Influence of a Quantitative Trait Locus on Mouse Chromosome 19 to the Light-Adapted Electroretinogram

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Purpose. Both implicit time and amplitude of the cone-mediated electroretinographic (ERG) b-wave differ significantly between the C57BL/6JOlaHsd and 129S2/SvHsd inbred mouse strains. The purpose of this work was to undertake a quantitative genetics study to localize the gene or genes involved.

Methods. Implicit time and amplitude of the a- and b-waves of the single-flash and flicker cone-mediated ERG were recorded as the quantitative traits in reciprocal backcrossed populations. A genome-wide scan was performed with 106 polymorphic markers. Map Manager (release QTXb20) was used to analyze the data and make phenotype–genotype correlations.

Results. A quantitative trait locus (QTL) of major effect in controlling variation in both implicit time and amplitude of the cone-mediated ERG localized to the middle of chromosome 19.

Conclusions. Mapping of a QTL influencing both implicit time and b-wave amplitude of the light-adapted ERG represents an initial step toward identifying the gene(s) responsible for this phenotypic variation. (*Invest Ophthalmol Vis Sci.* 2008;49: 4058–4063) DOI:10.1167/iovs.07-1620

Electroretinography (ERG) is a long-established diagnostic tool that has been invaluable in the study of normal retinal function and retinal disease. Light-induced changes in retinal electrolyte concentrations (potassium, sodium, and chloride ions) result in the generation of multiple electrical currents that are conducted through the vitreous, lens, and aqueous and may be detected on the corneal surface.[1] Data from experimental work involving intraretinal and intracellular recording of light-induced currents, as well as pharmacologic,[2,3] and more recently, genetic ablation[4] of putative cellular and biochemical contributors to the mass response have expanded our knowledge of the possible sites of origin of the various components of the electroretinogram.

Manipulation of the subject (dark- or light-adapted) and/or of the stimulus (e.g., flash intensity, flash wavelength, and flicker frequency) allows isolation of ERG responses arising predominantly from the rod or cone photoreceptors. Cone-dominated responses are measurable in a light-adapted retina. In both the single-flash and flicker cone ERG, cone phototransduction and postphotoreceptor OFF-bipolar cells contribute to the light-adapted a-wave[5,6] and postphotoreceptor ON-bipolar cells contribute significantly to the light-adapted b-wave.[7] The b-wave of the cone ERG response is largely determined by inputs from ON-center bipolar cells, but this influence is opposed by OFF-center bipolar cells—the so-called push–pull hypothesis.[8,9]

Marmor suggested that cone b-wave implicit time may have prognostic significance.[10] Patients with a diagnosis of retinitis pigmentosa (RP) and normal cone b-wave implicit time, termed delimited RP, had a better long-term visual outlook than did those with delayed cone implicit time.

Natural variation in the ERG, between different mouse strains and between albino and pigmented mice, has been reported.[11,12] However, to date, no studies have reported on genetic parameters that influence the ERG waveform. We report the first example of a quantitative trait-mapping study designed to examine variation observed in the cone ERG between two pigmented strains of inbred mice: C57BL/6JOlaHsd and 129S2/SvHsd. The observed differences comprise a delay in the implicit time of the cone a- and b-waves and an increase in the amplitude of the cone b-wave in 129S2/SvHsd compared with C57BL/6JOlaHsd. A search was performed for quantitative trait loci (QTLs). A QTL is a region of the genome containing a gene, or genes that influences the phenotype. Because the cone b-wave is thought to be a result of processing by second-order retinal neurons, specifically ON-bipolar cells, identifying the source of such variation is of intrinsic scientific interest, as it may lead to the identification of novel genes or pathways involved in the processing of the visual signal at this level.

Materials and Methods

Mice

All procedures involving mice were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, approved by the Trinity College Dublin ethics committee, and performed under license. C57BL/6JOlaHsd (B6) and 129S2/SvHsd(129) mice (Harlan UK, Oxon, UK) were bred on site for several generations before this study. Henceforth, we will refer to these strains as B6 and 129. All mice were kept in a 12-hour light–12-hour dark cycle, with an ambient room temperature between 19°C and 22°C. For this quantitative study, reciprocal backcrosses were performed, with F1 hybrid animals backcrossed to either the B6 or 129 strains. An initial comparison of parental and F1 mice showed that B6 was dominant for most traits of the ERG, suggesting that an F1 × 129 backcross would be more statistically powerful and require fewer animals.[13] An F1xB6 backcross was performed to identify potentially additive or dominant 129 alleles. The total number of B6, 129, and F1 mice analyzed for this

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study was 29, 34, and 52, respectively. For the backcrosses, 138 F1xB6 mice and 166 F1x129 mice were produced.

Quantitative Traits

Amplitudes and implicit times of the light-adapted electroretinogram (ERG) were the quantitative traits measured for this study. In total, eight parameters of the 0.5-Hz flash cone (called single flash) and 10-Hz flicker (called flicker) light-adapted ERG responses were analyzed. Specifically, these eight parameters were single-flash a-wave implicit time, a-wave amplitude, b-wave implicit time, and b-wave amplitude and flicker a-wave implicit time, a-wave amplitude, b-wave implicit time, and b-wave amplitude.

Electroretinography

We used a previously published ERG protocol[14] that followed standard ISCEV protocols for humans[15] adapted for use in mice.

Mouse Genomic DNA

Genomic DNAs were extracted from 0.5-cm tail snips by a standard method.[16] DNA concentration was determined by using spectrophotometry and diluted to 200 ng/μL.

Genotyping

Selective genotyping was performed on backcross progeny of high ($n = 10$) and low ($n = 10$) phenotypic extremes, with the single-flash cone b-wave implicit time trait used to select these groups. We chose this trait as there was a highly significant difference in single-flash b-wave implicit time between the two strains (Table 1). We did not choose an amplitude trait, as these were more likely to be influenced by environmental traits.[17]This screening was performed to reduce sample size, cost, and time and to increase statistical power.[18] A genome scan was performed with markers spaced at no more than 30 cM apart along each chromosome and at most 15 cM from centromeres and telomeres. Exceptions to this rule were the most proximal markers on Chr 2 (27.5 cM), Chr 7 (26 cM), Chr 9 (21 cM), Chr 10 (20 cM), and Chr 18 (20 cM) and the most distal markers on Chr 1 (21 cM), Chr 7 (21 cM), Chr 14 (21 cM), and Chr 15 (19 cM). One hundred twelve SNP markers were genotyped commercially (Kbioscience, Herts, UK) with SNP detection assays (Amplifluor, Ashby de la Zouch, UK) from a published panel of informative markers[19,20]; 86 were informative. In addition, 19 informative microsatellite markers were genotyped in-house. Additional genotyping was performed, using all backcrossed DNAs to verify any suggestive linkage. Two additional microsatellite markers (*D19Mit88* and *D19Mit1*) were genotyped within a QTL on Chr 19 in all backcrossed DNAs. All informative markers used in the genome scan are available on request.

Data Analysis

The quantitative trait-mapping program Map Manager QTXb20 (http://www.mapmanager.org)[21,22] was used to detect and localize QTLs by comparing phenotype (trait) and genotype data, to determine whether there was an association between the two. Flanking marker analyses are performed for detection of QTLs by the least-squares method.[23,24] The program estimates the size (in centimorgans) of a 95% confidence interval (CI) for each QTL with a standard method,[25] and "add," the additive regression coefficient. For a backcross, regression is performed according to an additive model, which fits a single regression coefficient for the additive component, by using the least squares method[23,24] to estimate the coefficients of a linear regression. Each backcross was treated as a separate experiment. For every ERG trait examined, marker regression and interaction testing was performed with DNA samples from the 20 mice selectively genotyped for the single flash ERG b-wave delay in each backcross. Any markers showing suggestive or significant association with traits in the selectively genotyped set were genotyped in the entire backcrossed population. Localization of the QTL was performed using simple interval mapping. Bootstrap resampling,[26] involving resampling (with replacement) of multiple pseudoreplicate datasets, was used to estimate CIs.

For analysis of additional Chr 19 data, empiric genome-wide permutation tests could not be used as a test of significance. Instead, prior-determined genome backcross values were used[27] to determine the logarithm (base 10) of odds (LOD), a statistical test used for linkage analysis, where suggestive LOD $\geq$ 1.9, significant LOD $\geq$ 3.3, highly significant LOD $\geq$ 5.4. Suggestive means that for every three quantitative genetics backcross studies, one QTL of 1.9 would occur by chance; significant means that only one QTL of LOD 3.3 would occur by chance in 20 such studies; and highly significant means that only 1 QTL in 1000 studies would have a LOD score of 5.4 by chance. The Map Manager program provides a likelihood ratio statistic (LRS) that can be converted to a LOD score by dividing by 4.6 (twice the natural logarithm of 10).

In addition, differences in pigmentation (coat color), gender, and Y-chromosome genotype (in males) were analyzed (Datadesk 6.0; Data Description, Inc., Ithaca, NY), with an unpaired two-sample *t*-test, to see whether they have an effect on the light-adapted ERG. Boxplots were plotted for all traits, to ensure normal distribution of data (data not shown).

Table 1. Differences between B6, 129, and F1 Mice in the Light-Adapted ERG

Trait	129S2/Sv Hsd	C57BL/6J OlaHsd	*P** 129 vs. B6	F1	*P** B6 vs. F1	*P** 129 vs. F1	129 Backcross	B6 Backcross
0.5 Hz-a-ms†	22.0 ± 4.1 ms	20.1 ± 1.4 ms	0.0161	20.7 ± 2.3 ms	NS	NS	23.6 ± 3.2 ms	20.7 ± 1.9 ms
0.5 Hz-a-μV‡	3.4 ± 2.2 μV	6.8 ± 2.4 μV	≤0.0001	6.1 ± 2.5 μV	NS	≤0.00001	6.4 ± 4.4 μV	8.1 ± 3.2 μV
0.5 Hz-b-ms†	59.7 ± 4.6 ms	51.3 ± 4.7 ms	≤0.0001	51.6 ± 3.9 ms	NS	≤0.00001	58.8 ± 5.2 ms	50.9 ± 3.8 ms
0.5 Hz-b-μV‡	69.6 ± 10.3 μV	62.4 ± 23 μV	0.13	79.3 ± 15.8 μV	0.0017	0.0051	78.1 ± 21 μV	78 ± 16.5 μV
10 Hz-a-ms†	25 ± 4.4 ms	21.3 ± 1.7 ms	≤0.0001	21.7 ± 1.7 ms	NS	0.0003	24. ± 3 ms	21.6 ± 1.6 ms
10 Hz-a-μV‡	5.1 ± 3.9 μV	6.9 ± 2.3 μV	0.0291	6.2 ± 2.4 μV	NS	NS	8.6 ± 3.8 μV	9.5 ± 3.8 μV
10 Hz-b-ms†	57.7 ± 5 ms	46.6 ± 8.1 ms	≤0.0001	47.9 ± 3.3 ms	NS	≤0.00001	53.3 ± 4.9 ms	46.8 ± 3.5 ms
10 Hz-b-μV‡	30.3 ± 5.2 μV	44 ± 17.9 μV	0.0004	49.6 ± 11.7 μV	NS	≤0.00001	43.1 ± 15.5 μV	56.9 ± 12.2 μV

The means and standard deviations of eight traits of the light-adapted ERG: single-flash cone (0.5 Hz) and flicker (10 Hz).

* The corresponding probability for 0.5 Hz b-wave amplitude for 129 vs. F1 is $P = 0.0051$. This shows that while B6 and 129 wild-type values for single-flash b-wave amplitude do not differ significantly from one another, the wild-type strains both differ significantly from F1 mice. The means and standard deviations for the 129 backcross population ($n = 166$) and the B6 backcross population ($n = 138$) are given so that comparisons can be made.

† The a- or b-wave implicit time (in milliseconds).

‡ The a- or b-wave amplitude (in microvolts) in C57BL/6JOlaHsd ($n = 29$) and 129S2/SvHsd ($n = 34$) strains, together with an associated probability, generated by performing an independent two-sample *t*-test to compare B6 animals with 129 animals and then to compare wild-type animals with F1 animals. For the 129-F1 and B6-F1 comparisons, equal numbers of F1 animals were needed, to prevent bias ($n = 34$ 129 mice; $n = 29$ B6 mice; and $n = 34$ or $n = 29$ F1 mice).

RESULTS

Phenotypic Variation in the Mouse Light-Adapted Electroretinogram

We observed that the light-adapted ERG varied substantially between B6 and 129 mice. In both the single-flash and flicker light-adapted ERGs, the a- and b-waves had increased implicit times, and the amplitude of the b-wave was greater in the 129 strain than in the B6 strain (Fig. 1). A two-sample *t*-test performed with ERG traits in wild-type B6 and 129 mice (Table 1) showed statistically significant differences.

Contribution of Alleles in the 129 Strain to Light-Adapted ERG

The F1 ERG implicit time traits were not significantly different from those of the B6 strain, showing that the B6 allele(s) governing implicit time are dominant to the 129 allele(s). This finding was true of the single-flash a-, flicker a-, and flicker b-wave amplitudes (Table 1). The backcross design of the experiment means that additive and dominant effects cannot be extracted. As there was no significant phenotypic variation between B6 and F1 mice in a- or b-wave implicit time and the three amplitude characteristics just mentioned, only those animals backcrossed to the 129 strain were used for all further analyses of these traits. For the eighth trait, Figure 1 shows that the single-flash b-wave amplitude was greater in the F1 offspring than in either the 129 or B6 parental strain.

Identification of a QTL on Chr 19 in the [B6×129]-F1×129 Backcross

A genome scan was performed in 20 animals of the 129 backcross selected for the shortest and the longest implicit times[10] for the single-flash cone b-wave ERG implicit time trait. Genome scan analysis with marker regression over the entire mouse genome identified three peaks of high LOD score: one each on chromosomes 19 (significant), 9 and 17 (not significant; data not shown). The genome scan was repeated in 20 animals for all the traits of the light-adapted ERG. Five of the light-adapted ERG traits (flicker cone a-wave implicit time, and both single-flash and flicker b-wave implicit times and amplitudes) showed an association with *D19Mit10* at 47 cM on Chr 19, two of which (both b-wave amplitudes) showed a highly significant association. The same five light-adapted ERG traits showed an association with a neighboring marker, rs3023517 at 57 cM on Chr 19. The only other marker associations across the genome were on chromosomes 4 (rs3667635 at 22 cM, suggestive association to flicker a-wave implicit time) and 17 (rs4231722 at 56 cM, suggestive association to flicker b-wave implicit time). However, the Chr 4 and 17 associations disappeared when all 166 progeny of the 129 backcross were analyzed. A second set of marker regression analyses was performed, by masking the most significant primary marker association (in most cases, *D19Mit10* on Chr 19) and repeating the analysis. In these cases, both single-flash and flicker b-wave amplitudes showed suggestive marker associations with the Synuclein alpha locus (*Snca*) at 29 cM on chromosome 6. The flicker a-wave implicit time showed an additional suggestive association with a marker on chromosome 13 at 44 cM (rs3693589). However, none of these associations remained when all 166 mice of the 129 backcross were genotyped and analyzed.

Several additional comparisons were performed to see whether they had an effect on the light-adapted ERG. Neither pigmentation (coat color) nor Y-chromosome genotype had an effect on these traits. Of interest, gender did make a difference, with male mice in the 129 backcross having an approximately 2 ms faster single-flash b-wave than their female counterparts ($P = 0.018$). Despite this, no suggestive or significant marker associations were made to either the X- or Y-chromosome.

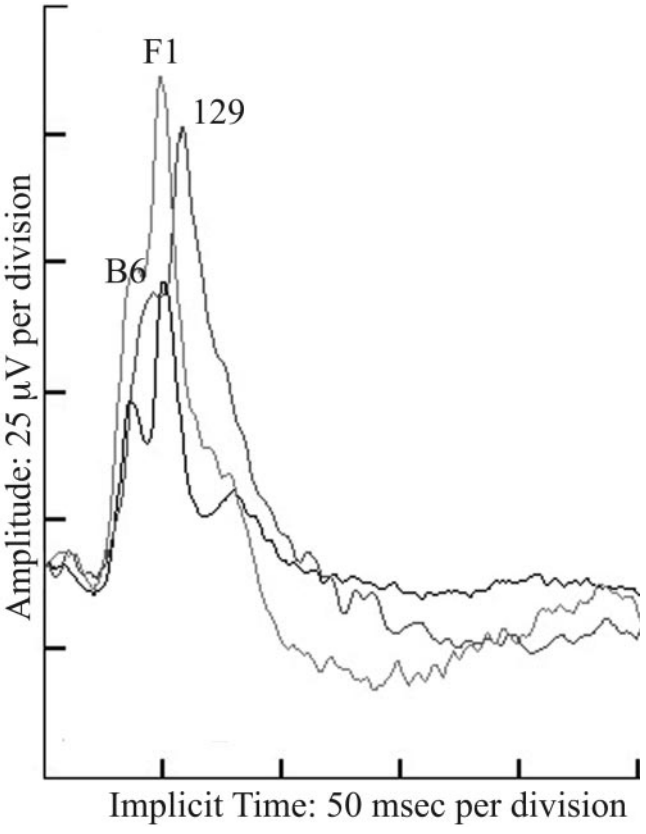

FIGURE 1. Representative light-adapted, single-flash ERG in a B6, 129, and heterozygote F1 mouse.

Confirmation of a QTL on Chr 19

After all suggestive, significant, or highly significant marker associations in the genome scan were genotyped through the full population of 166 mice backcrossed to the 129 strain, the only marker association to remain was the QTL on Chr 19. Two additional markers were genotyped on Chr 19, and marker regression was performed. The maximum LOD score for each trait is shown in Table 2. The highest marker association was still at *D19Mit10*, where LOD scores varied from 30.3 to 9.3, depending on the light-mediated ERG trait examined. Table 2 also gives a percentage value, which is the amount of phenotypic variation explained by genetic variation, in this case between 57% and 24%. CI is the estimate of the size (in cM) of a 95% CI for a QTL of this strength and significance, calculated by a standard method.[25] "Add"(itive) is the effect on the mean of having a second 129 allele at this marker (i.e., homozygous 129 vs. heterozygous). For example, in the single-flash b-wave implicit time, a second 129 allele at *D19Mit10* increases the implicit time by 7.18 ms. The maximum marker association was observed at *D19Mit10* for five of the eight light-adapted ERG traits and at *D19Mit63* (33 cM) for flicker a-wave implicit time. No marker associations were observed for either single-flash or flicker a-wave amplitude.

Interval Mapping of a QTL on Chr 19

The localization of a QTL can be undertaken by using simple interval mapping, which involves an estimate of the genetic map, based on the number of recombinations, to locate a QTL and estimate its effects. It is possible to travel through an

TABLE 2. Chr 19 Marker Associations in a 129 Backcross Studying the Light-Adapted ERG

Chromosome	Marker	LOD	%	CI (cM)	Add	Trait
19 (47 cM)	D19Mit10	30.3	57	6	−22.7 μV	10 Hz-b-μV*
		22.9	47	7	−29.97 μV	0.5 Hz-b-μV*
		20.5	43	7	+7.18 ms	0.5 Hz-b-ms†
		22.9	47	7	+7.72 ms	10 Hz-b-ms†
		9.3	24	13	+3.23 ms	0.5 Hz-a-ms†
19 (33 cM)	D19Mit63	16.8	37	9	+3.62 ms	10 Hz-a-ms†

Marker regression for chromosome 19 only, using 166 mice from the 129 backcross [B6×129]F1×129 and five polymorphic markers. Max LOD scores and the markers at which they were recorded are shown. There were no significant associations for either single-flash or flicker a-wave amplitude. %, percentage that a QTL surrounding this marker contributes to variation. Add, the actual amount (in milliseconds or microvolts) that a gene or genes within the QTL contributes to the trait (for example, the presence of two 129 alleles within this QTL decreases flicker b-wave amplitude by 22.7 μV). CI, the estimate of the size (in centimorgans) of a 95% CI for a QTL of this strength and significance by a standard method.[25]

* The a- or b-wave amplitude (microvolts); 0.5 Hz = single flash and 10 Hz = flicker.

† The a- or b-wave implicit time (milliseconds).

interval (between markers) at 1-cM increments and fit a regression equation for a hypothetical QTL at the position of each cM using the outline of the markers for which there is data. Interval mapping was performed for all six light-adapted ERG traits for which there was a marker association on Chr 19. Results are presented for single-flash and flicker a-wave implicit times (Figs. 2a, 2b), b-wave implicit time (Figs. 2c, 2d), and b-wave amplitude (Figs. 2e, 2f). All interval maps showed that the maximum LOD score (solid line) occurred between *D19Mit63* (33 cM or 36.1 Mbp) and *D19Mit1* (52 cM or 54.9 Mbp), with *D19Mit10* (47 cM) at the peak, as validated by bootstrap resampling (histogram); the added effect of 129 alleles is shown by the dotted line. Thus, the 129 alleles increased the implicit time between stimulus and the a- and b-waves and decreased the b-wave amplitudes.

DISCUSSION

B6 and 129 inbred mouse strains are commonly used in eye research, particularly in the generation of transgenic, knockout, and knock-in mice. These strains are often used as control subjects in electroretinography experiments when examining retinal function in transgenic mice. An observation of phenotypic variation in the ERG between these strains presented an ideal opportunity to elucidate genetic parameters that cause such variation.

We observed strain-specific variation in ERGs between B6 and 129 mice (Table 1; Fig. 1). 129 mice exhibited slower cone a- and b-wave single-flash and flicker implicit times and decreased b-wave flicker amplitude than did B6 mice. The net effect of B6 alleles was dominant for all implicit time traits of the cone ERG (Table 1), and analysis of progeny from an F1xB6 backcross showed no evidence for a QTL (data not shown). Thus, only the 129 backcross could be used for analysis of implicit time traits. One exception occurred: When heterozygote F1 mice were analyzed for the single-flash b-wave amplitude trait, the mean of the heterozygote was found to fall outside the range of the B6 and 129 homozygote parental strains. When the heterozygote lies outside the range of the respective homozygotes, it is described as "overdominance."[28] Overdominance, also known as heterozygote advantage, is a condition in which the phenotype of the heterozygote is fitter than the phenotype of either homozygote. This cannot be analyzed well in quantitative trait programs, because the effect of one B6 plus one 129 allele is significantly different from the effect of homozygous B6 and 129 alleles. A comparison of genotype and phenotype, to find significant marker associations in the 129 backcross, identified a region of Chr 19 as being significantly associated with the cone single-flash b-wave implicit time (LOD = 4.0) in the selectively genotyped sample. When this study was expanded to look at the other traits of the cone ERG in the entire population of mice backcrossed to the 129 strain (n = 166), the presence of a highly significant QTL of major effect on Chr 19 was confirmed for five traits, with LOD scores ranging from 9.3 to 30.3. None of the marker associations on other chromosomes was confirmed when the entire population was examined, suggesting that most of the variation seen between these strains in the cone ERG is due to differences in a gene or genes within the Chr 19 QTL.

As a reduction in b-wave amplitude can indicate a defect in synaptic transmission between photoreceptor and bipolar cells, one hypothesis is that the gene(s) responsible for the observed variability is (are) involved in this aspect of retinal neurotransmission. Realistically, polymorphisms in any gene involved in the photoreceptor presynaptic or ON-bipolar postsynaptic terminals could explain variation between these two strains of mice. Genes involved in lateral inhibition, negative feedback loops, and cone photoreceptor and ON-bipolar homeostasis can also be considered candidates.

The QTL interval on Chr 19 contains none of the classes of genes, such as GABA, glutamate, AMPA, or kainate receptors, expressed in the synaptic terminal, which may explain the variation in the cone ERG. A functional assessment was made of the ~150 genes within the Chr 19 QTL (from *D19Mit63* to *D19Mit1*, a region identified by interval mapping), to identify candidate quantitative trait genes (QTG). Several candidate QTGs with functions of interest were identified and included those implicated in axon guidance (slit homolog 1 and semaphorin 4g), ion channels (Kv channel-interacting protein 2), and retinal development (paired box 2), as well as those involved in cone phototransduction (phosphodiesterase 6C) and homeostasis (retinol binding protein 4). Studies of animal models of these genes have not reported an ERG phenotype comparable to that reported in this study.[29–31] In a separate study, retinal expression levels were compared between B6 and 129 mice. Two genes with lower expression in 129 retinas when compared with B6 retinas were identified in this region of Chr 19: programmed cell death 11 and survival of motor neuron domain containing 1. Evaluation of any QTG must await narrowing of the QTL interval, to include a manageable number of candidates.

The QTLs that control phenotypic variation in vision-related traits have been successfully identified in other studies[32–36]; however, none of these QTLs maps to Chr 19. Only one successful mapping of a QTG controlling a vision-related trait has been reported: identification of the *Rpe65* gene as a QTG controlling resistance to light-induced apoptosis,[37,38] where the protective allele produces lower levels of RPE65 protein, ensuring a slower regeneration of rhodopsin during exposure to light intensities likely to cause retinal damage.[39]

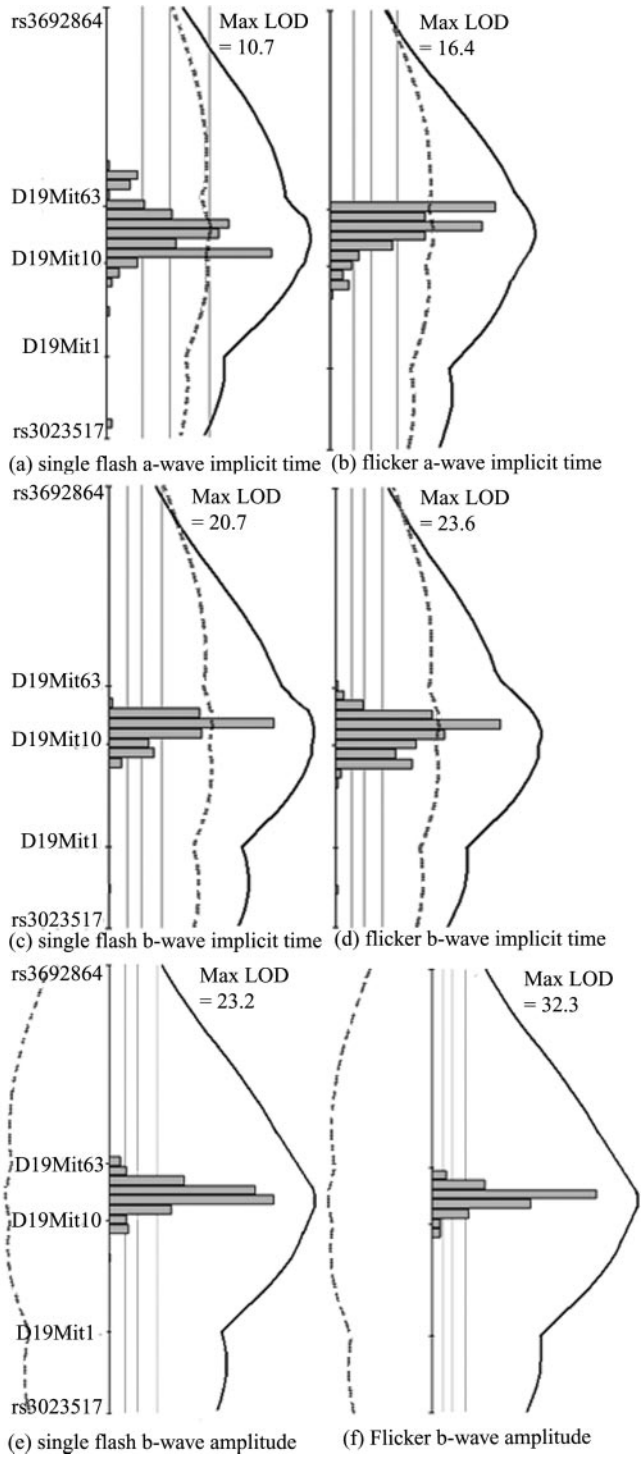

FIGURE 2. Interval maps of Chr 19 for six traits of the light-adapted ERG.

In every QTL analysis, the ultimate end point is to find the gene or genes that contain a variation-controlling polymorphism. This is one of the most difficult steps of QTL analysis, and while many research groups have narrowed QTLs to given genomic regions, very few have made the final step in identifying the gene(s) that causes the variation.[40] Finding those is dependent on refining the interval size of a QTL. The QTL on Chr 19 is highly significant, containing a gene or genes that influence the six traits of the light-adapted ERG (Fig. 2). QTGs within the interval control between 24% and 57% (depending on trait, the gene/allele has a greater or lesser influence on each of the six traits) of the variation observable between 129 and B6 mice in the cone a- and b-waves of the ERG. The remaining variation can be, and often is, explained by a large number of genes that influence phenotype with such a small effect as to be undetectable and environmental factors, such as differences in the microenvironments in which the mice are raised. By employing rational breeding schemes, 129 mice congenic for B6 recombinant regions within the Chr 19 QTL interval and the reverse can be generated. Thus, identification of the novel or functionally novel gene(s) influencing the cone ERG that lie within this interval may be accomplished.

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