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Construction of a BALB/c Congenic Mouse, C.C3H-*Lps*^{d}, That Expresses the *Lps*^{d} Allele: Analysis of Chromosome 4 Markers Surrounding the *Lps* gene

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Development of a congenic BALB/c mouse strain that contains a segment of chromosome 4 including the *Lps*^{d} allele of the lipopolysaccharide (LPS)-hyporesponsive C3H/HeJ strain is presented. On the basis of LPS-induced spleen cell mitogenesis, macrophage tumor necrosis factor secretion, and tyrosine phosphorylation in vitro and lethality in galactosamine-sensitized mice in vivo, the C.C3H-*Lps*^{d} strain provides a model of LPS hyporesponsiveness that is comparable to that of the parental C3H/HeJ strain. Analysis of markers in this region indicates that length of the donor fragment is ~5.5 centimorgans. Thus, the C.C3H-*Lps*^{d} strain provides an important genetic tool for analysis of markers in this region and for examining functional effects of *Lps*^{d} expression on the BALB/c background.

Some time between 1960 and 1965, a spontaneous mutation occurred within the C3H/HeJ colony at the Jackson Laboratory that rendered this strain profoundly refractory to the toxic effects of lipopolysaccharide (LPS) from gram-negative bacteria, more specifically, to the lipid A component of this complex structure (reviewed in references 22 and 28). Analyses of responses to LPS in C3H/HeJ versus other fully LPS-responsive strains were consistent with control by a single autosomal gene with two allelic forms: *Lps*^{n}, for normal LPS responsiveness, and *Lps*^{d}, for hyporesponsiveness (reviewed in reference 28). An enormous amount of functional information has been elucidated by using this defective strain in conjunction with fully LPS-responsive strains to study the cellular and molecular pathways involved in LPS signaling, the role of cytokines in inflammation, susceptibility to infection (i.e., particularly those that involve intracellular replication in macrophages), macrophage differentiation, and an array of other functions. The nature of the *Lps* gene has not been delineated, although recently reported data suggest a role in the transduction of signals following the binding of LPS to the membrane (reviewed in references 22 and 28). A noncomplementary defect within the *Lps* locus has also been reported in the C57BL/10ScCR strain (6, 18), as well as in its predecessor strain, C57BL/10ScN (29). For the most part, these mice have been used in conjunction with *Lps*^{n} mice on a C57BL/10 background to confirm and extend functional analyses carried out with C3H/HeJ and *Lps*^{n} C3H strains.

Analyses of inheritance of LPS responsiveness in recombinant inbred strains derived from C57BL/6J and C3H/HeJ parental strains (BXH) and in backcross linkage analyses between C57BL/6J and C3H/HeJ mice established the gene order and approximate distance in centimorgans (cM) as *Mup1*-6 ± 2 cM-*Lps*-13 ± 7 cM-*Ps* (30, 32, 33). On the basis of previous analyses that mapped the *Mup1* and *Ps* genes on the centromeric and distal sides of the brown coat color (*b*) gene, respectively (7 and 8 cM for the two intervals; 9, 13, 14), Watson and colleagues (30, 31) suggested that *Lps* is probably near *b*. *Ifa*, the locus that encodes a large number of closely related alpha interferon genes, was subsequently estimated to be 13.6 ± 3.6 and 5.3 ± 2.9 cM distal to *Mup1* in two separate backcross linkage analyses (7), suggesting the possibility of physical overlap of the *Lps* and *Ifa* loci; however, by using BXH recombinant inbred strains of mice, these two genes were shown to be physically distinct (10). Since these genetic analyses were carried out, relatively little has been done to map more precisely the position of the *Lps* gene, in large part because of a paucity of markers in that particular region of chromosome 4.

Among the five most widely used inbred strains, the BALB/c mouse has distinguished itself as a model for susceptibility to infection and neoplastic disease (reviewed in reference 23). As with the C3H/HeJ strain, the macrophage and the genes that regulate the functions of this cell type appear to be the focus of many of their functional abnormalities, including susceptibility to intracellular pathogens (23). In addition, BALB/c mice are highly susceptible to induction of plasmacytomas and other tumors by tumorigenic retroviruses, paraffin oils, and other substances (23, 24), although they carry the *Lps*^{n} allele. For this reason, we felt that development of a congenic BALB/c mouse strain that expresses the *Lps*^{d} allele might provide a unique model with which to study pathogenesis or the induction of plasmacytomas, which requires a conventional environmental setting for quantitative development (4). Moreover, the availability of such a strain could provide an important genetic tool for the mapping of genes within this region. The construction and functional analysis of the C.C3H-*Lps*^{d} congenic strain are presented herein.

MATERIALS AND METHODS

Mice. BALB/cAnPt, C3H/HeJ, and C.C3H-*Lps*^{d} mice were bred and housed at Organon Teknika (Rockville, Md.). C3H/HeJ mice were also obtained from Jackson Laboratory (Bar Harbor, Maine). Male and female mice were tested at 5 to 7 weeks of age.

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Reagents. *Escherichia coli* K235 LPS was prepared by the method of McIntire et al. (19). D(+)-galactosamine was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Spleen cell mitogenesis. To assess the LPS phenotype of mice, LPS-induced spleen cell proliferation was measured. Briefly, single-cell spleen cell suspensions were prepared from splenectomized or hemisplenectomized mice at a final concentration of 8×10^6/ml in serum-free RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*2-ethanesulfonic acid), and 0.3% sodium bicarbonate. Spleen cells (100 μl per well) were cultured in 96-well plates (Falcon, Oxnard, Calif.). To each well was added 100 μl of medium only or the indicated concentrations of LPS in medium. Cells were incubated for 48 h at 37°C. During the last 4 to 6 h of incubation, 0.5 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; NEN, Boston, Mass.) was added per well. Cells were harvested onto glass filter paper with a cell harvester, and [^{3}H]thymidine incorporation was measured by scintillation counting techniques.

Macrophage culture. Peritoneal exudate macrophages were isolated by peritoneal lavage 4 days after intraperitoneal injection of 2 ml of sterile 3% thioglycolate broth. Cells were washed and resuspended in RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 10 mM HEPES, 0.3% sodium bicarbonate, and 2% heat-inactivated fetal calf serum as described previously (17). For tumor necrosis factor (TNF) secretion studies, $\sim 2 \times 10^5$ cells per well were plated into 96-well microtiter plates (Becton Dickinson). For tyrosine phosphorylation studies, 10^6 cells per well were plated into 12-well polystyrene tissue culture plates (Costar, Cambridge, Mass.). Nonadherent cells were removed by washing ~4 h after plating. Adherent macrophages were then stimulated as described in the text.

Assays for macrophage LPS responsiveness. Culture supernatants were assayed for TNF bioactivity in a standard cytotoxicity assay using actinomycin D-treated L929 cells as described previously (12). Macrophage lysates were prepared, and protein tyrosine phosphorylation was evaluated by Western blot (immunoblot) analysis as described elsewhere (17), with an anti-phosphotyrosine monoclonal antibody (clone 4G10; UBI, Lake Placid, N.Y.) and the enhanced-chemiluminescence detection method (Amersham, Amersham, United Kingdom).

LPS-induced lethality. Galactosamine-induced sensitization to LPS was carried out as described by Galanos et al. (11).

Marker analysis of the C.C3H-*Lps*^{d} genome. Restriction fragment length polymorphism and simple sequence length polymorphism (SSLP) analyses of a series of chromosome 4 markers surrounding the *b* and *Lps* loci were performed with DNAs isolated from strains BALB/cAnPt and C3H/HeJ and the C.C3H-*Lps*^{d} congenic strain at several different backcross generations (N6F38, N10F7, N10F11, N20F9, and N20F10), as described by Mock et al. (20) and Dietrich et al. (8). Microsatellite primer pairs were purchased from Research Genetics (Huntsville, Ala.). The allele sizes of these SSLPs are available from the Massachusetts Institute of Technology Genome Center database. Probes for *CD301, D4Lgm4, Mup1, Orm2, Jun, Ifa*, and *Scl* have been previously described (3, 15, 16, 20, 21, 25, 27).

RESULTS AND DISCUSSION

Breeding history of the C.C3H-*Lps*^{d} congenic mouse strain. The C.C3H-*Lps*^{d} congenic mouse strain was begun in 1977 by crossing a BALB/cAnPt male with a C3H/HeJ female obtained from the colony of David Sachs (National Institutes of Health). The F_1 mouse was introgressively backcrossed to the BALB/cAnPt strain for six generations, always selecting for black (*B*), the dominant, allelic form of the brown (*b*) coat color gene, which is closely linked to the *Lps* locus on chromosome 4 according to independent analyses of *b* and *Lps* with respect to *Mup1* and *Ps* (30). Beginning with N6 [i.e., N(backcross) 6], the line was made homozygous for *B* (i.e., *B*/*B*) and tested for the *Lps*^{d} allele which was present and operative. The N6 line was inbred for four generations (N6F4), backcrossing was continued in the same manner to N10, and then the N10 line was made homozygous for *B* (and, presumably, *Lps*^{d}/*Lps*^{d}). However, when N10F2-4 mice were tested, it was discovered that the N10 line was not homozygous for *Lps*^{d}; thus, *B* and *Lps*^{d} are not as closely linked as originally suggested (30). A new N10 line was advanced from N6F22 and was inbred. At the same time as the inbreeding of the N10 line was occurring, backcrossing was continued to N20 and completed as a homozygous (for *B* and *Lps*^{d}) congenic strain in 1993. In the course of development of the N20 line, LPS sensitivity was repeatedly tested on the heterozygous mice; separation of *B* and *Lps*^{d} loci never occurred again.

Analysis of C.C3H-*Lps*^{d} LPS hyporesponsiveness in vitro and in vivo. LPS-stimulated interleukin 1 production by thioglycolate-elicited macrophages (as measured in the thymocyte costimulation assay; 26) was originally used to distinguish LPS-hyporesponsive mice from those that were either heterozygous or homozygous for *Lps*^{n}; however, this was extremely cumbersome to carry out on individual mice and was often insufficiently sensitive to discriminate heterozygotes from fully LPS-responsive mice. Therefore, we adopted a new strategy for screening of the mice for LPS sensitivity. Initially, mice were bred and once the litters were weaned, the mother was sacrificed and the spleen was removed and tested in a standard spleen cell mitogenesis assay (26, 29). As the breeding continued, hemisplenectomies were performed on potential breeders and, again, the spleens were subjected to LPS-induced mitogenesis in vitro. This allowed us to eliminate as breeders those progeny that did not carry the *Lps*^{d} allele. Figure 1 shows the data from three individual experiments in which a total of 23 individual N20 mice were assessed for the capacity to respond to LPS in the spleen cell mitogenesis assay. In each of the three experiments shown, three BALB/cAnPt and three C3H/HeJ spleens were included as positive and negative controls, respectively. None of the N20 splenocytes responded to LPS to incorporate [^{3}H]thymidine above unstimulated control levels, even at 10 μg of LPS per ml. Thus, at N20, the C.C3H-*Lps*^{d} mice expressed the *Lps*^{d} allele homozygously.

It has been shown previously that the spleen cell mitogenic response to LPS is also linked to LPS responsiveness in macrophages (26). Therefore, we tested macrophages derived from C.C3H-*Lps*^{d} mice for the ability to produce TNF in vitro. As can be seen in Table 1, pooled BALB/cAnPt macrophages made TNF in response to LPS, in contrast to pooled C3H/HeJ macrophages and macrophages derived from 11 individual C.C3H-*Lps*^{d} macrophages. An even earlier marker of LPS-induced signaling in macrophages is the induction of phosphorylation and dephosphorylation on tyrosine (17, 34) that peaks ~15 min following stimulation of macrophages with LPS and results in the detection of three newly phosphorylated proteins (~40 to 47 kDa). Figure 2 illustrates that only BALB/cAnPt macrophages responded to LPS to express newly phosphorylated proteins.

Since the splenocyte and macrophage responses of the C.C3H-*Lps*^{d} congenic mouse strain indicated a level of in vitro

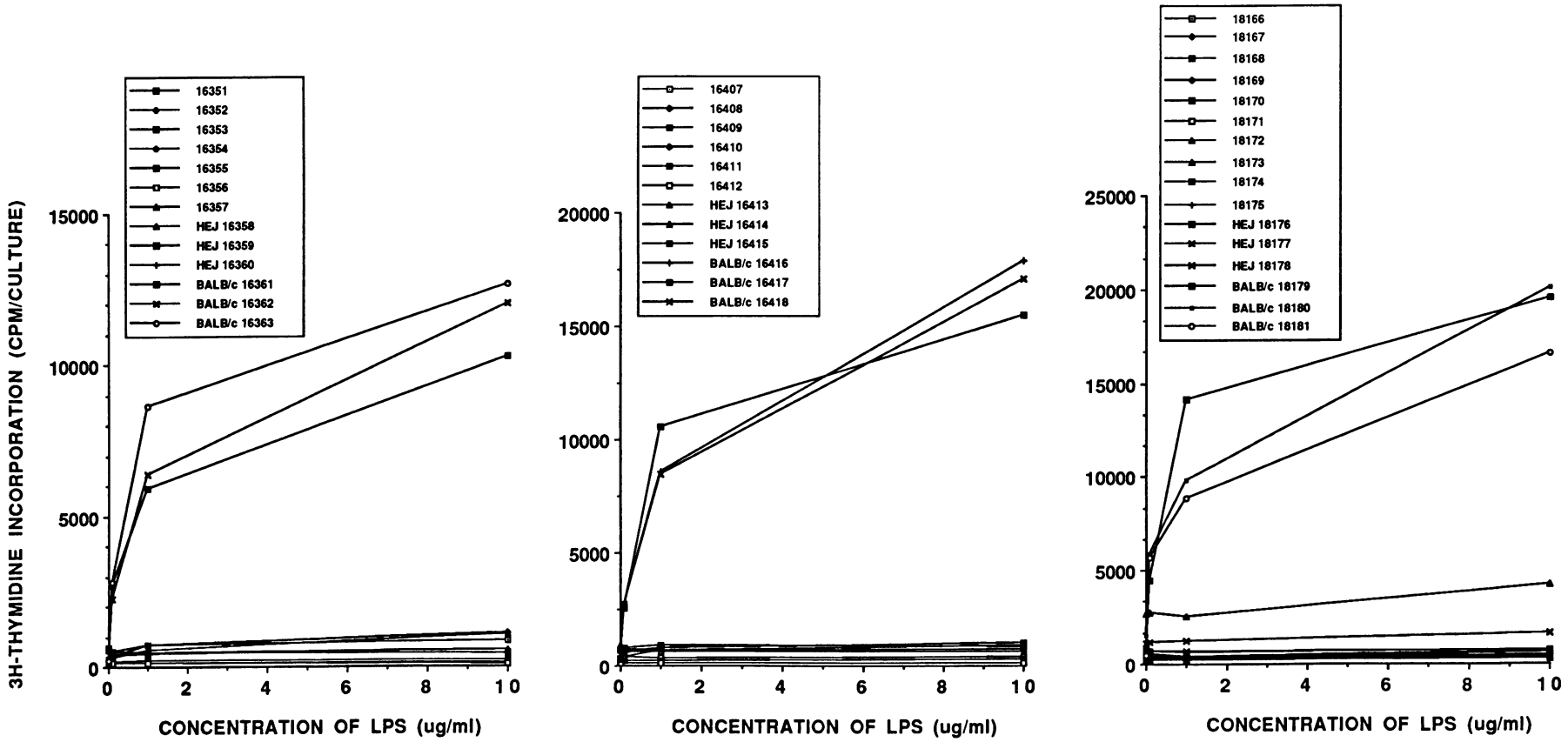

FIG. 1. LPS-induced spleen cell mitogenesis in LPS-sensitive and -resistant mouse strains. The three panels represent three separate experiments in which individual N20 C.C3H-*Lps*^{d} spleens were compared with C3H/HeJ (*Lps*^{d}) and BALB/AnPt (*Lps*^{n}) splenic responses to LPS. Individual mouse identification numbers are shown.

TABLE 1. LPS-induced TNF secretion in BALB/cAnPt, C3H/HeJ, and C.C3H-*Lps*^{d} macrophages

LPS treatment[a] (ng/ml)	TNF activity (U/ml)[b]		
	BALB/cAnPt	C3H/HeJ	C.C3H-*Lps*^{d}
0	<80	<80	<80
1.0	1,920	<80	<80
10.0	20,480	<80	<80
100.0	7,680	<80	<80

[a] Thioglycolate-elicited murine peritoneal macrophages were prepared as described in Materials and Methods. For the BALB/cAnPt and C3H/HeJ cultures, peritoneal exudate cells derived from three mice were pooled. The results of the C.C3H-*Lps*^{d} congenic strain were obtained with 11 individual mice.
[b] TNF activity was measured as described in Materials and Methods. The lower limit of detection in this assay is 80 U/ml.

TABLE 2. Lethal effect of LPS on BALB/c mice but not on C.C3H-*Lps*^{d} congenic mice[a]

Strain	No. of mice	LPS treatment (μg)	% Dead
BALB/cAnPt A	12[b]	2	75
BALB/cAnPt B	12	2	75
BALB/cAnPt C	12	2	50
C.D2-*Idh1 Pep3*	12	2	50
C.C3H-*Lps*^{d}	6	2	0
C.C3H-*Lps*^{d}	6	20	0

[a] Mice were injected intraperitoneally with 20 mg of D(+)-galactosamine and the indicated concentration of LPS simultaneously, and deaths were recorded for 72 h.
[b] In this representative experiment, all of the BALB/c A, B, and C mice were females. Equal numbers of male and female C.D2-*Idh1 Pep3* and C.C3H-*Lps*^{d} congenic strain mice were injected with no significant difference in mortality (data not shown).

LPS hyporesponsiveness equivalent to that of the parental C3H/HeJ strain, we next assessed the level of LPS hyporesponsiveness exhibited by these mice in vivo. By using the well-characterized model of sensitized endotoxin shock developed by Galanos et al. (11), mice of three substrains of BALB/cAnPt, BALB/c congenic strain C.D2-*Idh1 Pep3*, and C.C3H-*Lps*^{d} were injected with D(+)-galactosamine and LPS and percent lethality was measured. A dosage of LPS was chosen that was found in preliminary experiments to kill ~50% of BALB/cAnPt mice (data not shown). Indeed, among the mice of the three BALB/cAnPt strains (A, B, and C) and BALB/c congenic strain C.D2-*Idh1 Pep3*, 50 to 75% died within 48 h of injection of 2 μg of LPS and galactosamine (Table 2). In contrast, none of the galactosamine-sensitized C.C3H-*Lps*^{d} mice died, nor did any exhibit any symptoms of endotoxicity (e.g., ruffled fur, conjunctival discharge, or diarrhea) when injected with either 2 or as much as 20 μg of LPS per mouse. Taken collectively, the in vitro and in vivo data indicate that BALB/c congenic mouse strain C.C3H-*Lps*^{d} appears to be as refractory to LPS as the parental C3H/HeJ strain.

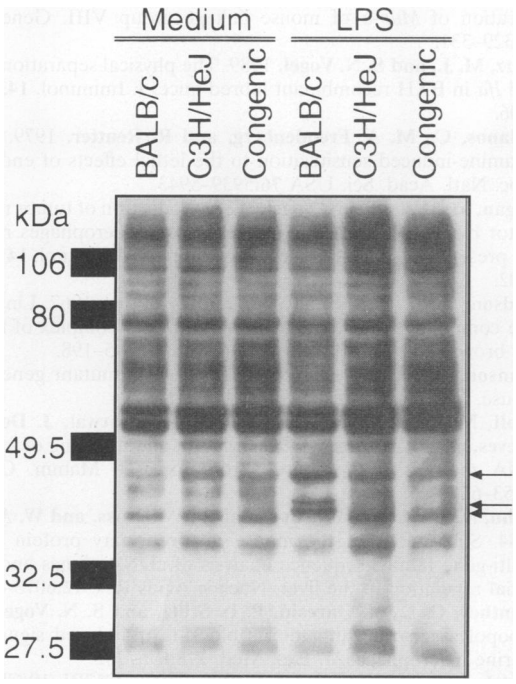

FIG. 2. LPS induces tyrosine phosphorylation in BALB/cAnPt macrophages but not in C3H/HeJ or C.C3H-*Lps*^{d} macrophages. Pooled macrophage cultures were stimulated with 1 μg of LPS per ml for 15 min prior to extraction of lysates for Western analysis as previously described (17). The arrows indicate the approximate positions of the three LPS-inducible phosphoproteins.

Analysis of chromosome 4 markers to delineate the size of the C3H/HeJ donor fragment within the C.C3H-*Lps*^{d} mouse strain. Restriction fragment length polymorphism and SSLP analyses (performed as described by Mock et al. [20] and Dietrich et al. [8]) of a series of background markers and chromosome 4 markers surrounding the *b* and *Lps* loci were performed with DNAs isolated from the C.C3H-*Lps*^{d} congenic strain at several different backcross generations (N6F38, N10F7, N10F11, N20F9, and N20F10). SSLP analysis of the C.C3H-*Lps*^{d} congenic strain confirmed that it carries the following non-chromosome 4 BALB/c alleles: D1Mit17, D2Mit51, D2Nds3, D3Mit21, D5Nds2, D6Mit8, D7Mit14, D8Mit78, D9Mit11, D10Mit15, D11Mit2, D11Mit41, D12Nds2, D13Mit13, D13Mit78, D14Mit1, D15Mit46, D15Mit58, D16Mit5, D17Mit16, D18Mit7, D19Mit13, DXMit31, and DXMit36. We detected no restriction fragment length polymorphisms between strains BALB/cAnPt and C3H/HeJ for the following chromosome 4 markers by utilizing a series of 18 different restriction enzymes: *D4Lgm4, Mup1, Orm2, Jun, Ifa,* and *Scl*. In addition, a series of D4Rck microclones (described and analyzed as reported by Bahary et al. [2]), were also found to be nonpolymorphic between strains BALB/cAnPt and C3H/HeJ; these were D4Rck4, D4Rck9, D4Rck12, D4Rck28, D4Rck49, D4Rck52, D4Rck58, D4Rck65, D4Rck83, D4Rck122, D4Rck137, D4Rck140, and D4Rck151. The only restriction fragment length polymorphisms found between BALB/cAnPt and C3H/HeJ were with the markers *Cd301* (CD30 ligand) and D4Rck130 (DNA segment, Chr 4, Rockefeller University, no. 130). *Cd301* hybridized to 20-, 18-, and 3-kb *Taq*I fragments in C3H/HeJ and only the 180 and 3-kb *Taq*I fragments in BALB/cAnPt. Similarly, the probe for D4Rck130 recognized a doublet near 12 kb in BALB/cAnPt and a doublet near 14 kb in C3H/HeJ. The C.C3H-*Lps*^{d} congenic strain retained the C3H/HeJ allele for both of these markers.

In addition, the D4Mit SSLPs shown in Fig. 3 were also polymorphic between BALB/cAnPt and C3H/HeJ. These markers were utilized to determine the extent of C3H/HeJ chromatin in the C.C3H-*Lps*^{d} congenic strain. No differences in the allelic composition of any of these polymorphic markers were found among the various backcross, intercross genera-

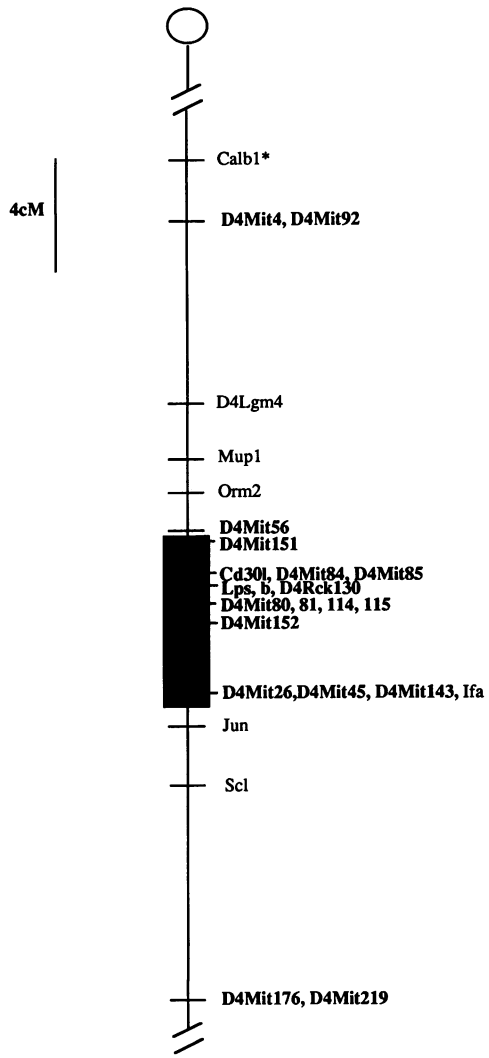

FIG. 3. Map of the proximal portion of mouse chromosome 4. The markers in boldface were found to be polymorphic between BALB/cAnPt and C3H/HeJ. The other markers were nonpolymorphic with at least 18 enzymes; a series of 13 D4Rck markers from this region of chromosome 4 were also found to be nonpolymorphic (see the text). *, the marker *Calb1* (calcium-binding protein 1) was not examined. The marker locations shown are according to Copeland et al. (5), Abbott et al. (1), and the Massachusetts Institute of Technology Genome Center database. Data from the current study positioned D4Mit151 distal to D4Mit56. The region of chromosome 4 highlighted by the black box delineates the portion of chromosome 4 in the C.C3H-*Lps*^{d} congenic strain which is of C3H/HeJ origin. The markers D4Mit4, D4Mit92, D4Mit56, D4Mit176, and D4Mit219 were all of BALB/c origin in the C.C3H-*Lps*^{d} congenic strain.

tions of the *Lps*^{d} congenic strain. Thus, a minimum of 5.5 cM of C3H/HeJ chromatin was retained after 20 backcross generations. Interestingly, data from the current study positioned D4Mit151 distal to D4Mit56 since the congenic strain carries the BALB/c allele of D4Mit56 and the C3H/HeJ allele of D4Mit151. The delineation of this region defines a series of markers to use in isolation of the *Lps* gene.

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