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Chromosome Mapping of *Rmp-4*, a Gonad-Dependent Gene Encoding Host Resistance to Mousepox

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DBA/2 (D2) mice are susceptible and C57BL/6 (B6) mice are resistant to lethal mousepox. A congenic resistant strain, D2.B6-*Rmp-4*^{r} (D2.R4), was developed by serially backcrossing male mice that survived ectromelia virus infection with D2 mice, beginning with (B6 × D2)F_1 mice. Male D2.R4 mice were at least 300-fold more resistant to lethal mousepox than male D2 mice. Female D2.R4 mice were 100-fold more resistant than male D2.R4 mice and 500-fold more resistant than female D2 mice. Neonatal gonadectomy prevented development of resistance in D2.R4 mice of both sexes. Differences in resistance between strains and between sexes correlated with restriction of virus replication in spleen and liver, but gender differences were less evident in liver than in spleen. High-resolution interval mapping of the 19 autosomes of D2.R4 mice using dispersed informative microsatellites as marker loci revealed a segment of distal chromosome 1 to be of B6 origin. Haplotypes for a marker locus, *D1Mit57*, from the differential segment were determined in (D2.R4 × D2)F_1 × D2 backcross mice, which were then infected with ectromelia virus. Significantly more heterozygotes than homozygotes survived ectromelia virus infection in both sexes. Whereas nearly all surviving males were heterozygotes, 44% of surviving females were homozygotes. These results indicate that resistance in D2.R4 mice is determined by a gonad-dependent gene on distal chromosome 1, provisionally named *Rmp-4*, and by an ovary-dependent factor that is not genetically linked to *Rmp-4*.

Orthopoxviruses cause localized or generalized infections in a variety of species (14). Systemic orthopoxvirus infections, which include smallpox (variola) in human beings and mousepox (ectromelia) in laboratory mice, are among the most lethal infections known (15, 16). Recent studies indicate that the large orthopoxvirus genomes encode many potential host-interactive proteins, some of which are homologs of host proteins, which allow certain orthopoxviruses to thwart host defenses (9, 25, 40, 41). The term virokines has been applied to these proteins (42). Deletional analyses have shown that the repertoire of virokines is an important determinant of orthopoxvirus dissemination and lethality (1, 8, 31).

Ectromelia virus is a natural pathogen of murid rodents (15). Susceptibility to the lethal effects of ectromelia virus is widespread in *Mus* species of murid rodents indigenous to Asia, including *Mus caroli*, *Mus cookii*, and *Mus cervicolor*, whereas *Mus* species from Europe, including *Mus spretus* and *Mus musculus*, are variably resistant (10). It has been proposed that resistance in European murids is a consequence of selection pressure exerted by ectromelia virus or a closely related virus on wild murid populations (10). The genomes of inbred strains of laboratory mice are mosaics of the genomes of European and Asian *M. musculus* subspecies (3). Most inbred strains, including DBA/2 (D2), are susceptible to the lethal effects of ectromelia virus, but several, including C57BL/6 (B6), are highly resistant (43). Resistance to mousepox is inherited as a polygenic trait (7, 43). The genes that control resistance to mousepox are of interest because they are dominant over ectromelia virus genes that encode virokines and other virulence factors and because they may be some of the best examples of mouse genes that have responded to selection pressure by a highly lethal naturally occurring virus.

At least four autosomal dominant genes, named *Rmp-1*, *Rmp-2*, *Rmp-3*, and *Rmp-4*, control resistance to mousepox in B6 mice. We have localized *Rmp-1*, *Rmp-2*, and *Rmp-3* to chromosomes 6, 2, and 17, respectively (6, 12). The mapping of these genes has been aided by the development of a series of congenic resistant strains which have individual resistance genes from B6 mice on a susceptible D2 background (5). Three congenic resistant strains have B6 haplotypes for marker loci linked to *Rmp-1*, *Rmp-2*, and *Rmp-3*, respectively (5, 12). A fourth strain has D2 haplotypes for these marker loci and is presumed to carry at least one unmapped resistance gene (5). Assuming that this strain has a single resistance gene, we have named this gene *Rmp-4* and the strain D2.B6-*Rmp-4*^{r} (D2.R4). D2.R4 mice are notable because females are substantially more resistant than males to lethal mousepox (5). Gender has previously been shown to be an important determinant of resistance to mousepox in (B6 × D2)F_1(F_1) × D2 backcross mice but not in B6, D2, or F_1 mice (6, 43). Neonatal gonadectomy prevents development of this gender difference in backcross mice by increasing the proportion of females that are susceptible more than that of males but has no demonstrable effect on resistance in B6 mice (6).

In this study, we compared resistance and ectromelia virus replication in intact and gonadectomized, male and female D2.R4, B6, and D2 mice; used high-resolution interval mapping to delineate chromosomal segments of the congenic strain that were of B6 origin; and mapped all of the male and some of the female resistance to a gene on distal chromosome 1.

MATERIALS AND METHODS

Mice. Specific-pathogen-free DBA/2NCr (D2) and C57BL/6NCr (B6) mice were obtained from the Frederick Cancer Research Center, Frederick, Md. D2.R4 mice were developed as previously described (5). Briefly, first-backcross (N_2) mice were produced from male D2 and female F_1 mice. The subsequent N_3

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to N_8 generations were produced by crossing male mice that survived subcutaneous challenge infection with 10^3 PFU of the Moscow strain of ectromelia virus with D2 females. Approximately 30% of male mice in generations N_3 to N_8 survived challenge infection. From 4 to 14 males served as progenitors in generations N_3 to N_8. This was followed by brother-sister intercrosses for five generations. Because female mice were required for intercrosses, challenge infection with ectromelia virus was restricted to second filial (F_2) and F_4 generations to avoid passive protection of progeny by maternal antibodies (2). Male D2.R4 mice were bred to D2 female mice, and the male F_1 mice were bred to female D2 mice to produce first-backcross mice. Microisolator (Lab Products, Maywood, N.J.) cages, changed under a laminar flow hood, were used to house mice. Mice were specific pathogen free and maintained under specific-pathogen-free conditions. Serological monitoring confirmed that the mice used in this study were negative for serum antibodies to ectromelia virus prior to experimental inoculation. Mice were used at 8 to 10 weeks of age.

Gonadectomy. Mice were anesthetized by immersion in ice chips for 3 min and castrated or ovariectomized at 4 to 6 days of age as previously described (6).

Virus. Stocks of the Moscow strain of ectromelia virus were prepared, counted, and scored as previously described (2). The titer of the stock virus was 2×10^9 PFU/ml, as determined on BSC-1 cell culture monolayers.

LD_{50}. Mice were infected by intravenous tail vein injection under light methoxyfluorane anesthesia with serial 10-fold dilutions of virus ranging from 10^0 to 10^6 PFU in 100 μl. Mortality was scored daily for 21 days. Survivors were bled, and serum was analyzed for antibodies to vaccinia virus. Only survivors with serum antibodies to vaccinia virus were included in determining median lethal doses (LD_{50}). LD_{50} values were determined by the method of Reed and Muench (33). Ten groups of mice were studied: intact and gonadectomized male and female B6 and D2.R4 mice, and intact male and female D2 mice. Each group contained five mice per virus dilution tested.

Quantification of ectromelia virus DNA. Spleen and liver DNA was extracted and purified by the method of Krieg et al. (22) and brought to a concentration of 10 μg in 100 μl of 10 mM Tris (pH 8)–1 mM EDTA. DNA samples were heated to 95°C for 4 min and chilled on ice. An equal volume of 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) was added, and samples were spotted onto Hybond-N+ nylon membranes (Amersham Life Sciences, Arlington Heights, Ill.) prewetted with 10× SSC with a commercial dot blotting apparatus. Membranes were denatured, neutralized, and fixed according to the manufacturer's recommendations. Each dot blot included 10 μg of spleen and liver DNA from uninfected mice and serial twofold dilutions of purified ectromelia virus DNA ranging from 5 to 160 ng.

Ectromelia virus DNA was detected with a ^{32}P-labeled probe consisting of the cloned *Hin*dIII J fragment of the WR strain of vaccinia virus as described previously (4). This fragment contains the highly conserved viral thymidine kinase gene (34, 44). A commercial random prime labeling kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used to label the probe according to the manufacturer's recommendations. Nylon membranes were prehybridized for 2 h at 65°C in 10× Denhardt's reagent (1× Denhardt's reagent is 0.02% Ficoll type 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin fraction 5), 5× SET (1× SET is 1.5 M NaCl, 0.3 M Tris [pH 8], and 20 mM EDTA), 500 μg of salmon sperm DNA per ml, and 1% sodium dodecyl sulfate (SDS). Membranes were hybridized overnight at 65°C in 5× Denhardt's reagent–5× SET–200 μg of salmon sperm DNA per ml–200 μg of heparin sulfate per ml. Hybridized membranes were washed for 30 min at 65°C twice with 5× Denhardt's reagent–2× SET, four times with 2× SET–0.5% SDS, and twice with 0.1% SET–0.1% SDS.

Hybridized membranes were dried and autoradiographed overnight to check technical quality. Dots were punched out from hybridized membranes and mounted in a dot holder, and radioactivity was counted with a Bioscan (Washington, D.C.) QC-2000 direct high-energy beta counter. There was a linear regression of $\log_{10}$ counts per minute (cpm) on $\log_{10}$ nanogram ectromelia virus DNA standards, with *r* values equal to or greater than 0.95. The amount of viral DNA in test samples was determined by plotting $\log_{10}$ cpm against $\log_{10}$ nanograms of viral DNA with the linear regression equation. The limit of detection was 2.5 ng of viral DNA per 10 μg of DNA, which was determined to be equivalent to a titer of $10^{3.6}$ PFU/0.1 g of spleen or liver in preliminary experiments. A 1.0 $\log_{10}$ difference in virus titers (PFU per 0.1 g) was also determined to be equivalent to a 0.37 $\log_{10}$ difference in virus DNA (nanograms per 10 μg).

Ten groups of mice were studied: intact and gonadectomized male and female B6 and D2.R4 mice, and intact male and female D2 mice. Mice were infected by intravenous tail vein injection, and spleens and livers were harvested on days postinfection (PID) 2, 3, 4, and 6. There were five mice per group per interval.

Microsatellite analysis. DNA was isolated from mouse tail biopsy specimens by a standard method (19) and dissolved in 10 mM Tris (pH 8)–1 mM EDTA at a concentration of 1 μg/μl. PCR primers for mouse microsatellites were obtained commercially (Research Genetics, Huntsville, Ala.). Microsatellites were selected based on provisional map positions and maximum allelic size differences to allow them to be distinguished in NuSieve agarose gels. Linkage maps and allele sizes for these markers were obtained from the Whitehead/MIT Genome Center, Cambridge, Mass.

A 1-μg aliquot of genomic DNA was amplified in a 25-μl reaction with AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's specifications. The primer concentrations were 1.0 μM. The reaction mixes were overlaid with 50 μl of light mineral oil. Reactions were amplified on a Perkin Elmer model 480 DNA thermal cycler with the following thermocycling protocol: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, and finally followed by a single cycle of 72°C for 7 min. PCR products were diluted with loading buffer and electrophoresed on 3.5% NuSieve GTG agarose gels in TAE buffer at 6 V/cm for 2 to 3 h. Gels were stained with ethidium bromide, and PCR products were viewed on a long-wave UV light box. Allelic size differences of 8 bp could be distinguished in preliminary studies. Microsatellites were ordered on gels based on the reported allelic size for D2 mice, from smallest to largest. Alleles for D2.R4 mice that did not follow the expected size progression for D2 mice were run against B6 and D2 alleles for the same marker locus. A microsatellite that did not follow the size progression of D2 alleles and that was of a size that was indistinguishable from the B6 allele and distinguishable from the D2 allele was judged to be of B6 origin.

Statistical analysis. Main effects on mean $\log_{10}$ values for virus DNA between groups were tested by two- or three-way analyses of variance with a microcomputer-based statistical program (SuperANOVA, version 1.1; Abacus Concepts, Berkeley, Calif.). Pairwise comparisons were then made by using Duncan's new multiple-range test. Mortality rates between heterozygous and homozygous backcross mice were compared with *z* tests. Comparisons that yielded *P* values of less than 0.05 were considered statistically significant.

TABLE 1. LD_{50} values for intact and gonadectomized B6 and D2.R4 mice and intact D2 mice

Strain	Sex	Gonadectomized	LD_{50}[a] ($\log_{10}$ PFU)
B6	Female	No	5.7
		Yes	6.0
	Male	No	5.5
		Yes	6.0
D2.R4	Female	No	4.5
		Yes	1.4
	Male	No	2.5
		Yes	—[b]
D2	Female	No	1.8
	Male	No	—

[a] LD_{50} value in $\log_{10}$ PFU of ectromelia virus calculated by the method of Reed and Muench (34).

[b] —, no mice survived infection at any infectious dose.

RESULTS

Relative resistance of intact and gonadectomized D2.R4 mice. Intact and gonadectomized male and female B6 and D2.R4 mice and intact male and female D2 mice were infected intravenously with serial dilutions of ectromelia virus, and LD_{50} values were determined (Table 1). Male and female intact and gonadectomized B6 mice were equally resistant to lethal mousepox. By contrast, female D2.R4 mice were more resistant than male mice, and resistance in both sexes was largely prevented by gonadectomy. Some ovariectomized but no castrated D2.R4 mice survived infection with very low infectious doses of virus. The resistance of intact D2.R4 mice was less than that of B6 mice but greater than that of D2 mice. Some female D2 mice resisted very low infectious doses of virus, whereas no male mice survived infection at any dose.

Restriction of virus replication in spleen. Intact B6, D2, and D2.R4 mice and neonatally gonadectomized B6 and D2.R4 mice of both sexes were infected intravenously with 10^4 PFU of ectromelia virus at 10 to 12 weeks of age, and ectromelia virus DNA (E-DNA) was quantified in spleens and livers at selected intervals. The dose of virus used was below the LD_{50} value for female but not male D2.R4 mice. The amount of virus DNA that was equivalent to 10^4 PFU of input ectromelia virus and expected to be deposited in the spleen (less than 4%) was at least fivefold below the detection limit of the assay used. The E-DNA assay therefore measured replicating virus. Initially, gender effects were discounted (Fig. 1A and B). As observed in

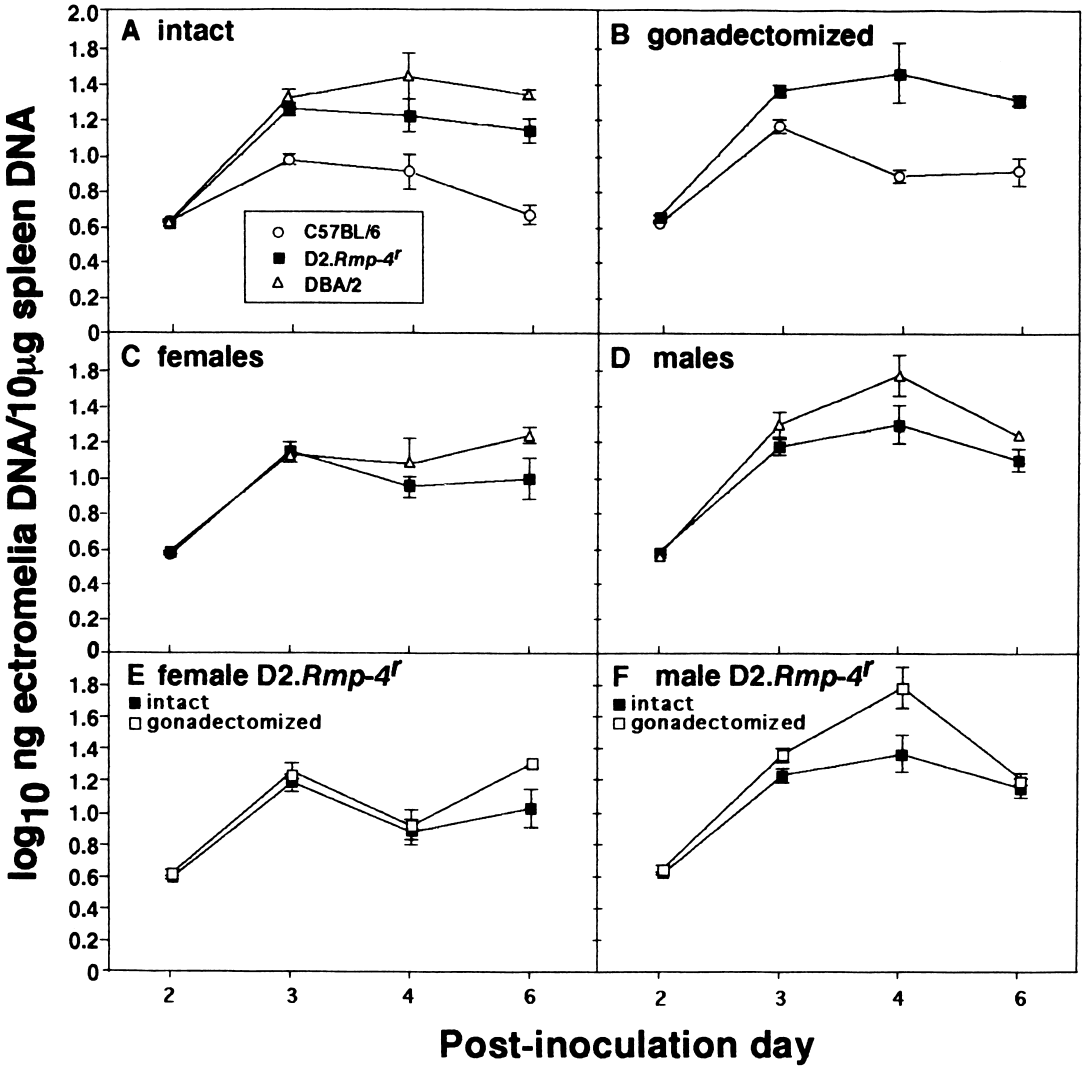

FIG. 1. E-DNA quantified in spleens of intact and gonadectomized male and female mice of three strains after intravenous infection with 10^4 PFU of ectromelia virus. Each point represents the $\log_{10}$ mean of 10 (A and B) or 5 (C to F) animals, with standard errors shown. Where error bars are not shown, error bars lie within symbols. Strain symbols are the same in panels A to D.

a previous study (4), there was significantly less E-DNA in B6 spleens than in D2 spleens beginning on PID 3. There was 2.7-fold, 6.1-fold, and 6.9-fold more viral DNA in the spleens of D2 mice than in the spleens of B6 mice on PID 3, 4, and 6, respectively ($P < 0.001$). This was equivalent to differences in infectious virus titers of 16-fold, 125-fold, and 200-fold, respectively. D2.R4 mice also restricted virus replication more than D2 mice, but the magnitude of the restriction was less than that observed in B6 mice. D2 mice had 1.2-fold, 2.3-fold, and 1.8-fold more spleen viral DNA than D2.R4 mice on PID 3, 4, and 6, respectively ($P < 0.01$), which was equivalent to differences in infectious virus titers of 1.6-fold, 10-fold, and 5-fold, respectively. *Rmp-4*^{r} therefore had a significant but small effect on ectromelia virus replication in the spleen compared with the effects of the full complement of resistance genes carried by B6 mice.

Gonadectomized B6 and D2.R4 mice did not restrict virus replication in the spleen as efficiently as intact mice (Fig. 1A and B). Gonadectomized B6 mice had more E-DNA than intact B6 mice ($P < 0.05$) but less E-DNA than intact D2 mice ($P < 0.001$). Gonadectomized D2.R4 mice had more E-DNA than intact D2.R4 mice ($P < 0.025$), similar to E-DNA levels in intact D2 mice ($P > 0.05$).

These results, combined with those of the LD_{50} study, showed that gonad-dependent factors were required for resistance and for restriction of virus replication in D2.R4 mice and contributed to restriction of virus replication in B6 mice but were not required for B6 mice to express the fully resistant phenotype.

Because female D2.R4 mice were more resistant to lethal mousepox than male mice, levels of E-DNA in intact male and female mice were compared for all strains. The gender of D2.R4 mice, like the gender of D2 mice, played an important role in restricting virus replication. Male D2 and D2.R4 mice had more E-DNA than females of the same strain ($P < 0.01$, Fig. 1C and D). However, the differences in E-DNA levels between female D2 and D2.R4 mice (Fig. 1C) and between male D2 and D2.R4 mice (Fig. 1D) were similar. Male and female B6 mice had similar E-DNA levels at all intervals (data not shown, $P > 0.05$).

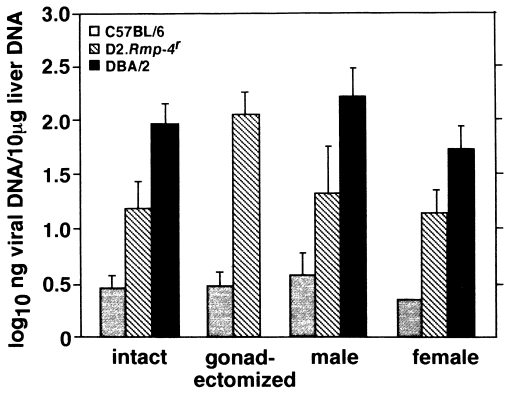

FIG. 2. E-DNA quantified in livers of intact and gonadectomized male and female mice of three strains 6 days after intravenous infection with 10^4 PFU of ectromelia virus. Each bar represents the $\log_{10}$ mean of 10 (intact and gonadectomized) or 5 (male and female) animals, with standard errors shown.

Gonadectomized D2.R4 mice of both sexes did not restrict virus replication as efficiently as intact mice, but surprisingly, the gender difference remained. E-DNA levels in ovariectomized D2.R4 mice were increased relative to those in intact females (Fig. 1E), similar to E-DNA levels in intact female D2 mice (Fig. 1C). Likewise, E-DNA levels in castrated D2.R4 mice were increased relative to those in intact males (Fig. 1F), similar to E-DNA levels in male D2 mice (Fig. 1D).

Restriction of virus replication in liver. We quantified E-DNA in the livers to determine if the effects of *Rmp-4*, gender, and gonads on virus replication were specific to the spleen or had broader tissue expression. Generally, differences in liver E-DNA levels between strains and between gonadectomized and intact mice mirrored differences in the spleen E-DNA levels, but significant differences were delayed until PID 6 (Fig. 2). Excluding gender effects, E-DNA levels were 43 times lower in B6 than in D2 mice on PID 6 ($P < 0.001$), with intermediate levels in D2.R4 mice. Gonadectomized B6 mice restricted virus replication in the liver as effectively as intact B6 mice ($P > 0.05$), which differed from results for spleen, and gonadectomized D2.R4 mice had more E-DNA than intact D2.R4 mice ($P < 0.01$), as was the case for spleen. Gender effects were not as evident in the liver as they were in the spleen. Male and female B6 mice had similar levels of E-DNA ($P > 0.05$), as did male and female D2.R4 mice ($P > 0.05$). Male D2 mice had more E-DNA than females ($P < 0.01$). Gender differences in resistance in D2.R4 mice therefore correlated more with control of virus replication in the spleen than in the liver up to PID 6.

High-resolution interval map of chromosomes 1 to 19 in D2.R4 mice. As a first step toward mapping *Rmp-4*, we sought to identify autosomal regions in D2.R4 mice that were of B6 origin. Only autosomes were considered because the selection of female F_1 mice in the N_1 generation leading to the development of this strain eliminated all but the pseudoautosomal region of the Y chromosome of B6 mice, and the selection of male mice in subsequent generations eliminated all but the pseudoautosomal region of the X chromosome of B6 mice. After eight backcross generations, a selected locus is carried on a chromosome segment that averages about 25 centimorgans (cM), derived from the donor strain (17). The consensus genetic length of the 19 autosomes is 1,371 cM (13). Of this, an average of about 11 cM is expected to contain donor strain genetic material that is unlinked to the selected locus based on eight backcross generations (17). We selected a panel of 422 provisionally mapped informative microsatellites dispersed throughout the 19 autosomes, with an average spacing of 3.2 cM. All of the microsatellites in D2.R4 mice were of D2 origin except for five consecutive microsatellites spanning a distance of 9.1 cM on distal chromosome 1 which were of B6 origin (Fig. 3A and B).

Mapping resistance to distal chromosome 1. To determine if a resistance gene was contained within the segment of chromosome 1 defined by microsatellites *D1Mit104*, *D1Mit33*, *D1Mit106*, *D1Mit57*, and *D1Mit110*, we backcrossed (D2.R4 × D2)F_1 mice to D2 mice, determined haplotypes for *D1Mit57* in individually identified mice, and infected them with ectromelia virus. Ninety male and 88 female mice were studied. Among the males, 58 mice were heterozygous and 32 were homozygous for the marker locus, which deviated from the expected 1:1 ratio ($P < 0.005$, chi-square analysis with Yate's correction). This indicated that male backcross mice that were heterozygous at the *D1Mit57* locus had a survival advantage over homozygous littermates prior to challenge infection with ectromelia virus. By contrast, females were equally divided between heterozygotes and homozygotes. At 8 weeks of age, mice were infected with 10^3 PFU of ectromelia virus, and mortality was scored daily for 21 days. This dose exceeded the LD_{50} value for male D2.R4 mice and was below the LD_{50} value for female D2.R4 mice. As expected, mortality rates were higher in males than in females (Table 2). Nearly all male resistance was determined by a gene within the differential segment of chromosome 1. Forty percent of heterozygous males (23 of 58) survived challenge infection, whereas only 1 of 32 homozygotes survived infection.

Female resistance was also controlled by a gene on chromosome 1, but it was not the only resistance factor. *D1Mit57* heterozygotes had nearly half the mortality rate of homozygotes. Unlike males, however, half of the female homozygotes survived infection. This result indicated that female D2.R4 mice were also protected by a gender-specific factor that was not linked to the differential segment of chromosome 1.

DISCUSSION

A previous study indicated that D2.R4 mice carried a resistance gene that was not one of the three that we had localized to specific chromosomes (5, 12). This study confirmed that all of the male resistance and some of the female resistance of D2.R4 mice was determined by a new gene on distal chromosome 1. None of the previously localized resistance genes map to this region (5, 6, 12). We provisionally designate this resistance gene on chromosome 1 *Rmp-4*, assuming that resistance is mediated by a single gene within the differential segment.

A reason for mapping *Rmp-4* and other resistance genes is to identify candidate genes which map to the same region, have obvious or potential antiviral effects, and are polymorphic between mouse strains. The most proximal and distal of the five chromosome 1 microsatellites of D2.R4 mice inherited from B6 mice have been provisionally mapped to 78.0 and 87.9 cM distal to the centromere, respectively, as reported by the 1994 Chromosome Committee of the Mouse Genome Informatics Project maintained by the Jackson Laboratory, Bar Harbor, Maine (Table 3). The differential segment extends beyond these markers, however, to include regions bracketed by the adjacent marker loci with the D2 haplotype. Thus, *Rmp-4* maps distal to *D1Mit103* at 73.0 cM and proximal to *D1Mit206* at 95.8 cM. In Table 3, we list candidate genes for *Rmp-4*. These have been mapped between 73.0 and 95.8 cM on chromosome 1 and encode host defense proteins, and most are polymorphic. Most candidate genes map proximal to *D1Mit104* or distal to

A

Chromosome									
1 prox.	1 dist.	2	3	4	5	6	7	8	9
Marker locus/genotype									
118D	87 D	6 D	149D	149D	42 D	86 D	178D	124D	90 D
67 D	163D	81 D	54 D	50 D	123D	138D	57 D	3 D	91 D
231D	191D	32 D	130D	101D	1 D	50 D	114D	95 D	67 D
211D	54 D	82 D	118D	172D	75 D	116D	117D	4 D	129D
70 D	94 D	152D	167D	91 D	11 D	159D	55 D	63 D	154D
170D	139D	203D	151D	55 D	81 D	74 D	78 D	64 D	95 D
171D	218D	156D	152D	111D	184D	93 D	145D	24 D	22 D
122D	193D	61 D	65 D	139D	15 D	175D	86 D	125D	4 D
213D	30 D	91 D	69 D	151D	134D	17 D	84 D	129D	162D
214D	194D	37 D	51 D	17 D	135D	16 D	181D	25 D	142D
156D	103D	94 D	40 D	142D	113D	9 D	31 D	31 D	48 D
178D	104B	126D	49 D	178D	93 D	102D	148D	135D	106D
19 D	33 B	42 D	28 D	81 D	157D	132D	38 D	106D	75 D
180D	106B	58 D	12 D	152D	24 D	103D	98 D	45 D	155D
181D	57 B	102D	106D	185D	188D	105D	40 D	50 D	8 D
46 D	110B	63 D	42 D	116D	136D	39 D	66 D	81 D	146D
134D	206D	133D	14 D	146D	65 D	107D	12 D	41 D	111D
216D	150D	17 D	15 D	76 D	138D	55 D	94 D	33 D	50 D
80 D	221D	164D	127D	37 D	161D	150D		113D	12 D
49 D	56 D	223D	17 D	12 D	98 D	61 D		88 D	115D
48 D	209D	166D	86 D	16 D	99 D	25 D		56 D	53 D
84 D	222D	194D	147D	148D		59 D			51 D
45 D	210D	57 D	128D	54 D		137D			18 D
157D	155D	59 D	129D	170D		15 D			
135D		50 D	89 D	189D					
		229D	19 D	33 D					
		147D		190D					
		200D							
Consensus length of chromosome (cM)									
	98	101	100	81	84	74	89	81	70
Average distance between microsatellites (cM)									
	2.0	3.6	3.8	3.0	4.0	3.1	4.9	3.9	3.0

B

Chromosome										
10	11	12	13	14	15	16	17	18	19
Marker locus/genotype										
75 D	148D	38 D	57 D	99 D	13 D	9 D	57 D	110D	43 D
28 D	77 D	1 D	17 D	10 D	53 D	100D	113D	83 D	42 D
51 D	79 D	83 D	60 D	110D	111D	88 D	46 D	68 D	31 D
3 D	82 D	84 D	63 D	126D	7 D	103D	81 D	94 D	28 D
44 D	53 D	136D	91 D	50 D	5 D	58 D	135D	14 D	61 D
38 D	135D	46 D	94 D	54 D	26 D	12 D	30 D	17 D	41 D
61 D	108D	63 D	139D	56 D	63 D	84 D	28 D	58 D	16 D
42 D	51 D	88 D	13 D	129D	90 D	5 D	22 D	74 D	45 D
11 D	174D	36 D	66 D	60 D	29 D	65 D	11 D	52 D	40 D
41 D	20 D	34 D	99 D	18 D	71 D	50 D	49 D	81 D	13 D
68 D	154D	14 D	9 D	101D	105D	6 D	66 D	33 D	19 D
133D	157D	118D	126D	142D	33 D	70 D	139D	79 D	66 D
14 D	177D	79 D	107D	122D	72 D		70 D	7 D	53 D
102D	4 D	141D	145D	5 D	96 D		88 D		10 D
	30 D	133D	128D	102D	34 D		89 D		36 D
	90 D	18 D	148D	39 D	39 D		53 D		1 D
	178D		76 D	115D	41 D		3 D		34 D
	121D		77 D	35 D	42 D		120D		33 D
	38 D		35 D	75 D			93 D		
	41 D			131D			39 D		
	179D			136D			142D		
	67 D						122D		
	99 D						41 D		
	132D						96 D		
	166D						129D		
	181D						123D		
	48 D								
	104D								
Consensus length of chromosome (cM)										
78	78	73	72	49	56	58	36	57	36	
Average distance between microsatellites (cM)										
5.6	2.8	4.6	3.8	2.3	3.1	4.8	1.4	4.4	2.0	

FIG. 3. Interval map of the 19 autosomes of D2.R4 mice based on 422 informative microsatellites. (A) Interval map of chromosomes 1 to 9. (B) Interval map of chromosomes 10 to 19. Listed in columns by chromosome number are microsatellites, abbreviated from the nomenclature proposed by the Committee on Standardized Genetic Nomenclature for Mice as applied to those described by the Whitehead Institute/MIT Center for Genome Research, Cambridge, Mass. The full designations are D-chromosome number-Mit-number shown in figure. Microsatellites are listed in their provisional order from proximal to distal relative to the centromere, as reported in the eighth release of the Whitehead Institute/MIT Genome Center Genetic Map of the Mouse. Microsatellite designations are followed by letters corresponding to the strain of origin based on allele size as described in Material and Methods: D, D2; B, B6. Microsatellites inherited from B6 mice are outlined. Consensus lengths for the 19 autosomes are taken from reference 14. Average spacing was calculated by dividing consensus lengths by the number of microsatellites.

D1Mit110. It is not known, therefore, if the differential segment contains the B6 alleles for most of these candidate genes. Loci for lymphotactin and the selectin family, however, provisionally fall within the B6 marker loci. An additional candidate gene has been mapped close to the *Fcgr3* locus but has not been assigned a provisional map location. This is the locus that encodes the 2B4 alloantigen which is expressed selectively by natural killer (NK) cells and T cells that mediate non-major histocompatibility complex-restricted killing (18, 26). Because resistance controlled by *Rmp-4* is gonad dependent, the expression of candidate genes might be expected to be sex hormone responsive. With the exception of complement factor H (28), important roles for sex hormones in the expression of the other candidate genes have not been described.

Because resistance to lethal mousepox is genetically complex, it is important to determine the relationships, if any, between resistance genes. A comparison of the candidate genes for *Rmp-4* with the candidate genes for *Rmp-1* on distal chromosome 6 and *Rmp-2* on proximal chromosome 2 reveals that all map near genes that are important in innate immunity. A gene within the NK cell gene complex, *Ly55*, is a likely candidate gene for *Rmp-1* (12), as is the *Listeria* resistance gene, *Lsr1*, for *Rmp-2* (6). The *Ly55* and *Lsr1* genes are expressed through cells that are important in natural immunity. The *Ly55* genes encode lectin-like receptors on the surface of NK cells, including the NK cell-specific NK1.1 alloantigen, that play a role in recognition and lysis of virus-infected targets (36, 46). We have shown that NK cells of B6 mice restrict ectromelia virus replication in vivo, whereas those of D2 mice do not (12). The *Lsr1* locus controls natural resistance to *Listeria monocytogenes*, an intracellular bacterial pathogen (39). The cellular basis of resistance controlled by *Lsr1* is an augmented ability to recruit macrophages and neutrophils to sites of infection (11).

TABLE 2. Ectromelia virus-induced mortality in (D2.R4 × D2)F_1 × D2 backcross mice based on gender and haplotypes for *D1Mit57* on distal chromosome 1

Sex	*D1Mit57*[a]	No. dead/total (% dead)	*P*[b]
Male	DD	31/32 (97)	
	BD	35/58 (60)	<0.001
Female	DD	21/46 (46)	
	BD	10/42 (24)	<0.05

[a] DD, homozygous for D2 haplotype; BD, heterozygous for D2 and B6 haplotypes.
[b] Results of *z* test comparisons of proportion dead between genotypes.

The list of candidate genes for *Rmp-4* includes several with features in common with the *Ly55* and *Lsr1* loci. The selectin gene complex consists of genes that encode type I integral membrane proteins expressed by a variety of leukocytes and endothelial cells that function as cell adhesion molecules (38). Like the product of the *Lsr1* locus, selectins are important in leukocyte recruitment to sites of infection (32). The locus that encodes the 2B4 antigen, like the *Ly55* complex, has been implicated in NK cell-mediated cytotoxicity of virus-infected targets (26).

In the linkage study of (D2.R4 × D2)F_1 × D2 backcross mice, *Rmp-4* was the sole source of protection in male mice, whereas female mice were protected by *Rmp-4* and an addi-

TABLE 3. Candidate genes for *Rmp-4*

Position[a] (cM)	Marker locus (haplotype)[b]	Candidate gene	Polymorphism[c]		Reference
			B6 allele	D2 allele	
73.0	*D1Mit103* (D)	Complement factor H	*Cfh*^{a}	*Cfh*^{0}	35
74.1					
78.0	*D1Mit104* (B)				
81.6	*DiMit33* (B)				
85.0	*D1Mit106* (B)				
86.6		Selectin gene complex	*Sell*^{b}	*Sell*^{d}	38
87.8		Lymphotactin	ND	ND	21
87.9	*D1Mit57* (B)				
87.9	*D1Mit110* (B)				
92.3		Low-affinity IgG Fc receptors	*Fcgr2*^{b}	*Fcgr2*^{a}	37
93.3		Lymphocyte antigen 9	*Ly9.2*	*Ly9.1*	24
93.3		CD48 antigen	*Cd48*^{b}	*Cd48*^{s}	45
94.2		Serum amyloid P-component	*Sap*^{b}	*Sap*^{d}	27
95.2		Interferon-induced proteins	*Ifi203*^{b}	*Ifi203*^{a}	30
95.8	*D1Mit206* (D)				

[a] Map positions in distance from the centromere are from the 1994 Chromosome Committee Report of the Mouse Genome Informatics Project maintained by the Jackson Laboratory, Bar Harbor, Maine. Marker loci positions are underlined.
[b] D, D2 haplotype; B, B6 haplotype.
[c] ND, not determined.

tional resistance factor. Both resistance factors appeared to be gonad dependent because of the near absence of resistance in gonadectomized D2.R4 mice of both sexes. The nature and origin of the female resistance factor is obscure. The dose of virus that elicited 54% survival in homozygous female (D2.R4 $\times$ D2)F_1 $\times$ D2 backcross mice was 1.2 logs higher than the calculated LD_{50} value for female D2 mice. Thus, it would appear to have originated from B6 mice. High-resolution interval mapping did not, however, identify any autosomal segment, other than the segment of chromosome 1, that was of B6 origin. A small differential segment containing the female resistance factor could have escaped detection, but because this strain was developed by selecting male backcross mice that survived ectromelia virus infection, we were not selecting for a female resistance factor that was unlinked to *Rmp-4*. The probability of retaining a small random passenger segment of the B6 genome that contained a female resistance gene was exceedingly small. Female D2 mice were, however, slightly more resistant than their male counterparts and restricted virus replication in the spleen to a significantly greater extent than males. By contrast, no gender differences were observed in B6 mice either in resistance to lethal infection or in restriction of virus replication, although gonadectomized mice did not restrict virus replication as efficiently as intact mice. It is likely, therefore, that female D2.R4 mice expressed a resistance factor that originated from a gene(s) in the D2 background. The reason that it provided greater protection to female D2.R4 mice than to female D2 mice is not known.

In linking resistance to the differential segment of chromosome 1, we found that male but not female backcross mice that carried the B6 marker exceeded the expected 1:1 ratio. Since we also used male backcross mice to produce the D2.R4 congenic line, it is possible that this segment of chromosome 1, in addition to containing a resistance gene, contains a gene(s) that provides a developmental advantage to male but not female mice when the allele is of B6 origin.

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