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The Bxs6 Locus of BXSB Mice Is Sufficient for High-Level Expression of gp70 and the Production of gp70 Immune Complexes

Joanna Rankin,* Joseph J. Boyle,‡ S. Jane Rose,* Luisa Gabriel,* Margarita Lewis,* Vasuky Thiruudaian,* Nicola J. Rogers,† Shozo Izui,§ and Bernard J. Morley2*

High levels of the retroviral envelope protein gp70 and gp70 immune complexes have been linked to a single locus on chromosome 13 (Bxs6) in the BXSB model, to which linkage of nephritis was also seen. Congenic lines containing the BXSB Bxs6 interval on a non-autoimmune C57BL/10 background were bred in the presence or absence of the BXSB Y chromosome autoimmune accelerator gene (Yaa), which accelerates disease in male mice. In these mice, we have shown that Bxs6 is sufficient to cause high-level expression of gp70 and the production of gp70 autoantibodies, independently of Yaa, with gp70 immune complex levels enhanced by Yaa. In the presence of Yaa, Bxs6 also causes mild nephritis, and interestingly the sporadic production of high levels of anti-DNA Abs in some mice. Fine mapping using rare recombinant mice suggested that Bxs6 lies between 59.7 and 74.8 megabases (Mb), although the interval of 0.6 Mb between 73.6 and 78.6 Mb on chromosome 13 cannot be excluded in this study.


*Rheumatology Section, †Immunology Department, and ‡Histopathology Department, Imperial College, London, United Kingdom; and §Department of Pathology and Immunology, Centre Médical Universitaire, Geneva, Switzerland

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2 Address correspondence and reprint requests to Dr. Bernard J. Morley, Rheumatology Section, Faculty of Medicine, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0NN, U.K. E-mail address: b.morley@imperial.ac.uk
3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; ANA, anti-nuclear Ab; gp70IC, gp 70 immune complex; SNP, single nucleotide polymorphism; Mb, megabase; Yaa, Y chromosome autoimmune accelerator gene.

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maintained in both Geneva and Imperial College. All congenic strains were bred using a speed congenic approach (18), with microsatellite marker typing at each generation, and fixed by brother-sister mating. B10.Yaa.Bxs6 (B10.Yaa.Chr13) congenic mice were bred through multiple backcrosses of (B10 × BXS6)F1 males to B10 females. B10.Yaa.BXS6.Bxs6 (B10.Yaa.Bxs6) congenic mice were bred through crosses between B10.Yaa.Chr13 congenic mice and B10 mice. B10.BXS6-Chr13 (B10.Chr13) and B10.BXS6-Bxs6 (B10.Bxs6) congenic mice were bred by creating an F1 between male B10 mice and female B10.Yaa.Chr13 or B10.Bxs6.Yaa mice, respectively. The resulting male F1 mice were then crossed back to female B10.Yaa.Chr13 or B10.Yaa.Bxs6 mice, respectively. All B10.Yaa and BXS6 control data are from male mice.

**Fine mapping of the Bxs6 locus**

BXS6 × (B10 × BXS6)F1 mice were genotyped with microsatellite markers across the Bxs6 region, and serum gp70 and gp70IC levels were measured at 8 or 12 mo of age for recombinant mapping. SNP data was obtained from www.well.ox.ac.uk/mouse/INBREDS. The genotypes of C57BL/6 (B6), SB/Le, BXS6, and BXS6/Il mice were compared at each SNP to determine which regions of the BXS6/Il strain were derived from B6 and which from BXS6 mice.

**Serological analyses**

As previously described, levels of ANA were measured by indirect immunofluorescence using Hep-2 cells and a FITC-conjugated IgG Fe-specific anti-mouse Ab (5). Levels of gp70 and gp70IC were measured by ELISA as published (19). Anti-dsDNA Abs and anti-ssDNA Abs were measured by ELISA as described previously (5).

**Autopsy analyses**

Mice were sacrificed at 9, 10, or 12 mo of age. Kidneys were removed into 10% formalin solution, sectioned, and H&E stained. As previously described, kidney sections were scored under a light microscope according to the degree of hypercellularity and mesangial matrix increase of glomeruli. A scale of 0–4 was used, where 0 equaled no histological abnormality, 1 equaled <25%, 2 equaled 25–50%, 3 equaled 51–90%, and 4 equaled >90% of the glomeruli were abnormal (5).

**Statistical analysis**

All statistical analysis was conducted using GraphPad Prism (version 3.00 for Windows). A two-tailed unpaired t test, Mann-Whitney U test, log-rank test, or two-way ANOVA was used to test for statistical significance of data between cohorts of mice. Statistical tests used for each group of data are described in the figure legends. If p < 0.05, the means were considered to be significantly different.

**Results**

**Breeding of congenic strains containing the BXS6 Bxs6 locus**

Four congenic strains were bred using a speed congenic approach (18), and for each generation of breeding, mice were selected by typing with a total of 17 microsatellite markers that distinguish between BXS6 and B10 on chromosome 13 (www.ensembl.org/Mus_musculus/index.html). In addition, for the first two generations of the backcross, microsatellites were used to exclude other chromosomes, at least five per chromosome (20, 21), with 25 on chromosome 1 (5), to ensure other Bxs loci were not present in the congenics. B10.Yaa.Chr13 and B10.Chr13 mice were BXS6 between microsatellite markers D13Mit13 (19.79 megabases (Mb)) and D13Mit78 (115.80 Mb), which are the markers closest to either end of the chromosome (Fig. 1). B10.Yaa.Bxs6 and B10.Bxs6 mice were BXS6 between D13Mit122 (58.99 Mb) and D13Mit233 (80.23 Mb) (Fig. 1). B10.Yaa.Chr13 and B10.Yaa.Bxs6 mice were Yaa positive and B10.Chr13 and B10.Bxs6 mice were Yaa negative. In all studies, Bxs6 congenic mice and Chr13 congenic mice were indistinguishable, and therefore only data for Chr13 congenic mice are shown.

The Bxs6 locus caused high-level expression of gp70 and production of gp70IC

B10.Yaa.Chr13 mice had significantly higher levels of gp70 than B10.Yaa mice at 9 mo (p < 0.0001) (Fig. 2a), although levels were still lower than in 6-mo-old BXS6 mice (median titer, 31.06 μg/ml; p < 0.0001) (data not shown). B10.Chr13 (p < 0.0001) also had significantly higher levels of gp70 than B10.Yaa mice, as did female B10.Yaa.Chr13 mice (p < 0.0001). However, the level of gp70 was significantly lower in female B10.Yaa.Chr13 mice than in male B10.Yaa.Chr13 (p < 0.0001) and male B10.Chr13 (p < 0.0001) mice.

B10.Yaa.Chr13 congenic mice had significantly higher levels of gp70IC than B10.Yaa mice at 9 mo (p < 0.0001) (Fig. 2b), but levels were still lower than those of 6-mo-old BXS6 mice (median titer, 6.0 μg/ml; p < 0.0001) (data not shown). B10.Chr13 congenic mice also had significantly higher levels of gp70IC than B10.Yaa mice (p = 0.0001), although significantly lower than those of male B10.Yaa.Chr13 mice (p = 0.0001). The level of gp70IC was not significantly different between B10.Chr13 and female B10. Yaa.Chr13 mice. A correlation was observed between gp70 and gp70IC levels (r = 0.4032, p = 0.0411 at 9 mo), which corroborates previously published data (17).

There is significant interaction between the Chr 13 locus and Yaa in the production of gp70 (p < 0.0001) and gp70IC (p = 0.0038) as determined by a two-way ANOVA.

**The Bxs6 locus accelerated mortality**

B10.Yaa.Chr13 mice had a marginally significant decrease in survival rate compared with B10.Yaa mice (p = 0.0387) (Fig. 2c). Mortality in B10.Yaa.Chr13 mice was 17% (4 of 24), as opposed to 0% (0 of 24) in B10.Yaa mice at 12 mo. Mortality in the
The B10.Chr13 strain was not significantly different to that of the B10.Yaa strain.

The Bxs6 locus caused increased incidence and severity of nephritis
B10.Yaa mice had nephritis grades between 0 and 1, which can thus be considered normal. Grade 2 therefore indicates a mild degree of nephritis, grade 3 a significant degree of nephritis, and grade 4 severe nephritis as seen in the Bxs6 parental strain (20). Of the B10.Yaa.Chr13 mice measured, 15% (3 of 20) developed mild nephritis and 10% (2 of 20) significant nephritis (Fig. 3d). For the female B10.Yaa.Chr13 mice, 0% (0 of 7) had a grade above 1, and for the B10.Chr13 congenic mice 0% (0 of 21) had a grade above 0.

The Bxs6 locus caused elevated levels of autoantibodies to nuclear components
It has previously been shown that B10.Yaa mice have higher spleen weights than B10 mice (5). B10.Yaa.Chr13 congenic mice had significantly larger spleens than B10.Yaa (p < 0.0001) mice at 12 mo (Fig. 3a). However, spleens from B10.Chr13 and female B10.Chr13.Yaa mice were not significantly different from the spleens of B10.Yaa mice.

It has also previously been shown that B10.Yaa congenic mice develop elevated levels of ANA compared with B10 mice (5). Our data showed that B10.Yaa.Chr13 congenic mice had significantly higher levels of ANA than B10.Yaa mice at 9 mo (p = 0.0001) (Fig. 3b). Furthermore, B10.Chr13 mice had significantly higher levels of ANA than B10 mice (p = 0.0236) (data not shown), although significantly lower levels of ANA than B10.Yaa mice (p = 0.0126). There was no significant difference between B10.Chr13 and female B10.Chr13.Yaa mice. A correlation was observed between ANA levels and gp70IC levels at 9 mo (r = 0.4299 and p = 0.0406).

There is significant interaction between the Chr 13 locus and Yaa in the production of splenomegaly and ANA (p < 0.0001) as determined by a two-way ANOVA.

No linkage of anti-DNA Abs had previously been found to the Bxs6 locus. Indeed, B10.Yaa.Chr13 mice and B10.Yaa mice did not have significantly different levels of anti-ssDNA Abs at 9 mo (Fig. 3c). However, although not statistically significant, there was clearly an upward trend observed for B10.Yaa.Chr13 mice. None of the B10.Yaa mice developed detectable levels of anti-ssDNA Abs (<39 ELISA units) at any time point, but for serially tested B10.Yaa.Chr13 mice, 6 mice of 23 (26%) developed detectable levels of anti-ssDNA Abs (>39 ELISA units) for at least one time point. Indeed, five of these mice, and thus 22% of the total, developed high levels of above 100 ELISA units. It would therefore appear that it is possible for occasional congenic mice to develop high levels of anti-ssDNA Abs. B10.Yaa.Chr13 mice also did not have significantly different levels of anti-dsDNA Abs to B10.Yaa mice at 9 mo (Fig. 3d).

Interestingly, however, a subset of B10.Yaa.Chr13 mice developed high levels of anti-dsDNA Abs. All of the B10.Yaa mice tested had undetectable levels of anti-dsDNA Abs (<250 ELISA units) at all time points, but for serially tested B10.Yaa.Chr13 mice, 10 of the
23 measured (43%) developed detectable levels of anti-dsDNA Abs for at least one time point. Indeed, three of these mice, and hence 13% of the total, developed very high levels of anti-dsDNA Abs of above 1,000 ELISA units.

A highly significant correlation was observed between the production of anti-ssDNA Abs and anti-dsDNA Abs at 12 mo (r = 0.9160 and p < 0.0001). It was generally found that it was the same mice that had high levels of anti-ssDNA Abs and anti-dsDNA Abs. Thus, the mouse shown in Fig. 3c, with the highest level of anti-ssDNA Abs (276 ELISA units) is the same as the mouse shown in Fig. 3d, with the highest level of anti-dsDNA Abs (5748 ELISA units).

The Bxs6 locus can be mapped to a total region of 15.7 Mb

Recombinant mapping was conducted using BXSB × (B10 × BXSB)F1 backcross mice. On measuring gp70 levels in 45 Bxs6 homozygous (BXSB and Chr13 consomic) mice and 11 heterozygous (F1) mice at 8 mo of age, it was determined that only Bxs6 heterozygous mice had levels of gp70 below 9.3 μg/ml (data not shown). For the backcross mice, we had previously demonstrated that for gp70 in 92 homozygotes and 59 heterozygotes at 12 mo of age, only Bxs6 heterozygous mice were found to have levels below 2.6 μg/ml (17). Therefore, any recombinant mice with gp70 titers below these levels (at the respective age points) could be assumed to be heterozygous for Bxs6. Two such mice (13.10 and 8.78) were identified as having informative recombinations. Mouse 13.10 had a gp70 level of 4.4 μg/ml, measured at 8 mo of age. Mouse 8.78 had a gp70 level of 1.3 μg/ml, measured at 12 mo of age. Thus, both were defined as low producers of gp70. Through the exclusion of any homozygous regions, these two mice narrowed down the Bxs6 locus from the 35 Mb identified by the linkage analysis, to the 18.9 Mb region between 59.7 and 78.6 Mb (Fig. 4a).

In addition to this approach, the recently published SNP map of the mouse genome (www.well.ox.ac.uk/mouse/INBREDS), with SNPs at every 10 kb, was used to define regions of the Bxs6 locus that could be excluded. The BXSB/ll strain is an alternative recombinant inbred strain derived from SB/Le and B6 mice (M. E. Haywood, L. Gabriel, S. J. Rose, N. J. Rogers, S. Izui, B. J. Morley, manuscript submitted for publication), but does not have the early mortality seen in BXSB mice. Interestingly, however, the BXSB/ll strain does develop significantly elevated levels of gp70 compared with B10. Yaa mice (p < 0.0065; data not shown), although levels are not as high as those seen in BXSB mice. It can therefore be assumed that the Bxs6 locus will map to intervals that were inherited from the same parental stock in BXSB and BXSB/ll mice. Hence, by comparison of the genotype of BXSB and BXSB/ll mice using the SNP map, regions of the Bxs6 locus could be eliminated.

Using the genotype of B6, SB/Le, BXSB, and BXSB/ll mice at each SNP as published, two discrete sections within the Bxs6 locus could be identified, as labeled in Fig. 4b. In Fig. 4b, section A (59.7–74.5 Mb), the BXSB/ll and BXSB both originate from SB/Le. In Fig. 4b, section B (74.8–78.0 Mb), BXSB/ll and BXSB differ in their origins with BXSB/ll from SB/Le and BXSB from...
B6. There are, however, three SNPs in section B that suggest an SB/Le origin for BXSB in this region and therefore indicate some heterogeneity for this interval, or some further mutation.

In Fig. 4b, section A, there are 47 SNPs that are informative between B6 and SB/Le of a total of 69, in a region of 14.8 Mb. The largest gap within this region with no informative markers is 1.4 Mb, but 80% of this section is within 0.5 Mb of an informative marker. For Fig. 4b, section B, there are 18 SNPs that are informative between B6 and SB/Le of a total of 22, in a region of 3.2 Mb. The largest gap within this region with no informative markers is 0.9 Mb, but 90% of this section is within 0.5 Mb of an informative marker.

Assuming that Fig. 4b, section B, between 74.8 and 78.0 Mb, can be eliminated from the Bxs6 region as BXSB/ll and BXSB differ in this region, the interval can be narrowed down to the 15.1 Mb between 59.7 and 74.8 Mb, although the 0.6 Mb from 78.0 to 78.6 Mb cannot be excluded, a combined region of 15.7 Mb.

**Discussion**

From the data presented in this study, a model can be suggested for the role of Bxs6 and its interaction with the Yaa locus, as summarized in Fig. 5. First, Bxs6 causes the production of high levels of gp70, and Yaa is not involved in this process. However, the levels of gp70 are not as high as those seen in the BXSB strain, thus showing that there must be other contributory factors in the BXS6 genome.

Interestingly, the female B10.Chr13.Yaa mice, although they also had high levels of gp70, only had levels approximately half that of corresponding Yaa-negative or -positive congenic male mice. It would therefore seem that there is a gender effect for levels of gp70. This effect could be due to hormonal influences, but could also be due to genes within the Bxs6 locus that regulate the expression of proteins in a sex-dependent manner.

The high levels of gp70 appear to result in the production of anti-gp70 Abs and therefore the development of gp70IC. Thus, high-level autantigen titer in this model does direct the production of autoantibodies as previously reported (17). However, the production of gp70IC is not solely dependent on high levels of gp70.
gp70, because levels are accelerated by the presence of Yaa. This result is consistent with the finding that certain non-autoimmune strains develop high levels of gp70, equal to those of SLE-prone strains, but do not produce gp70IC (11). It can therefore be hypothesized that Yaa provides a further break in tolerance above that caused by Bxs6 alone, allowing higher levels of gp70IC to be produced. It has been proposed that the acceleration of disease can be attributed to a duplication of Tlr7 on the Y chromosome of Yaa-bearing mice (22, 23). TLR7 is one of four nucleic acid-sensing Toll receptors, specifically recognizing ssRNA. Although this process provides no direct link to the enhancement of Bxs6-driven gp70IC production by Yaa, triggering of TLR7 is known to stimulate the production of large amounts of proinflammatory cytokines. These include IFN-α, which has been shown to enhance Ag presentation capacity of both B cells and dendritic cells driving T cell development and activation, as well as B cell hyperresponsiveness and increased deposition of immune complexes in the glomeruli (24).

Even in the presence of Yaa, the level of gp70IC caused by Bxs6 in the congenic mice is significantly lower than that seen in BXSB mice. Therefore, there must be further factors in the BXSB genome that have an effect on the production of gp70IC. One such example is the interval Gpl on chromosome 9, which was identified by linkage analysis in Bxs6 homozygotes as an additional locus that contributed to gp70IC levels (17).

A proportion of the B10.Chr13 mice developed elevated levels of ANA even in the absence of Yaa, therefore showing that Bxs6 is sufficient for ANA production. However, the Bxs6 locus was not able to cause high levels of anti-DNA Abs alone, implying that Yaa provided a necessary further break in tolerance.

A number of B10.Yaa.Chr13 mice produced high-titer anti-ssDNA and anti-dsDNA Abs. It has been demonstrated that, in addition to anti-gp70 Abs, Abs to nuclear material can also bind to NZB xenotropic retroviruses (14), due to the presence of this material on the surface of the retrovirus. The correlation between gp70IC and ANA levels, consistent with both phenotypes being reliant on high levels of gp70, provides support for this theory.

Our data has also shown that Bxs6 can cause mild nephritis in the presence of Yaa. There was no correlation observed between the level of nephritis and any other traits. However, the likelihood is that the observed nephritis is caused by the presence of gp70IC, because levels of gp70IC correlated most strongly with nephritis in the BXSB backcross studied for the linkage analysis (17). Indeed, there is substantial evidence for the involvement of gp70IC in the development of nephritis. It has been shown that gp70IC is deposited in the kidneys of SLE-prone mice (1), and in many crosses of autoimmune mice levels of gp70IC are significantly correlated with nephritis (6–8). However, the data presented in this study demonstrate that Bxs6 cannot cause the development of nephritis in the absence of Yaa, possibly due to the reduction in gp70IC.

Fine mapping of the Bxs6 locus using recombinant BXSB × B10 × BXSB/F2 backcross mice suggested that Bxs6 lies in the 18.9 Mb between 59.7 and 78.6 Mb on chromosome 13. Although this result is based on the genotype of only two mice with low levels of gp70 and gp70IC, our studies have demonstrated that mice with low levels such as these are reliably heterozygous for Bxs6. To extend these mapping data, we used the recently available SNP database, which enabled us to eliminate a further region in which the Bxs6 gene is unlikely to lie. This assumption could be made because BXSB/ll and BXSB mice are genotypically different in this region, which would not be expected at the Bxs6 locus as both strains develop elevated levels of gp70. The entire region from 74.8–78.0 Mb (Fig. 4b, section B) could therefore be eliminated. Based on this assumption, the Bxs6 region can be narrowed down to the 15.1 Mb between 59.7 and 74.8 Mb, and the 0.6 Mb between 78.0 and 78.6 Mb on chromosome 13, which is a total region of 15.7 Mb.

Congenic lines have been bred containing the NZW Sgp3 locus, which lies in the same region as the Bxs6 locus, on a B6 background (25). The Sgp3 locus was identified in a B6 × (NZW × B6. Yaa)F2 backcross as linked to gp70, with a trend toward linkage to gp70IC. Like the Bxs6 locus, there was no linkage to anti-DNA Abs. The Sgp3 locus was mapped to between D13Mit142 (60.7 Mb) and D13Mit254 (76.5 Mb) on chromosome 13 using overlapping congenic strains, which is an interval of 15.8 Mb. The gp70 phenotype of Bxs6 and Sgp3 congenic mice is very similar. This similarity suggests that it is most likely the same gene for both Sgp3 and Bxs6 that controls levels of gp70. The largest of the two regions that comprise the 15.7 Mb now suggested for the Bxs6 locus is predominantly contained within the region identified for the Sgp3 gene. It is therefore highly likely that, given the similarity between the effects of Bxs6 and Sgp3, the 0.6 Mb interval between 78.0 and 78.6 Mb can be eliminated and the gene mapped to a 14.1 Mb interval between 60.7 and 74.8 Mb.

Also mapped to the same interval (56.6–77.5 Mb) is Gv1 (26), which, along with the gene Gv2, is required for the expression of G3sa, an antigenic determinant of gp70, on normal lymphoid cells (27, 28). Gv1 encodes a trans-acting factor, which regulates retroviral expression but has not been cloned. However, functionally and genetically, it is a very strong candidate and it is likely that Bxs6, Sgp3, and Gv1 are the same locus.

Interestingly, the Sgp3 congenic mice did not produce gp70IC, despite having equally high levels of gp70 as the Bxs6 congenic mice. It is therefore feasible that the Bxs6 locus contains a second gene that causes the development of gp70IC, which is not present in the Sgp3 locus. Although a B10 locus has been identified as important in gp70IC production (29), this locus is BXSB in the B10.Chr13.Yaa mice and so is not a contributory factor. It may thus be the case that high levels of gp70 allow the production of gp70IC, but that a further factor within the Bxs6 locus is required, but not sufficient, for production to occur. This possibility is under investigation.

Disclosures

The authors have no financial conflict of interest.

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