecular underpinnings of systems properties as well as the systems context in which molecular features function. Ultimately these integrated studies will provide a picture of the genetic and phenotypic architecture of complex conditions and an ability to modulate phenotypic and disease outcomes. A broad array of genetic resources, phenotyping technologies, and analytical methods are needed to support these discovery and experimental studies.

Comprehensive collections of naturally occurring, chemically induced, and genetically engineered variants are needed to test specific hypotheses about the role of particular genes and DNA sequence variants and to discover the unexpected contributions of genes and variants to organismal biology (Nadeau et al. 2001; Glazier et al. 2002; Beutler and Moresco 2008; Probst and Justice 2010; Aitman et al. 2011; Dow and Lowe 2012). Reliable, quantitative, and cost-effective phenotyping assays are essential for systematically interrogating the various levels of molecular activities and organismal functions (Ayadi et al. 2012; Fuchs et al. 2012; Laughlin et al. 2012; Mallon et al. 2012). In many instances, genetically defined resources are useful for replication, time-course, and perturbation studies. These resources should be readily available for a variety of studies ranging from small-scale projects such as a graduate student in a conventional research laboratory to large-scale comprehensive and systematic community endeavors.

The remarkable diversity of public resources makes the laboratory mouse an invaluable model for biomedical research. Hundreds of inbred strains are available to test associations between genetic variants and phenotypic differences (Ghazalpour et al. 2012). Specialized resources such as panels of recombinant inbred strains, including the Collaborative Cross, and chromosome substitution strains (CSSs) are permanent resources for quantitative trait locus (QTL) mapping, tests for gene interactions, and analysis of the systems consequences of genetic and environmental perturbations (Nadeau et al. 2001 Aylor et al. 2011; Churchill et al. 2012; Welsh et al. 2012). Both gene-based and phenotype-based chemical mutagenesis surveys are ongoing to identify genes affecting specific phenotypes (Beutler and Moresco 2008; Probst and Justice 2010). Novel techniques enable genetic engineering of specific conditional and reporter variants (Dow and Lowe 2012). These diverse resources have both overlapping and complementary attributes, but together they represent the power of the laboratory mouse in biomedical research (Paigen 1995; Nadeau et al. 2001; Glazier et al. 2002; Aitman et al. 2011).

CSSs are a distinct paradigm for complex trait analysis. In contrast to the conventional approach based on segregating populations and heterogeneous genetic backgrounds, CSSs partition the genome of the donor strain in a defined and reproducible manner so that each segment of the genome can be independently tested on a constant, uniform, and identical genetic background. In the early days of the Human Genome Project, Eric Lander and J. H. Nadeau anticipated that the availability of large numbers of readily genotyped markers would soon enable surveys for genetic variants that control phenotypic variation and disease risk. They also recognized that because of the inherent limitations of study designs and

statistical power, genetic variants with weak effects would tend to be lost in the background noise of many other segregating genes and phenotypes and would therefore elude discovery. Based on prior work in Drosophila (Seiger 1966; Ratty and Lovellette 1967) and plants (Aksel and Kuspira 1968; Sherrard et al. 1976), Lander and Nadeau thought that CSSs might enable discovery of these elusive variants because QTLs could be tested on a constant background where the noise of other segregating variants could be controlled. With these thoughts, construction of the first complete CSS panel was undertaken in mammals. Subsequently, three new panels of CSS have been reported for mice (Gregorova et al. 2008; Takada et al. 2008; Burgio et al. 2007) as well as a panel for rats (Malek et al. 2006).

In this review we outline the principles for making CSSs, summarize the key features of the three primary CSS panels, and summarize the ways in which CSSs are being used and the discoveries that have been made with these unique and powerful genetic resources.

Making CSSs

Methods

Making a CSS is somewhat time-consuming but relatively easy because the process is based on standard genetic principles, namely, at least ten sequential backcrosses with progeny genotyping at each generation, followed with intercrosses to homozygose the substituted chromosome. The resulting CSS can be maintained simply with brother–sister mating and without further genotyping. Detailed breeding and genotyping protocols were recently published (Hill et al. 2006), and additional information can be found in the original reports of the CSS panels (Nadeau et al. 2000; Singer et al. 2004; Malek et al. 2006; Burgio et al. 2007; Gregorova et al. 2008; Takada et al. 2008).

Briefly, an inbred strain is selected to serve as the donor for chromosome substitution and another to serve as the host. These strains are intercrossed and the resulting F1 hybrids are backcrossed to the host strain. Progeny of these backcrosses that inherit a nonrecombinant chromosome are identified and used for the next backcross. To identify mice that carry a nonrecombinant chromosome, DNA markers that are located near both telomeres are used to make certain that the ends of each chromosome are transferred. Several intervening markers are used to select against mice that carry double crossovers and other complex chromo-some exchanges. To homozygose the autosomes and the X chromosome, intercrosses are made between heterosomic mice at the tenth backcross generation (or later). CSSs to substitute mitochondria and the Y chromosome are made by using female and male mice, respectively, as breeders (Hill et al. 2006). Finally, brother–sister matings are used to maintain the CSSs.

Based on genotyping more than 50,000 mice for many CSSs, we found that for any chromosome, ~40 % of backcross progeny are nonrecombinant and ~20 % carry the nonrecombinant chromosome of interest (Nadeau et al. 2000; Singer et al. 2004; A. E. Baskin-Hill and J. H. Nadeau, unpublished). Thus, on average, one mouse in a litter of five will carry the desired nonrecombinant chromosome.

The most laborious step involves homozygosing the substituted chromosome. Intercrosses between heterosomic mice are expected to yield homosomic mice at a rate of ~4 % [= $(20\ \%)^2$], which corresponds to the rate of transmitting the nonrecombinant chromosome of interest from both parents to the same offspring. Needing female and male homosomic mice to establish a breeding colony compounds the logistical challenge. An alternative approach involves backcrossing homosomic mice to heterosomic sibs to obtain additional homosomic progeny at the 20 % rate. In this way, homosomic mice are produced more quickly and a breeder colony is established more readily.

CSS panels

B6-Chr#$^{A/J}$. The primary criteria for making the first complete panel of CSSs was to ensure that each chromosome could be substituted intact. While single CSSs are valuable for studying specific genetic questions, a complete panel, with every chromosome fully substituted, is essential for a comprehensive genome survey. Thus, a compatible combination of strains was desirable. Although the genetic constitution of established inbred strains confers sufficient viability and fertility to ensure that they can be maintained under laboratory conditions, many gene combinations derived from these inbred progenitors are incompatible, as demonstrated by the loss of many incipient recombinant inbred and recombinant congenic strains (Fortin et al. 2001; Threadgill and Churchill 2012). Lander and Nadeau noted that the largest panel of recombinant inbred and recombinant congenic strains was derived from the C57BL/6 J (B6) and A/J inbred strains (Marshall et al. 1992; Fortin et al. 2001; Nadeau et al. 2000), suggesting that the compatibility of these strains might suffice for a complete CSS panel. B6 and A/J are two classic inbred strains that were established just over 100 years ago from Castle's mice (Beck et al. 2000). After genotyping more than 17,000 mice over 7 years, the first complete CSS panel was reported, with loss of only one small chromosome segment located beyond the most telomeric marker of chromosome 1 (Singer et al.). With the increased availability of genetic markers (Keane et al. 2011; Yalcin et al. 2011), loss of unmarked segments is now less likely.

Although selected based solely on their perceived genetic compatibility, the B6 and A/J strains show a remarkable number and variety of phenotypic differences (Singer et al. 2004; Shao et al. 2008; phenome.jax.org), many of which have already been studied with this CSS panel (see below). The extensive phenotypic diversity between this pair of relatively closely related inbred strains is remarkable given that substantial portions of their genomes are identical by descent (Keane et al. 2011; Yalcin et al. 2011). Many reports show that at least one CSS differs significantly from the B6 host strain for most traits. These phenotypic surveys have led to identification of several QTLs and to evidence for gene interactions and non-Mendelian inheritance, including parent-of-origin effects and heritable epigenetic changes.

B6-Chr#^{MSM}. The MSM/Ms (MSM) inbred strain was established from the Japanese wild mouse, *M. m. molossinus*, which is a naturally occurring hybrid of *M. m. musculus* and whose range extends across much of Asia, and *M. m. castaneus*, which is found in Southeast Asia (Yonekawa et al. 1980; Moriwaki 1994; Moriwaki et al. 2009). Many studies have shown that *M. m. molossinus* is genetically distant from *M. m. domesticus* (Moriwaki 1994; Silver 1995; Schalkwyk et al. 1999; Abe et al. 2004; Sakai et al. 2005). Traditionally, classical inbred strains, particularly “Castle's stocks,” were thought to originate from Japanese fancy mice (Klein 1975). However, recent SNP data suggest that *M. m. molossinus* contributes only 6–10 % to the genome of the classical inbred strains (Frazer et al. 2007; Yang et al. 2007). The considerable divergence time between the two subspecies, which is estimated to be approximately 0.5–1.0 million years (Yonekawa et al. 1980; Moriwaki 1994), has resulted in a considerable genetic differentiation (~1 % DNA sequence divergence), with more than ten million SNPs differing between B6 and MSM (Abe et al. 2004).

As a consequence of its genetic divergence from B6 and other classical inbred strains, MSM has many unique phenotypes (Yonekawa et al. 1980; Moriwaki 1994; Yonekawa et al. 1994; Takada et al. 2008; Moriwaki et al. 2009). For example, MSM has an extremely low susceptibility to various tumor types (Moriwaki et al. 1999). Significant resistance to age-dependent hearing loss has been reported (Nemoto et al. 2004; Morita et al. 2007). Unique wild-derived behavioral traits, including very high locomotor activity, are also characteristic of MSM (Koide et al. 2000; Takahashi et al. 2008; Nishi et al. 2010; Takahashi et al. 2010).

The MSM strain likely bears additional intriguing phenotypes that are specific to *M. m. molossinus*.

Based on the large genetic distance and the corresponding phenotypic differences between B6 and MSM, MSM was chosen as the chromosome donor strain and B6 as the recipient strain for the B6-Chr^{MSM} CSS panel. In the resulting panel, 14 chromosomes (1, 3, 4, 8, 9, 11, 14–19, Y and the mitochondrial genome) are intact, eight chromosomes (2, 5, 6, 7, 10, 12, 13, and X) are maintained as congenic strains as a result of reduced fertility and viability, and two chromosome segments are missing (the central portion of chromosome 13 spanning 18 Mb between markers D13Mit311 and D13Mit9, and the centromeric region of chromosome 10 between the centromere and 63.2 Mb). These segments are maintained in heterozygous state and can be homozygosed, although mice with these missing segments are infertile on the B6 background.

MSM shows many unique behavioral traits, such as emotionality and temporal patterns of home-cage activity, compared with the classical inbred strains, including B6. To clarify the genetic basis of strain differences in these behavioral traits, systematic behavioral phenotyping of the full CSS panel of B6-Chr#^{MSM} was undertaken. These studies identified several chromosomes associated with anxiety-like behaviors (Takahashi et al. 2008), home-cage activity (Nishi et al. 2010), and social interaction behavior (Takahashi et al. 2010).

B6-Chr#^{PWD}. The primary intent for making the inter-subspecific B6-Chr^{PWD} CSS panel was to analyze genetic interactions resulting from the independent evolution of closely related subspecies (Gregorova et al. 2008). The B6 and PWD inbred strains were chosen as genetically defined representatives of the *Mus m. domesticus* and *Mus m. musculus* subspecies, respectively (Gregorova and Forejt 2000; Jansa et al. 2005; Fernandes et al. 2004). Their subspecific status was recently verified in studies comparing a set of trapped wild mice as well as wild-derived and classical laboratory inbred strains (Yang et al. 2007, 2012). Taking advantage of over 600,000 SNPs detectable with high-density oligonucleotide array technology (Wang et al. 2012), most of the B6 genome derives from *Mus m. domesticus*, whereas most of the PWD genome originates from *Mus m. musculus*, with only minimal admixture from *Mus m. domesticus* (Yang et al. 2012).

All of the chromosomes except for three are intact; the exceptions are chromosomes 10, 11 and X, each of which is represented as three overlapping congenic segments that together span the length of each chromosome.

The inter-subspecific B6-Chr#^{PWD} CSSs were made primarily to study the genetic architecture of hybrid male sterility associated at early stages of speciation of the house mouse. *Mus m. domesticus* and *Mus m. musculus* are young subspecies that diverged from their last common ancestor less than 500,000 years ago (Geraldes et al. 2011). In Europe, their narrow hybrid zone suggests reproductive isolation in the region of secondary contact (Forejt et al. 2012). Indeed, male hybrids between *Mus m. musculus^{PWD}* females and *Mus m. domesticus^{B6}* males are azoospermic with a pachytene block of spermatogenesis. Analysis of a (PWD × B6)F1 × B6 backcross revealed two strong hybrid sterility loci, one on chromosome 17 overlapping with Hybrid sterility 1/*Prdm9* (Mihola et al. 2009), and the other on chromosome X overlapping with the *Hstx1* hybrid sterility locus (Storchova et al. 2004) and three to five weak hybrid sterility loci still to be mapped. Male sterility is under strong epistatic control since only chromosome X^{PWD} together with heterozygous *Prdm9$^{PWD/B6}$* on the F1 hybrid background results in male meiotic arrest. Epistasis was further confirmed in crosses employing CSSs instead of B6 to make F1 hybrid males. In particular, (PWD × B6.Chr17^{PWD}) F1 males are fully fertile because of *Prdm9$^{PWD/PWD}$* homozygosity. By contrast, fertile (B6 × PWD) reciprocal hybrids became sterile and

displayed pachytene arrest when B6.ChrX^{PWD} females were used instead of B6 (Dzur-Gejdosova et al. 2012). By using CSSs to genetically dissect the multigenic hybrid sterility phenotype, high-resolution mapping of individual components of the network together with the ability to control gene interactions in a precise manner was highly feasible.

Public access to phenotyping information. B6, A/J, and PWD were selected for systematic analysis as part of the Mouse Phenome Project and the accumulated phenotypic data are available online (Paigen and Eppig 2000; Grubb et al. 2008). Results for many traits in the corresponding CSS panels are also available in the Phenome Database (phenome.jax.org). The SNP data for MSM as well as phenotype data of the B6-Chr^{MSM} CSSs are publicly available (NIG Mouse Functional Genomics Database: http://molossinus.lab.nig.ac.jp).

Analytical issues

To test whether a CSS harbors a QTL, trait values for each CSS and the corresponding host strain are compared, with a significant difference, after correction for multiple-hypotheses testing, implicating at least one QTL on the substituted chromosome. Because each CSS corresponds to a single independent test, the penalty for testing multiple hypotheses corresponds simply to the number of chromosomes in the genome, which for the mouse is 22, one for each of the 19 autosomes, the X and Y chromosomes, and the mitochondria. This penalty for a genome survey is considerably smaller than that for segregating crosses and other genetically heterogeneous populations (Lander and Kruglyak 1995; Belknap 2003). Alternatively, permutation tests can be used to establish study-wide significance thresholds for CSSs (Doerge and Churchill 1996; Burrage et al. 2010; cf. Churchill and Doerge 2008; Nadeau et al. 2003).

We note that in general the statistical threshold needed to detect a given QTL is 30-fold lower in CSSs than in F2 intercrosses ($p = 0.003$ vs. $p = 0.0001$, respectively), and therefore the necessary sample size is also smaller in a CSS survey than in conventional genetic surveys (Belknap 2003; Singer et al. 2004). As with other inbred genetic resources, arbitrarily large sample sizes can be obtained for each CSS.

Because CSSs are inbred, replication is reliably undertaken at different times and places with genetically identical mice. We recently found strong replication for diet-induced obesity that was conducted several times in two animal facilities; only 3 of 69 tests showed discordant results (Burrage et al. 2010, J. H. Nadeau, in preparation).

We note that absence of a significant difference between a CSS and the host strain has three interpretations. First, the phenotypic effect of a QTL may be less than the statistical threshold needed to declare significance (probably common); second, at least two loci whose phenotypic effects are in opposite directions may be present on the substituted chromosome (not unusual); and third, QTLs that affect the trait of interest are not located on the chromosome (unusual) (Buchner et al. 2008; Burrage et al. 2010).

The unique genetic constitution of CSS panels complicates estimation of the portion of the total phenotypic variance that is attributable to each CSS in a complete genome survey. In particular, because they are inbred, phenotypic variation within a CSS results from environmental, measurement, and stochastic effects, but not from genetic factors. Belknap (2003) showed how variance can be estimated for a comparison of a single CSS and its host strain. However, similar estimates across a panel are not possible because the total phenotypic variance is not known. An alternative is to estimate phenotypic effect sizes (measured in their natural units) that are attributable to each CSS or QTL (Shao et al. 2008). However, even here these measures are not directly comparable between CSSs and segregating populations because the nature of the study populations differs profoundly. As

Wright (1968), Gruneberg (1963), and others have shown, features such as dominance, penetrance, expressivity, pleiotropy, and phenotypic effect sizes depend on genetic background and therefore cannot be legitimately compared among study populations that have distinct genetic features.

Obviously, the initial genetic resolution is not as great as with conventional QTL or haplotype mapping paradigms, with QTLs assigned to a chromosome rather than to genetic intervals. Nevertheless, results are highly reproducible, both CSS crosses and congenic strains are readily made for regional localization, and QTLs regularly persist with further genetic dissection in contrast to many other circumstances where QTL effects sometimes disappear (Legare et al. 2000), presumably because of context-dependent effects and low penetrance (Shao et al. 2008, 2010; Yazbek et al. 2011). We now consider these points in detail.

Applications

QTL mapping with single CSSs Single CSSs have been made to verify prior evidence implicating particular loci and then used as a resource for further genetic and functional studies. Because Y chromosome and mitochondrial substitutions are made so readily, they have been used for many years to study a variety of traits in many strain combinations (for recent examples, see Barrick et al. 2009; Llamas et al. 2009; Suto 2009; Nelson et al. 2010; Hines et al. 2011; Case et al. 2012; Kotarska and Styrna 2012). In other instances, an autosomal CSS was made specifically to verify and in some cases localize QTLs with crosses and congenic strains (see, for example, Matin et al. 1999; Ochiai et al. 2003; Hollis-Moffatt et al. 2005; Kumazawa et al. 2007; Wang et al. 2010; Anderson et al. 2009; Trammell et al. 2012). In other cases, evidence from crosses and other mapping panels has been validated with a CSS selected from an existing panel (Torres et al. 2005; Ajioka et al. 2007; Matthews et al. 2008; Bryant et al. 2009; Whitney et al. 2009; Yang et al. 2009; Chai et al. 2011; Hillhouse et al. 2011; Winawer et al. 2011).

QTL mapping with a CSS panel Often because prior information is not available, surveys of a CSS panel are used to obtain information about chromosomal localization and to create new models for genetic and functional studies (Singer et al. 2004; Gregorova et al. 2008; Takada et al. 2008; Boyle and Gill 2009; Hessel et al. 2009; Keum and Marchuk 2009; Ahn et al. 2010; Peltz et al. 2011).

The logistics for using CSS panels is not complicated. For a complete survey, only 22 strains need to be tested if the Y chromosome and mitochondrial substitutions are included. Typically an average of about eight CSSs, and sometimes many more, differ significantly from B6 across a variety of developmental, morphological, physiological, and behavioral traits (Singer et al. 2004; Gregorova et al. 2008; Takada et al. 2008).

If the goal is to identify a CSS affecting a trait of interest, a survey of as few as three CSSs could suffice, given that an average of eight CSSs with significant effects in a panel of 22 strains (or approximately one in three randomly selected CSSs) differ from the host strain across a broad range of traits (Singer et al. 2004; Gregorova et al. 2008; Takada et al. 2008). For example, we recently tested resistance to diet-induced nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC) in three randomly selected CSSs and found susceptibility in one CSS and resistance in the other two (A. Baskin-Hill, C. Croniger, and J. H. Nadeau, unpublished), which shows that even a small survey has a good chance of finding interesting CSSs.

B6-Chr#$^{A/J}$ The original survey involved 53 traits and revealed 150 CSSs that differed significantly from the B6 host strain with respect to various blood, bone, and metabolic features (Singer et al. 2004).

B6-Chr#^{MSM} The systematic phenotyping survey of these inter-subspecific CSSs focused on 40 morphological and physiological traits related to reproduction, growth, and energy metabolism and successfully detected many QTLs that have statistically significant phenotypic effects (Takada et al. 2008). The number of significant QTLs detected in this study was 74 for males and 53 for females. In particular, 208 CSSs with significant effects across 26 traits were detected for an average of eight with a signifi-cant effect per trait, a result that is remarkably similar to results for the B6.Chr#$^{A/J}$ panel (cf. Singer et al. 2004; Shao et al. 2008). Many QTLs were detected only for males or for females, and less than half (34.1 %) of the significant QTLs were observed in both males and females. Furthermore, evidence for simple additive effects was found for body weight, body length, fat pad weight, relative ratios of kidney and heart weight to body weight, and blood constituents such as triglycerides, amylase, and blood urea nitrogen.

B6-Chr#^{PWD} The C57BL/6J-Chr#^{PWD} panel was used for genetic analysis of male fertility of hybrids of PWD females and CSS males. While (PWD × B6)F1 hybrid males show mid-late pachytene apoptosis and are completely sterile, replacing B6 with B6-Chr 17 and B6-Chr 19 strains resulted in complete and partial release of meiotic arrest. No other chromosomes displayed similar effects (Dzur-Gejdosova et al. 2012). The C57BL/6J-Chr#^{PWD} panel was also used to study 31 phenotypes, including clinical blood chemistry, hematology, bone density and body composition. The survey is ongoing and the number of studied phenotypes is expanding (Svenson et al. 2012; phenome.jax.org).

Strategies to locate QTLs

An important motivation for making CSSs was to facilitate discovery and characterization of the genetic variants that underlie phenotypic variation (Nadeau 2001). After identifying a CSS that differs significantly from the host strain, the challenge is finding an efficient strategy for localizing the QTL to a small number of candidate genes so that proof-of-concept studies can be undertaken. Two strategies have been pursued to localize QTLs to a small number of candidate genes: one involves CSS crosses and the other involves panels of congenic strains, with the latter appearing to be a more general and powerful approach.

An obvious way to localize QTLs is to cross a selected CSS with the host strain and then use the resulting F1 hybrids in backcrosses or intercrosses (Matin et al. 1999; Kumazawa et al. 2007; Matthews et al. 2008; Boyle and Gill 2009; Keum and Marchuk 2009; Ahn et al. 2010; Burrage et al. 2010; Wang et al. 2010; Winawer et al. 2011. Yazbek et al. 2011). In these cases, the segregating genome is limited to a single chromosome, and therefore the threshold to declare a significant effect is less than for a complete genome survey.

CSS crosses have at least three limitations, however. The first is that localizations are not precise in part because multiple QTLs are often located on a substituted chromo-some. The second is that QTL boundaries are defined statistically, e.g., 95 % confidence limits, rather than precisely, as is possible with congenic strains (see below). The third is that insufficient crossovers are usually found to localize QTLs precisely, unless mice carrying a recombinant chromosome are specifically selected for genetic and phenotypic analysis.

An important example of CSS crosses involves transfer of the X chromosome from the MSM donor strain to the B6 background that resulted in reproductive failure characterized by male sterility and caused by an incompatibility between the MSM-derived genes on the X chromosome and other genes in the B6 background (Oka and Shiroishi 2012; Oka et al.

2004, 2007, 2010). QTL analysis was conducted to find interacting loci with the MSM X chromosome to elicit reproductive failure. This analysis successfully detected QTLs with significant phenotypic effects on chromosomes 1 and 11 that interact with the X chromosome (Oka et al. 2007).

Congenic strains from a CSS

Because they are readily made from a CSS and share similar experimental and analytical attributes for many genetic, functional, and systems studies, congenic strains and their derivatives (subcongenic and sub-subcongenic strains) are ideal resources for dissecting relationships between genotypes and phenotypes. Because the substituted chromosome has already been introgressed onto the host strain, making a congenic strain simply involves a series of four crosses, namely, (1) intercrossing the CSS and the inbred host strain to make F1 hybrids, (2) back-crossing these hybrids to the host strain and selecting backcross progeny that carry a recombinant chromosome, (3) backcrossing these recombinant mice to the host strain, and (4) intercrossing mice with the recombinant chromo-some to homozygose the congenic segment. Given these logistics, making a congenic strain, and even a panel of congenic strains, is relatively easy and efficient and can typically be completed in 1 year. Shao et al. (2010) provides many examples of congenic strains that have been made from CSSs and reviews their use in complex trait analysis; they also propose a way to analyze results for congenic panels that is similar in principle to CSS analysis.

Despite remarkable advances in the analytical methods for complex trait genetics, developing formal methods for studying congenic strains has been largely overlooked. Historically, chromosome segments are sought that are strongly associated with phenotypic variation, and in the ideal circumstance alternative alleles segregate perfectly with contrasting phenotypes. Implicit in this “common segment” method are the assumptions that relatively few genes control phenotypic variation, gene interactions are negligible, and allelic variants are highly penetrant. Violations of these assumptions probably account for the seemingly inexplicable vagaries of congenic strain analysis, where strong QTLs detected in a cross or population “disappear” in congenic strains (Legare et al. 2000; Shao et al. 2010). In general, if the genetic control of a trait is simple then the common segment method is reliable, but if the control is complex, which is often the case, then the common segment method misses much of the complexity of phenotypic variation.

Shao et al. (2010) proposed an alternative method for analyzing phenotypes in congenic strains. Their method is based on the principles of CSS analysis, where each CSS, which represents an independent segment of the donor strain genome, is compared to the host strain. With the sequential method, strains in the congenic panel are ordered beginning with the strain with the smallest congenic segment, progressing with those with increasingly large overlapping segments, and ending with the strain that has the largest segment. Then to test whether the chromosome segment from the donor strain carries a QTL that affects the phenotype of interest, the strain with the smallest congenic segment is compared to the CSS from which the congenic strains were derived. Then the strain with the next larger segment is compared to the previous congenic strain to test whether the introduced segment affects the phenotype of the strain with the shorter segment. This process is continued sequentially through the panel of congenic strains in an ordered manner. This method, which is based on standard principles of graph theory, makes no assumptions about the number of QTLs, the nature of their interactions, or their penetrance.

Shao et al. (2010) applied the sequential method to a variety of traits in both mouse and rat panels of congenic strains. In every case, unambiguous evidence for QTL effects was found, usually involving multiple genes with strong epistasis and highly variable penetrance. By

contrast, the common segment method failed to detect many of these QTLs. Subsequent genetic studies that have been undertaken in several cases typically validate results of the sequential method (Shao et al. 2008; Yazbek et al. 2010).

Two studies illustrate the merits of congenic strains versus CSS crosses. Matin et al. (1999) used the first autosomal CSS 129-Chr19^{MOLF} (CSS-19) in a cross to map a TGCT susceptibility gene that had been previously mapped to chromosome 19 (Collin et al. 1996). This CSS-19 cross revealed a peak near the middle of the genetic map but with broad confidence limits that spanned most of the chromosome. Subsequently, Youngren et al. (2003) made a panel of 13 single and double congenic strains derived from CSS-19 to determine the number and location of these susceptibility genes. The genetic complexity that this study revealed was remarkable. The five QTLs that were detected showed pervasive epistatic effects depending on the genetic constitution of the congenic strain. Subsequent expression studies with these congenic strains revealed strong candidate genes for several of these QTLs (Zhu et al. 2010). Presumably, the large number of susceptibility genes and their interactions account for the broad QTL localization in the CSS cross.

In addition, two related studies involve diet-induced obesity in B6-Chr6$^{A/J}$ (CSS-6) males (Shao et al. 2008; Burrage et al. 2010). Analysis of a panel of 15 congenic strains revealed at least four QTLs that contribute significantly to diet-induced obesity (see also Yazbek et al. 2011). Each of these QTLs had unexpectedly large phenotypic effects, accounting for, on average, 66–102 % of the difference between the parental strains, depending on the trait. A CSS-6 cross revealed multiple QTLs, but remarkably the portion of the genetic map where no significant evidence was found was precisely the region where the two QTLs with the strongest phenotypic effects were located in the congenic strains. Inspection of the results showed too few mice carried crossovers to provide sufficient independent evidence for these two QTLs (L. C. Burrage and J. H. Nadeau, unpublished). Thus, congenic strains provided definitive evidence for QTLs in intervals that were defined with recombination breakpoints rather than sometimes ambiguous evidence with statistically defined boundaries of the intervals in which they are located.

Congenic–consomic strains

Many kinds of gene interactions can be tested in a highly reproducible way by combining either a single-gene mutation or a QTL in a congenic chromosome segment together with a substituted chromosome. Congenic–consomic strains are readily made if the single-gene mutation and the substituted chromosome are on the same host strain background. (However, for logistical reasons, the mutant gene and the substitution usually involve different chromosomes.) In these cases, a simple intercross between mice carrying the mutant gene or the QTL segment is made to the CSS, and the resulting F1 hybrids are backcrossed to the CSS to rehomozygose the substituted chromosome. The resulting mice are homosomic for the substituted chromo-some and segregate for the mutant gene or congenic segment, thereby providing test and control mice in the same cross. Given the ease of statistically powerful follow-up genetic, mechanistic, and functional studies, congenic–consomic strains are ideal for studying modifier effects and gene interactions.

Another relevant study showed that a primary determinant of late-onset hearing loss in the MSM strain is located on chromosome 17 (Nemoto et al. 2004; Morita et al. 2007). Further genetic dissection with congenic–consomic strains showed that a region in the vicinity of D17Mit119 harbors a susceptibility gene, designated *Ahl3*, for age-related hearing loss-3 (Morita et al. 2007). This study clearly demonstrated that congenic–consomic strains facilitate localization of QTLs without the influence of genetic noise of other chromosomes and the substituted chromosome.

Other examples of studies based on congenic–consomic strains include modifiers of the spectrum and latency of spontaneous tumors in p53-deficient CSS-19 mice (Ochiai et al. 2003), the impact of apslenia on lipid metabolism in CSS-Y strains with the dominant hemimelia *Dh* mutation (Suto 2009), and modifiers of tumor resistance in NF1-, p53-deficient mice (Barrick et al. 2009).

Double CSSs

Combinations of intercrosses and backcrosses can be used to test interactions among multiple substituted chromosomes in the homosomic state on the same host strain background. These strains can also be used to test questions about molecular mechanisms and about functional interactions between particular phenotypes. Examples involve genetic variants on chromosomes 2 and 6 that interact to cause airway hyperresponsiveness in a mouse model of asthma (Ackerman et al. 2005), and genetic variants on chromosomes 11 and 14 that interact in the Nagoya-Shibata-Yasuda model of type 2 diabetes (Babaya et al. 2010).

Gene discovery

Discovery of at least seven genes and other functional genomic elements has been reported with considerable circumstantial if not formal proof. These discoveries include the splicing factor 1 in control of TGCT susceptibility (Zhu et al. 2010); solute receptor *Slc35b4* as a regulator of diet-induced obesity, insulin resistance, and gluconeogenesis (Yazbek et al. 2011); the TNNi3 kinase *Tnni3k*, fucose-1 phosphate guanylyltransferase *Fpgt*, or *H28* genes in control of viral myocarditis (Wiltshire et al. 2011); tomosyn-2 (syntaxin binding protein 5-like, *STXBL5L*) as a negative regulator of insulin secretion (Bhalnagar et al. 2011); lymphocyte antigen 6 complex, locus A *Ly6a* as a regulator of novelty responsiveness (de Jong et al. 2011); transcription factor AP2 alpha *Tcfap2a* in control of voluntary physical activity through a dopaminergic pathway (Yang et al. 2012); a long noncoding RNA *(Rubie)* upstream of Bmp4 in vestibular development (Roberts et al. 2012); and the voltage-gated potassium *Cntnap2* gene in control of diet-induced obesity (Buchner et al. 2012). These early results clearly show the efficacy of gene discovery with CSSs.

Genetic architecture of complex traits in CSSs

Epistasis, nonadditive, and context-dependent effects

The nature of the genetic constitution of CSSs and the way that results are analyzed suggested that QTLs that have additive effects primarily would be readily detected, whereas those with epistatic effects would tend to elude discovery (Nadeau 2001). Typically, interaction tests focus onnonadditive effects in double- versus single-gene variants. However, this test is generally not possible with CSSs, unless double CSSs or congenic–consomic congenics are used. Shao et al. (2008) used several alternative approaches to test for interactions. For example, they noted that for each trait the sum of the signed additive effects, measured in their natural units (rather than variance, see Belknap 2003), should not exceed 100 %. Remarkably, the median cumulative effect for 777 CSS phenotypes was 803 % (range = 164–1,397 %) across more than 40 traits. Interestingly, the median cumulative effect for CSSs with nonsignificant phenotypic differences was also highly nonadditive for the majority of CSS phenotypes. Several other tests for epistasis yielded similar results. Together these results suggest that phenotypic effects tend to be highly non-additive and that epistasis is pervasive. This discovery is consistent with the pervasive nature of modifier and genetic background effects on single-gene mutations (Nadeau 2001).

Another example involves the use of the B6.Chr#^{PWD} CSS panel to probe the evolutionary divergence of the genetic architecture of mandible shape, a classical model of quantitative

genetics (Boell et al. 2011). If the effects of individual QTLs were primarily additive, then relatively fast evolution of the genetic network would be expected because changes in one locus could be compensated by changes in another (Boell et al. 2011). Significant effects were found for all chromosomes except 10 and 11. Moreover, pervasive epistasis was found between loci controlling the shape of the mandible because the sum of individual chromosome effects was several times higher than the difference between parental strains.

Systems properties

CSSs led to many unexpected discoveries about systems properties related to genetically complex traits (Shao et al. 2008; Spiezio et al. 2012; Boell et al. 2011). These discoveries include the following: Too many variants had phenotypic effects that were too large. The average phenotypic effect was ~75 % of the phenotypic difference between the parental strains, across hundreds of CSSs for more than 100 traits. Interestingly, the sum of all signed effects for all CSSs for 40 of 41 traits exceeded 100 %, with a median cumulative effect of 803 % (range = 164–1,397 %). Similar results were found for a separate analysis of significant effects and separately for nonsignificant effects only. The level of plasma cholesterol in mice on a regular diet nicely illustrates nonadditivity.

Another unusual property was that phenotypes in CSS tended to be bounded by traits in the CSS progenitor strains. This is an example of ceiling/floor effects. These physiological boundaries are genetically determined, as demonstrated by the observation that other inbred strains have more extreme phenotypes (phenome.jax.org). Phenotypic effects also show strong directionality, with 92 % of the 342 CSSs that show significant phenotypic effects shifting toward the A/J donor strain. A strong bias was also found among nonsignificant CSSs (66 % of 435 CSSs). Remarkably, these patterns are independent of genetic divergence. A/J and B6 diverged ~100 years ago from a common pool of noninbred stocks, whereas *M. m. musculus* and *M. m. molossinus* diverged more than 500,000 years ago from a common ancestor. The wild-derived strains show fourfold more DNA sequence differences versus the difference between the classical inbred strains. Together, these observations provide an unexpected picture of the genetic and phenotypic architecture of traits, with epistasis buffering strong phenotypic effects, parental genome representing bistable states, and physiological boundaries limiting the range of phenotypic variation, independent of genetic divergence.

Parent-of-origin and transgenerational effects

CSSs are especially useful for detecting parent-of-origin effects and heritable epigenetic changes. In study populations such as segregating crosses and heterogeneous stocks where conventional, parent-of-origin effects and heritable epigenetic changes segregate simultaneously, distinguishing their independent action is extremely difficult. By fixing genetic variants in substituted strains, and with crosses with CSSs and congenic strains where gender of ancestral generations can be precisely controlled, non-Mendelian effects emerge. Parent-of-origin effects have been reported for cardiac response to pressure overload (Barrick et al. 2009), alcohol preference in B6.Chr2$^{A/J}$ mice (Lesscher et al. 2009), open-field activity in B6.Chr1$^{A/J}$ mice (de Mooij-van Malsen et al. 2009), resistance to peripheral nerve sheath tumors in hemizygous Nf1-, p53-deficient B6.Chr19$^{A/J}$ mice (Walrath et al. 2009), and insulin resistance and metabolic hepatocarcinogenesis (Hines et al. 2011).

One of the most exciting recent discoveries in complex trait analysis is evidence for heritable epigenetic changes (Nelson and Nadeau 2010; Daxinger and Whitelaw 2012). Key to many of these discoveries was use of genetically defined mice. By precisely controlling conventional genetic effects with inbred strains and by greatly limiting environmental

variation, phenotypic variation attributable to epigenetic effects has emerged. In these cases, epigenetic changes that arise in one generation, whether from environmental factors or genetic variants, are transmitted through meiosis and gametogenesis to contribute to phenotypic variation in subsequent generations. These discoveries demonstrate that inherited molecules, in addition to DNA sequence differences, control phenotypic variation. Examples of environmental influences include folate effects on variation in coat color in agouti viable A^{vy} mice (Cooney et al. 2002), high-fat-diet effects on pancreatic β-cell function (Ng et al. 2010), and low-protein-diet effects transmitted through the paternal germ lineage (Carone et al. 2010). Genetic variants can also induce heritable epigenetic changes, with examples including white-spotting paramutation effects of some but not all Kit receptor mutants (Rassoulzadegan et al. 2006), various modifier genes such as p53 in the parental generation and the Deadend1 *(Dnd1)* gene in the offspring generation on testicular cancer risk (Lam et al. 2007), paternal Kit ligand effects on testicular cancer risk among wild-type male offspring (Heaney et al. 2008), Cdk9 deficiency on heart development (Wagner et al. 2008), miR-124-Sox9 effects on embryonic and adult growth (Grandjean et al. 2009), susceptibility to diet-induced obesity and eating habits in subcongenic strains derived from B6-Chr6$^{A/J}$ (Yazbek et al. 2010), and paternal Y chromosome effects on daughters in host and CSS-Y strains (Nelson and Nadeau 2010; Kotarska and Styrna 2012). In each case, the same strains that led to these discoveries can also be used as test and control groups to search for the molecular basis for these novel inheritance patterns.

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