# Genetic Analysis of the Distribution of Corticosterone-Containing Cells in Mouse Adrenal Cortex (43062)

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*Abstract.* **Strains A/J and C57BL/6J (B6) differ in susceptibility to many neoplasms and infectious agents, with B6 mice generally being more resistant. Glucocorticoids protect against some of these pathologies. We examined the distribution of adrenocortical corticosterone (CS), the major endogenous glucocorticoid in mice, in these strains, using anti-CS serum. A distinct strain difference was found. B6 adrenals exhibited abundant CS-positive cells in cord-like arrays while A/J adrenals contained fewer, randomly arranged CS-positive cells. To quantify these results, each adrenal cortex was divided into eight sectors and each sector was classified as to phenotype. Ninety-three percent of the sectors of B6 cortices exhibited the cord-like pattern, whereas only 15% of the sectors of A/J cortices exhibited this pattern. These differences are consistent with a hypothesis that A/J mice are relatively deficient in the prophylactic activities of endogenous glucocorticoids. Adrenal glands from (C57BL/6J × A/J)$F_1$ hybrid mice had approximately equal proportions of areas exhibiting each phenotype, indicating codominant alleles for this trait. We propose the name *Cor* for this gene. Thirty AXB and BXA recombinant inbred (RI) lines of mice derived from A/J and B6 progenitors were examined for CS immunostaining. Twenty-eight of them had either predominantly A/J-like or predominantly B6-like phenotypes. These RI data support either of two hypotheses. Hypothesis 1 emphasizes the nearly complete concordance of the RI lines with progenitor phenotypes and proposes that a single *Cor* gene regulates the distribution of CS-positive cells. Using this model, the strain distribution among RI lines implies linkage of *Cor* to a region on chromosome 6, 27-37 cM from the centromere. Hypothesis 2, which gives greater weight to the two RI lines with intermediate numbers of CS-positive cells, postulates an epistatic interaction between two *Cor* loci.**

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A/J and C57BL/6J (B6) mice vary in their relative susceptibilities to cancer and to infection by bacteria and viruses, with A/J mice generally being more sensitive (1). For example, most A/J mice develop at least one lung adenoma spontaneously within their lifetime (2) and many tumors after treatment with a chemical carcinogen, e.g., urethan (3). B6 mice, on the other hand, develop less than one lung tumor per mouse even after treatment with a carcinogen (4, 5). Three *Pas* (pulmonary adenoma susceptibility) genes (*Pas 1–3*) account for this difference (6).

Adrenal insufficiency, produced by adrenalectomy, increases lung tumor multiplicity 50% to 2-fold in both strains (7). Chronic treatment with pellets containing corticosterone (CS), the major endogenous glucocorticoid in the mouse (8, 9), reduced the respective multiplicities to or below those of sham-adrenalectomized mice (7). Differences in the number of CS-secreting cells in the adrenal cortex could provide a partial basis for the differing susceptibilities of these two strains to tumorigenesis.

Herein, we describe an immunohistochemical process for detecting CS-containing cells. Upon application of this procedure to A/J and B6 mice, a strain difference was found which supported our hypothesis that higher numbers of CS-containing cells are present in the mouse strain resistant to tumorigenesis. $F_1$ hybrid mice and recombinant inbred (RI) lines were examined to characterize the inheritance of CS staining patterns in the adrenal cortex.

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## Materials and Methods

**Animals.** Male 8-week-old A/J and C57BL/6J (B6) mice, and (B × A)$F_1$ hybrids produced from these parent strains, were purchased from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate at the University of Colorado for 2 weeks before use. All mice were housed in accordance with the National Institutes of Health guidelines for the care of laboratory animals. They were kept on hardwood bedding, given Wayne Lab Blox and water *ad libitum*, and maintained on a 12-hr light/dark cycle. RI lines derived from A/J and B6 progenitors (1) were similarly housed at the University of California. Adrenal samples from additional AXB and BXA RI lines were generously provided by Dr. Robert Erickson (Department of Human Genetics, University of Michigan School of Medicine, Ann Arbor, MI); AXB1 and AXB2) and Dr. Ellen Goldberg (Department of Immunology, University of New Mexico School of Medicine, Albuquerque, NM; BXA12). Adrenals from progenitor A/J and B6 mice were included along with those of the RI lines obtained from each laboratory, to serve as controls for possible environmental effects on CS immunostaining patterns.

**Tissue Preparation.** Adrenal glands were removed during the second hour of the light cycle to minimize circadian effects on hormone secretion. Mice were sacrificed by cervical dislocation and, to minimize stress-induced changes in CS, excision of the glands was completed within 3 min of the time the mice were first disturbed. Adrenal glands were collected from a total of 23 A/J, 24 B6, 9 (B × A)$F_1$, and 2–6 mice from each of 30 AXB and BXA RI lines. Adrenals from the RI lines were processed and sent to Boulder from San Diego, Ann Arbor, or Albuquerque.

Excised adrenal glands were placed into 4% formaldehyde buffered in 2% calcium acetate (pH 7.5) and cleaned of extraneous connective tissue within the first 10 min of fixation. After 2 hr in fixative, they were rinsed for 2 hr in running water to remove excess formaldehyde, dehydrated through a graded series of ethyl alcohol, cleared in toluene, and embedded in paraffin.

**Corticosterone Immunohistochemistry.** Sections 4-$\mu$m thick were cut and immunostained by the streptavidin-biotin procedure using the Histostain-SP kit (Zymed, South San Francisco, CA). Briefly, sections were hydrated in phosphate-buffered saline (PBS, 1.48 g of $Na_2HPO_4$, 0.43 g of $KH_2PO4$, 8.77 g of NaCl/liter; pH 7.3) for 5 min, treated with 3% $H_2O_2$ for 15 min to minimize endogenous peroxidase activity, rinsed in PBS (three times for 3 min each), and incubated in nonimmune goat serum for 15 min to block nonspecific antibody binding. Rabbit anti-CS serum (Western Chemical Research, Ft. Collins, CO) was applied as a 1/100 dilution in PBS for 30 min at 37°C. This serum has been thoroughly characterized for its monospecificity to CS (10). Normal rabbit serum (1/100) was used in place of the anti-CS on some samples as a control for serum specificity and background staining in the adrenal cortex. PBS rinses (three for 3 min) were used between all subsequent steps. Biotinylated goat anti-rabbit IgG serum was applied for 30 min at room temperature, horseradish peroxidase-streptavidin conjugate for 5 min, and the Zymed aminoethyl carbazole substrate for the peroxidase enzyme for 15 min. Because the aminoethyl carbazole reaction product is alcohol soluble, sections were embedded in Crystal Mount permanent aqueous mounting medium (Biomeda, Foster City, CA) before coverslips were mounted with Permount for photomicrography using a Nikon Microphot-FX.

**Quantitation.** After identifying a distinct difference in staining patterns between A/J and B6 mice (see Results), the following method of quantitation was used to determine the extent to which mice adhered to a homogeneous staining pattern. We used only sections cut so as to expose both cortex and medulla; tangentially cut sections were ignored. Each stained specimen was divided arbitrarily into eight equal sections around the circumference of an entire gland. Each sector was then classified as having an A/J-like pattern or a B6-like pattern. Slides were read "blind" so that at the time of diagnosis it was not known from which experimental group each section came. Using one gland from each of five animals per strain, we accumulated a total of 40 measurements for each group. In a few cases a gland was partially damaged during dissection and fewer than eight sectors in that gland were then examined. For some RI lines varying numbers of mice were available, and we used sections from both adrenal glands, as indicated in Results.

**Statistical Methods.** Confidence limits for the estimates of gene linkage in the RI lines were determined according to Silver (11).

## Results

CS-positive cells were readily identified immunohistochemically in all adrenal cortical samples. Normal rabbit serum yielded virtually no background staining (Fig. 1).

**A/J and B6 Parental Strains.** There were no readily apparent differences in the gross or microscopic anatomy of the adrenal glands themselves. The major cortical layers, the zona glomerulosa (outermost zone just under the capsule), fasciculata (middle zone), and reticularis (inner zone, next to the medulla) were similarly represented in both strains. CS immunostaining was present in the zona fasciculata, which comprises most of the cortical width. Adrenal glands from B6 mice showed abundant immunostained cells in cord-like arrangements (Fig. 1A). In contrast, adrenal cortex

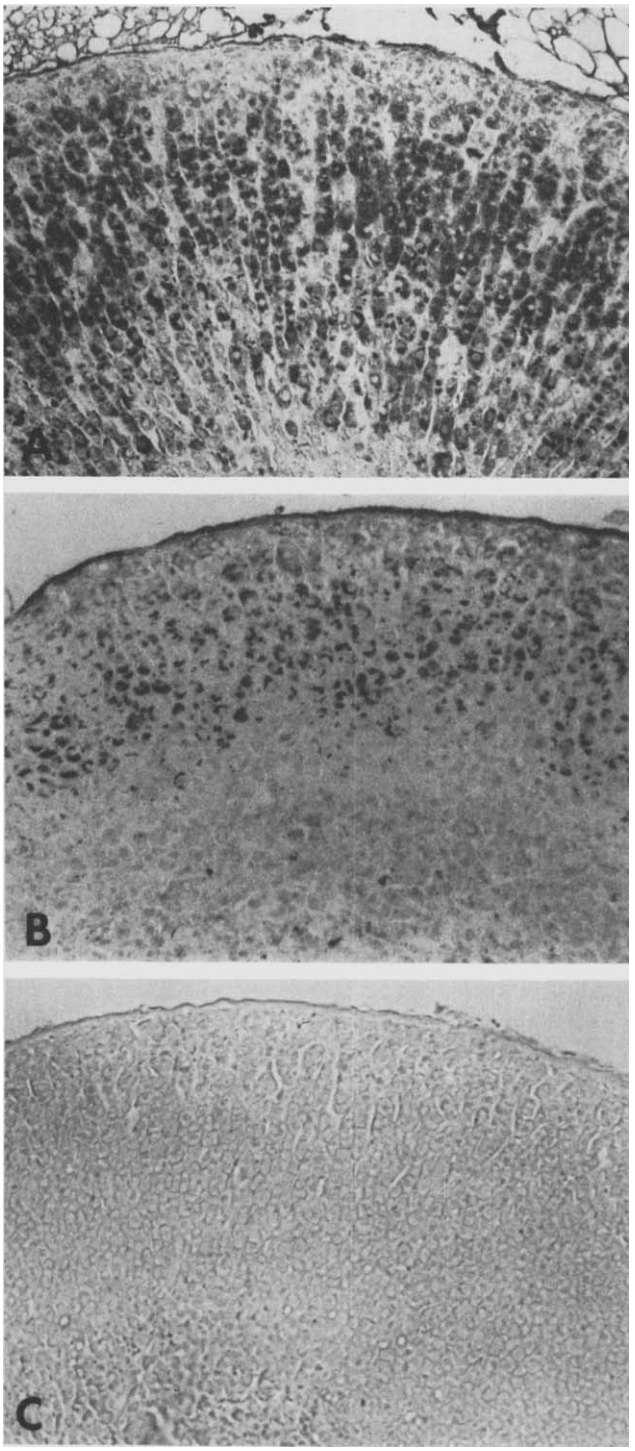


**Figure 1.** Adrenal sections immunostained for corticosterone (original magnification ×250). (A) The B6 mouse adrenal cortex contains many CS-positive cells in the zona fasciculata. These are arranged in parallel rows or cords, indicating that a large proportion of cortical cells contain this hormone. The medullary cells are uniformly negative. (B) Adrenal cortex from the A/J mouse exhibits fewer CS-positive cells. The stained cells appear to have a random distribution in the zona fasciculata and are few enough that the cord-like arrangement of cells in this zone is not readily discernible without counterstaining. (C) Adrenal cortex from a B6 mouse after substitution of nonimmune rabbit serum for anti-CS in the immunohistochemical procedure. No staining is evident in any of the adrenal cells.

from A/J mice exhibited sparse, randomly arranged CS-positive cells (Fig. 1B). The cells in the zona fasciculata of both strains are arranged in cords. The cord-like arrangement in A/J adrenal glands was not easily discernible after immunostaining for CS due to a low number of positive cells per cord. In B6 glands, however, sufficient numbers of cells were CS positive so that the cord-like arrangement of cells was clearly visible. Whether the cells in a cord are clonally derived has not yet been established (12, 13). Our results suggest that CS positivity is not clonally arranged, since CS-positive cells are not contiguous within many cords in each of the mouse strains examined. No apparent difference in the intensity of staining in individual cells was observed between strains. No differences in CS immunostaining patterns were present in adrenals of A/J and B6 mice received from different laboratories.

Quantitation (Table I) revealed that 85% of A/J cortical sectors had sparse staining patterns and 93% of B6 sectors had abundant stained cells. The 95% confidence intervals for B6 were 81–99% "B6-like" sectors and for A/J, 3–26% B6-like sectors.

**$F_1$ Hybrids.** In contrast to the parental strains, CS immunostaining of the (BxA)$F_1$ hybrids did not yield homogeneous staining patterns. For each mouse, some cortical regions were A/J-like while other regions were B6-like. Quantitatively, they displayed 55% A/J-like patterns and 45% B6-like patterns, with little variation between individuals (Table I). These results suggest genetic codominance for this trait.

**AXB and BXA RI Lines.** Quantitation of the RI lines by sector analysis yielded a range of values (Table I), which suggests a bimodal distribution of phenotypes when arranged graphically (Fig. 2). Of the 30 lines examined, 16 were A/J-like and 12 resembled B6. Only two lines, AXB13 and AXB17, were truly intermediate between the phenotypes of the progenitors.

Some sectors from cortices of RI mice contained abundant but randomly distributed CS-positive cells. In a few adrenal specimens, positively stained cells extended across the full width of the cortex and appeared to include all three cortical zones. In a few others, positively stained cells were localized mainly in the outer cortex, including both the zona glomerulosa and approximately the outer half of the zona fasciculata. Although in these cases the staining extended to zones not usually observed to contain CS, the A/J-like and B6-like CS-positive cell distribution patterns could still be discerned and classified. These novel patterns of CS-positive cell distribution were observed in only a few specimens and were not characteristic of any individual RI line. They were not noted in A/J, B6, or $F_1$ hybrid mice. When both glands from one mouse were examined, the two adrenals from the same animal did not show any greater similarity than two glands from different mice of the same RI line.

**Table I.** Quantitation of the Immunocytochemical Distribution of Corticosterone in the Adrenal Cortices of A/J, B6, and (B × A)$F_1$ Hybrids and AXB and BXA RI Lines

| Strain or RI Line | No. of sectors analyzed[a] | Percentage of B6-like patterns[b] | RI Line | No. of sectors analyzed | Percentage of B6-like patterns |
|---|---|---|---|---|---|
| A/J | 40 | 15 | AXB18 | 36 | 83 |
| B6 | 40 | 90 | AXB24 | 24 | 100 |
| (B × A)$F_1$ | 40 | 45 | AXB25 | 40 | 83 |
| AXB1 | 8 | 25 | BXA1 | 16 | 19 |
| AXB2 | 16 | 75 | BXA4 | 64 | 14 |
| AXB3 | 32 | 3 | BXA7 | 24 | 33 |
| AXB4 | 95 | 84 | BXA8 | 40 | 18 |
| AXB5 | 51 | 29 | BXA9 | 44 | 18 |
| AXB6 | 32 | 31 | BXA10 | 39 | 15 |
| AXB7 | 37 | 24 | BXA11 | 36 | 89 |
| AXB8 | 32 | 91 | BXA12 | 32 | 22 |
| AXB9 | 62 | 84 | BXA13 | 46 | 17 |
| AXB10 | 64 | 20 | BXA14 | 40 | 3 |
| AXB12 | 32 | 3 | BXA19 | 40 | 83 |
| AXB13 | 12 | 50 | BXA24 | 22 | 73 |
| AXB15 | 40 | 75 | BXA25 | 52 | 98 |
| AXB17 | 38 | 58 | | | |

[a] Sector analysis of these phenotypes is described in Materials and Methods.
[b] B6-like is represented by positive immunostaining of abundant adrenal cortical cells in cord-like arrangements.

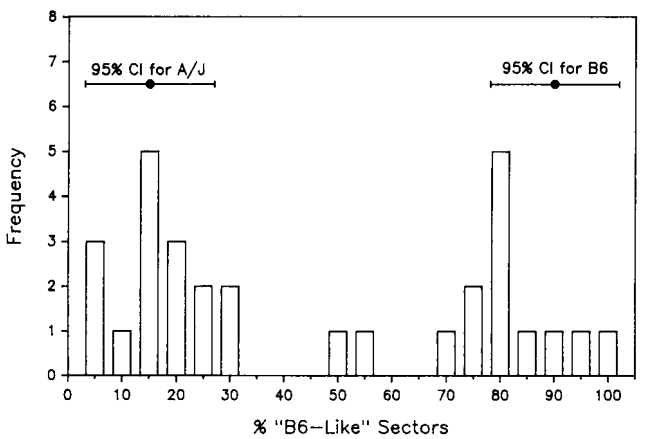


**Figure 2.** Frequency analysis of the percentage of adrenocortical sectors exhibiting a B6-like CS immunostaining pattern (defined as positive immunostaining of abundant adrenal cortical cells in cord-like arrangements) among 30 AXB and BXA RI lines.

## Discussion

Our immunohistochemical results reveal a strain difference in the distribution of CS-positive cells in the mouse adrenal cortex.

There are two clearly defined zones in the adrenal cortex of adult male mice (14). The outermost zona glomerulosa is composed of small cells arranged in arches and varies in size among several inbred strains (15). The zona fasciculata contains radial columns of cells; inbred strains vary in the size and number of cells in this cortical region (16) although no comparisons between A/J and B6 have been described. CS, the major circulating glucocorticoid in the mouse, is synthesized primarily in the zona fasciculata. It also serves as a precursor for aldosterone synthesis in the zona glomerulosa (17, 18). A third region, the zona reticularis, is sometimes observed in mice and lies between the fasciculata and the centrally located medulla. Additionally, a transitory X zone, next to the medulla, is present only in immature males and in females before their first pregnancy (19). Since we used only mature male mice for this study, the X zone was not a consideration in our analyses.

Adrenocortical phenotypic differences between A/J and B6 mice have been reported. These include structural variations such as the incidence and rate of regeneration of the zona reticularis (20), and physiologic differences such as the amounts of CS released into the circulation following stimulation by electric shock (21), ethanol (22), and nicotine (23). Normal (unstimulated) circulating CS levels for these two strains have been reported by three different laboratories (7, 22, 23), including our own, and no significant differences have been found. Plasma CS levels are not necessarily linked primarily to the number of CS-positive cells in the adrenal cortex. The two strains may differ in the amount of CS per cell and/or in the temporal ability of the CS-producing cells to respond to stress, questions which this study was not designed to address. Unpublished results from our laboratory indicate that the number of CS-positive cells in A/J adrenals does not increase following ACTH stimulation, and that A/

J adrenocortical cells may not maintain as sustained a response as those in B6 mice.

We propose the name *Cor* for the locus governing the distribution of CS-producing cells in the adrenal cortex. The purpose of investigating the strain distribution patterns of the *Cor* alleles among the RI lines was to map them in order to gain insight into the molecular identity of this gene. We used two models in attempting to map the loci which generate the CS staining pattern. The first hypothesis, represented by Model A, postulates that the phenotype is determined by a single locus. Model A emphasizes the apparent bimodal distribution shown in Figure 2. With the exception of AXB13 and AXB17, we assigned allele *b* (B6-like) to all of the high RI lines and allele *a* (A/J-like) to all the low ones. This model predicts that a single major locus affects the phenotype, but that a minor locus may also exist whose effects can cause a strain to fall slightly higher or lower than the parent whose major allele is present. This model does not exclude the possibility that AXB13 and AXB17 result from recombination of two closely linked genes (i.e., *Cor-1* and *Cor-2*). A strain distribution analysis in which recombination between *Cor* and other genes where polymorphisms are known to exist between A/J and B6 suggests that the major *Cor* locus affecting the CS pattern is linked to the *Hox-1* (8 cM, 29 cM upper limit of the 95% confidence interval) and *Ggc* (9 cM, 35 cM upper limit) genes on chromsome 6. These results are based on 6 mismatches in 27 RI lines for which both the *Cor* and *Hox-1* loci are known, and 6 mismatches in 25 RI lines for which both the *Cor* and *Ggc* loci are known (M. Nesbitt, unpublished results). *Hox-1* is the gene family homologous to homeotic genes in Drosophila (24), while *Ggc* is the structural gene for γ-glutamyl cyclotransferase (25). Model A is a single gene model consistent with 28 of the 30 RI lines examined. AXB13 and AXB17 would be among the 5% artifacts outside the 95% confidence limit statistic.

The second hypothesis (Model B) proposes an epistatic interaction of two *Cor* loci. RI lines are useful for determining the number of genes which control a phenotypic difference between two inbred strains (26, 27). A single locus is indicated if each RI line exhibits the same phenotype as one or the other progenitor strain. When some of the RI lines in a series have characteristics intermediate between those of the parent strains, it indicates that more than one gene determines the characteristics, unless the intermediate lines are heterozygous at a single locus determining the character. It is highly unlikely that the intermediate lines are heterozygous at a single locus affecting CS staining, because the lines used ranged between F27 and F59 of brother-sister inbreeding at the time of the study. If all of the RI data are used without omitting any lines, a single locus model for the inheritance of the CS staining pattern is untenable. There was a limited supply of AXB13 mice, so only a few sectors from a few mice were analyzed. Several AXB17 mice were examined, however, and most of the mice within this line resembled each other in having a phenotype similar to (B × A)$F_1$ hybrids; both A/J- and B6-like sectors were present in each mouse. Thus, the phenotypes were not segregating. If two loci determine a trait in a simple additive fashion, one expects a 1:2:1 ratio of A/J:intermediate:B6 phenotypes among the RI lines. If we interpret A/J-like and B6-like strictly as falling within the 95% confidence intervals of the parental strain means, then our data reveal a 13 A/J:8 intermediate:9 B6 ratio. This 2:1:1 A/J:intermediate:B6 ratio obtained when all mice are included in the analysis would arise if two loci determined the trait in an epistatic manner. AA and AB genotypes might both have the A/J phenotype, while the BA genotype produces an intermediate phenotype and BB produces a B6 phenotype. Since we can only define the genotype of lines at the first locus, the A/J-like lines were assigned the allele *a* and all intermediate and B6-like lines the allele *b*. This second model, involving epistasis, does not indicate significant linkage to any mapped locus. Interestingly, Eleftheriou and Bailey (28) found that plasma CS levels in C57BL/6By and BALB/cBy mice and in the CXB RI lines derived from these progenitors were controlled by two as yet unmapped genetic loci, *Cpl-1* and *Cpl-2*, exhibiting an epistatic interaction.

We have previously published the strain distribution patterns for the *Pas* genes which control urethan-induced tumor multiplicity (6). Extensive recombination between *Cor* and *Pas-1*, the major *Pas* gene, has occurred, indicating that variations in the number of adrenocortical CS-containing cells are not the major factor responsible for the difference in lung tumor susceptibility. Interestingly, one of the *Pas* genes, the protooncogene *Kras-2* (29), is also on chromsome 6 but at the distal end of the chromosome (30). *Hox-1* is in the proximal portion (16) which provides sufficient distance between these genes for extensive recombination.

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