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Genetic Bases of Estrogen-Induced Pituitary Tumorigenesis: Identification of Genetic Loci Determining Estrogen-Induced Pituitary Growth in Reciprocal Crosses Between the ACI and Copenhagen Rat Strains

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ABSTRACT

Estrogens stimulate proliferation and enhance survival of the prolactin (PRL)-producing lactotroph of the anterior pituitary gland and induce development of PRL-producing pituitary tumors in certain inbred rat strains but not others. The goal of this study was to elucidate the genetic bases of estrogen-induced pituitary tumorigenesis in reciprocal intercrosses between the genetically related ACI and Copenhagen (COP) rat strains. Following 12 weeks of treatment with the synthetic estrogen diethylstilbestrol (DES), pituitary mass, an accurate surrogate marker of absolute lactotroph number, was increased 10.6-fold in ACI rats and 4.5-fold in COP rats. Composite interval mapping analyses of the phenotypically defined F_2 progeny from the reciprocal crosses identified six quantitative trait loci (QTL) that determine the pituitary growth response to DES. These loci reside on chromosome 6 [***E**strogen-induced **p**ituitary **t**umor* (*Ept*) *1*], chromosome 3 (*Ept2* and *Ept6*), chromosome 10 (*Ept9*), and chromosome 1 (*Ept10* and *Ept13*). Together, these six *Ept* loci and one additional suggestive locus on chromosome 4 account for an estimated 40% of the phenotypic variance exhibited by the combined F_2 population, while 34% of the phenotypic variance was estimated to result from environmental factors. These data indicate that DES-induced pituitary mass behaves as a quantitative trait and provide information that will facilitate identification of genes that determine the tumorigenic response of the pituitary gland to estrogens.

ESTROGENS play a central role in the regulation of cell proliferation and survival in numerous mammalian tissues and are implicated in the etiology of several types of cancer (Shull 2002). The prolactin (PRL)-producing lactotroph of the anterior pituitary gland provides a well-defined cell model for studying estrogen action. It is well established that estrogens enhance transcription of the PRL gene, stimulate lactotroph proliferation, and promote lactotroph survival (Spady *et al.* 1999b). Continuous treatment with either naturally occurring or synthetic estrogens induces rapid and sustained growth of the anterior pituitary gland in male or female rats of the Fischer 344 (F344) (Segaloff and Dunning 1945; Wiklund *et al.* 1981a,b; Wiklund and Gorski 1982), A × C Irish (ACI) (Segaloff and Dunning 1945; Holtzman *et al.* 1979; Shull *et al.* 1997; Spady *et al.* 1999c,d), Copenhagen (COP) (Spady *et al.* 1998a, 1999d), and several other inbred rat strains (Noble *et al.* 1940; Furth *et al.* 1973; Spady *et al.* 1999b). The grossly enlarged pituitary glands, commonly referred to as pituitary tumors, exhibit diffuse lactotroph hyperplasia histologically and result in marked hyperprolactinemia (Spady *et al.* 1998b, 1999b,c). In contrast, continuous estrogen treatment induces very little pituitary growth in other rat strains, such as the outbred Holtzman strain (Wiklund *et al.* 1981b; Wiklund and Gorski 1982) or the inbred Brown Norway (BN) strain (Wendell *et al.* 1996; Wendell and Gorski 1997; Spady *et al.* 1999d).

The genetic bases of sensitivity of the F344 rat strain to estrogen-induced pituitary growth have been investigated in crosses to the insensitive Holtzman and BN strains. Following 8 weeks of treatment with the synthetic estrogen diethylstilbestrol (DES), average pituitary mass was increased 7.8-fold in female F344 rats but was unaffected in female Holtzman rats (Wiklund *et al.* 1981a). Average pituitary mass was increased 1.8- and 2.2-fold in DES-treated (Holtzman × F344)F_1 and (F344 × Holtzman)F_1 rats, respectively, suggesting that sensitivity to DES-induced pituitary growth is recessive or incompletely dominant in these crosses. More recently, Wendell and colleagues (Wendell *et al.* 1996, 2000; Wendell and Gorski 1997) have evaluated DES-induced pituitary growth in crosses between the F344 and BN

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strains and have mapped six genetic loci, each of which harbors one or more genes that control the pituitary growth response to DES in these crosses. The effects of two of these loci have now been evaluated using congenic rat lines (Wendell *et al.* 2002; Pandey *et al.* 2004). Together, these studies indicate that estrogen-induced pituitary growth behaves as a quantitative trait in these crosses between the F344 and BN rat strains.

We are studying the genetically related ACI and COP rat strains to define the genetic bases of the differing sensitivities of these strains to estrogen-induced pituitary growth (Spady *et al.* 1999d; Harvell *et al.* 2003) as well as susceptibility to estrogen-induced mammary cancer (Shull *et al.* 1997; Spady *et al.* 1998a; Harvell *et al.* 2000; Shull *et al.* 2001; Gould *et al.* 2004). The goals of this study were to characterize DES-induced pituitary growth in F_1, F_2, and backcross (BC) progeny from reciprocal crosses between the ACI and COP rat strains and to map the genetic loci that determine the differing pituitary growth responses of the ACI and COP rat strains to DES. We demonstrate that sensitivity to DES-induced pituitary growth behaves as a complex trait determined by at least six quantitative trait loci (QTL). For the most part, these loci are distinct from those mapped previously by Wendell *et al.* in crosses between the F344 and BN rat strains (Wendell *et al.* 1996, 2000; Wendell and Gorski 1997). Moreover, these loci are distinct from loci that we mapped previously that determine susceptibility to estrogen-induced mammary cancer in reciprocal crosses between the ACI and COP rat strains (Gould *et al.* 2004). These data indicate that the genes that determine the tumorigenic potential of estrogens act in a rat strain- and tissue-specific manner.

MATERIALS AND METHODS

Analysis of phenotypes: The Institutional Animal Care and Use Committee of the University of Nebraska Medical Center approved all procedures involving live animals. ACI rats were obtained from Harlan Sprague Dawley (Indianapolis). COP rats were obtained from the breeding program of the National Cancer Institute. Animals were housed in a barrier facility under controlled temperature, humidity, and 12-hr light/12-hr dark conditions. This facility was accredited by the American Association for Accreditation of Laboratory Animal Care and operated in accordance with the standards outlined in the *Guide for the Care and Use of Laboratory Animals* (DHHS Pub. 85-23). The animals were caged and fed as described previously (Spady *et al.* 1999d). Female COP rats were mated to male ACI rats to produce (COP × ACI)F_1 progeny, F_1 progeny were mated to generate (COP × ACI)F_2 progeny, and ACI females were mated to F_1 males to produce (COP × ACI)BC progeny. Pups were weaned at 20–24 days of age. Implants, either empty or containing 5 mg of DES (Sigma, St. Louis), were made from Silastic tubing and medical adhesive (Dow Corning, Midland, MI) and were inserted subcutaneously in the interscapular region as described previously (Spady *et al.* 1998a). Treatment with DES was initiated when the animals were 63 ± 4 days of age. Small populations of male rats of each genetic type received empty implants. The rats were killed by decapitation after 12 weeks of DES or sham treatment. The pituitary gland was immediately removed and weighed. Pituitary mass in estrogen-treated rats correlates highly with pituitary DNA content (Wiklund *et al.* 1981b; Wendell *et al.* 2000) and with circulating PRL (Spady *et al.* 1999d), indicating that pituitary mass serves as an accurate surrogate indicator of absolute lactotroph number. The spleen was collected as a source of DNA and stored at −80°.

Analysis of genotypes: The phenotypically defined (COP × ACI)F_2 population described herein and the (ACI × COP)F_2 population described by us previously (Spady *et al.* 1999d) were subjected to genotypic analyses. DNA was isolated from the spleen of 162 of 163 (COP × ACI)F_2 rats and 102 of 103 (ACI × COP)F_2 rats using DNeasy columns (QIAGEN, Valencia, CA). One rat from each cross was excluded from analysis because tissue was not available for isolation of DNA. The marker panel for QTL mapping consisted of simple sequence length polymorphisms (SSLP) that were selected at ~20-cM intervals from those distributed across the 20 autosomes (Rat Genome Database, http://rgd.mcw.edu). Oligonucleotide primers specific to each SSLP marker were obtained from Invitrogen (Carlsbad, CA). Genotypes were determined at each of the 178 SSLP markers for the F_2 progeny from each cross that exhibited the highest and lowest pituitary mass values. For the remaining F_2 progeny, genotypes were determined at the 58 SSLP markers that resided on the six chromosomes that yielded evidence of the presence of a QTL. Each DNA sample was amplified in a 10-μl reaction containing 30 ng of DNA; 1.25 units Taq polymerase (Invitrogen); 20 mm Tris (pH 8.4); 1.5 mm $MgCl_2$; 50 mm KCl; 198 nm of each of the forward and reverse primers; 200 μm each of dATP, dGTP, dCTP, dTTP; and 1.0 μCi [α-^{32}P]dATP (Amersham, Arlington Heights, IL). The reaction mixtures were incubated at 94° for 5 min and subjected to 30 cycles of PCR as follows: (1) 94° for 30 sec; (2) 55° for 30 sec; (3) 72° for 1.5 min, with the final cycle followed by incubation at 72° for 3 min. The DNA products were denatured, resolved on 5, 6, or 8% polyacrylamide gels, and visualized using a PhosphorImager and ImageQuant 5.0 for Windows NT software (Molecular Dynamics, Sunnyvale, CA).

Interval mapping: Genetic maps were estimated and interval mapping (IM) analyses were performed using MapManager QTX version 0.29 (Manly *et al.* 2001). The IM function of MapManager QTX is most accurate when the phenotypic data are normally distributed (K. F. Manly, personal communication). Therefore, the pituitary mass data were $\log_{10}$ transformed, resulting in a normal distribution of values, prior to IM analyses. Two subpopulations consisting of the 45 (COP × ACI)F_2 and the 44 (ACI × COP)F_2 rats that exhibited extreme phenotypes were selected for the initial IM analyses (Lander and Botstein 1989). Experiment-wise threshold values were calculated by performing 1000 permutations of the phenotypic data (Churchill and Doerge 1994). IM was performed using data for 178 markers distributed across the 20 autosomes. Because similar regions of the genome were indicated to harbor QTL during the initial analyses of the two phenotypically extreme populations and there was no significant difference in the pituitary mass phenotype between the two F_2 populations, these two populations were combined to provide additional power to detect QTL. Permutation-derived thresholds were again calculated and IM was performed on all autosomes for the combined selective population of 89 F_2 animals. The remaining 117 (COP × ACI)F_2 and 58 (ACI × COP)F_2 animals were subsequently genotyped across those chromosomes that exhibited at least suggestive evidence of a QTL upon analysis of the phenotypically extreme F_2 animals. Permutation-derived thresholds were then calculated and IM was performed using the data from the 264 F_2 animals.

Composite interval mapping: Composite interval mapping (CIM) was performed using MapManager QTX to reduce the

effect of unlinked and linked QTL on each locus mapped (Zeng 1993, 1994; Teuscher *et al.* 1999; Doerge 2002). The markers included as cofactors in the CIM analyses were identified using a stepwise selection process and the marker regression function of MapManager QTX. For each step, marker regression was performed across the six chromosomes of interest using the $\log_{10}$-transformed pituitary mass as the phenotype, and the marker exhibiting the most statistically significant likelihood-ratio statistic (LRS) was added to the background. This process was repeated until no additional marker exhibited a statistically significant LRS value (Manly *et al.* 2001). For each CIM analysis, a marker (for QTL near a chromosome end) or markers (for QTL not near a chromosome end) flanking the test interval as well as the peak marker associated with the five other significant QTL were added to the model as cofactors. The permutation-derived threshold was then calculated by performing 1000 permutations of the phenotypic data and CIM was conducted to determine the effect of the test interval with these cofactors included in the model. This process was performed for each significant QTL. To determine the effect of the significant QTL on the suggestive QTL, CIM was performed with the peak marker associated with the six significant QTL added to the model as cofactors. The confidence interval for each significant QTL identified by CIM analysis was estimated using bootstrapping analysis (Visscher *et al.* 1996), which is part of the interval-mapping function of MapManager QTX. For each significant QTL, the percentage of trait variance explained and the additive effect was determined by CIM analysis (Manly *et al.* 2001). Degree of dominance was calculated using CIM analysis and the method of Falconer and Mackay (1996).

Evaluation of genetic interactions: Potential interaction between the 58 markers resident on the six chromosomes that yielded suggestive or significant evidence of a QTL was evaluated pairwise using MapManager QTX. For this analysis, the threshold for significance was obtained by performing 1000 permutations of the phenotypic data using the interaction model with the level of significance set at $P = 0.01$ (Manly *et al.* 2001). Interaction testing was performed with the probability of a type I error set at $\leq 10^{-5}$ (Manly *et al.* 2001).

Statistical analyses: Differences in pituitary mass between experimental groups were assessed using the Wilcoxon rank sum test (GB-STAT, version 6.5; Dynamic Microsystems, Silver Spring, MD). *P*-values ≤0.05 were considered to be indicative of statistical significance. The distribution of pituitary mass and $\log_{10}$-transformed pituitary mass values within the combined F_2 population was tested for normality using SPSS version 12.0 (SPSS, Chicago). The contribution of environmental factors to phenotypic variance was estimated using the method of Wright (1968).

RESULTS

Phenotypic characterization of progeny from a COP × ACI cross: Treatment with the synthetic estrogen DES for 12 weeks resulted in significant increases in pituitary mass in each of the experimental groups evaluated in the context of the COP × ACI (strain of the female is indicated first) cross, relative to that observed in untreated male rats (Figure 1). However, the magnitude of the pituitary growth response to DES was rat strain specific and genetically determined. DES increased pituitary mass in male ACI rats 10.6-fold, from 7.7 mg [standard deviation (SD) = 0.9] in untreated male ACI rats to 81.7 mg (SD = 10.3). By contrast, DES increased pituitary mass only 4.4-fold in male COP rats, from 7.6 mg (SD = 1.0) to 34.2 mg (SD = 5.9). The pituitary growth response of the DES-treated (COP × ACI)F_1 progeny was intermediate to that exhibited by the parental ACI and COP strains, indicating that this phenotype behaves as an incompletely dominant trait. In these F_1 progeny, DES increased pituitary mass 6.9-fold, from 8.2 mg (SD = 1.2) to 56.6 mg (SD = 13.2). Male (COP × ACI)F_2 rats exhibited a 7.8-fold increase in pituitary mass when treated with DES, whereas DES treatment increased pituitary mass 9.2-fold in male (COP × ACI)BC rats. Pituitary mass in untreated rats did not differ significantly between groups (Figure 1).

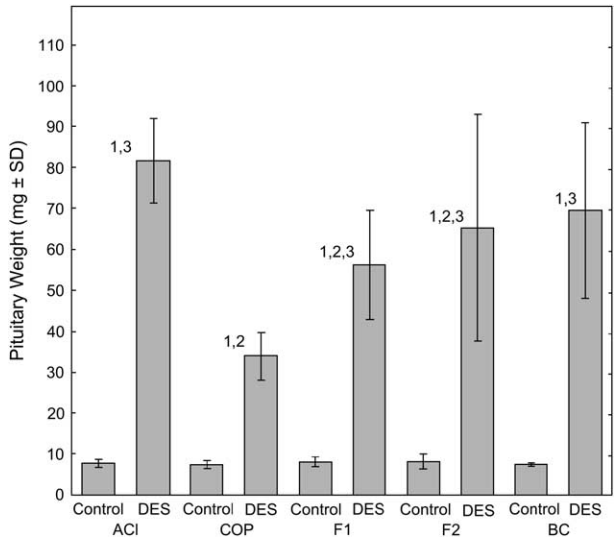

Figure 1.—Sensitivity to DES-induced pituitary growth in progeny from a COP × ACI cross is genetically determined. Male ACI, COP, F_1, F_2, and BC rats from a cross originating with COP females and ACI males were generated and treated with DES for 12 weeks beginning at 9 weeks of age as described in materials and methods. Each data bar represents the mean mass of the anterior pituitary gland in milligrams ± the standard deviation of the mean. Each of the untreated control groups consisted of 5–6 rats. The numbers of DES-treated rats were: ACI, 14; COP, 14; F_1, 18; F_2, 163; and BC, 49. The Wilcoxon rank sum test was performed to assess differences in pituitary mass between experimental groups. *P*-values ≤0.05 were considered indicative of statistical significance. Numeral 1 indicates a statistically significant difference between a DES-treated population and its corresponding sham-treated control population. Numeral 2 indicates a significant difference between the indicated population and the treatment-matched ACI population. Numeral 3 indicates a significant difference between the indicated population and the treatment-matched COP population.

The pituitary growth responses exhibited by the DES-treated male F_1, F_2, and BC populations evaluated in this COP × ACI cross were compared to the responses exhibited by DES-treated F_1, F_2, and BC populations evaluated in an ACI × COP cross that was described by us previously (Spady *et al.* 1999d). DES-induced pituitary growth was similar for each of the F_1, F_2, and BC populations from the two crosses (Table 1), indicating

TABLE 1

Pituitary weights in DES-treated male rats from reciprocal intercrosses between the ACI and COP rat strains

Progeny type	COP × ACI[a]	ACI × COP[a]	*P*-value[b]
F_1	56.6 ± 13.6 (18)	58.8 ± 7.5 (30)	0.81
F_2	65.3 ± 26.5 (163)	60.9 ± 23.9 (103)	0.15
BC	71.8 ± 25.1 (49)	68.2 ± 12.8 (19)	1.00

[a] Mean pituitary weight and standard deviation are presented. The number of animals in each group is in parentheses.

[b] *P*-values were calculated using the Wilcoxon rank sum test.

TABLE 2

Combined data from reciprocal crosses between the ACI and COP rat strains

Group	Untreated[a]	DES treated[a]	*P*-value *vs.* ACI[b]	*P*-value *vs.* COP[b]
ACI	8.1 ± 1.1 (7)	72.7 ± 14.6 (28)	—	<0.0001
COP	9.5 ± 2.7 (8)	36.2 ± 7.4 (28)	<0.0001	—
F_1	9.2 ± 1.5 (12)	58.0 ± 10.0 (48)	0.0003	<0.0001
F_2	8.9 ± 1.7 (8)	63.6 ± 25.6 (266)	0.0024	<0.0001
BC	7.8 ± 0.4 (5)	70.7 ± 22.2 (68)	0.2819	<0.0001

[a] Mean pituitary weight and standard deviation are shown for each of the pooled populations from the COP × ACI and ACI × COP crosses. The number of animals in each group is in parentheses.

[b] Pituitary mass in each group of DES-treated animals was compared to that observed in the parental ACI and COP strains using the Wilcoxon rank sum test.

that the parental orientation of the crosses generating the F_1 generation did not exert a discernible effect on the phenotypes exhibited by the DES-treated F_1, F_2, or BC progeny from these reciprocal crosses between the ACI and COP strains. Table 2 summarizes the combined data from the two crosses.

Mapping of QTL that control estrogen-induced pituitary growth: Genotypes were initially determined at 178 SSLP markers distributed across the 20 autosomes for 45 DES-treated (COP × ACI)F_2 and 44 DES-treated (ACI × COP)F_2 progeny that exhibited the extreme phenotypes, *i.e.*, the largest or smallest pituitary masses, within their respective populations. Interval-mapping analyses of each of these F_2 subpopulations provided suggestive evidence for the presence of QTL affecting pituitary mass on RNO3, RNO6, and RNO10 (data not shown). Because the (COP × ACI)F_2 and (ACI × COP)F_2 populations did not differ from one another phenotypically (Table 1) and the preliminary interval-mapping analyses of the two subpopulations of phenotypically extreme F_2 progeny were suggestive of similar QTL, the two subpopulations were combined to increase statistical power. Interval-mapping analysis of this combined population of 89 phenotypically extreme F_2 progeny revealed a total of eight regions on RNO1, RNO3, RNO4, RNO5, RNO6, and RNO10 where LRS values exceeded 10.6, the permutation-derived threshold indicative of suggestive evidence for the presence of a QTL affecting pituitary mass (data not shown). Genotypes were subsequently determined at 58 SSLP markers spanning these six chromosomes for the remaining 117 DES-treated (COP × ACI)F_2 progeny and the remaining 58 DES-treated (ACI × COP)F_2 progeny. Interval-mapping analyses of this combined population of 264 DES-treated F_2 progeny revealed one locus on RNO3 where the peak LRS value exceeded 20.9, the permutation-derived threshold indicative of highly significant evidence of a QTL; a total of four loci on RNO1, RNO3, RNO6, and RNO10 where the peak LRS values exceeded 14.0, the permutation-derived threshold indicative of significant evidence of a QTL; and three additional loci on RNO1, RNO4, and RNO5 where the peak LRS values exceeded 7.9, the permutation-derived threshold for suggestive evidence of a QTL (data not shown).

CIM was performed to evaluate these QTL further. The CIM analyses revealed six statistically significant QTL residing on RNO1, RNO3, RNO6, and RNO10 (Figure 2). These loci have been designated as ***E**strogen-induced **p**ituitary **t**umor* (*Ept*) loci: *Ept1*, *Ept2*, *Ept6*, *Ept9*, *Ept10*, and *Ept13*. The numbering of these QTL is not continuous because other *Ept* loci not described here have been mapped in other crosses between the ACI and Brown Norway strains (T. E. Strecker and J. D. Shull, unpublished data). *Ept1* is defined by a peak LRS value of 20.4 at *D6Rat80* and a confidence interval extending from *D6Rat150* to *D6Rat39* (Figure 2A). This locus is estimated to account for 8% of the phenotypic variance exhibited by the combined F_2 population (Table 3). *Ept2* is defined by a peak LRS value of 38.4 near *D3Rat26*, with the confidence interval defined by *D3Rat130* and *D3Rat21* (Figure 2B). *Ept2* is estimated to account for 14% of the phenotypic variance and exerts the strongest effect on DES-induced pituitary mass of the six QTL (Table 3). *Ept6* also maps to RNO3 (Figure 2C). This QTL is defined by a peak LRS value of 30.6 at *D3Mgh9*, a confidence interval extending from *D3Mgh16* to *D3Rat277*, and accounts for ~11% of the phenotypic variance (Table 3). *Ept9* is defined by a peak LRS value of 17.9 near *D10Mit7* and a confidence interval extending from *D10Rat27* to *D10Rat11* (Figure 2D). This QTL is estimated to account for 7% of the phenotypic variance (Table 3). Both *Ept10* and *Ept13* map to RNO1. *Ept10* is defined by an LRS peak of 17.4 near *D1Rat119* and a confidence interval extending from *D1Rat133* to *D1Rat81* (Figure 2E). *Ept13* is defined by a peak LRS value of 15.6 near *D1Rat192* and a confidence interval extending from *D1Rat192* to *D1Rat259* (Figure 2F). *Ept10* and *Ept13* are estimated to account for 7 and 6% of the phenotypic variance, respectively (Table 3).

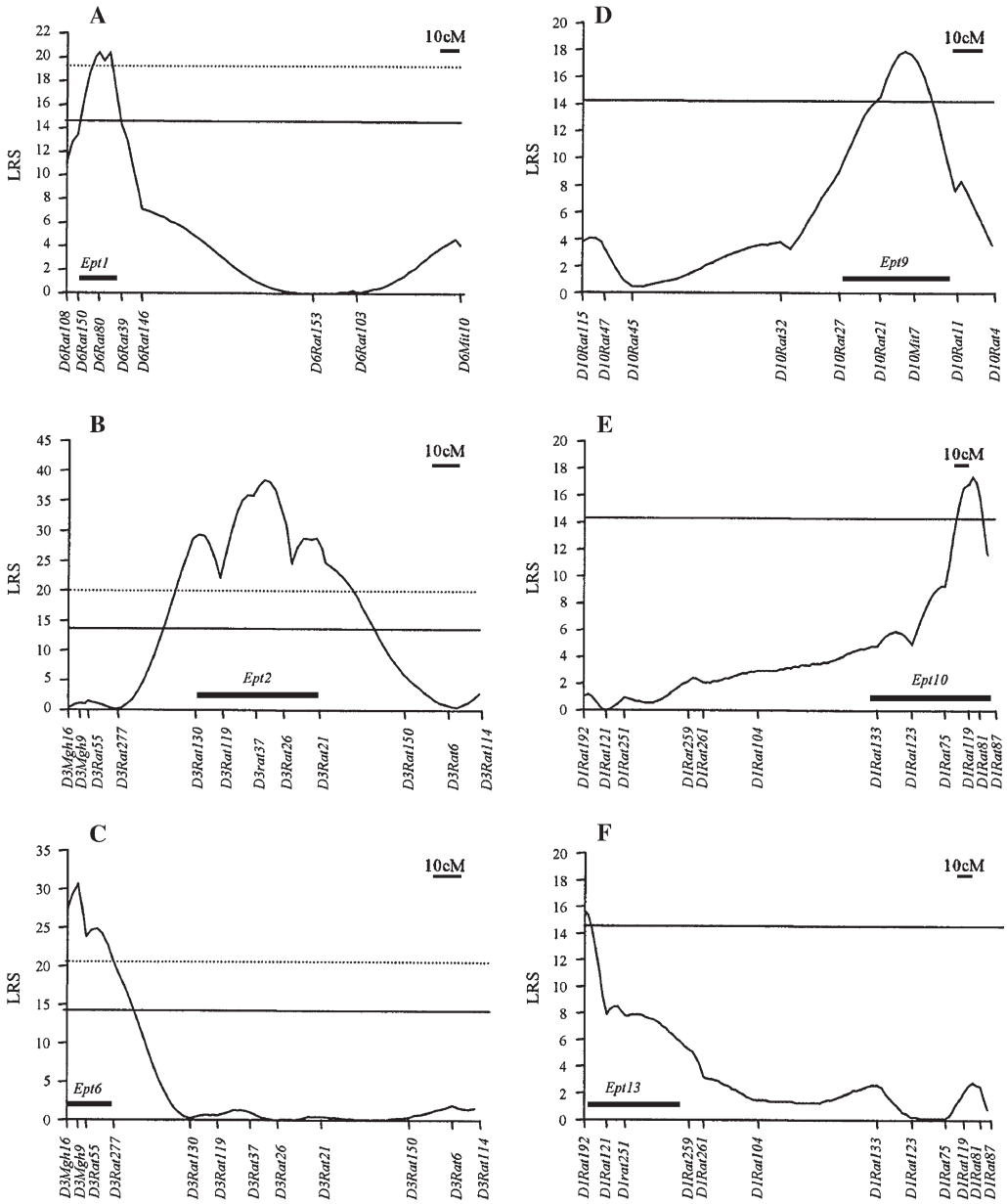

FIGURE 2.—Six quantitative trait loci determine sensitivity to DES-induced pituitary growth in F_2 progeny from reciprocal crosses between the ACI and COP rat stains. Genotypes were determined at the indicated polymorphic SSLP markers for a total of 264 phenotypically defined F_2 progeny from reciprocal crosses between the ACI and COP strains. Each horizontal axis represents the genetic map of the indicated rat chromosome in Haldane centimorgans and the markers at which genotypes were determined. Each vertical axis represents the LRS value for the correlation between $\log_{10}$-transformed pituitary mass and genotype along each chromosomal interval as determined by CIM. Each solid box represents the bootstrapping-derived confidence interval. (A) *Ept1* is defined by an LRS peak of 20.4 at *D6Rat80* and a confidence interval of ~15 cM extending from *D6Rat150* to *D6Rat39*. Permutation-derived thresholds for significant (14.3; solid line) and highly significant (19.6; dotted line) evidence of a QTL are shown. (B) *Ept2* is defined by an LRS peak of 38.4 between *D3Rat37* and *D3Rat26* and a confidence interval of ~48 cM from *D3Rat130* to *D3Rat21*. Permutation-derived thresholds for significant (13.7; solid line) and highly significant (20.0; dotted line) evidence of a QTL are shown. (C) *Ept6* is defined by an LRS peak of 30.6 at *D3Mgh9* and a confidence interval of ~17 cM from *D3Mgh16* to *D3Rat277*. Permutation-derived thresholds for significant (14.0; solid line) and highly significant (20.5; dotted line) evidence of a QTL are shown. (D) *Ept9* is defined by an LRS peak of 17.9 between *D10Rat21* and *D10Mit7* and a confidence interval of ~35 cM from *D10Rat27* to *D10Rat11*. The permutation-derived threshold (14.1) for significant evidence of a QTL is shown. (E) *Ept10* is defined by an LRS peak of 17.4 between *D1Rat119* and *D1Rat81* and a confidence interval of ~60 cM from *D1Rat133* to *D1Rat81*. The permutation-derived threshold (14.2) for significant evidence of a QTL is shown. (F) *Ept13* is defined by an LRS peak of 15.6 at *D1Rat192* and a confidence interval of ~57 cM from *D1Rat192* to *D1Rat259*. The permutation-derived threshold for significant (14.5) evidence of a QTL is shown.

When CIM analysis was performed for the suggestive QTL on RNO4, a peak LRS value of 11.1 was observed at *D4Mgh7*. Although this LRS value exceeded the permutation-derived threshold suggestive of a QTL (7.6), it did not exceed the threshold indicative of a significant QTL (14.0). By contrast, CIM-derived LRS values on RNO5 did not exceed the permutation-derived threshold suggestive of a QTL (7.6). The six significant *Ept* loci and the suggestive QTL on RNO4 together are estimated to account for 40% of the phenotypic variance, whereas environmental factors are estimated to account for 34% of the variance.

Impact of *Ept* loci on pituitary mass: Data presented above indicate that growth response of the pituitary gland to DES is determined by a minimum of six QTL. CIM analyses indicate that ACI alleles of *Ept1*, *Ept2*, *Ept9*, and *Ept13* confer increased pituitary mass in response to DES, whereas COP alleles for *Ept6* and *Ept10* confer

TABLE 3

Actions of *Ept* loci on DES-induced pituitary mass

QTL	Marker[a]	Position (cM)[b]	Peak LRS value[c]	% variance[d]	Additive effect[e]	Degree of dominance[f]	Phenotype A/A − C/C[g]
Ept1	*D6Rat80*	11	20.4	8	0.05	−0.41	16.3
Ept2	*D3Rat26*	73	38.4	14	0.07	0.00	24.2
Ept6	*D3Mgh9*	4	30.6	11	−0.07	−0.28	−19.7
Ept9	*D10Mit7*	104	17.9	7	0.05	0.00	13.8
Ept10	*D1Rat119*	212	17.4	7	−0.04	0.73	−15.8
Ept13	*D1Rat192*	0	15.6	6	0.04	−0.50	14.6

[a] SSLP marker nearest the LRS peak.
[b] Position of peak LRS score in Haldane units relative to position of first marker on the chromosome.
[c] Peak LRS value from CIM analyses.
[d] Percentage of phenotypic variance attributed to the QTL as determined by CIM analyses.
[e] Additive effect (Falconer and Mackay 1996) was generated using MapManager QTX and $\log_{10}$-transformed pituitary mass data. Positive values indicate the ACI allele is associated with increased pituitary mass. Negative values indicate the COP allele is associated with increased pituitary mass.
[f] Degree of dominance (Falconer and Mackay 1996). 0 indicates additive affects of the ACI and COP alleles; 1 indicates full dominance of the ACI allele; −1 indicates full dominance of the COP allele.
[g] Mean pituitary mass (milligrams) in the F_2 subpopulation that is homozygous for the ACI allele at the indicated marker minus mean pituitary mass in the F_2 subpopulation that is homozygous for the COP allele at that marker.

increased mass (Table 3). Whereas the ACI alleles for *Ept2* and *Ept9* appeared to act additively, the ACI alleles for the remaining four *Ept* loci deviated from an additive mode of action (Table 3). When the combined F_2 population was classified into subpopulations according to genotype at the marker closest to the LRS peak for each *Ept* locus, homozygosity for the ACI allele at *Ept1*, *Ept2*, *Ept9*, and *Ept13* was associated with 16.3, 24.2, 13.8, and 14.6 mg, respectively, of additional pituitary mass relative to rats homozygous for the COP allele at these loci (Table 3). By contrast, homozygosity for the COP allele at *Ept6* and *Ept10* was associated with 19.7 and 15.8 mg, respectively, of additional pituitary mass relative to rats homozygous for the ACI allele. For each of the six *Ept* loci, the average pituitary mass in F_2 rats homozygous for the ACI allele differed significantly from that observed in the F_2 rats that were homozygous for the COP allele.

Epistatic interaction between *Ept9* and *Ept10*: Map Manager QTX was used to evaluate potential pairwise interactions between the 58 markers residing on the six chromosomes demonstrated to harbor the six *Ept* loci or to have yielded suggestive evidence of a QTL during interval mapping. A single, statistically significant interaction was detected between *D10Mit7*, which resides within *Ept9*, and *D1Rat75*, which resides within *Ept10*. This interaction yielded an LRS value of 44.1, which exceeded the permutation-derived threshold, 42.0, indicative of highly significant evidence of interaction. To illustrate the nature of this interaction, the F_2 population was initially subdivided on the basis of genotype at *D10Mit7* and then further subdivided by genotype at *D1Rat75* (Figure 3). In F_2 rats homozygous for either the ACI allele (Figure 3A) or the COP allele (Figure 3C) at the *Ept9* marker *D10Mit7*, genotype at the *Ept10* marker *D1Rat75* had no significant effect on pituitary mass. By contrast, among the F_2 rats that were heterozygous at the *Ept9* marker *D10Mit7*, rats homozygous for the COP allele at the *Ept10* marker *D1Rat75* exhibited significantly increased pituitary mass, relative to that exhibited by F_2 rats that were either homozygous for the ACI allele or heterozygous at *D1Rat75* (Figure 3B). The biological significance of this interaction is not known at this time.

DISCUSSION

It has been known for >60 years that chronic treatment with estrogens, either naturally occurring or synthetic, leads to development of pituitary tumors in rats (Mceuen *et al.* 1936; Segaloff and Dunning 1945; Clifton and Meyer 1956) and mice (Gardner and Strong 1940; Gardner 1941; Spady *et al.* 1999b). Moreover, substantial evidence suggests that estrogens may contribute to development of pituitary tumors in humans (Landolt *et al.* 1984; Holmgren *et al.* 1986; Miyai *et al.* 1986; Gooren *et al.* 1988; Panteon *et al.* 1988; Bevan *et al.* 1989; Kovacs *et al.* 1994). However, the mechanisms through which estrogens induce pituitary tumor development remain poorly defined. Elucidation of the genetic bases of the differing sensitivities of the genetically related ACI and COP rat strains to DES-induced pituitary growth will likely provide novel insights into the mechanisms through which estrogens regulate pituitary lactotroph homeostasis and induce pituitary tumor development.

The data presented in this study indicate that estrogen-induced pituitary growth behaves as a quantitative trait in reciprocal crosses between the ACI and COP rat strains. Six QTL that determine sensitivity to DES-

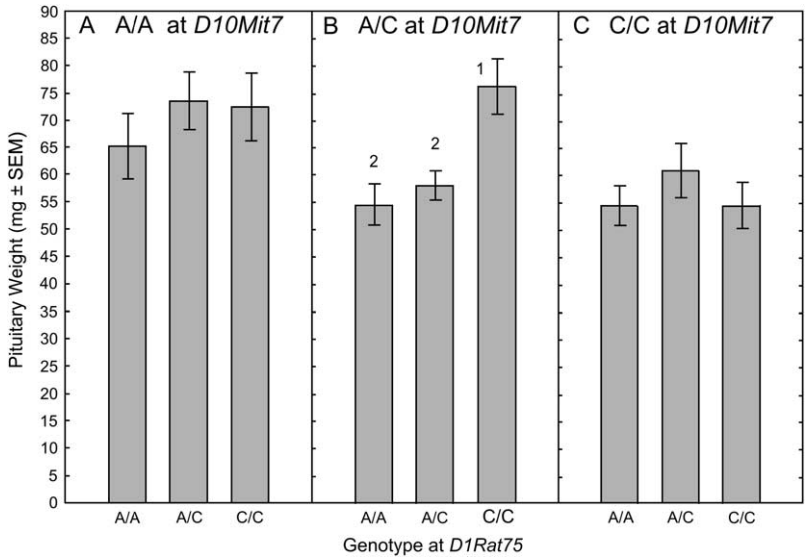

FIGURE 3.—Characterization of the interaction between *Ept9* and *Ept10*. Each of the 264 phenotypically defined F_2 rats from the reciprocal crosses between the ACI and COP rat strains was classified by genotype at the *Ept9* marker *D10Mit7* and then further classified by genotype at the *Ept10* marker *D1Rat75*. Each data bar represents the mean mass of the anterior pituitary gland in milligrams ± the standard error of the mean. The Wilcoxon rank sum test was performed to assess differences in pituitary mass between the genotypically defined subpopulations. *P*-values ≤0.05 were considered indicative of statistical significance. Numeral 1 indicates that pituitary mass differs significantly from that observed in the subpopulation homozygous for the ACI allele at *D1Rat75*. Numeral 2 indicates that pituitary mass differs significantly from that observed in the subpopulation homozygous for the COP allele at *D1Rat75*.

induced pituitary growth have been mapped through CIM analyses of the F_2 populations generated in these crosses. Thus, the genetic bases of estrogen-induced pituitary tumor development in these crosses are more complex than proposed by us previously (SPADY *et al.* 1999d). We have estimated that these six *Ept* loci, together with one suggestive locus, account for 40% of the phenotypic variance exhibited by the combined F_2 population from the two crosses. Environmental factors were estimated to account for an additional 34% of phenotypic variance. These data suggest that one or more unmapped *Ept* loci account for the estimated 26% of the phenotypic variance that remains unexplained. It is possible that an unmapped *Ept* locus may reside within a region of the rat genome too distant from the nearest polymorphic marker to allow the locus to be detected. Seven segments of the rat genome exist where an additional *Ept* locus, were it to reside there, would be 20–28 cM from the nearest polymorphic marker. These segments are located on RNO1, -2, -5, -6, -7, -10, and -17. Moreover, the X and Y chromosomes were not fully evaluated in this study. Therefore, we cannot exclude these chromosomes as determinants of the pituitary growth response to DES. Alternatively, the remaining 26% of the phenotypic variance could result from the actions of multiple unmapped *Ept* loci, each of which would exert an effect on pituitary mass that was too small to be detected in our analyses (VAN OOIJEN 1992; MURANTY and GOFFINET 1997).

The six *Ept* loci mapped in this study segregate and appear, for the most part, to function independently of one another. ACI alleles at *Ept1*, *Ept2*, *Ept9*, and *Ept13* are associated with increased DES-induced pituitary growth, relative to COP alleles at these loci. In contrast, COP alleles at *Ept6* and *Ept10* are associated with increased pituitary growth. Thus, the sensitivity of the COP strain to DES-induced pituitary growth is not simply due to the action of growth-conferring alleles shared with the genetically related ACI strain. Rather, the sensitivity of the COP strain to DES-induced pituitary growth is due, at least in part, to the action of growth-conferring alleles not carried by the ACI strain. Interestingly, we have previously demonstrated that a 40% restriction of dietary energy consumption inhibits estrogen-induced pituitary growth in COP, but not ACI, rats (SPADY *et al.* 1999a,c; HARVELL *et al.* 2001, 2002, 2003). Together these data suggest that the mechanism underlying estrogen-induced pituitary growth in the ACI rat strain differs, at least in part, from that in the COP strain.

Wendell *et al.* have mapped to RNO2, RNO3, RNO5, and RNO9 a total of six QTL, referred to as *Estrogen-dependent pituitary mass* (*Edpm*) loci, that affect DES-induced pituitary growth in female F_2 and BC progeny derived from crosses between the F344 and BN rat strains (WENDELL and GORSKI 1997; WENDELL *et al.* 2000). Data from our study of male F_2 progeny from crosses between the ACI and COP rat strains specifically exclude pituitary growth-controlling loci from the regions of RNO2, RNO5, and RNO9 to which Wendell *et al.* mapped five of the six loci that control DES-induced pituitary growth in the F344 × BN crosses. The *Ept2* locus mapped by us to RNO3 overlaps with the *Edpm3* locus mapped by Wendell *et al.* Thus, five of the six *Ept* loci mapped in the ACI × COP crosses are clearly distinct from those mapped by Wendell *et al.* in crosses between the F344 and BN strains. We hypothesize that the dissimilarities in the QTL mapped in our study compared to those of Wendell *et al.* result from differences in the inbred strains being evaluated. However, we cannot exclude the possibility that the differences in the QTL identified in the two studies are due to variation in experimental design, such as the gender of rats used for linkage analysis.

We observed highly significant evidence of an epistatic interaction between *Ept9* and *Ept10*. The potential interaction between *Ept9* and *Ept10* is particularly intri-

guing because *Jak2* resides within the *Ept10* interval, tightly linked to *D1Rat75*, and *Stat5a* and *Stat5b*, which are phosphorylated by *Jak2*, reside within the *Ept9* interval, linked to *D10Mit7*. Together, Jak2 and Stat5a/5b mediate PRL signaling through the PRL receptor to regulate lactotroph function and number (Bole-Feysot *et al.* 1998; Schuff *et al.* 2002). The known role of Jak2 and both Stat5a and Stat5b in mediating the effects of PRL signaling makes these genes attractive candidates for *Ept10* and *Ept9*, respectively.

In studies performed in parallel to those presented herein, analysis of female F_2 progeny from reciprocal crosses between the ACI and COP strains localized to RNO5 and RNO18 two loci, referred to as *Emca1* and *Emca2*, respectively, that determine susceptibility to 17β-estradiol-induced mammary cancer (Gould *et al.* 2004). Although the interval-mapping analyses performed in our study provided suggestive evidence of a QTL modifying the pituitary growth response to DES on RNO5, this putative QTL was not confirmed during CIM analyses. Similarly, the genetic analyses described herein indicate that RNO18 does not harbor a genetic determinant of DES-induced pituitary growth. It is also interesting that the regions of the genome in which the six *Ept* loci reside do not exert discernible effects on susceptibility to 17β-estradiol-induced mammary cancer in the female F_2 progeny (Gould *et al.* 2004). Thus, the QTL that determine sensitivity to DES-induced pituitary growth in male F_2 progeny are distinct from those that determine susceptibility to 17β-estradiol-induced mammary cancer in female F_2 progeny evaluated in these reciprocal crosses between the ACI and COP rat strains. Together, these data indicate that the genes that determine the tumorigenic response to continuous estrogen stimulation act, in large part, in a rat strain- and tissue-specific manner.

In summary, the data presented herein indicate that a minimum of six QTL control sensitivity to DES-induced pituitary growth in F_2 progeny produced in crosses between the ACI and COP rat strains. Congenic rat lines are under development that will allow the impact of each *Ept* locus on estrogen-induced pituitary growth to be assessed, both qualitatively and quantitatively, independently of the other loci. These congenic lines will also allow each *Ept* locus to be mapped with greater precision. The recent assembly of the draft sequence of the rat genome will facilitate the identification of those genes residing within each *Ept* locus that determine sensitivity to DES-induced pituitary growth, and this, in turn, should significantly enhance our understanding of how estrogens regulate lactotroph homeostasis and contribute to development of PRL-producing pituitary tumors.

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