Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7631–7635, November 1985
Developmental Biology

Chromosomal location of the gene encoding the neural cell adhesion molecule (N-CAM) in the mouse

(DNA homology/somatic cell hybrids/recombinant inbred mouse strains/neurological markers *Thy-1* and *sg*)

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ABSTRACT **The gene encoding the neural cell adhesion molecule, N-CAM, has been localized on mouse chromosome 9. A BALB/cJ mouse genomic library prepared in λ bacteriophage EMBL4 was screened by using a cDNA probe, pEC204, that corresponds to the coding region of the chicken N-CAM gene. Four weakly reactive and one strongly reactive recombinant phage were isolated. A region of the latter that was strongly homologous to pEC204 was subcloned to yield a new probe, pEC501. RNA transfer blots and nucleotide sequencing indicated that pEC501 encoded part of the mouse N-CAM gene. This probe defined a unique genetic locus, *Ncam*, associated with a restriction fragment length polymorphism that allowed the definition of two alleles. The locus could be provisionally assigned either to chromosome 9 or to chromosome 10 by correlating the presence or absence of mouse-specific DNA fragments reactive with the probe in a panel of somatic hybrid cell lines with the presence or absence of the various mouse chromosomes. Analysis of the inheritance of the *Ncam*-associated DNA polymorphism in recombinant inbred strains of mice revealed close linkage between *Ncam* and the *Lap-1*, *Sep-1*, and *Thy-1* loci on chromosome 9. This result suggests an additional linkage between *Ncam* and the locus for the cerebellar mutation staggerer (*sg*). The *Ncam* locus provides an important reference point for mapping the genes for additional cell adhesion molecules as well as genes for other molecules involved in neural development and function.**

Cell–cell adhesion is an important primary process in embryonic development. Over the past decade, specific cell adhesion molecules (CAMs) have been identified in embryonic and adult tissues (1, 2). These include two primary CAMs, the neural cell adhesion molecule N-CAM (3, 4) and the liver cell adhesion molecule L-CAM (5, 6), which are found very early in development, and one secondary CAM, the neuron–glia adhesion molecule, Ng-CAM, which appears on postmitotic neurons (7, 8). Studies of these cell surface glycoproteins have suggested that there may be few primary CAMs (9) and that regulation of the expression of these molecules and of additional secondary CAMs may be pivotal processes in morphogenesis and histogenesis (10).

The two primary CAMs are dynamically expressed in definite sequences during development (9, 11) and both appear at least transiently on derivatives of all three germ layers (12). At sites of embryonic induction there are two modes of CAM expression at the cell surface: in migrating mesenchyme, N-CAM first diminishes at the cell surface and then reappears, whereas in epithelia, both N-CAM and L-CAM appear together and one or the other subsequently disappears. Local signals are thus probably responsible for modulation of CAM expression in a fashion that is coordinated with key morphogenetic events.

In embryonic tissues N-CAM is distinguished by a high content of polymeric sialic acid (4), which decreases during development (13, 14) with a concomitant increase in the efficacy of homophilic N-CAM binding (15). In the chicken, the molecule consists of two polypeptide chains with molecular weights of 130,000 and 160,000 that are extremely similar to each other (16); a smaller third species, which is much more prominent in mice, is also detected (14).

Recently, we have prepared cDNA probes to chicken N-CAM genes in plasmid vectors and in the phage expression vector λgt11 (ref. 17; unpublished data). RNA transfer and Southern blot analyses indicate that the two N-CAM polypeptides are probably specified by two mRNA species derived by differential splicing of a single transcript. Because of the role of N-CAM in development and its altered appearance in the mouse cerebellar mutant staggerer (18, 19), it was of interest to determine whether linkage existed between the N-CAM gene and other genetic loci, especially those associated with the development or function of the nervous system. Such mapping would also provide an important step towards defining elements that might regulate CAM expression.

We have now mapped the gene for N-CAM in the mouse, using a probe derived from a mouse genomic clone detected on the basis of its homology to a chicken cDNA probe that is known to contain coding sequences. Two independent methods were used: somatic cell hybrids were first used to assign the gene to a unique site present on either chromosome 9 or chromosome 10. A restriction site polymorphism in the N-CAM gene then allowed a precise mapping to be made with recombinant inbred strains of mice. The results place the gene close to two loci on chromosome 9 whose expression has been related to the nervous system, namely the *Thy-1* gene and the staggerer locus *sg*.

MATERIALS AND METHODS

The chicken N-CAM cDNA clone, pEC204, a 3.5-kilobase (kb) cDNA fragment inserted into λgt11, has recently been isolated by immunological screening with anti-N-CAM antibodies (unpublished results). A mouse genomic DNA library was constructed by partially digesting BALB/cJ liver DNA with *Mbo* I, purifying 15- to 20-kb DNA fragments by sucrose density gradient centrifugation, and ligating these to purified arms from *Bam*HI-digested λ bacteriophage EMBL4 (ref. 20 and ref. 21, pp. 269–286). The ligation mixture was packaged *in vitro* (ref. 21, pp. 256–263) and amplified in *Escherichia coli* LE392. Phage plaques were transferred to nitrocellulose filter discs (Schleicher & Schuell) and screened to identify recombinant phage homologous to the pEC204 chicken N-CAM probe (ref. 21, pp. 326–328). Hybridizations were performed in 20% (vol/vol) formamide and 0.9 M NaCl at 42°C, and

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Abbreviations: kb, kilobase(s); cM, centimorgan; CAM, cell adhesion molecule; L-CAM, liver CAM; N-CAM, neural CAM; Ng-CAM, neuron–glia CAM.

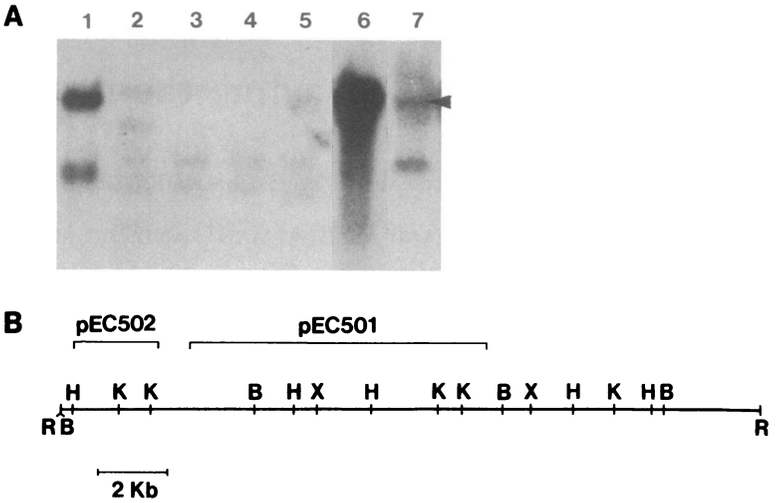

FIG. 1. Mouse genomic DNA clones homologous to the chicken N-CAM cDNA clone pEC204. (*A*) DNA purified from each of the five BALB/cJ genomic DNA clones homologous to pEC204 was digested with *Pst* I and analyzed by Southern blotting using pEC204 as a probe. The hybridization was carried at 42°C in 20% (vol/vol) formamide and the filter was washed at 58°C in 0.3 M NaCl/0.03 M Na citrate/0.1% sodium dodecyl sulfate. Lane 1, λmN1 (mouse N-CAM 1); lane 2, λ3A; lane 3, λ3B; lane 4, λ7A; lane 5, λ7B. The two strongly reactive *Pst* I fragments from λmN1 were subcloned in pBR328, yielding plasmid clones pEC501 and pEC502. *Pst* I-digested DNA from these plasmids, analyzed as above, is shown in lanes 6 and 7, respectively (arrowhead indicates hybridization to incompletely cut plasmid). (*B*) Restriction map of the mouse DNA insert in λmN1. The *Eco*RI sites (R) at the two ends of the insert and the *Bam*HI (B) site at the left end are derived from the polylinker at the cloning site in EMBL4 (20). Cleavage sites within the insert are indicated: H, *Hin*dIII; K, *Kpn* I; X, *Xho* I. Neither *Sal* I nor *Eco*RI cleaves the insert. The locations of the inserts of pEC501 and pEC502 are indicated.

filters were washed at 58°C in 0.3 M NaCl/0.03 M Na citrate/0.1% sodium dodecyl sulfate. These conditions were determined empirically from preliminary Southern blots of total mouse genomic DNA, and they permitted DNA duplex formation with approximately 30% mismatching (22). Positive recombinant phage were plaque purified and grown in large-scale culture. Phage DNA was extracted, analyzed by restriction mapping, and subcloned in plasmid vectors by using standard procedures (ref. 21, pp. 363–402).

Mouse–Chinese hamster and mouse–rat hybrid cell lines were generated, maintained, and characterized as previously described (23, 24). Hybrid cell lines ABm11, ABm14, and F(11)U were a gift from R. E. K. Fournier. Mice were obtained from the Salk Institute (CXJ strains) or from The Jackson Laboratory (all others). Genomic DNA was purified as previously described from mouse livers and from cultured hybrid cell lines and analyzed by Southern blotting (ref. 21, pp. 382–389, and refs. 23 and 25).

RESULTS

N-CAM structure appears to be well conserved between the chicken and the mouse: antibodies to one protein react specifically with the other (26), and the chicken N-CAM cDNA clone pEC204 hybridizes to specific DNA fragments in Southern blots of mouse genomic DNA (unpublished observations). To isolate the murine N-CAM gene, a genomic library prepared from BALB/cJ liver DNA was screened with pEC204. Five independent phages were identified, and two *Pst* I subfragments from the phage (λmN1) that hybridized strongly to pEC204 (Fig. 1) were inserted into the *Pst* I site of the plasmid vector pBR328, yielding the recombinant plasmids pEC501 and pEC502. pEC501 was selected for further study because it was free of detectable reiterated sequences. Two lines of evidence suggested that pEC501 contained DNA sequences encoding murine N-CAM. First, in RNA transfer blots of brain and liver poly(A)$^+$ RNA from neonatal mice, pEC501 hybridized to two brain-specific RNA species with estimated sizes of 7.0 and 4.7 kb. These sizes are similar to those of two of the transcripts reported by others (27). Second, the nucleotide sequence of a 180-base-pair fragment of pEC501 showed 84% homology to a region of pEC204. Moreover, a reading frame open for the full length of the fragment specified an amino acid sequence 88% homologous to part of the predicted chicken N-CAM protein sequence (unpublished results).

Genomic restriction mapping experiments indicated that pEC501 corresponded to a unique genetic locus and that the locus was associated with restriction fragment length polymorphisms. DNA isolated from livers of C57BL/6J and BALB/cJ mice was digested with each of the restriction endonucleases *Eco*RI, *Pst* I, *Bam*HI, *Hin*dIII, and *Msp* I and analyzed by Southern blotting with pEC501. In each case, the numbers and sizes of homologous genomic DNA fragments were as predicted by the restriction map of λmN1 (Fig. 1). No additional genomic DNA fragments were observed. To search for polymorphisms associated with the locus, DNA from each of the inbred mouse strains AKR/J, C57L/J, SWR/J, DBA/2J, C57BL/6J, C3H/HeJ, SJL/J, and BALB/cJ was digested with *Msp* I and analyzed by Southern blotting (Fig. 2). Three fragments, 2.2, 2.6, and 3.2 kb, were present in all strains. In addition, DNA from AKR/J (lane A), C57L/J (lane L), SWR/J (lane S), C57BL/6J (lane B), and

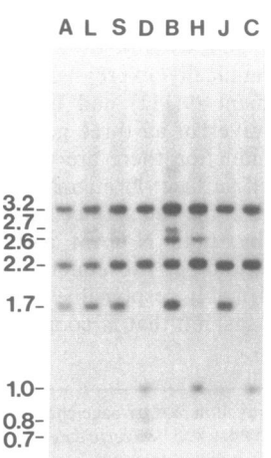

FIG. 2. Restriction fragment length polymorphism associated with *Ncam*. Liver DNA from the inbred mouse strains AKR/J (A), C57L/J (L), SWR/J (S), DBA/2J (D), C57BL/6J (B), C3H/HeJ (H), SJL/J (J), and BALB/cJ (C) was digested with *Msp* I restriction endonuclease and analyzed by Southern blotting using pEC501 as a probe (ref. 21, pp. 382–389, and ref. 25). DNA fragment sizes in kb are shown, calculated on the basis of fragment mobility relative to fragments of λcI857 DNA digested with *Hin*dIII.

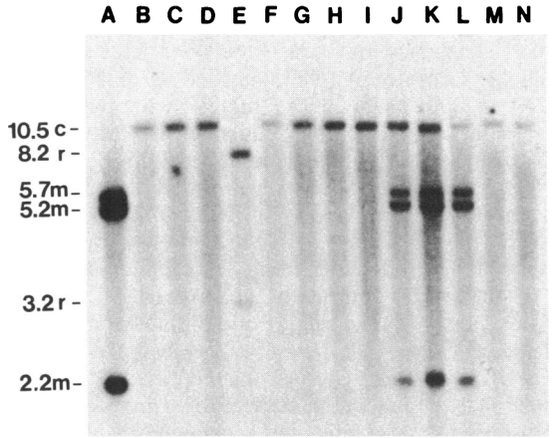

FIG. 3. Detection of mouse N-CAM-specific DNA fragments in interspecies somatic hybrid cell lines. DNA from C57BL/6J mouse liver (A), the Chinese hamster cell line E36 (B), and the hybrid cell lines ABm11 (C), ABm14 (D), F(11)U (E), BEM1-4 (F), MAE28 (G), MACH4A63 (H), MACH4B31Az3 (I), MACH2A2B1 (J), MACH2A2C2 (K), MACH2A2H3 (L), ECm4e (M), and R44-1 (N) was digested with *Hin*dIII restriction endonuclease and analyzed as described in the legend to Fig. 2. Somatic hybrid cell lines contained various numbers of mouse chromosomes on the background of a complete rat genome [F(11)U, lane E] or Chinese hamster genome (all others). Sizes of fragments are shown in kb, and the species of origin of each is indicated: m, mouse; c, Chinese hamster; r, rat.

SJL/J (lane J) yielded 1.7- and 2.7-kb fragments, while DBA/2J (lane D), CH3/HeJ (lane H), and BALB/cJ (lane C) gave 0.7-, 0.8-, and 1.0-kb fragments.

To determine the chromosomal location of N-CAM-encoding sequences in the mouse, interspecies somatic cell hybrids were examined. A panel of 12 somatic hybrid cell lines carrying various combinations of mouse chromosomes on the background of either a complete rat genome [hybrid F(11)U] or a complete Chinese hamster genome (all others) was scored for the presence or absence of mouse-specific N-CAM DNA fragments by Southern blotting (Fig. 3). The complete set of mouse-specific DNA fragments was present in three of the hybrids (see lanes J, K, and L). The only mouse chromosomes present in these hybrids at a frequency ≥0.15 copy per cell [the approximate limit of detection of the assay (23)] were numbers 9 and 10. All other hybrids lacked both of these chromosomes (23, 24).

To confirm this result and to localize the N-CAM-encoding sequence more precisely, five sets of recombinant inbred mice (28, 29) were scored for inheritance of the restriction fragment length polymorphism (Fig. 2) associated with the locus (Table 1). All mice were homozygous for one of the progenitor forms of the locus. Comparison of these strain distribution patterns with those previously determined for other polymorphic loci in these strain sets showed linkage between the N-CAM gene restriction fragment length polymorphism and the *Sep-1* (apolipoprotein 1) and *Lap-1* (leucine aminopeptidase 1) loci on the proximal portion of chromosome 9 (6 recombinants among 63 strains and 9 recombinants among 40 strains, respectively). In the BXD strain set, linkage was found as well between N-CAM and *Env-2* (2 recombinants among 24 strains typed) (31), and in the AKXD set, linkage was found between N-CAM and *Thy-1* (4 recombinants among 25 strains typed).

The odds due to chance alone of finding this degree of concordance between unlinked loci are less than 0.001 in each of the cases (30). Conversely, comparison of the N-CAM strain distribution patterns with those for more than 80 other loci typed in these five recombinant inbred strain

Table 1. Inheritance of *Ncam* and other chromosome 9 markers in recombinant inbred strain sets

Locus	Allele at locus																											
	AKXD strain:																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	21	22	23	24	25	26	27	28	
Thy-1	D	D	A	—	—	A	D	A	D	A	A	A	D	D	D	A	D	A	D	D	D	A	A	D	A	D	A	
Sep-1	D	D	A	A	D	A	D	—	D	—	A	A	D	D	D	—	D	A	D	D	D	A	A	D	A	D	A	
Ncam	D	D	A	—	—	D	D	D	A	A	A	A	D	D	D	D	D	A	D	D	D	A	A	D	A	D	A	
	BXD strain:																											
	1	2	5	6	8	9	11	12	13	14	15	16	18	19	20	21	22	23	24	25	27	28	29	30	31	32		
Lap-1	B	D	B	D	D	D	D	D	B	B	B	D	D	D	B	D	D	D	D	D	B	B	D	D	—	—		
Sep-1	B	D	B	D	D	D	B	D	B	D	B	D	D	D	B	D	B	D	D	D	B	B	D	D	B	—		
Ncam	B	D	B	B	D	D	B	D	B	D	B	D	D	D	—	—	B	D	D	D	B	B	D	B	B	D		
Env-2	B	D	B	B	D	D	B	D	B	D	B	D	D	D	B	D	B	D	D	D	B	D	D	D	B	D		
	BXH strain:														CXB strain:													
	2	3	4	5	6	7	8	9	10	11	12	14	19		D	E	G	H	I		J	K						
Lap-1	B	B	H	B	B	B	B	H	B	H	B	B	H		C	C	C	B	B		B	C						
Sep-1	B	B	H	B	—	B	B	H	H	H	H	B	H		C	C	C	B	B		B	C						
Ncam	B	B	H	—	B	H	B	H	H	H	H	B	H		C	B	C	B	B		B	C						
	CXJ strain:																											
	1	3	6	8	9	10	11	15																				
Ncam	J	J	J	C	C	C	J	J																				

Recombinant inbred strains of mice were derived (28, 29) by inbreeding pairs of F_2 hybrid mice from a cross between two inbred progenitor strains: AKXD, AKR/J × DBA/2J; BXD, C57BL/6J × DBA/2J; BXH, C57BL/6J × C3H/HeJ; CXB, BALB/cBy × C57BL/6By; CXJ, BALB/cJ × SJL/J. Mice were typed at *Ncam* by scoring the presence or absence of the strongly hybridizing 1.7-kb fragment in *Msp* I-digested liver DNA, as shown in Fig. 2. AKXD mice were typed at *Thy-1* by scoring the presence of either a 4.7-kb fragment (AKR/J form) or a 4.2-kb fragment (DBA/2J form) in Southern blots of *Pvu* II-digested liver DNA probed with the pcT108 mouse Thy-1.2 genomic clone (39). Alleles at each locus are indicated according to the progenitor strains from which they were derived. A = AKR/J, D = DBA/2J, B = C57BL/6J, H = C3H/HeJ, C = BALB/cJ, and J = SJL/J.

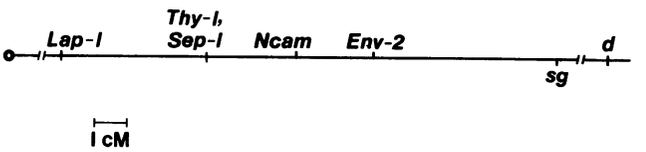

FIG. 4. Linkage of *Ncam* to other markers on chromosome 9. Estimated linkage distances in cM were calculated from the data in Table 1 according to Taylor (28). These distances are in agreement with those estimated from conventional backcross experiments involving *Thy-1*, *Lap-1*, *Sep-1*, and other markers of the chromosome. The marker *sg* has been interpolated into the map on the basis of its segregation with respect to the more distal dilute (*d*) locus in backcross experiments (32–34).

sets and known to map elsewhere in the genome revealed no concordances better than expected from chance alone. Using the fractions of recombinants found among the loci to estimate distances between them in centimorgans (cM) yielded the map: centromere–*Lap-1*–4.4 ± 2.7 cM–(*Thy-1*, *Sep-1*)–2.8 ± 1.3 cM–*Ncam*–2.4 ± 1.8 cM–*Env-2* (Fig. 4) (28). No recombinants were found between *Thy-1* and *Sep-1* among 22 AKXD strains, indicating at a 95% confidence level that these two loci are within 3.9 cM of each other (30).

DISCUSSION

N-CAM has been extensively characterized in terms of both its function and its chemistry, and cDNA clones corresponding to large portions of the chicken N-CAM mRNA have recently been isolated (ref. 17; unpublished results). To map the location of the gene or genes encoding N-CAM in relation to other genetic markers with potential roles in neural development, we have identified a murine genomic clone homologous to a portion of the chicken N-CAM gene. RNA transfer blots and DNA sequencing indicate that the clone encodes part of the mouse N-CAM gene.

The clone defines a novel genetic locus on mouse chromosome 9, which we designate *Ncam*. This chromosome contains two other loci that may influence neuronal development, *Thy-1*, a well-conserved neuronal marker, and *sg*, responsible for a cerebellar connectional defect. On the basis of the restriction fragment length polymorphisms associated with *Ncam*, two alleles can be distinguished: *a*, found in DBA/2J, C3H/HeJ, and BALB/cJ; and *b*, found in C57BL/6J, AKR/J, SJL/J, C57L/J, and SWR/J.

The locus defined by the probe is present as a single copy, suggesting that there is only one gene for mouse N-CAM. This implies that the three N-CAM polypeptides found in the mouse are all derived by alternative splicing, as previously suggested for chicken N-CAM (17). With pEC501 (a mouse genomic DNA fragment strongly homologous to the chicken N-CAM cDNA clone pEC204) in Southern blot analysis of genomic DNA from several mouse strains, all DNA fragments that were visualized (Figs. 2 and 3) were of intensities appropriate for single-copy DNA sequences, and all could be accounted for from the restriction map of the one N-CAM genomic clone, λmN1 (Fig. 1). Furthermore, the patterns of segregation of *Ncam*-associated DNA fragments both in somatic cell hybrids (Fig. 3) and in recombinant inbred mouse strain sets (Table 1) indicate that these fragments are clustered at a single locus in the mouse genome. The isolation of additional weakly hybridizing phages in the original screening of the mouse genomic library with the chicken N-CAM cDNA probe raises the possibility that other evolutionarily related DNA sequences might exist. Further work will be required to explore this possibility.

The *Ncam* locus is associated with a complex restriction fragment length polymorphism. This polymorphism affects several recognition sites for the enzyme *Msp* I and thus cannot be due to a single point mutation. There are no obvious neurological differences between mice carrying the two allelic forms of *Ncam* identified in this study, and therefore the DNA sequences affected by the polymorphism may fall outside of coding sequence. Description of the intron–exon structure of the gene will test this hypothesis. A complex pattern of restriction fragment length polymorphism has been observed previously in the mouse, in association both with transcriptionally active loci and with random DNA fragments and pseudogenes (e.g., see refs. 25 and 35). This pattern is in striking contrast to the apparent preponderance of polymorphisms due to single point mutations in the human (e.g., see ref. 36).

There are two other genes associated directly or indirectly with neural development that appear to be linked to *Ncam* on chromosome 9. *Thy-1* encodes a cell-surface glycoprotein specific to synaptic vesicles in brain white matter and also found on some epidermal cells, thymocytes, and T lymphocytes (32–34), and *sg* is a recessive mutation affecting cerebellar development. Data from breeding experiments place *Thy-1* approximately 2 cM proximal and *sg* approximately 7 cM distal to *Ncam* (Table 1 and Fig. 4; refs. 37 and 38). Because of the sampling fluctuations associated with the estimates of the linkage distances, however, these data alone are insufficient to determine a precise location of *Ncam* relative to *Thy-1* and *sg*.

Several other lines of evidence indicate that the three loci are distinct but keep open the possibility of functional interactions. The *Thy-1* gene product is a cell-surface glycoprotein of unknown function, which differs from the *Ncam* product in size, chemical composition, tissue distribution, and appearance during development (37, 38). Moreover, the existence of recombination events between *Thy-1* and *Ncam* (Table 1) argues convincingly against identity of the two loci.

The locus defined by the *sg* mutation likewise appears distinct from *Ncam*. In normal mice, the N-CAM polypeptide undergoes age- and tissue-specific alterations in its glycosylation pattern (14). These alterations do not occur at the normal time or to the normal extent in *sg/sg* homozygous animals (19). Nevertheless, apparently normal adult forms of the N-CAM glycoprotein can be detected in homozygous mutant mice, although in reduced amounts. Taken together, these facts suggest that *sg* defines a locus different from *Ncam* that directly or indirectly affects N-CAM glycosylation.

The possibility that three loci such as *Thy-1*, *Ncam*, and *sg*, all of which may be involved in neural development, might be tightly linked is an intriguing one. Breeding experiments will be necessary to confirm the independence of these loci and to begin to assess the possible functional consequences of their linkage.

We thank Drs. J. Silver and R. E. K. Fournier for gifts of probes and cell lines. This work was supported by U.S. Public Health Service Grants HD-09635 and HD-16550, a Basil O'Connor Starter Award from the National Foundation–March of Dimes, and a Leukemia Society of America Scholar Award.

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