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Fragmentation of Two Quantitative Trait Loci Controlling Collagen-Induced Arthritis Reveals a New Set of Interacting Subloci[1]

Emma Ahlqvist,[2] Robert Bockermann,[3] and Rikard Holmdahl

Linkage analysis of F_2 crosses has led to identification of large numbers of quantitative trait loci (QTL) for complex diseases, but identification of the underlying genes has been more difficult. Reasons for this could be complications that arise from separation of interacting or neighboring loci. We made a partial advanced intercross (PAI) to characterize and fine-map linkage to collagen-induced arthritis in two chromosomal regions derived from the DBA/1 strain and crossed into the B10.Q strain: *Cia7* on chromosome 7 and a locus on chromosome 15. Only *Cia7* was detected by a previous F_2 cross. Linkage analysis of the PAI revealed a different linkage pattern than the F_2 cross, adding multiple loci and strong linkage to the previously unlinked chromosome 15 region. Subcongenic strains derived from animals in the PAI confirmed the loci and revealed additional subloci. In total, no less than seven new loci were identified. Several loci interacted and three loci were protective, thus partly balancing the effect of the disease-promoting loci. Our results indicate that F_2 crosses do not reveal the full complexity of identified QTLs, and that detection is more dependent on the genetic context of a QTL than the potential effect of the underlying gene. ***The Journal of Immunology,*** **2007, 178: 3084–3090.**

Collagen-induced arthritis (CIA)[4] is the most extensively studied model of rheumatoid arthritis in mice (1). Just like rheumatoid arthritis, CIA is a complex disease with both genetic and environmental components (2). Identifying the disease-regulating genes would give invaluable information about the largely unknown disease mechanisms to date. Great efforts have been made to do so, mainly in humans, but also in animal models; however, despite this, very few of the responsible genes have been identified (3).

Studying complex diseases in inbred rodents has many advantages compared with doing linkage studies in humans. Through strict breeding schemes and a fixed environment, much of the variation that causes problems in human studies can be avoided (4). The most commonly used method to detect quantitative trait loci (QTL) in animals is to perform a gene-segregating cross, usually on the F_2 level, of strains with different susceptibility to disease, followed by linkage analysis. This method has led to the identification of many genes controlling monogenetic or near-monogenetic diseases. It has also been very successful in identifying major loci controlling various complex disease models, but identification of the underlying genes has proven to be more difficult. Even though large numbers of QTL have been located for many complex diseases (27 for CIA in the mouse (www.informatics.jax.org)), only a few loci have had high probability candidate genes tied to them (5). Increased understanding of the architecture of complex traits is therefore desirable because it would allow better comprehension of the complications that arise when using the traditional methods, which are developed for genetic analysis of Mendelian traits, to study complex diseases.

Already, it is apparent that the actual number of disease-modifying loci can be expected to be higher than the number of loci that are reported today. Several loci for complex diseases, initially found in two-generation crosses, have later been found to be composed of clusters of weaker subloci adding up to the total effect, or even subloci acting in opposite directions (6–9). In addition, some loci are known to be dependent on interactions with other loci (7, 10). Detecting such loci requires large sample sizes, explaining why not all of them have been found (11). So, the small number of recombinations that arise on each chromosome in a single meiosis is not sufficient to separate closely positioned loci in a linkage analysis. This means that groups of weaker loci that act in the same direction are detected as one strong locus. It is reasonable to believe that it also means that groups of loci that act in opposite directions are detected as very weak loci or not detected at all, an assumption that is supported by studies of complex traits in other model organisms, such as *Drosophila* and *Arabidopsis*, which lend themselves to investigation more easily (12, 13).

So, if the chance of detecting a locus is more dependent on the genetic context of the gene than the potential phenotypic difference between the alleles, what is missing in the results we obtain from traditional crosses? To what extent do the results of F_2 intercrosses reflect the total number of disease-modifying loci, and how does this affect the subsequent process of cloning the underlying genes?

We used an alternative approach, a partial advanced intercross (PAI) in combination with subcongenic strains, to characterize and

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[4] Abbreviations used in this paper: CIA, collagen-induced arthritis; CII, collagen type II; EAE, experimental autoimmune encephalomyelitis; LOD, logarithm of odds; LODint, interactive LOD; LODjnt, joint LOD; PAI, partial advanced intercross; QTL, quantitative trait loci.

fine-map linkage to CIA in two regions on chromosomes 7 and 15. Congenic strains with fragments from the highly susceptible strain DBA/1 on the relatively resistant B10.Q background (referred to as BQ.DQ7 and BQ.DQ15) were made for the two chromosomes, and subsequently intercrossed for seven generations. The chromosome 7 region contained a previously known locus (*Cia7*) that had been defined in the same strain combination in an F_2 intercross (14). The chromosome 15 region was not linked to CIA in this F_2 intercross, but crosses made in another strain combination (B10.RIII × RIII.S/J) had found several loci, affecting both CIA and experimental autoimmune encephalomyelitis (EAE), in this region, as well as an EAE locus (*Eae2*) that interacted with a locus close to *Cia7* (15, 16). If the locus discovered in the F_2 intercross was the result of a single polymorphism, we would expect the PAI approach to return a stronger and narrower logarithm of odds (LOD)-score peak over the *Cia7* locus, whereas chromosome 15 remained unlinked. We would then have an advantageous starting point for identifying the *Cia7* gene using the small congenic fragments generated in the PAI cross. Would this be the case or would the increased number of recombinations allow new loci to appear and thereby reveal possibilities that could clarify the cloning strategy?

Materials and Methods

Production of congenic strains

The mice were bred and kept in a climate-controlled environment in the animal house of Medical Inflammation Research, Lund University. The mice were kept at a 12-h light/dark cycle, fed with regular rodent chow, and given water ad libitum.

The BQ.DQ7 and BQ.DQ15 congenic mice were produced as speed congenics by marker-assisted backcrossing of DBA/1 (shortnamed DQ) mice to the recipient strain C57BL/10.Q (i.e., B10.Q, shortnamed BQ) for six generations, followed by intercrossing. The founders of the C57BL/10.Q mice came from J. Klein (University of Tübingen, Tübingen, Germany). DBA/1J mice were originally obtained from The Jackson Laboratory. The mice were bred so that both the Y chromosome and mitochondria originated from the BQ strain by crossing first to BQ females and then to BQ males. All animal experiments were approved by the local ethical committee, Malmö-Lund (M70-04).

PAI intercrossing

Mice with a congenic fragment on chromosome 7 (BQ.DQ7), including markers D7Mit69-D7Mit371 (D7Mit225 and D7Mit333 excluded), were crossed to mice with a congenic fragment on chromosome 15 (BQ.DQ15), including markers D15Mit13-D15Mit42 (upper border unknown, D15Mit79 excluded). The mice were intercrossed for seven generations. From each generation, mice were selected for breeding to collect as many recombinations as possible while avoiding early fixation of regions. Starting from generation four, the animals that were not used for breeding were used for experiments. In total, 576 mice completed experiments. Subcongenic strains were produced by marker-assisted backcrossing of PAI animals with appropriate DQ fragments onto BQ. All experiments on congenic mice were made using littermate controls to minimize effects caused by contaminating fragments.

Induction of CIA and scoring

CIA was induced by an s.c. injection of an Ag-adjuvant emulsion at the base of the tail (100 μg of rat collagen type II (CII) in 50 μl of CFA (Difco)). A boost (50 μg of CII in 25 μl of IFA (Difco)) was given 35 days later. The CII was isolated by pepsin digestion of SWARM chondrosarcoma, purified, and dissolved in 0.1 M acetic acid, as previously described (17). Clinical disease was quantified once per week according to a scoring system based on the number of inflamed joints, as follows: 1 point for each inflamed toe or knuckle plus 5 points for an inflamed wrist or ankle, giving a maximum score of 15 per limb or 60 per animal. Male mice were scored until day 49, whereas female mice, which had a later onset, were scored until day 70. Experiments on subcongenic strains were scored approximately twice every week and terminated after a significant difference between the groups had been confirmed. The CIA phenotypes used for the PAI were defined, as follows: incidence, whether the animal contracted disease or not during the observation period, scored as 1 or 0; onset, the first day after immunization the animal first developed visible signs of arthritis; maximum score, the highest score reached by each mouse during the disease course; accumulated score, the sum of the scores from all scoring days for each mouse.

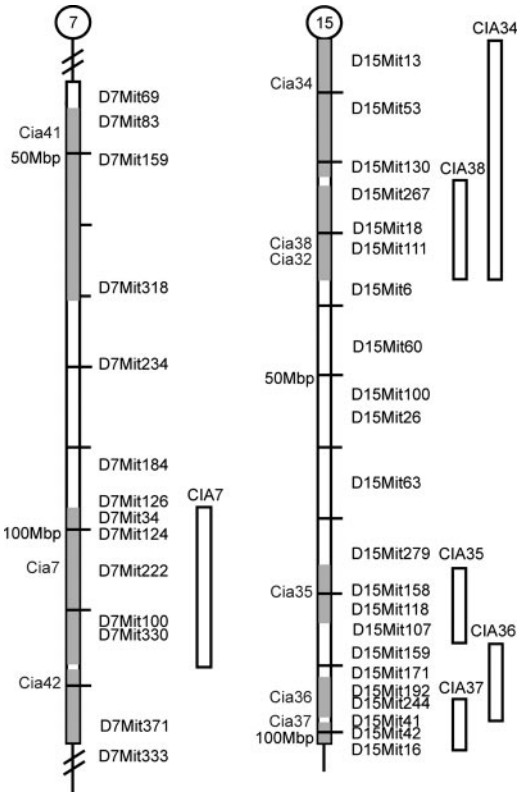

FIGURE 1. Physical map of the BQ.DQ7 and BQ.DQ15 regions, found loci, and subcongenic fragments. Map of chromosomes 7 and 15 showing the BQ.DQ7 and BQ.DQ15 congenic fragments that were the origin of the PAI. The approximate positions of all loci that we mapped to the region are shaded in gray. The subcongenic fragments that were produced to confirm loci are shown as □. Markers are positioned according to the National Center for Biotechnology Information m36 mouse assembly (April 2006, strain C57BL/6J).

Ab data

Serum was collected on day 14 after immunization and stored in −20°C until assayed. ELISA was performed to determine levels of anti-CII Abs. Plates (Nunc MaxiSorp) were coated with 10 μg/ml CII in PBS (pH 9) and blocked with 1% BSA. Levels of IgG1, IgG2a, IgG2b, and IgG3 were measured using the following biotinylated secondary Abs: goat anti-mouse IgG1 (1070-08), goat anti-mouse IgG2a (1080-08), goat anti-mouse IgG2b (1090-08), and goat anti-mouse IgG3 (1100-08) (Southern Biotechnology Associates). Total anti-CII Ab levels were measured using peroxidase-conjugated goat anti-mouse IgG (H + L) (115-035-062; Jackson ImmunoResearch Laboratories). Biotinylated Abs were incubated with extravidin peroxidase (E-2886; Sigma-Aldrich). Plates were developed with ABTS (Roche).

Genotyping

Genomic DNA was prepared by dissolving 1 mm tail in 300 μl of 50 mM NaOH at 95°C for 1–2 h, and subsequently neutralizing with 30 μl of 1 M Tris-HCl (pH 8). A 1-μl sample was used to perform a standard 10-μl PCR. Microsatellite variants were dissolved using a MegaBACE DNA analysis system 1000 (Amersham Biosciences).

Statistical analysis

CIA mean score and Ab levels were compared using the Kruskal-Wallis or Mann-Whitney *U* test depending on the number of groups. Linkage analyses were performed using R (The R Foundation for Statistical Computing, version 2.0.1) and R/qtl (18). Linkage was calculated for males and females separately, as well as for all animals together. The imputation

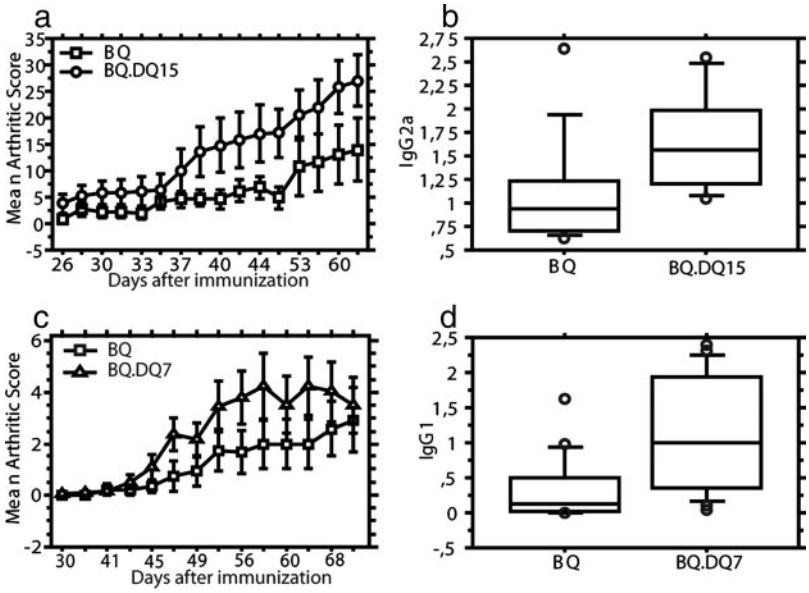

FIGURE 2. Arthritis susceptibility of the BQ.DQ7 and BQ.DQ15 congenic strains. Before the PAI, the BQ.DQ7 and BQ.DQ15 congenics were tested for arthritis susceptibility. *a*, BQ.DQ15 mice ($n = 14$) had higher disease scores than BQ littermates ($n = 13$). $p = 0.02$ for accumulated score. *b*, The BQ.DQ15 mice also produced more anti-CII Abs, in which the most significant difference was for IgG2a production. $p = 0.004$ for IgG2a. *c*, In accordance with the previous F_2 intercross, BQ.DQ7 mice ($n = 17$) were more susceptible to arthritis than their littermate BQ controls ($n = 21$). $p = 0.02$ for accumulated score. *d*, BQ.DQ7 mice also produced higher levels of Abs. $p = 0.001$ for IgG1. Ab levels are plotted as absorbance values at 405 nm.

model (2-cM steps) was used for all calculations. The R/qtl two-locus model calculates a joint LOD (LODjnt) score that compares a full model (if including covariates) ($y = \mu + \beta q1 + \beta q2 + \beta q1 \times q2 + A\gamma + Z\delta q1 + Z\delta q2 + Z\delta q1 \times q2 + \varepsilon$) with a null model ($y = \mu + A\gamma + \varepsilon$), and an interactive LOD (LODint) score that compares the full model with an additive model ($y = \mu + \beta q1 + \beta q2 + \beta q1 \times q2$). The variables q1 and q2 are unknown QTL genotypes at two different locations, A is a matrix of additive covariates, and Z is a matrix of interacting covariates. Significance thresholds were determined using permutation tests ($n = 1000$ for single-locus scans, and $n = 300$ for two-locus scans), in which $p < 0.05$ was considered significant. The genetic map was generated in the R/qtl environment and based on the recombinations collected in the cross. It is therefore skewed and does not correspond to any other genetic map.

Results

Both of the original congenic fragments increased CIA susceptibility

The two congenic strains BQ.DQ7 and BQ.DQ15 (Fig. 1) were tested for CIA susceptibility. Both fragments significantly increased arthritis susceptibility and production of anti-CII Abs (Fig. 2). This confirmed both the previously found linkage on chromosome 7 and the assumption that chromosome 15 harbors disease-modifying loci (14–16). The *Cia7* locus could also be reproduced in a subcongenic strain covering only the locus region. A single copy of this fragment significantly increased CIA susceptibility and anti-CII IgG production in male mice (Fig. 3).

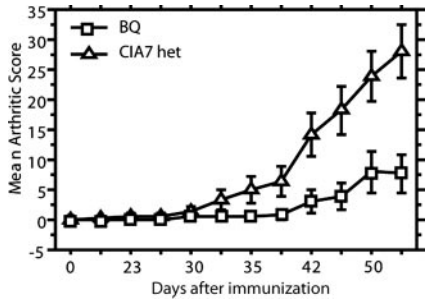

FIGURE 3. The previously found *Cia7* locus was confirmed in a congenic strain. The *Cia7* locus could be reproduced in a subcongenic strain. A heterozygous *Cia7* congenic fragment increased both incidence and severity of arthritis in males ($n = 19$) compared with BQ littermates ($n = 16$). $p = 0.0023$ for accumulated score. Error bars show SE.

The PAI could not reproduce Cia7*, but allowed the detection of three new loci*

The BQ.DQ7 and BQ.DQ15 congenic strains were then intercrossed for seven generations in a PAI. In total, 576 mice from generations 4–7 were immunized and tested for CIA susceptibility. Four arthritis

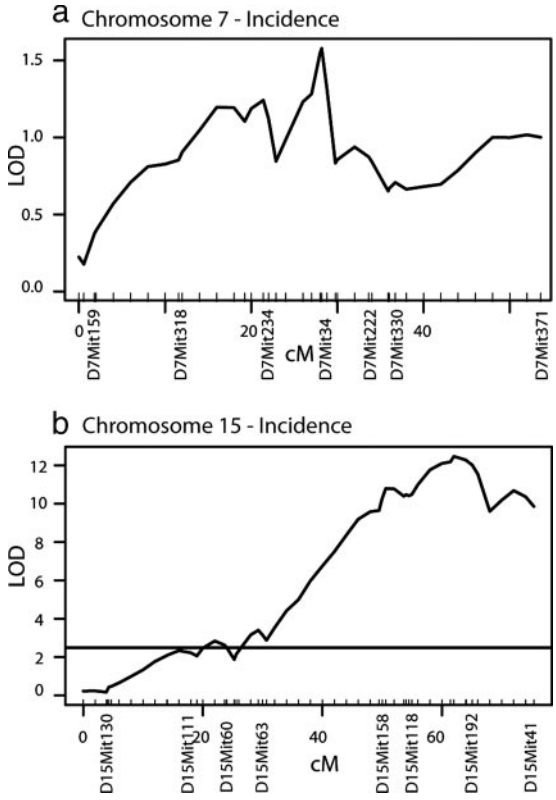

FIGURE 4. Chromosome 15, but not chromosome 7, was linked to CIA in the PAI. A one-locus scan was performed on all animals in the PAI. *a*, Chromosome 7 was not significantly linked to any subphenotype ($p = 0.05$ was LOD 2.5 for incidence). *b*, There was a strong linkage to the telomeric end of chromosome 15 for all tested phenotypes. The highest LOD score was for incidence (LOD 12.5). The line marks the $p = 0.05$ significance level (LOD 2.5).

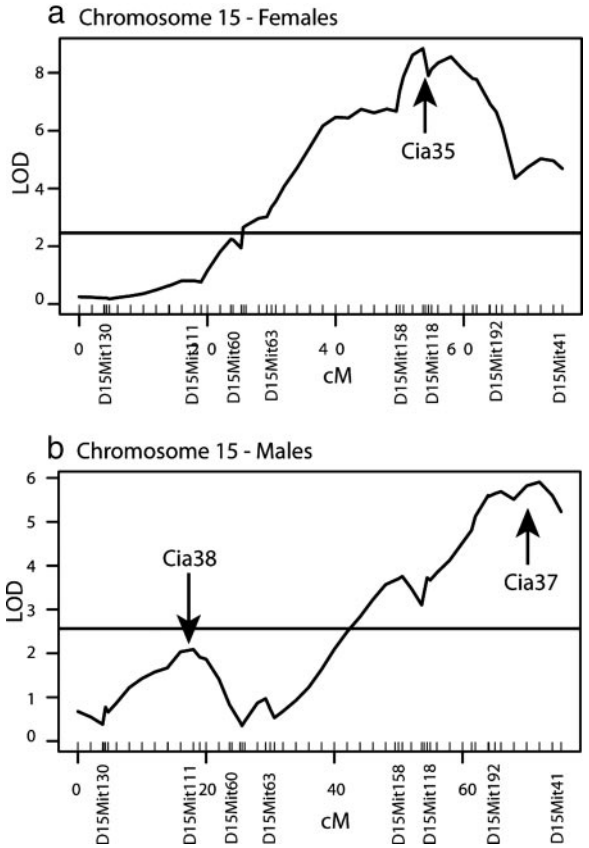

FIGURE 5. Dividing the animals by sex separated the locus on chromosome 15 into two loci. Separating the animals by sex divided the chromosome 15 peak into two separate loci. *a*, In females, the strongest linkage was to marker D15Mit118, which was significantly linked to all phenotypes examined. *b*, In males, marker D15Mit41 was linked to all disease phenotypes, whereas the Ab phenotypes were linked to the nearby marker D15Mit192. The line marks the $p = 0.05$ significance level (LOD 2.5 for females; LOD 2.6 for males).

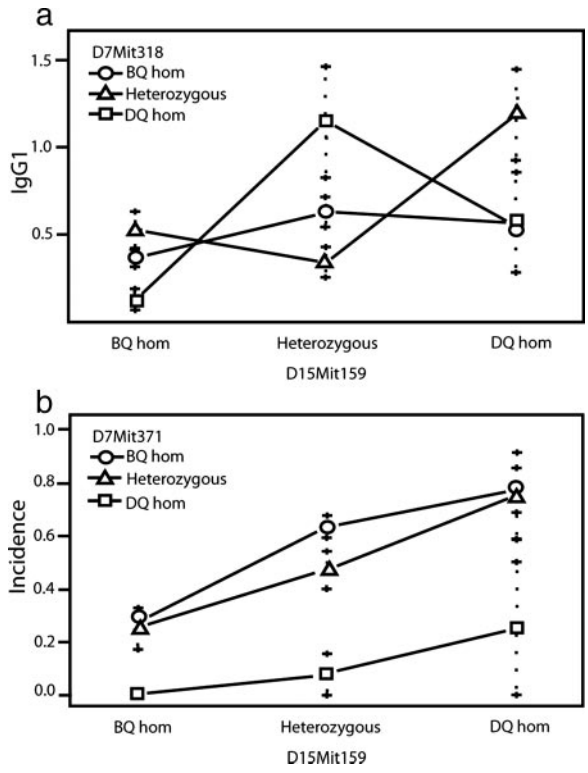

FIGURE 6. A search for interactions revealed two new loci on chromosome 7. Including the possibility of interacting loci revealed two new loci on chromosome 7. *a*, A locus close to marker D7Mit318, designated *Cia41*, affected Ab production in interaction with *Cia35* (marker D15Mit159) in an epistatic fashion (LODint was 5.26, whereas $p < 0.05$ was LOD 5.04). *b*, Another locus, *Cia42*, close to marker D7Mit371, protected the mice from arthritis. The effect was seen as an additive interaction with any of the disease-promoting loci. The *Cia41-Cia35* interaction plot for incidence is shown (LODjnt 17.7, whereas $p < 0.05$ was LOD 5.7).

phenotypes (incidence, onset, maximum score, and accumulated score) were investigated, as well as anti-CII IgG levels (IgG1, IgG2a, IgG2b, IgG3, and total IgG). Because the PAI collected data from twice as many mice as the F_2 intercross and contained many more recombinations, it was expected to have a significantly higher power and resolution and thus return a higher, narrower, LOD-score peak in the *Cia7* region. However, we found that the linkage pattern was considerably changed compared with the F_2 cross. *Cia7* could not be reproduced in the PAI (Fig 4*a*), whereas a strong linkage to chromosome 15 was seen, even though there had been no detectable linkage to chromosome 15 in the original F_2 cross. A one-locus scan performed on all animals resulted in a broad peak in the telomeric half of chromosome 15, with a high LOD score for all subphenotypes (Fig. 4*b*). In addition, marker D15Mit111 was significantly linked to Ab production. The locus was assigned *Cia38*. By separating females (Fig. 5*a*) and males (Fig. 5*b*), the broad telomeric peak could be split into one female-specific peak (*Cia35*) close to marker D15Mit118 and one male-specific peak (*Cia37*) close to D15Mit41. The effect of *Cia38* was more pronounced in males than females, and the locus was significantly linked to onset of disease in males. Interestingly, the disease phenotype was strongest in heterozygous animals, whereas the IgG3 phenotype was dominant.

Including interactions revealed two additional loci

Because an interaction between chromosome 15 and a locus close to *Cia7* had been suggested, we thought that the *Cia7* locus might be dependent on this interaction, and that a two-locus scan considering both additive (LODjnt) and epistatic (LODint) interactions would allow the locus to be detected. However, *Cia7* remained undetectable. Instead, two new loci were found on chromosome 7. *Cia41*, close to marker D7Mit318, affected Ab production in interaction with one of the loci on chromosome 15 (*Cia35*) (Fig. 6*a*) in an epistatic fashion. The LOD score supporting an epistatic interaction between these loci (LODint) was 5.26, in comparison with $p = 0.05$ determined to be at LOD 5.04 using a permutation test. Another locus, named *Cia42*, close to marker D7Mit371, protected mice from arthritis, which was seen as an additive interaction with any disease-promoting locus (Fig. 6*b*). Neither of these loci seemed to correspond to the *Cia7* locus nor to the previously identified interacting locus. In total, five new loci were identified using the PAI approach.

Confirmation of loci in subcongenic strains

Subcongenic strains were produced, by backcrossing of PAI animals with suitable fragments, to confirm the newly found loci. The approximate span of each subcongenic fragment can be seen in Fig. 1. All loci that were found in the single locus scan were tested in subcongenics.

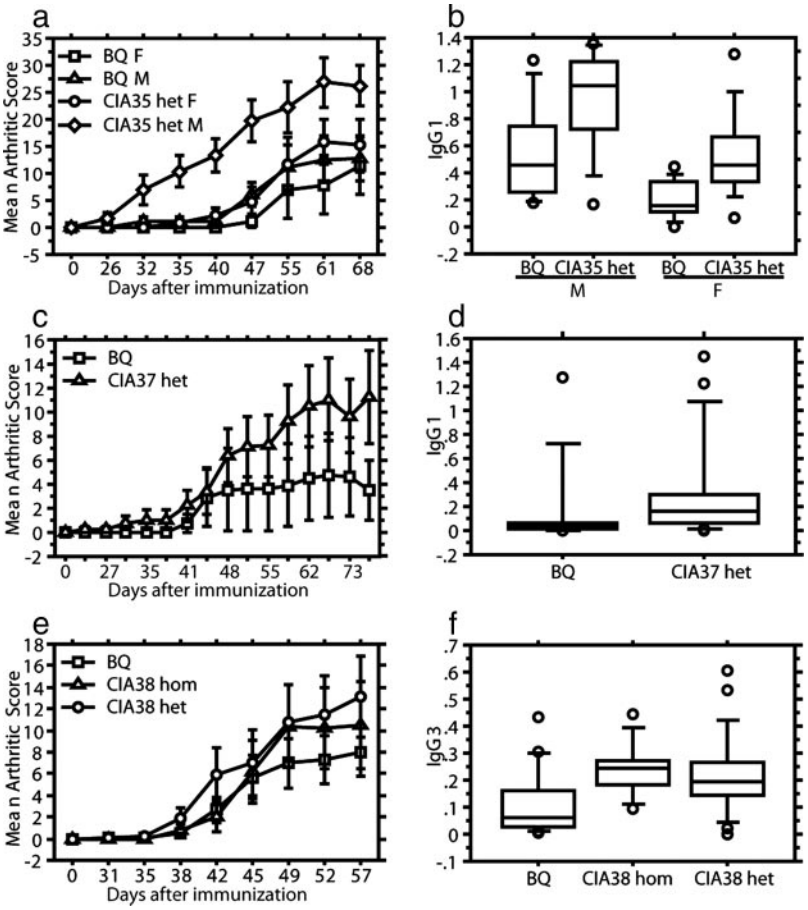

FIGURE 7. All loci found in the one-locus scan were confirmed in subcongenic strains. Subcongenic strains were produced by backcrossing of mice with appropriate fragments generated in the PAI. Disease curves for subcongenic lines compared with BQ littermates are shown. Maximum score is 60. Significance values are calculated on accumulated score at the end of the experiment. Ab levels are plotted as absorbance values at 405 nm. *a*, The *Cia35* locus was most predominant in females in the PAI, but once isolated in a congenic strain it appeared to be sex independent. *b*, The effect on Ab production was significant in both males and females ($p = 0.004$ and 0.03, respectively), whereas the effect on arthritis was significant in males ($p = 0.01$), with only a tendency in females. *c*, The effect of *Cia37* on arthritis could not easily be confirmed; only a tendency toward higher mean disease score was seen. *d*, The Ab production was significantly higher in males ($p = 0.03$ for anti-CII IgG1, reproduced twice), indicating that the *Cia37* locus was within the congenic region. No effect was seen in females, indicating that the locus is male specific. *e*, In accordance with the PAI results, a subcongenic fragment covering the *Cia38* region had little effect on the disease phenotypes, but had a strong effect on anti-CII IgG3 production ($p = 0.006$) specifically in males (*f*). Error bars show SEs.

Cia35

Cia35 was strongly linked to all phenotypes tested and appeared to be female specific in the PAI. The LOD score was 8.8 for incidence of arthritis in females ($p < 0.05$ at LOD 2.5), but only 3.7 in males ($p < 0.05$ at LOD 2.7). However, when a subcongenic line was tested, the locus had a strong effect on Ab production ($p = 0.004$) and CIA ($p = 0.01$) (Fig. 7, *a* and *b*) in male mice. Female congenic mice also had significantly higher Ab levels ($p = 0.03$). They did not have significantly higher disease scores, but followed the same trend as the males.

Cia37

Cia37 was strongly linked to all CIA subphenotypes as well as Ab production in the PAI. LOD score in the PAI was 5.9 for incidence

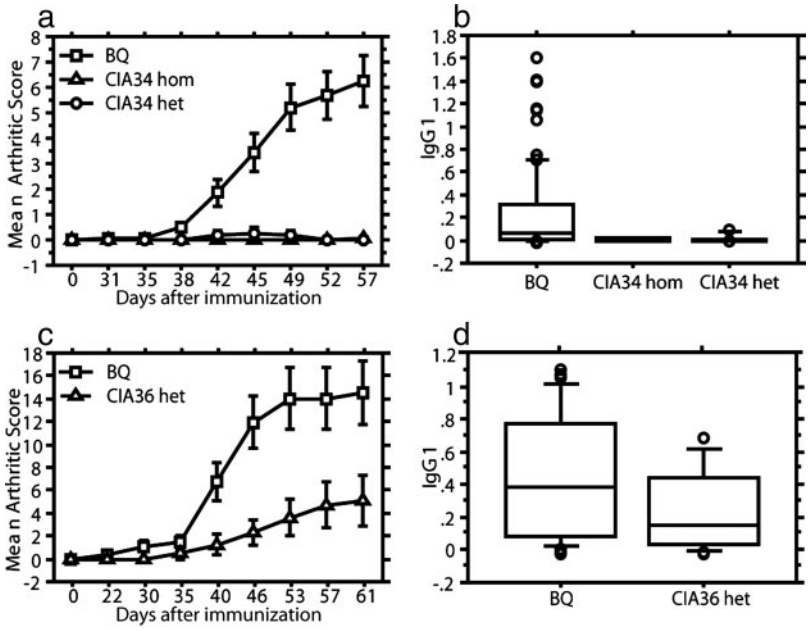

FIGURE 8. Two additional loci were found in subcongenic strains. When isolated in subcongenic strains, two additional loci were found to affect CIA. Both loci were protective. *a*, *Cia34* in the centromeric end of chromosome 15 offered almost complete protection from arthritis in both males and females ($p = 0.007$). *b*, *Cia34* also decreased anti-CII IgG production ($p = 0.002$). *c*, *Cia36* was found between *Cia35* and *Cia37* and conferred resistance to disease ($p = 0.03$). *d*, The *Cia36* locus also decreased production of anti-CII IgG3 ($p = 0.01$) and IgG1 ($p = 0.05$). IgG1 is shown. Ab levels are plotted as absorbance values at 405 nm.

of arthritis ($p = 0.05$ at LOD 2.6). The effect of the locus appeared to be stronger in males, although the LOD-score curve for the female mice indicated that there was a smaller effect of the locus also in females. In a subcongenic line, a single copy of the *Cia37* fragment significantly increased Ab production in males ($p = 0.03$), whereas no difference could be detected in females. The disease phenotype could not be significantly reproduced in the subcongenic line, neither in this experiment nor in a similar experiment that included homozygous animals, but there was a tendency for higher disease in male mice (Fig. 7, *c* and *d*).

Cia38

Cia38 had a LOD score in the PAI that just barely passed the significance level for onset of arthritis in males, but had a more convincing LOD score for anti-CII IgG3 production (4.9, in which $p = 0.05$ was at LOD 2.6). In accordance, the effect on IgG3 production could be confirmed in a *Cia38* subcongenic line. The *Cia38* animals also had slightly, but not significantly, higher disease scores (Fig. 7, *e* and *f*).

Two protective loci could be identified in subcongenic strains

While testing congenic fragments to confirm the identified loci, two fragments were found to contain loci that had not been detected by the PAI. Both of these loci were acting in the opposite direction to the others, i.e., DBA/1 alleles being protective. *Cia34* was identified in the centromeric end of the BQ.DQ15 fragment. It decreased both CIA susceptibility and Ab production (Fig. 8*a*). *Cia36* was located between *Cia35* and *Cia37*. The effect was seen in male mice, but sex specificity was hard to determine because female BQ mice have a low incidence and severity of disease (Fig. 8*b*).

Discussion

In this study, we followed up the results of a previous F_2 intercross (14) by using the results of a PAI in combination with complementary experiments performed in congenic strains. By this fine mapping, we identified seven new loci that controlled incidence and severity of CIA as well as production of CII-specific Abs, in the B10.Q × DBA/1 cross. None of these loci were found in the F_2 intercross. Five of the new loci were identified by using a PAI approach, whereas two loci remained undetectable in the PAI, but were found when using a congenic approach. Oddly, the *Cia7* locus, which was detected in the F_2 cross and reproduced in a congenic strain, was not seen in the PAI.

The results of the study emphasize the importance of defining a favorable genetic context before proceeding from a genome-wide search for QTLs to attempt positional cloning of the underlying genes. The PAI method is an efficient way to accomplish this with a restricted number of animals, starting from the congenic strains that are used to confirm the loci. The PAI approach defines the position of the studied loci while collecting valuable recombinations needed for successful positional cloning. The number of mice needed to split the congenic fragments is also reduced because recombinations during the meiosis will operate independent on both chromosomes. In this study, we have also shown that the approach allows detection of loci that are not seen in an ordinary F_2 intercross.

It is apparent that the results from the F_2 intercross reflected very few of the disease-modifying loci in these regions. The reasons for this are probably several. One reason is of course that the PAI experiment included almost twice as many animals as the F_2 cross. However, at least the *Cia35*/*Cia37* loci were strong enough to be detected with a sample size of corresponding size (data not presented). A PAI also differs from an F_2 intercross in other ways that affect the power of linkage analyses. One is that a limited region of the genome is studied, which decreases the total variance. This also means that most of the interactions with other genes are fixed because all genes outside the region have a fixed genotype. The complexity is thereby greatly reduced. Another reason for the discrepancies between the two crosses could be the difference in resolution. In an F_2 intercross, all recombinations arise in a single meiosis. This means that each animal will have only a few recombinations on each chromosome, and thus, that closely positioned genes will be inherited together in all but a few cases. In a PAI, a larger number of recombination events are collected by intercrossing the animals for several generations, thus allowing neighboring genes to segregate independently. Chromosome 15 harbored five loci spread over the entire chromosome. Three of these were disease promoting, whereas two, one in each end of the chromosome, were protective. Thus, in an F_2 cross, each promoting locus would be accompanied by a protecting locus that balanced the effect.

The occurrence of multiple loci on chromosome 15 was proposed based on linkage analysis of B10.RIII × RIIIS/J crosses using arthritis and encephalomyelitis traits (8, 15, 16). In fact, two of the loci we found correspond to such loci. *Cia34* and *Cia38* were linked to the same regions as *Cia30* and *Cia32*, respectively. In addition, a barely significant linkage to marker D15Mit60 corresponds to *Cia26*, also a locus identified in the B10.RIII × RIIIS/J cross.

The reasons that the *Cia7* locus could not be detected in the PAI remain obscure. Because the locus could be reproduced in a congenic strain that also had fixed BQ alleles on all other chromosomes, it does not seem to be dependent on interactions with other loci. Neither is it dependent on interactions with the mitochondria because these were all of BQ origin. This is important because the *Cia7* locus contains the *ucp2* gene that codes for a mitochondrial membrane protein, which has been suggested to play a role in both multiple sclerosis and the multiple sclerosis model EAE (19, 20). However, genetic mutations/contaminations or changing environmental factors can never be completely excluded in this type of experiment, although we have no evidence for this. A remaining possibility, but which is difficult to prove, is that the difference is due to interactions involving multiple genes.

There is always an uncertainty of whether an effect seen is due to one gene or several. For example, in the B10.RIII × RIIIS/J cross, a protective locus was found very close to *Cia32*/*Cia38*, which suggests that there might be yet another protective locus also in our investigated regions. This would explain why only the Ab phenotype, which seems to have a dominant inheritance pattern, could be reproduced in the subcongenic strain, whereas the disease phenotype is very weak and appears as a heterozygosity effect. Interactions could also explain other differences between the results of the PAI and the subcongenic strains. *Cia37* might need some of the close-by loci to enhance its effect, and the apparent sex dependency of the *Cia35* locus in the PAI is probably due to sex-specific effects of loci such as *Cia36* and *Cia37*. There is an overlap in confidence intervals for the *Cia35* and *Cia37* loci. However, even though the *Cia37* locus had a rather weak effect on disease in the *Cia37* congenic strain, there was a significant increase in Ab production for this congene. Thus, we believe that there is a locus also within this region. In addition, the overlap is covered by the *Cia36* congenic that has a protective effect instead of the promoting effect expected from the PAI. There are no overlapping regions between the *Cia35* and *Cia37* congenic strains. The fact that our investigations led us to loci that correspond to previously found loci from other strain combinations bodes well for the identification of common important disease-regulating loci. However, it is

apparent that the methods used to map loci for arthritis to date have been largely inadequate to handle the extent of complexity with which we are dealing. Based on our results, it is reasonable to believe that the access to recent methods and strategies, such as PAI, advanced intercross lines, recombinant inbred lines, heterogeneous stock mice, and chromosome substitution strains, will lead to a dramatic increase in the number of known loci for many complex diseases (21). Considering that our experimental set-up included less than two chromosomes, two strains (equivalent with two homozygous individuals), and excluded all loci that were dependent on interactions with other chromosomes, the total number of CIA loci can be expected to be very large. Hopefully, these new approaches will also lead to many more quantitative trait genes being identified.

In conclusion, our results indicate that the output of traditional F_2 intercross approaches only includes a small fraction of the disease-modifying QTL and that detection is more dependent on the context of a QTL than the potential effect of the underlying gene. Clarification of the genetic environment at which a single polymorphism operates to mediate its phenotype will facilitate its eventual identification.

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Disclosures

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