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Congenic strains reveal effects of the epilepsy quantitative trait locus, *El2*, separate from other *El* loci

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Abstract. Congenic mouse strains made by transferring epilepsy predisposing alleles *El1, El2,* and *El3* from the EL/Suz strain to the ABP/Le recipient were tested for seizure frequency following gentle rhythmic stimulation. Mice homozygous for *El2,* but not *El1* or *El3,* experienced seizures much more frequently than ABP controls, while respective *El1* homozygotes and *El2* heterozygotes had only a modest increase over ABP, and *El3* homozygotes showed no increase. Association between marker genotypes and seizure frequency in small intra-strain crosses showed that the phenotypic effects of *El2* map to the selected interval, and that segregation of *El2* accounts for virtually all genetic effects. However, in separating *El2* from other EL susceptibility alleles, the seizure frequency phenotype was weaker and less heritable than in crosses between parental strains. These results confirm *El2* as an important QTL and show that it has significant phenotypic effects in the absence of other EL-derived alleles, including *El1.* In addition, the present localization of *El2* on Chr 2 suggests several potential candidate genes for *El2,* including the β subunit of phospholipase-C. The approach to dissecting complex traits by making congenic strains for individual QTL is discussed.

Introduction

Efforts to understand genetic factors that underly multigenic traits require a systematic dissection of the complex array of genetic and environmental factors into separate components. Although there has been much recent technological advancement in mammalian genetics, a simple and directed approach to take with mapped loci is to utilize congenic mouse strains, first developed for the study of histocompatibility in 1948 by George Snell at The Jackson Laboratory (Snell 1948). In essence, a piece of the genome from one mouse strain ("donor") is simply transferred to another strain background ("recipient") by successive backcrosses and selection for phenotype or genotype (for a review, see Flaherty 1981). The resulting congenic strain offers much greater genetic control than two parental strains, as the only effective genetic differences between the congenic and the recipient strain are within the selected segment. Hence, one can study the effects of particular loci that influence a complex trait in isolation from other segregating genetic factors. A recent example of the successful use of congenic strains in this manner is in dissecting out genetic factors that influence insulin-dependent diabetes of the NOD mouse (Leiter 1989; Prins et al. 1993; Serreze et al. 1994).

We are utilizing a congenic strain approach to dissect the inheritance of genetically complex epilepsy in certain mouse strains. The EL/Suz (EL) inbred mouse strain exhibits a form of epilepsy similar to temporal lobe epilepsy in humans (Imaizumi et al. 1959; Seyfried et al. 1992; Suzuki and Nakamoto 1977a, 1977b; Suzuki et al. 1982). In EL mice, seizures can be readily induced with a gentle stimulation protocol, and susceptibility can be measured as a highly heritable, semi-quantitative trait, seizure frequency (Frankel et al. 1995; Fueta et al. 1986; Rise et al. 1991). In crosses of EL with the inbred strain ABP, several EL-derived seizure frequency alleles were previously identified as quantitative trait loci (QTL): *El1* (distal Chr 9), *El2* (Chr 2), *El3* (Chr 10), and recently, *El4* (proximal Chr 9) (Frankel et al. 1995; Rise et al. 1991). In these crosses, it was possible to estimate the genetic location of each QTL, its effect on the trait variance, and also its mode of inheritance. However, the individual characteristics of each locus are likely to be muddied by effects of other QTL. Thus, we sought to separate the known EL-derived susceptibility alleles by transferring the respective chromosomal region onto the ABP strain background, and testing each resultant strain for seizure frequency.

Materials and methods

Mice. The mouse strain EL/Suz was obtained in Fall 1991 from T. Seyfried (Boston College) at $F76^+$ and is maintained as an inbred strain at The Jackson Laboratory by brother-sister matings. ABP/Le mice were obtained from JAX production at F102. ABP females were mated to $F76^+$ EL males, and the resulting F_1 hybrids (males or females) were successively backcrossed to ABP (males or females) to generate congenic strains, as discussed further in Results. Individual mice were identified by an ear marking system. Mice were raised on acid-treated water and fed 96WA grain (Old Guilford) ad libitum, depending on breeding success. All procedures were done according to National Institute of Health (NIH) guidelines for the humane treatment of animals and were approved by the Institutional Animal Care and Use Committee (IACUC). The Jackson Laboratory is fully accredited by the American Association of Accreditation for Laboratory Animal Care (AAALAC).

Genetic marker analysis. Genomic DNA was prepared from mouse tail tips by a modified salt-out procedure as described by Taylor and associates (1993). Tail DNAs were typed by the polymerase chain reaction (PCR) with SSR (simple sequence repeat) markers purchased from Research Genetics, Inc, according to Dietrich and colleagues (1992) with minor modifications (Frankel et al. 1994). Markers were radiolabeled with ^{32}P by direct incorporation into the PCR reaction, and PCR products were electrophoresed on denaturing polyacrylamide gels. Gels were exposed to X-ray film for 4–24 hours to analyze SSLP (simple sequence length polymorphism).

Seizure tests. Mice were weaned from parents at 21 days, males and females housed separately, and, at 30 days of age, mice were subjected to gentle rhythmic tossing (vertical displacement of ~1 cm, 256 cycles per minute), for up to 30 s or until seizure development on a quiet, mechanically driven plastic mouse cage as described recently (Frankel et al. 1994). Mice were scored as either plus or minus for seizure. The qualitative features of seizure in EL mice and ABP mice and criteria for scoring them

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are very similar to those recently described for the epileptic SWXL-4 strain (Frankel et al. 1994). For convenience, multiple cagemates were tested simultaneously. Testing was done once every 3 days for a total of 20 tests, to arrive at the trait "seizure frequency per 20 tests."

QTL analysis. Genotype and trait data were stored and sorted with Excel (Microsoft, Inc). MAPMAKER/QTL was used as previously described for interval mapping (Lander et al. 1987; Paterson et al. 1988). Analyses of variance (ANOVA) were carried out with Statistica 4.1 (StatSoft, Inc.) or Excel.

Results

Construction of congenic strains for El1, El2, El3 and El4 by using marker-assisted selection. The epilepsy loci *El1* and *El2* were defined initially as quantitative trait loci (QTL) that influence seizure frequency in (ABP × EL) F_1 × ABP and various reciprocal backcross progeny (Rise et al. 1991). To develop congenic strains ABP.EL-*El1*^{e} and ABP.EL-*El2*^{e}, (where the "e" allele is derived from the EL strain and "a" from ABP), genetic markers were chosen to readily distinguish ABP from EL, spanning a broad, 30- to 40-cM interval around the most likely position of each QTL. For *El1* on Chr 9, the simple sequence length polymorphisms (SSLP) *D9Mit2, D9Mit4, D9Mit32, D9Mit35,* and *D9Mit16* were chosen as flanking markers. *D9Mit2* and *D9Mit4,* though well centromeric to the suspected location of *El1,* were added to include a new QTL we mapped recently to this interval, *El4* (Frankel et al. 1995). Thus, the five markers should span both *El1* and *El4.* For *El2* on Chr 2, *D2Mit11, D2Mit43, D2Mit30, D2Mit21,* and *D2Mit55* were similarly chosen. In addition, a new QTL on Chr 10, *El3,* noted as an association with *Mpmv26* in Rise and coworkers (1991), and confirmed by us (Frankel et al. 1995) was transferred by use of markers *D10Mit42, D10Mit12,* and *D10Mit14.* To construct congenic strains, (ABP × EL) F_1 hybrids were backcrossed to ABP, and individuals heterozygous for SSLP for one chromosomal interval, but homozygous (ABP) for the other two, were selected separately for repeated backcrossing to ABP. At the fifth backcross generation (N6), individuals heterozygous for *El1* or *El2* were intercrossed, and subsequent progeny homozygous for the EL allele of the selected interval were maintained by brother-sister mating. This resulted in two incipient congenic strains, ABP.EL-*El1*^{e} and ABP.EL-*El2*^{e}. At the N6 generation, these strains should be approximately 98.5% ABP-like ($1/2^N \times 100$), in all unselected intervals. A homozygous ABP.EL-*El3*^{e} strain was not derived until the tenth backcross generation (N11).

The two incipient and one full congenic strains were then tested for sensitivity to epileptic seizures induced by gentle rhythmic stimulation, as previously described (Flavin et al. 1991; Frankel et al. 1994), and compared with our data accumulated for ABP and EL parents, and for (ABP × EL) F_1 hybrids. Seizures exhibited by EL, hybrid, or ABP mice are comprised of a progression of behavioral abnormalities from twitching and forelimb clonus, to loss of postural control, to generalized, tonic-clonic seizures and Straub tail (stiff tail with anterior curvature), and finally post-ictal automatisms such as forepaw grooming and salivation. Although the mean seizure frequencies of ABP and EL strains are very different, qualitatively the seizures are similar. Seizures were scored as all-or-none, with the minimum criterion being the loss of postural control. Seizure frequencies for individual mice were then plotted as frequency distributions (Fig. 1), and differences between genotypic means tested by ANOVA for statistical significance (Table 1). The EL inbred strain was the most sensitive, and the ABP strain the most resistant. As described previously, (ABP × EL) F_1 hybrids were sensitive, but less so than EL. The mean seizure frequency of ABP.EL-*El3*^{e}/*El3*^{e} congenic mice was not significantly different from that of ABP (Fig. 1, Table 1). However, the seizure frequencies of either ABP.EL-*El1*^{e}/*El1*^{e} or ABP.EL-*El2*^{e}/*El2*^{e} mice were significantly higher than those of ABP, and differed from each other as well (Table 1). Seizure frequencies of F1 hybrids between ABP and ABP.EL-*El1* (That is, *El1*^{e}/*El1*^{a} or heterozygous genotype) were not different from ABP, although *El2* heterozygotes exhibited a marginal increase, suggesting an additive mode of inheritance.

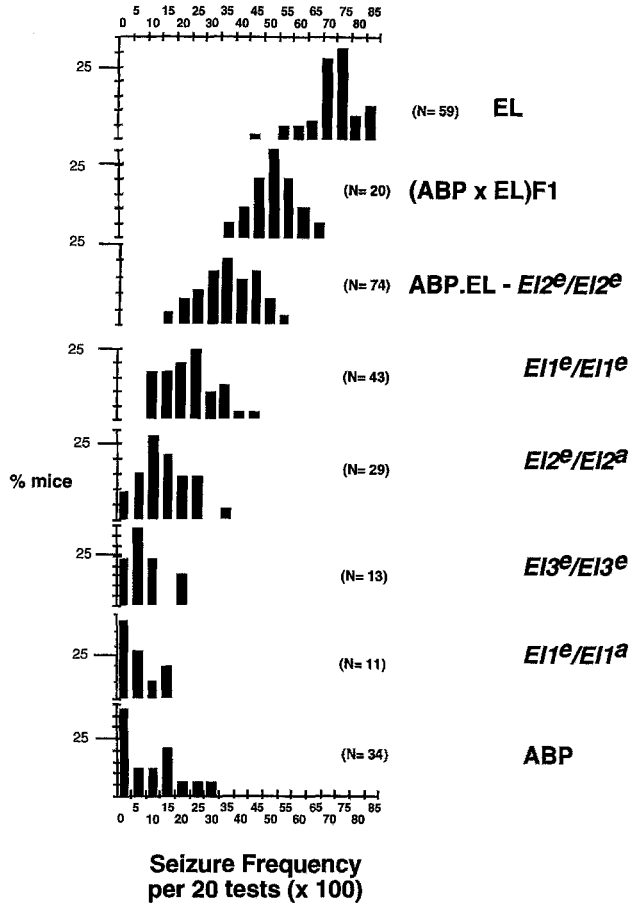

Fig. 1. Seizure frequency distribution of ABP.EL congenic and parental strains. Histograms show the distribution of seizure frequency per 20 tests (×100) on the X-axis, the percentage of mice on the Y axis (25% shown), and the sample sizes of each group (N). Group means are given in Table 1. The *El1* congenic also contains *El4.*

Segregation of El2 in a backcross and intercross. To confirm that the higher seizure frequency of the ABP.EL-*El2*^{e} congenic strain maps to the selected interval on Chr 2, 39 (ABP.EL-*El2*^{e} × ABP) F_1 × ABP.EL-*El2*^{e} backcross progeny were seizure tested and genotyped. In addition, the "full" *El2* congenic strain (N10F2 or higher) was similarly tested by analyzing 19 progeny from intercrosses between heterozygotes. The frequency distributions of the crosses and parents are shown in Fig. 2. Chr 2 markers *D2Mit11, D2Mit30,* and *D2Mit55* were then typed in all cross progeny, and the intervening markers *D2Mit43, D2Mit106, D2Mit258, D2Mit259,* and *D2Mit21* were typed in progeny that contained obligate crossover events. The inheritance of EL versus ABP alleles of these markers was then examined for association with seizure frequency by interval mapping, by use of MAPMAKER/QTL, in each cross (Fig. 2). In the 39 backcross progeny, the maximum LOD score was 2.6, between *D2Mit258* and *D2Mit259.* In the 19 intercross progeny, the maximum LOD score was 4.0, near *D2Mit106* and *D2Mit258.* In the intercross where additivity could be examined, the most likely mode of inheritance was recessive, followed by additivity (ΔLOD 1.6), then dominance

Table 1. Seizure frequency differences between *El* locus genotypes[a]

Genotype	N	Mean ± SD	*p* (ANOVA) ABP	*El1*^{e}/*El1*^{e}	*El1*^{e}/*El1*^{a}	*El2*^{e}/*El2*^{e}	*El2*^{e}/*El2*^{a}
ABP	34	0.09 ± 0.10					
El1^e/*El1*^{e}	43	0.22 ± 0.09	2.77×10^{-8}	–			
El1^e/*El1*^{a}	11	0.05 ± 0.06	(.17)	1.53×10^{-7}	–		
El2^e/*El2*^{e}	74	0.35 ± 0.10	5.22×10^{-23}	3.44×10^{-10}	2.10×10^{-15}	–	
El2^e/*El2*^{a}	34	0.13 ± 0.08	(.11)	8.23×10^{-6}	0.007	5.77×10^{-20}	
El3^e/*El3*^{e}	13	0.07 ± 0.07	(.50)	7.65×10^{-7}	(.38)	2.35×10^{-15}	(0.04)
EL	59	0.72 ± 0.08					
(ABP × EL)F1	20	0.50 ± 0.07					

p values in parentheses are not significant.

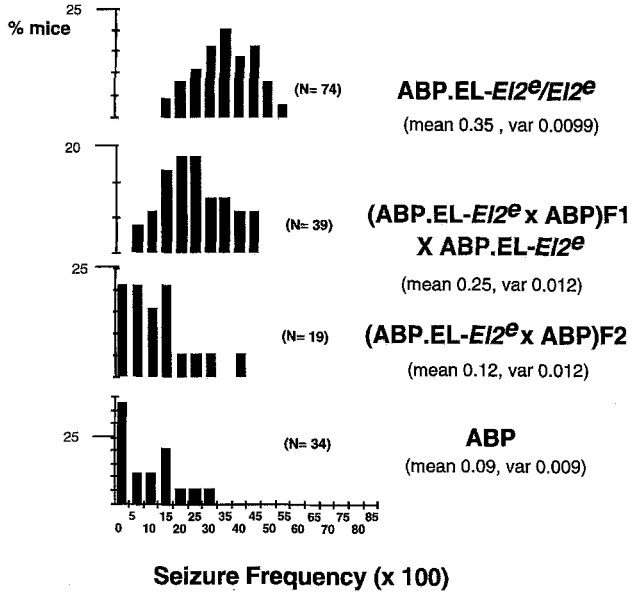

Fig. 2. Seizure frequency distribution crosses between ABP.EL-*El2* and ABP strains, compared with parents. As in Fig. 1. Also shown are the mean seizure frequencies and the total variance. To estimate heritability, the environmental variance, V_E (from *El2*^{a}/*El2*^{a}, *El2*^{e}/*El2*^{a}, and *El2*^{e}/*El2*^{e} populations), was subtracted from total variance V_T (from backcross or intercross) and divided by the total variance. V_E was 0.008. Heritability was estimated to be 0.29.

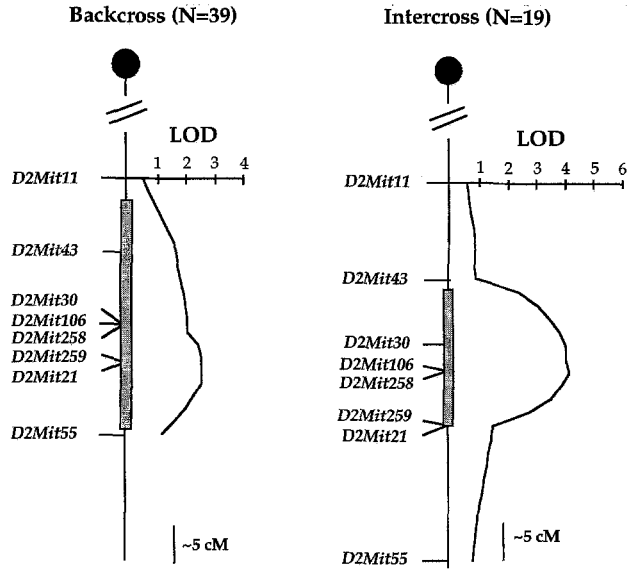

Fig. 3. Association between Chr 2 markers and seizure frequency in crosses of the ABP.EL-*El2* strain with ABP. Genetic map of Chr 2, with map distances in cM. Likelihood curves for *El2* were generated by MAPMAKER/QTL. *D2Mit11, D2Mit30,* and *D2Mit55* were typed in all backcross and intercross progeny, recombinants were identified, and the remaining markers were typed only in recombinants (non-recombinant genotypes inferred for others). 2-LOD confidence intervals for the location of *El2* are shown by a thick bar. Probabilities (ANOVA) for association between seizure frequency and individual marker genotypes from the crosses combined were: *D2Mit11*, $p < 0.003$; *D2Mit43*, $p < 0.003$; *D2Mit30*, $p < 0.00004$; *D2Mit106*, $p < 0.00003$; *D2Mit258*, $p < 0.00003$; *D2Mit259*, $p < 0.00008$; *D2Mit21*, $p < 0.00008$; *D2Mit55*, $p < 0.003$.

(ΔLOD 3.6). We note that the significance level need not be as stringent compared with genome-wide QTL searches (Lander and Schork 1994), because our congenic analysis is restricted to a portion of Chr 2. Nevertheless, higher probabilities were obtained with ANOVA for marker genotypes in both crosses combined, peaking at $p < 0.00003$ for *D2Mit106* and *D2Mit258* (Fig. 3 legend). The higher association in the intercross compared with the backcross, the rejection of dominance within the intercross, and the original mapping of *El2* as a dominant or additive factor are all supportive of the additive mode of inheritance deduced from Fig. 1. By estimating the environmental component of variance as an average from *El2*^{a}/*El2*^{a}, *El2*^{a}/*El2*^{e}, and *El2*^{e}/*El2*^{e} populations (0.008), the overall heritability of seizure frequency in either cross was estimated to be 0.29. The total variance fraction explained by *El2* in the backcross to the incipient congenic strain was 0.27, or 93% of the genetic effect. The total variance fraction explained by *El2* in the intercross (utilizing the full congenic) was 0.63, or virtually 100% of the genetic effect (that the actual genetic variance estimated as >100% is an artifact of the small sample size and indirect means by which the environmental variance is estimated). Thus, it seems reasonable to conclude that *El2* accounts, as expected, for most, if not all of the genetic contribution to seizure frequency in these crosses.

Discussion

Our approach to dissecting the genetically complex EL mouse model for epilepsy has been to select for a chromosomal genotype that spans the most likely position of a particular QTL and to separate it from unlinked QTL by successively backcrossing the susceptible allele to a relatively resistant strain background. Because QTL that underly seizure frequency in the EL and similar models depend heavily on genetic background (Frankel et al. 1994; Paigen 1995), it was necessary to use the same partner strain as used to define the QTL initially. Thus, we have bred the EL alleles of *El1* (distal Chr 9, along with the provisional *El4*, on proximal Chr 9), *El2* (Chr 2), and *El3* (Chr 10), which were defined initially in backcross or intercross progeny between EL and ABP, into the ABP strain. In the original backcross to ABP, *El2* had the largest association and effect, followed by *El1* and *El3*, respectively (Frankel et al. 1995; Rise et al. 1991). Together in the backcross, these loci accounted for most genetic effects. In the (ABP × EL) F_2 intercross, however, only *El3* and the provisional *El4* had large effects, with little effect from the presumed location of *El1* and *El2*

(Frankel et al. 1995). We hypothesized in a recent study that the expression of *El1* and *El2* requires the presence of several recessive modifier alleles from ABP; such modifiers would have been better represented in a backcross than in an intercross population (Frankel et al. 1995). Our results here for *El2,* which has significant effects on a homozygous ABP background, confirm that it was "real" and at the same time support our hypothesis invoking epistasis with recessive, ABP modifier loci. Presumably the lesser effects of one copy of the $El2^e$ allele here compared with the original backcross reflect additional interaction with EL-derived alleles. Finally, the comparatively higher mean seizure frequency of ABP.EL-*El2* compared with ABP.EL-*El1* congenic strain is consistent with the relative effects of these two loci in the original backcross.

It was fortunate that *El2* had measurable effects separate from other known epilepsy QTL of EL origin, though perhaps not surprising in view of the large effect of *El2* in the original backcross. Nevertheless, the low mean seizure frequency of ABP.EL-*El2* mice (35%, compared with 51% for F_1 hybrids, and 72% for EL itself) has clearly reduced the reliability of the phenotype so that the within-strain variance, which cannot be due to genetic effects, is similar to that of inter-strain crosses which include genetic effects. Nevertheless, *El2* seems to explain virtually all of the genetic effects in congenic strain crosses. Also, from our results it is clear that restricting the genetic variance to a single QTL does not necessarily narrow the location of the gene; the confidence interval for the location of *El2* is still about 20 cM (Fig. 3). Because of the additive effect and the large nongenetic contribution to variance, it would be difficult to determine which individual recombinants in backcross or intercross mice are predictive for the location of *El2;* this shortcoming would ultimately hold true even for larger crosses. Thus, our strategy is not simply to increase sample size, but to select cross progeny that contain specific recombinants, to breed each to homozygosity, and to test each recombinant in larger groups of isogenic mice. Thus, by comparing phenotypes of recombinants we should be able to delimit the location of *El2* to successively smaller regions. This approach should also address whether *El2* is a single gene or two or more closely linked genes—alternatives which cannot be distinguished presently.

One strategy for improving the heritability of the seizure phenotype in this congenic strain may be to analyze the segregation of *El2* on a background that is fixed for other EL susceptibility alleles. This approach is consistent with our long-term goal of learning how several QTL contribute to a high seizure frequency, and we are in the process of testing multiple ("double" and "triple") congenic strains to determine if seizure frequency is higher/more reproducible than that of the *El2* congenic strain. In addition, it may be of value to seek a "sub-phenotype" for which *El2* is uniquely responsible. Although this approach seems to work for complex traits that are manifest at the systemic level, such as insulin-dependent diabetes (Leiter 1989; Serreze et al. 1994), it could be more difficult for a complex, central nervous system disorder such as that exhibited by EL mice. Finally, it might be possible to find other means of inducing seizure differences between the strain pairs, for example by chemical means; in addition to giving a more sensitive assay, a directed chemical induction approach might give hints as to the specific role of *El2* in increasing seizure frequency.

There are several possible candidate genes near the confidence interval of *El2.* One candidate is *Slc1a3,* a high-affinity glutamate transporter that is expressed in glia (Kirschner et al. 1994). Although this locus was suggested previously as a candidate for *El2,* we note that the map position for *Slc1a3* is nearer *D2Mit43* and not the likelihood peak. Mapping nearer the likelihood peak is *Snap25,* a 25-kDa protein expressed in various neuronal populations correlating with the onset of synaptogenesis during development (Hess et al. 1992) However, sequence of the coding region revealed no differences between EL and ABP, and Northern blot analysis showed no gross differences in expression (unpublished results). Also near the likelihood peak is *Plcb,* encoding the β-subunit of phospholipase-C, an important signaling molecule (Hess et al. 1992). *Plcb* may be of particular interest because of recent results in knockout mice implicating the role of serotinergic (5-HT) neurotransmission in seizure suppression (Tecott et al. 1995), possibly in the EL model itself (Frankel et al. 1995) and also in that 5-HT2 receptors are coupled to phospholipase-C β (Julius et al. 1990). However, additional candidates include *Pcsk2,* a neuroendocrine convertase, and several neuropeptide genes including prodynorphin (Siracusa and Abbot 1994). Given this abundance of potential candidates and that many genes have yet to be discovered, coupled with the difficulty of testing candidates in a system for which there is no coisogenic control strain, sporadic cases, or simple means by which to test alleles in various other inbred strains (because of the genetic complexities), we will endeavor to systematically eliminate these as candidates for *El2* with individual recombinant strains. The congenic strain pairs may also serve as subjects for differential gene expression approaches to identifying new candidates for *El2.*

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