Genetic Interactions Among Sex-Determining Genes in the Fern *Ceratopteris richardii*

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ABSTRACT

Haploid gametophytes of the fern Ceratopteris are either male or hermaphroditic. The determinant of sex type is the pheromone antheridiogen, which is secreted by the hermaphrodite and directs male development of young, sexually undetermined gametophytes. Three phenotypic classes of mutations that affect sex-determination were previously isolated and include the *hermaphroditic* (*her*), the *transformer* (*tra*) and *feminization* (*fem*) mutations. In the present study, linkage analysis and tests of epistasis among the different mutants have been performed to assess the possible interactions among these genes. The results indicate that sex determination in Ceratopteris involves at least seven interacting genes in addition to antheridiogen, the primary sex-determining signal. Two models describing how antheridiogen may influence the activity states of these genes and the sex of the gametophyte are discussed.

HAPLOID gametophytes of many homosporous ferns, including *Ceratopteris richardii,* develop as either males or hermaphrodites. The sexual fate of the developing gametophyte is determined by pheromone antheridiogen (DÖPP 1950; NĀF 1979; NĀF *et al.* 1975), which is referred to as A_{CE} in Ceratopteris. In the absence of A_{CE}, individual Ceratopteris spores develop as hermaphroditic gametophytes that produce egg-bearing archegonia, sperm-bearing antheridia and a well-organized multicellular meristem (*i.e.,* are meristic). The hermaphrodite produces and secretes A_{CE} into its surroundings when it is no longer competent to respond to it. When grown in the presence of A_{CE}, spores develop as male gametophytes with numerous antheridia but no archegonia or organized multicellular meristem (*i.e.,* are amerisitic). Because hermaphrodites form a meristem and males do not, the two sexes can be easily distinguished by their size and shape as well as type of sex organ produced.

Physiological studies of the A_{CE} response in Ceratopteris (BANKS *et al.* 1993) have shown that the sex of the gametophyte is initally determined early in development (stage 2) as the spore wall cracks and the cell inside begins to divide. If A_{CE} is absent during this stage of development, the gametophyte will develop as a hermaphrodite even if exposed to exogenous A_{CE} at later stages of development. If A_{CE} is present during this and subsequent stages of development, the gametophyte will develop as a male. However, if A_{CE} is withdrawn from a male gametophyte, undifferentiated cells of the male thallus divide and form a hermaphroditic thallus. The capacity of the male to switch its sex in response to changes in the primary sex signal indicates that the male program of sex expression, unlike the hermaphroditic program of sex expression, is reversible during development and that A_{CE} is required for both initiating and maintaining the male program of sex expression. Although the structure of A_{CE} is unknown, the A_{CE} response can be blocked by inhibitors of gibberellin biosynthesis as well as abscisic acid (ABA), indicating that A_{CE} either is or is similar to a gibberellin (HICKOK 1983; WARNE and HICKOK 1989).

Genetic and molecular studies of sex determination and differentiation in animals, particularly *Drosophila melanogaster* and *Caenorhabditis elegans,* have shown that the control of sex type in these organisms involves a number of genes that interact within a well-defined regulatory heirarchy, which has at its apex the primary sex-determining signal (reviewed in BAKER 1989; HODGKIN 1990; CLINE 1993). Although the primary sex-determining signal in Ceratopteris (a pheromone) is clearly different from that in Drosophila and Caenorhabditis (the *X*:A ratio), Ceratopteris has many useful features in common with Drosophila and Caenorhabditis that make it well suited for the study of sex determination in plants. First, the determinant of sex type is known. Second, individuals are sexually dimorphic, a property of Drosophila and Caenorhabditis that has expedited the study of the differences between two individuals of different sexes. Third, Ceratopteris is amenable to the genetic analysis of mutations that affect a variety of developmental processes (reviewed in CHASEN 1992; HICKOK *et al.* 1995) including sex determination (BANKS 1994; EBERLE *et al.* 1995). A principal advantage of Ceratopteris as a genetic system is that mutations affect-

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ing haploid gametophyte development, either dominant or recessive, are apparent immediately after mutagenesis of the single-celled spore and growth of the gametophyte under selective conditions. While the haploid nature of the gametophtye facilitates the selection of mutations, it is also a limitation of the system because traditional complementation experiments cannot be performed in a haploid organism. We have used a genetic approach similar to that used in Drosophila and Caenorhabditis to understand how the pheromone A_{CE} governs the sexual phenotype of the Ceratopteris gametophyte (BANKS 1994). This analysis has led to the identification of three phenotypic classes of sex-determining mutants that exhibit insensitivity to A_{CE} as well as an altered sexual phenotype. These include the *hermaphroditic* (*her*) mutants that develop as meristic hermaphrodites, the *transformer1* (*tra1*) mutant that develops as an amerisitic male, and the *feminization1* (*fem1*) mutant that develops as a meristic female.

The mutant phenotype of each sex-determination gene, in conjunction with information from physiological and developmental studies of sex determination in this species, have been used to construct a model to describe how genetic and epigenetic factors might interact to form a regulatory hierarchy controlling the sex of the gametophyte (BANKS 1994). This model was based on the assumption that *TRA1* and *FEM1* act in a positive manner to direct differentiation of female and male traits, respectively, and is therefore referred to as the positive model. This model makes specific, testable predictions about the epistatic interactions among the sex-determining genes. The goals of the present study were to test the predictions of the positive model and to determine the number of gene loci represented by these mutations. While the results of this study support many predications of the positive model, they do not support them all. An alternative model that is consistent with the genetic evidence is described.

MATERIALS AND METHODS

The medium and conditions used for growing gametophytic and sporophytic Ceratopteris plants are described in BANKS (1994). The origin of the wild-type inbred strain of *Ceratopteris richardii* (Hnn) is described in SCOTT and HICKOK (1987). The antheridiogen insensitive mutations HαC23-12 (designated here as *her1*) and Hα575 (designated here as *her3*) were selected from X-irradiated Hnn spores of Ceratopteris by WARNE *et al.* (1988). All other *fem* and *her* mutants were isolated by selecting hermaphrodites that developed from EMS mutagenized Hnn spores in the presence of A_{CE}, as described in BANKS (1994). The *tra1* mutant was selected as a male from EMS mutagenized *her1* spores (BANKS 1994).

When choosing female donors for crossing, 9–12-day-old individual *fem* females, wild-type hermaphrodites or *her* hermaphrodites were placed in microtiter wells with agar solidified medium. For crosses requiring wild-type or *tra* males as male gamete donors, spores were plated on medium containing A_{CE} and maintained at 29° which results in >95% males. At 11–13 days after spore inoculation, room temperature water was added to the males. The water and decrease in temperature cause the sperm to be released from the antheridia within minutes. Drops of water containing sperm were then added to each individual female or hermaphrodite. A cross was considered successful only if the sperm swarm to the archegonia. To obtain sperm from a population of hermaphrodites, room temperature water was added to 3–4-week-old cultures previously maintained at 29°. Water containing the sperm released from hermaphrodites was removed within minutes before the sperm could swarm to the hermaphrodites from which they were being removed then added to the appropriate female donor. Because less sperm is obtained when using a hermaphrodite than a male gametophyte, there is a greater chance that the hermaphrodite serving as a female donor could be self-fertilized. To insure that sperm was present from a population of hermaphrodites, the water containing the sperm was visually inspected for sperm and was also added to *fem1* females since *fem1* females cannot self-fertilize.

Antheridiogen, which is not available in purified form, was obtained as a crude aqueous filtrate of medium that had supported the growth of gametophytes from wild-type Hnn spores (40 mg/liter medium) for 14 days. Each batch of medium was assayed for A_{CE} activity according to BANKS *et al.* (1993) and only batches of A_{CE} that induced >90% Hnn wild-type males grown in isolation were used. Because the number of wild-type hermaphrodites that form in a population of gametophytes grown in the presence of A_{CE} is negligable, the calculated expected gene frequencies of wild-type males grown in the presence of A_{CE} was assumed to be 100%. Batches of medium lacking A_{CE} were also tested to insure that the medium was not contaminated with A_{CE} by sowing wild-type spores in this medium in isolation. Only medium that resulted in 100% hermaphroditic development was used.

The presence and abundance of antheridia, particularly in the intersexual gametophytes, was assessed by staining gametophytes with Hoechst's dye and then viewing under UV fluorescence on a Zeiss Axiophot microscope according to BANKS *et al.* (1993).

RESULTS

Linkage analysis among the *her* and *fem1* mutants: The *her* mutants were originally selected as hermaphrodites that grow in the presence of A_{CE}, a condition that normally promotes male development (BANKS 1994). Most *her* mutations are completely insensitive to A_{CE}; *i.e.*, when a *her* hermaphrodite is self-fertilized to produce a homozygous diploid *her* sporophyte, all progeny haploid gametophytes develop as hermaphrodites on medium containing A_{CE} (Table 1). One exception is *her14*, which produces some ameristic males (3%) among the progeny of self-fertilized *her14* hermaphrodites (Table 1). Because the gametophyte is haploid, it is not possible to perform complementation analysis between two mutant *her* lines to test allelism. Linkage analysis was therefore performed to test whether two *her* mutations are tightly linked (and possibly allelic), linked but genetically separable, or unlinked by crossing two *her* mutant gametophytes to produce diploid sporophytes heterozygous for both mutations. The two mutations are considered to be tightly linked if all F_1 gametophytes are hermaphroditic (0% male). If 25% of the progeny are male, the two mutations are consid-

TABLE 1

Linkage analysis of the *her* mutants

	her1	*her3*	*her5*	*her7*	*her9*	*her10*	*her11*	*her13*	*her14*	*her15*	*her19*
her1	0										
her3	26 (2)	0									
her5	25 (2)	0 (4)	0								
her7	23 (1)	26 (5)	26 (7)	0							
her9	25 (1)	0 (6)	0 (2)	29 (1)	0						
her10	26 (1)[a]	25 (4)	20 (1)	0 (4)	ND	0					
her11	28 (1)	27 (1)	25 (3)	0 (8)	25 (7)	0 (8)	0				
her13	24 (3)	25 (3)	23 (3)	22 (4)	25 (3)	24 (2)	25 (4)	0			
her14	27 (2)	25 (2)	31 (2)	5 (2)	ND	6 (2)	11 (1)	26 (5)	3 (4)		
her15	29 (1)[b]	25 (3)	23 (4)	0 (6)	25 (3)	ND	0 (4)	24 (6)	10 (6)	0	
her19	ND	26 (4)	25 (3)	0 (1)	ND	0 (3)	0 (7)	26 (4)	6 (3)	0 (9)	0

The maternal and paternal genotypes are given on the top row and left-most column. The numbers indicate the average percentage of male gametophytes in the progeny of each cross when grown on media containing A_{CE}. The numbers in parentheses indicate the number of crosses analyzed. Unless otherwise noted, at least 280 gametophytes were scored per cross. ND, not determined.

[a] Progeny scored: 148.

[b] Progeny scored: 154.

ered to be unlinked. Between 0–25% male progeny would indicate that the two mutations are linked but genetically separable.

The results of the linkage analysis among 11 independently derived *her* alleles are shown in Table 1. In all cases, each paired cross and its reciprocal, where performed, gave similar results (data not shown). Because the progeny of a sporophyte obtained by self-fertilizing a *her* hermaphrodite and a *her* hermaphrodite crossed by sperm harboring a tightly linked *her* allele will all be hermaphroditic, it is not possible to determine whether a *her* gametophyte was self-fertilized or crossed by another source of *her* sperm in the linkage analysis. We assume that if a given type of cross produced no males in some cases and 25% males in other cases, those crosses producing no males were due to a self-fertilization event and were thus not considered in the analysis. The results of these crosses indicate that the 11 *her* alleles tested represent at least five loci tentatively assigned to four linkage groups. Three loci are represented by only one allele (*her1, 13* and *14*) and the other two loci are represented by three (*her3, 5* and *9*) and five (*her7, 10, 11, 15* and *19*) alleles. The locus represented by *her14* is likely to be closely linked to the locus represented by *her 7, 10, 11, 15* and *19*, as these pairwise crosses produced an average of 7.6% of male progeny. This is greater than the percentage of males produced by a *her14* self (3%) and much less than that expected for independent assortment (25%).

Similar crosses of *fem1* females by *her* hermaphrodite sperm were done to determine linkage between *fem1* and the *her* mutations. The ratio of ameristic male to meristic nonmale progeny gametophytes grown in the presence of A_{CE} was scored. For all crosses except one (*fem1* by *her14*), their progeny segregated males and meristic nonmale gametophytes in a 1:3 ratio (Table 2). Because the *her14* mutation is not fully penetrant in a population of *her14* gametophytes (see Table 1), the observed percentage of males in the progeny of the *fem1* × *her14* cross should be and is slightly greater than the expected 25%. This data indicates that none of the *her* mutations tested are allelic to *fem1*.

The *her fem1* double mutant: The *her* and *fem1* mutant gametophytes differ only in the presence or absence of antheridia, as illustrated in Figure 1. The *fem1* mutant gametophyte, like the *her* mutants, is insensitive to A_{CE} (BANKS 1991). To determine the epistatic relationships between the *fem1* and the *her* alleles, diploid sporophytes heterozygous for each of these mutations were generated by crossing *fem1* females by sperm from each of the different *her* hermaphrodites. The F_1 progeny should segregate equal numbers of *her, fem1, her fem1* and wild-type haploid gametophytes given that *fem1* and all *her* mutations segregate independently. If *her* is epistatic to *fem1,* the ratio of hermaphrodites to females should be 2:1 among the nonmale meristic progeny, whereas if *fem1* is epistatic to *her,* hermaphrodites and females should segregate 1:2. To test the ratio of hermaphrodites to females, some meristic gametophytes were removed from other members of the population and allowed to grow for 4 weeks before their phenotype was scored. This was necessary to prevent self-or cross-fertilization of the gametophyte, which halts gametophyte growth before antheridia might form on the hermaphrodite. The results shown in Table 2 show that among the meristic progeny of all *fem1* by *her* crosses, approximately one-third are hermaphroditic and two-thirds are female. The 1:2 segregation ratio of hermaphrodites to females indicates that the *fem1* single mutant and *fem1 her* double mutant gametophytes are female and that the *fem1* mutation is epistatic to all *her* mutants tested. The segregation ratios also

TABLE 2

Epistasis between the *fem1* and *her* genes

			Phenotype					
			All gametophytes + A_{CE}			Nonmales − A_{CE}		
Cross		*n*	Male	Nonmale	Summed data chi square, P^a	Hermaphrodite	Female	Summed data chi square, P^a
fem1 ×	*her1*	1	168 (150)	433 (451)	3.00, >0.05	51 (48)	93 (96)	0.28, >0.50
	her3	3	259 (241)	705 (723)	1.79, >0.10	75 (78)	159 (156)	0.17, >0.50
	her5	4	323 (309)	913 (927)	0.84, >0.30	96 (100)	203 (199)	0.24, >0.50
	her7	2	166 (156)	458 (468)	0.85, >0.30	38 (35)	68 (71)	0.39, >0.50
	her9	4	345 (326)	957 (975)	1.52, >0.20	72 (66)	127 (133)	0.82, >0.30
	her10	3	238 (237)	710 (711)	0.01, >0.90	52 (57)	119 (114)	0.66, >0.30
	her11	3	235 (237)	711 (710)	0.03, >0.70	32 (30)	57 (59)	0.20, >0.50
	her14	2	188 (162)	458 (485)	4.17, >0.05	37 (39)	80 (78)	0.15; = 0.70
	her15	3	219 (236)	728 (710)	2.30, p > 0.10	63 (66)	136 (133)	0.21, >0.50
	her19	4	304 (311)	941 (934)	0.21, >0.50	102 (103)	208 (207)	0.01, >0.90

The maternal and paternal genotypes are indicated at the left; *n* indicates the number of crosses analyzed. The numbers below each phenotype indicate the number of progeny displaying each phenotype. The numbers in parenthesis indicate the expected number for each phenotype assuming that the two mutations assort independently and that *fem1* is epistatic to *her* such that males and meristic nonmale progeny gametophytes segregated in a 1:3 ratio when all progeny are grown on medium containing A_{CE}. A 1:2 ratio of hermaphrodites to females is expected among the nonmale gametophytes which were grown on medium containing A_{CE} then transferred to medium lacking A_{CE} before assessing their phentoype.

[a] Homogenity chi squares were well within the accepted limit ($P > 0.05$) for all crosses except *fem1* × *her14*.

indicate that the *fem1* mutation is completely penetrant and expressive.

The *tra1* and *her tra1* mutants: The *tra1* mutation was originally isolated as a suppressor of *her1* (BANKS 1994). The *her1 tra1* male gametophyte develops as a male in the absence of A_{CE}, a condition that results in hermaph-

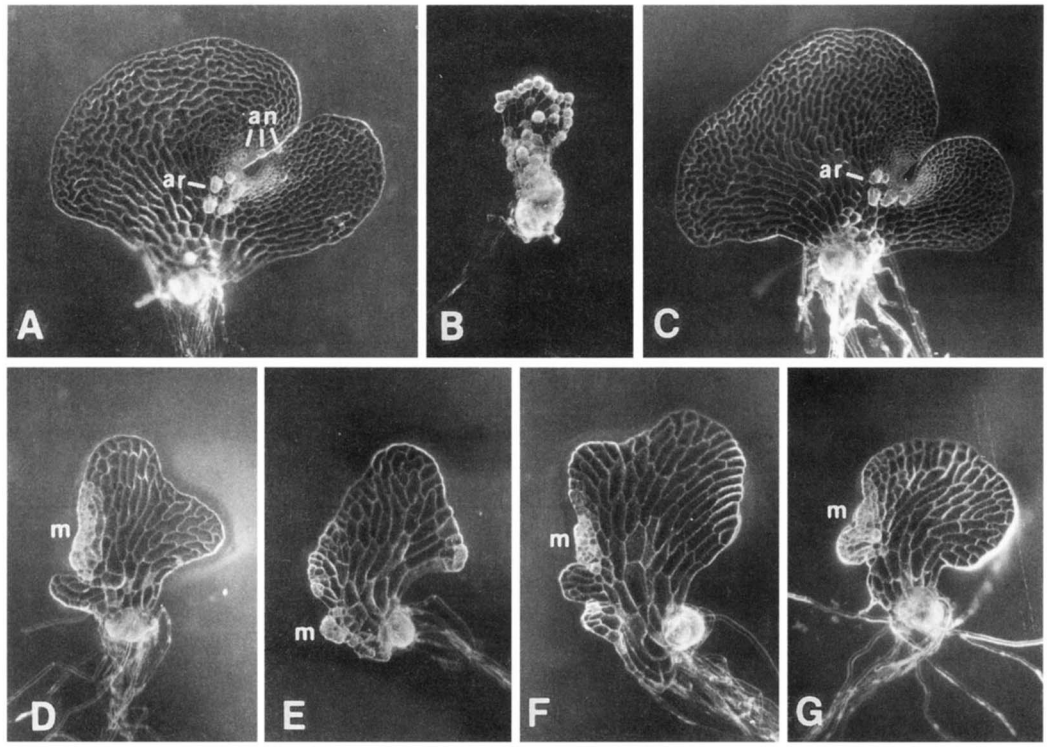

FIGURE 1.—Wild-type and mutant gametophyte phenotypes. (A) A wild-type hermaphrodite with archegonia, antheridia and a meristem. (B) A wild-type male gametophyte with numerous antheridia. (C) A mutant *fem1* female gametophyte with a meristem, archegonia but no antheridia. (D–G) Mutant *tra1 fem1* or *her1 tra1 fem1* intersexual gametophytes. ar, archegonia; an, antheridia; m, meristem.

TABLE 3

Segregation of progeny from a *her1 tra1* male double mutant crossed to a wild-type hermaphrodite

No. of crosses	Phenotype + A_{CE}		Summed data chi square, *P*	Homogeneity chi square, *P*
	Male	Hermaphrodite		
11	2620 (2604)	852 (868)	0.39, >0.80	3.99, >0.95

The values indicate the number of male and hermaphroditic progeny gametophyes when grown on medium containing A_{CE}. Values in parentheses indicate the expected number for each phenotype assuming that the two mutations assort independently and that *tra1* is epistatic to *her1* such that male and hermaphrodites segregate in a 3:1 ratio.

roditic development in wild-type gametophytes (BANKS 1994). To determine the penetrance and expressivity of the *tra1* single and *her1 tra1* double mutants, *her1 tra1* males, which were generated by crossing *her1* hermaphrodites by sperm from the original selected *her1 tra1* male gametophyte, were crossed to wild-type hermaphrodites. The resulting diploid sporophytes should be heterozygous for both *her1* and *tra1* and segregate equal numbers of *her1, tra1, her1 tra1,* and wild-type gametophytes in their progeny. If the phenotype of the *tra1* single and the *her1 tra1* double mutants are male, then the phenotypic ratio of progeny gametophytes obtained from this cross on media containing A_{CE} should be three male:one hermaphrodite. As shown in Table 3, only males and hermaphrodites were observed in the F_1 progeny and these segregated in a 3:1 ratio.

Phenotypic males from this cross should be wild-type, *her1 tra1,* or *tra1* gametophytes. To confirm their genotype, individual F_1 males were transferred to media lacking A_{CE}, because under these conditions genotypically wild-type male gametophytes will switch from male to hermaphroditic (BANKS 1994). Those F_1 males that switched after removal from A_{CE} (*i.e.,* A_{CE}-dependent males) were considered wild type and were not tested further. Those F_1 sibling gametophytes that remained male after removal from A_{CE} (*i.e.,* A_{CE}-independent males), presumably *her1 tra1* double mutants and *tra1* single mutants, were test crossed individually to wild-type hermaphrodites. The F_2 progeny of these crosses are expected to segregate as >95% male on media containing A_{CE} and as one male:one hermaphrodite on media lacking A_{CE} if the F_1 male parent is *tra1*. The F_2 progeny are expected to segregate three male:one hermaphrodite on media containing A_{CE} and one male:one hermaphrodite on media lacking A_{CE} if the F_1 male parent is *her1 tra1*. In addition, because the F_1 male parents were chosen at random, there should be equal numbers of *her1 tra1* double and *tra1* single mutants among the F_1 A_{CE}-independent males. As seen in Table 4, one class of crosses (class 1), involving four individual F_1 males, segregated almost exclusively as males on media containing A_{CE} and one male:one hermaphrodite in medium lacking A_{CE} in the F_2. This indicates that the F_1 male parent is genotypically *tra1*. The other class of crosses (class 2), involving four F_1 males, segregated as the F_0 did (three males:one hermaphrodite) on medium containing A_{CE} (Table 4). In medium lacking A_{CE}, the same progeny segregate males and hermaphrodites in a 1:1 ratio, confirming that the F_1 male parent in these crosses is *her1 tra1*. These results demonstrate that the *tra1* single mutant and *her1 tra1* double mutant gametophytes are A_{CE}-independent males and that both genotypes are completely penetrant and expressive.

The epistatic interactions between *tra1* and both *her11* and *her13* were assessed by crossing *her11* and *her13* hermaphrodites by *tra1* males. Diploid sporophytes derived from these crosses are heterozygous for *tra1* and *her11* or *her13* and should segregate in the progeny equal numbers of *tra1, tra1 her11* or *her13, her11*

TABLE 4

Test cross of A_{CE} independent males from Table 3

	$-A_{CE}$				$+A_{CE}$			
	No. of crosses	Male	Hermaphrodite	Summed data chi square[a], *P*	No. of crosses	Male	Hermaphrodite	Summed data chi square[a], *P*
Class 1	4	126 (282)	287 (282)	0.22, >0.50	4	>99%	<1%	
Class 2	2	125 (130)	135 (130)	0.40, >0.50	4	957 (940)	296 (313)	1.23, >0.20

The values indicate the number of progeny of each phenotype when grown on medium containing A_{CE} ("$+A_{CE}$") or lacking A_{CE} in single spore cultures ($-A_{CE}$). The numbers in parentheses indicate the number of progeny expected for each phenotype assuming that the male parent was genotypically either *tra1* (class 1), in which case all progeny should be male with A_{CE} and segregate males to hermaphrodites in 1:1 ratio without A_{CE}, or *her1 tra1* (class2), in which case the males and hermaphrodites should segregate in a 3:1 with A_{CE} and in a 1:1 ratio on medium without A_{CE}.

[a] Homogenity chi square were well within the accepted limit ($P > 0.05$) for all crosses.

TABLE 5

Epistasis between *tra1* and *her11* or *her13*

Cross	No. of crosses	Phenotype + A_{CE}		Summed data chi square, *P*	Homogeneity chi square, *P*
		Male	Hermaphrodite		
tra1 × *her11*	2	452 (458)	158 (152)	0.32, >0.50	1.27, >0.20
tra1 × *her13*	2	347 (340)	106 (113)	0.57, >0.30	0.09, >0.70

The maternal and patneral genotypes are shown at the left. The number of progeny of each phenotype are given in the table. The values in parentheses indicate the number of progeny expected for each phenotype assuming independent assortment of each mutation, that *tra1* is epistatic to *her*, and that the expected ratio of males to hermaphrodites is 3:1.

or *her13*, and wild-type gametophytes if *tra1* and the two *her* mutations segregate independently. Wild-type and *tra1* gametophytes are male in the presence of A_{CE} while *her11* and *her13* gametophytes are hermaphroditic in the presence of A_{CE}. The proportion of males to hermaphrodites in the progeny should therefore indicate the phenotype of the *tra1 her11* or *tra1 her13* gametophytes. As shown in Table 5, progeny gametophytes segregated males and hermaphrodites in a 3:1 ratio on medium containing A_{CE}, indicating that the phenotypes of *tra1 her11* and *tra1 her13* double mutant gametophytes are male and that *tra1* is epistatic to *her13* and *her11* in addition to *her1*.

The *tra1 fem1* double and *her1 tra1 fem1* triple mutants: To determine the phenotype of the *her1 tra1 fem1* triple mutant gametophyte and the epistatic interaction between *tra1* and *fem1*, diploid sporophytes heterozygous for each mutation were needed. These were generated by crossing *fem1* female gametophytes by sperm from *her1 tra1* double mutant male gametophytes. The resulting diploid sporophytes were simultaneously heterozygous for all three mutations. Because all three are unlinked, these sporophytes should segregate equal numbers of eight haploid genotypes (*her1, tra1, fem1, her1 tra1, her1 fem1, tra1 fem1, her1 tra1 fem1* and wild type). The phenotypes associated with these genotypes, except *her1 tra1 fem1* and *tra1 fem1*, on media with or without A_{CE}, were either known from previous studies (BANKS 1994) or have been determined in this study. Progeny spores from five triple mutant sporophytes were plated on media containing A_{CE}. Four phenotypically distinct gametophyte types were observed: males, females, hermaphrodites and a novel intersex phenotype. As shown in Table 6, these gametophytes segregate in a ratio of three males:two females:two intersexes:one hermaphrodite. Because the only genotypes for which a phenotype has not been determined are *her1 tra1 fem1* and *tra1 fem1*, the novel phenotype and its frequency indicate that the intersex phenotype is the result of being mutant at both *TRA1* and *FEM1*.

The intersex phenotype is characterized by the presence of one or more abnormal meristems, occasional rudimentary archegonia, and few antheridia which produce functional sperm, as illustrated in Figures 1 and 2. The wild-type and *her* hermaphrodites have a single lateral meristem; *i.e.*, a localized region of active cell division that forms the typical meristem notch of the gametophyte (Figures 1 and 2). Cells formed by the lateral meristem either differentiate as archegonial initials or gradually enlarge to form the two-dimensional sheet of cells that make up most of the hermaphrodite thallus. Some cells of the developing thallus may also differentiate as antheridia initials; however, antheridia always form distal to the archegonia and at the perimeter of the meristem (Figure 1). In comparison to the hermaphroditic meristem, the meristem of the intersex is smaller, made up of fewer cells and rarely forms a meristem notch (Figure 1). The lack of a meristem notch appears to be due to the inability of the intersexual meristem to maintain a size comparable with that of the hermaphrodite meristem. Antheridia in the intersex appear adjacent to the meristem and often directly below the meristem, a position where archegonia would form normally form in the hermaphrodite (Figure 2). Archegonia, if they form in the intersex, do not differentiate normally and are nonfunctional. The relative size and position of the meristem of intersexual gametophytes is so variable that no two intersexes appear the same (compare Figure 1, D–G).

The genotype of each phenotypic class in Table 6 was either confirmed or assessed to firmly establish the genotype of the intersex. The genotype of female gametophytes generated from the *fem1* by *her1 tra1* cross was confirmed by crossing 25 F_1 females by wild-type males. The results of these crosses appear in Table 7. The progeny of 12 test-crossed F_1 females segregated male and female gametophytes in a 1:1 ratio (class 1), indicating that the F_1 female parent was genotypically *fem1*. The progeny of 13 other test-crossed F_1 females segregated as male and nonmale gametophytes in a 1:3 ratio (class 2). Of the nonmale class 2 gametophytes, hermaphrodites and female gametophytes segregated in a 1:2 ratio indicating that the female donor was genotypically *her1 fem1*. These results confirm that phenotypically female gametophytes are genotypically *fem1* or *fem1 her1* and that *fem1* is epistatic to *her1*.

The genotype of the A_{CE}-insensitive hermaphroditic progeny from the *fem1* by *her1 tra1* cross (Table 6) should be *her1*. To test this, hermaphrodites were crossed by wild-type sperm and the progeny of this cross

TABLE 6

Segregation of progeny gametophytes from a sporophyte heterozygous for *tra1*, *fem1* and *her1*

	Phenotype + A_{CE}				
Cross	Male	Female	Intersex	Hermaphrodite	Chi square, *P*
1	156 (158)	117 (106)	108 (106)	41 (53)	2.71, >0.30
2	125 (125)	88 (83)	83 (83)	37 (42)	0.90, >0.80
3	147 (154)	111 (103)	112 (103)	40 (51)	4.10, >0.20
4	187 (167)	120 (112)	111 (112)	46 (56)	4.76, >0.05
5	160 (151)	103 (101)	94 (101)	45 (50)	1.57, >0.50

Values indicate the number of progeny of each phenotype. Values in parentheses indicate the number of progeny expected for each phenotype assuming a 3:2:2:1 ratio of males:females:intersexes:hermaphrodites.

assessed; the results are shown in Table 8. For all seven test crosses examined, the progeny segregated hermaphrodites and males in a 1:1 ratio, as expected.

The genotypes of male progeny gametophytes from the *fem1* by *her1 tra1* cross (Table 6) should either be *tra1*, *her1 tra1*, or wild type. Seven F_1 A_{CE}-dependent males that switch to hermaphrodites when removed from media containing A_{CE} were self-fertilized. Greater than 95% of their progeny were male (data not shown), confirming that the A_{CE}-dependent male progeny are wild type for all genes. The sibling F_1 males that did not switch when removed from medium containing A_{CE} (*i.e.*, A_{CE}-independent males) were crossed to wild-type hermaphrodites and their progeny assessed on media containing A_{CE}. As shown in Table 9, one class, represented by 10 crosses, produced >95% males in the presence of A_{CE}, indicating that the class 1 A_{CE}-independent male parent was genotypically *tra1*. A second class, represented by eight crosses, produced males and hermaphrodites in a 3:1 ratio (Table 9), confirming that the class 2 A_{CE}-independent male parent was *her1 tra1*.

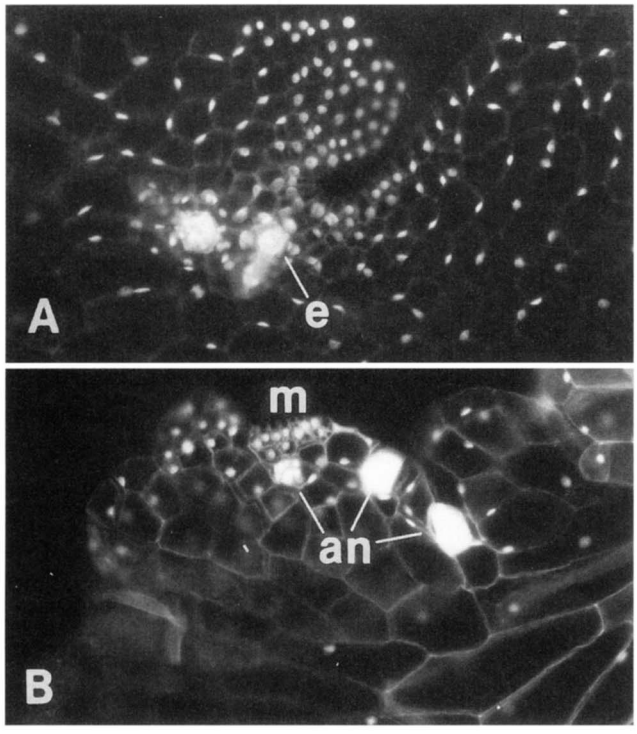

FIGURE 2.—The meristems of hermaphrodite and intersex gametophytes. Gametophytes were stained with Hoechst's dye and photographed under UV fluorescence. (A) The meristematic region of the hermaphrodite showing the typical meristem notch formed by the meristem and two archegonia, one with a prominent, brightly fluorescent egg cell. (B) The meristematic region of an intersexual gametophyte showing a relatively small meristem and three antheridia containing developing sperm cells. One antheridia is directly below the meristem, a position normally occupied by archegonia in the hermaphrodite. e, egg; m, meristem; an, antheridia.

The genotype of the F_1 intersex gametophytes from the *fem1* by *her1 tra1* cross (see Table 6) was tested by crossing *fem1* females by sperm from individual intersexes. If the intersex is genotypically *her1 tra1 fem1* or *tra1 fem1*, all of the resulting diploid sporophytes should produce progeny that segregate female and intersex gametophytes in a 1:1 ratio. The results shown in Table 10 demonstrate that, in six of six crosses assessed, all progeny gametophytes segregate in the expected ratio. These results, together with the segregation ratios of intersexes to other phenotypes in Table 6, confirm that the genotype of the intersex can either be *fem1 tra1 her1* or *fem1 tra1*.

DISCUSSION

The *HER* genes and the A_{CE} signal transduction pathway: The primary sex-determining signal in Ceratopteris is the pheromone A_{CE}, which directs the gametophyte to develop as a male. Gametophytes that are competent to respond to A_{CE} must perceive and transduce the A_{CE} signal to elicit the appropriate sexual response. The *HER* genes are likely to encode factors that constitute the A_{CE} receptor and/or signal transduction pathway because mutations of any of the *HER* genes result in a phenotype similar to wild-type gametophytes grown in the absence of A_{CE}. Antheridiogen is produced and secreted from *her* gametophytes (J. A. BANKS, unpublished results), indicating that these mutations have no effect on genes involved in A_{CE} biosynthesis and secretion. By genetic analysis, an approximation of the number of *HER* genes involved in this putative pathway can be determined (MOHLER 1977). The link-

TABLE 7

Test cross of female segregants from a heterozygous *her 1 tra1 fem1* sporophyte

	No. of crosses	All gametophytes + A_{CE}		Summed data chi square[a], *P*	Among nonmales − A_{CE}		Summed data chi square[a], *P*
		Male	Female		Female	Hermaphrodite	
Class 1	12	1901 (1941) Male	1980 (1941) Nonmale	1.6, >0.20	NA	NA	NA
Class 2	13	926 (955)	2895 (2866)	1.17, >0.20	170 (170)	85 (85)	0, = 1.00

The values indicate the number of progeny of each phenotype. Values in parentheses indicate the number of progeny expected for each phenotpye assuming independent assortment of each mutation. Class 1 includes those crosses that segregate 1:1 male to female in the presence of A_{CE}; class 2 includes crosses that segregate 1:3 male to nonmale in the presence of A_{CE}. Between 20 and 30 of the nonmale, class 2 meristic gametophytes were separated and grown for an addition 2 weeks to determine the frequency of females and hermaphrodites in the population.

[a] Homogenity chi squares were well within the accepted limit ($P < 0.05$) for all crosses.

age analyses of 11 independently derived *HER* alleles indicate that they represent at least five distinct loci, two of which are linked. The linkage analysis among the *fem1, tra1* and several of the *her* mutants also indicate that none of the *her* loci are linked to *fem1* or *tra1*. The actual number of *HER* loci involved in the putative signal transduction pathway is likely to be much larger than five since multiple alleles of a single *HER* locus exist for only two of the five loci. Several more *HER* alleles must be isolated before a good estimate of the complexity of the signal transduction pathway can be determined.

All *her* gametophytes are hermaphroditic and phenotypically indistinguishable with the exception of *her14*, which is not fully penetrant. The epistatic interactions among the *her* mutants are therefore impossible to assess. Assuming that the *her* mutations are recessive, loss-of-function mutations, there are three conceptual alternatives explaining how the *HER* genes form the A_{CE} signal transduction pathway. First, each *HER* locus may represent a step in a single linear pathway. That is, one *HER* activates or represses another, which in turn activates or represses another, and so on. Alternatively, each *HER* gene may act independently to activate or repress a common downstream gene. In this case, each *HER* gene must be activated by A_{CE} because mutation of only one *HER* gene is sufficient to block the signal transduction pathway. A third possibility is that the different *HER* genes form a single complex that must be complete to act on a downstream gene(s). A biochemical analysis of the *HER* gene products is required to determine which of these possibilities alone or in combination are operative in Ceratopteris.

It is assumed in these studies that the *her* mutations, as well as the *fem1* and *tra1* mutations, are recessive. In Ceratopteris, it is not possible to perform complementation analysis or determine dominance of a gametophytic mutation because the gametophyte is haploid. However, it is possible to generate a diploid fern gametophyte through a process known as apospory (reviewed in RAGHAVEN 1989). Apospory in ferns is the derivation of gametophytic tissue from sporophytic tissue without the intervention of meiosis. Because the gametophytic tissue derived through apospory is the product of mitosis rather than meiosis, the gametophyte is diploid. Using a recessive genetic marker in Ceratopteris, we have observed that self-fertilization of a heterozygous diploid gametophyte derived by apospory produces a tetraploid sporophyte, which, in turn, produces diploid gametophytes that segregate the recessive marker in the expected ratio (J. R. EBERLE and J. A. BANKS, unpublished results). On the basis of these results, it should be possible to determine dominance and allelism for each of the gametophytic mutations using this approach.

The positive model of sex determination: Previous studies have identified several classes of genes that alter the normal programming of sexual phenotype by A_{CE} (BANKS *et al.* 1993). To understand if and how these genes interact with one another to define a hierarchy

TABLE 8

Test cross of hermaphroditic segregants from a heterozygous *her1 tra1 fem1* sporophyte

No. of crosses	Phenotype + A_{CE}		Summed data chi square, *P*	Homogeneity chi square, *P*
	Male	Hermaphrodite		
12	1877 (1881)	1885 (1881)	0.02, >0.90	4.39, >0.90

The observed number of male and hermaphroditic progeny gametophytes when grown on medium containing A_{CE} is shown. Values in parentheses indicate the expected number of each phenotype assuming that males and hermaphrodites segregate in a 1:1 ratio.

TABLE 9

Test Cross A_{CE}-independent male segregants from a heterozygous *her1 tra 1 fem 1* sporophyte

	No. of crosses	Phenotpye + A_{CE}		Summed data chi square, *P*	Homogeneity chi square, *P*
		Male	Hermaphrodite		
Class 1	10	<5%	>95%		
Class 2	8	1784 (1769)	575 (590)	0.51, >0.30	2.17, >0.90

The observed number of male and hermaphroditic progeny gametophytes when grown on medium containing A_{CE} is shown. Values in parentheses indicate the expected number of each phenotype assuming that the male parent was genotypically either *tra1* (class 1), in which case all progeny should be male, or *her1 tra1* (class 2), in which case the males and hermaphrodites should segregate in a 3:1 ratio.

of regulatory genes that ultimately determines the sex of the Ceratopteris gametophyte, we tested epistasis among these genes by assessing and comparing the phenotypes of single, double, and triple mutant gametophytes. The outcome of these analyses are summarized in Figure 3.

A model of how the sex-determining genes in Ceratopteris form such a hierarchical network has been proposed (BANKS 1994; EBERLE *et al.* 1995). This model was based on the phenotypes of the *her1* and *fem1* single mutants and the *her1 tra1* double mutant and assumed that all known sex-determination mutants are loss-of-function mutants. This model makes specific, testable predictions about the epistatic relationships between the mutants. One goal of the present study was to test these predictions. The following discussion examines the previous model in light of the evidence confirming or contradicting the predictions of that model, as well as an alternative model of gene interaction that is also consistent with the available data. The previous model was based on the assumption that *TRA1* and *FEM1* act in a positive manner to direct differentiation of female and male traits, respectively, and is therefore referred to as the positive model.

According to the positive model diagrammed in Figure 4, *TRA1* and *FEM1* are the major sex-determining genes in the gametophyte. *TRA1* promotes the differentiation of female traits (meristem and archegonia) and *FEM1* promotes the differentiation of male traits (antheridia). The function of these genes was deduced from the phenotypes of *tra1* and *fem1* gametophytes. Because the *tra1* gametophyte is male in the presence or absence of A_{CE}, *TRA1* gene activity is necessary for the differentiation of the archegonia and meristem. Because the *fem1* gametophyte is female in the presence or absence of A_{CE}, *FEM1* gene activity is necessary for the differentiation of antheridia. Given that both hermaphrodites and males produce antheridia, the differences between the sexes can be regarded as the presence or absence of an organized multicellular meristem and archegonia, which are a consequence of *TRA1* gene activity. According to the positive model, A_{CE} acts by activating the *HER* gene(s) which, together with *FEM1*, represses *TRA1*. Thus, when A_{CE} is present, the *HER* genes and *FEM1* are active, *TRA1* is repressed and the gametophyte is an ameristic male. In the absence of A_{CE}, the *HER* genes are not active, *TRA1* is not repressed by the *HER* and *FEM1* genes, *FEM1* is active, and the gametophyte is a meristic hermaphrodite. A more detailed explanation of the positive model is given in BANKS (1993) and EBERLE *et al.* (1995).

TABLE 10

Back cross of intersex gametophytes from a heterozygous *her1 tra1 fem1* sporophyte to *fem1*

No. of crosses	Phenotype + A_{CE}		Summed data chi square, *P*	Homogeneity chi square, *P*
	Female	Intersex		
6	323 (335)	347 (335)	0.86, >0.30	1.27, >0.90

The values indicate the number of female or intersex progeny when grown on medium containing A_{CE}. The values in parentheses indicate the expected frequencies.

This model of sex determination makes three predictions that are amenable to genetic testing: that *fem1* is epistatic to *her1*; that there is no epistatic interaction between *tra1* and *fem1*; and that the phenotype of a *tra1 fem1* double mutant is asexual; *i.e.*, lacking archegonia, antheridia and a meristem. That *tra1* is epistatic to *her1* is not a prediction of this model because *tra1* was originally isolated as a suppressor of *her1*. However, the male phenotypes of the *her11 tra1* and *her13 tra1* double mutants assayed in this study indicate that *tra1* is also epistatic to two additional *her* genes.

To test the first prediction, that *fem1* is epistatic to *her1*, the phenotype of the *her1 fem1* gametophyte was assessed. As predicted, the *her1 fem1* gametophyte is female, indicating that *fem1* is epistatic to *her1*. Epistasis among all *fem1* and *her* alleles was also assessed, with the exception of *her13*, which was not tested. All *her fem1* double mutants are female indicating that *fem1* is epistatic to 10 *her* alleles representing four different *her* loci.

The second prediction of the model, that *tra1* and *fem1* act independently or in two branches of the sex-determining pathway, was tested by assessing the phenotype of the *tra1 fem1* double mutant. The lack of epistasis between *tra1* and *fem1* is evident in the novel phenotype

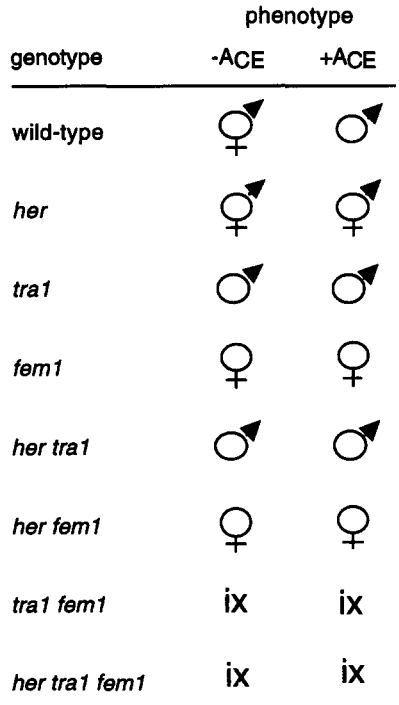

FIGURE 3.—The phenotypes of the sex determination mutants. The mutant phenotypes associated with various single, double, and triple mutant genotypes on media +/− A_{CE} are indicated. *her* represents all *her* alleles that have been analyzed and "ix" indicates an intersexual phenotype.

of the double mutant gametophyte, which is neither entirely female (like *fem1*) nor male (like *tra1*). The phenotypes of the double mutant and *her1 tra1 fem1* triple mutant confirm the predication that *tra1* and *fem1* act in two different branches of the sex-determination pathway.

The final prediction of the positive model is that the *tra1 fem1* double mutants should be unable to differentiate antheridia, archegonia or meristems. Phenotypically, such double mutants would appear as ameristic, asexual gametophytes devoid of antheridia and archegonia. The phenotype of the *tra1 fem1* double mutant is not asexual but intersexual where the gametophyte displays both male and female traits yet is clearly distinct from the hermaphrodite.

If the positive model is correct, the observation that *tra1 fem1* double mutants are intersexual, rather than asexual, could be accounted for if *tra1* and/or *fem1* are not completely expressive alleles. However, this seems unlikely because the results of this and one other study (BANKS 1994) demonstrate that each allele alone appears to be completely expressive and penetrant. The possibility that "stronger" *tra1* and *fem1* alleles could generate an asexual gametophyte will be addressed by comparing the apparent expressivity of additional mutant alleles in single and double mutant gametophytes. Six additional *tra* mutants have been isolated either as suppressors of *her13* or as abscisic acid (ABA) resistant mutants (J. A. BANKS, unpublished results); the phenotype of the six possible *fem1 tra* mutant gametophytes has yet to be determined.

The negative model of sex determination: The novel intersexual phenotype of *tra1 fem1* double mutant indicates that *TRA1* and *FEM1* might not be positive regulators of female and male traits but negative regulators of male and female traits, respectively. To account for this possibility, an alternative, negative model for the regulation of sex expression by sex-determining genes in Ceratopteris that is consistent with the available genetic data is proposed. As with the positive model, we assume for the negative model that all mutations are loss-of-function mutations. According to the negative model diagrammed in Figure 5, the sex of the gametophyte ultimately depends on the activity of the *TRA1* and *FEM1* genes; antheridia develop in the gametophyte only when *TRA1* is repressed, and meristem and archegonia develop only when the *FEM1* gene is repressed. The activites of *TRA1* and *FEM1* during gametophyte development are regulated by a number of factors, including the primary sex signal A_{CE}, the *HER* genes, and each other, as *TRA1* and *FEM1* negatively regulate and mutually exclude each other's expression according to this model. With the negative model of

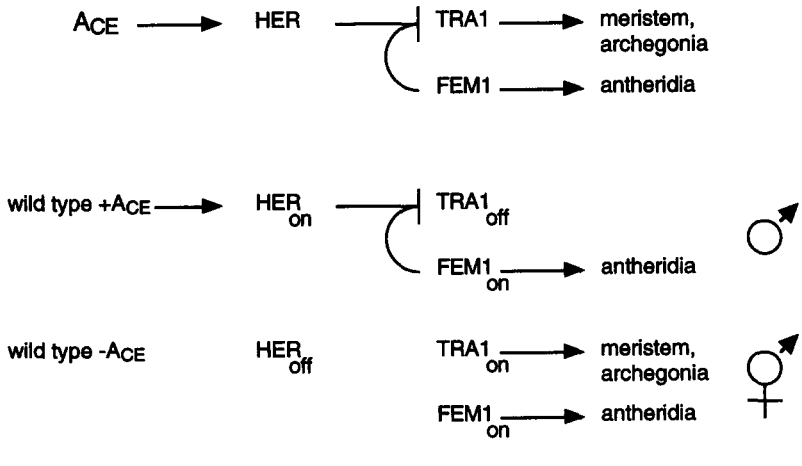

FIGURE 4.—A diagrammatic representation of the positive model of sex determination. The regulatory interactions among exogenous A_{CE} and the major sex-determination genes are shown at the top. The influence of the presence or absence of exogenous A_{CE} on the activity of these genes and the sex of the wild-type gametophyte is shown below. In this figure, "on" indicates a requirement for the wild-type gene product and "off" indicates that the wild-type gene product is not required. "Antheridia" indicates genes required for antheridia development and "meristem, archegonia" indicates genes necessary for meristem and archegonia formation. Lines ending in arrows indicate positive interactions whereas those ending in bars indicate repressing interactions. HER includes at least three *HER* loci defined by *her1*, *her11* and *her13* whose order in the pathway has not been established.

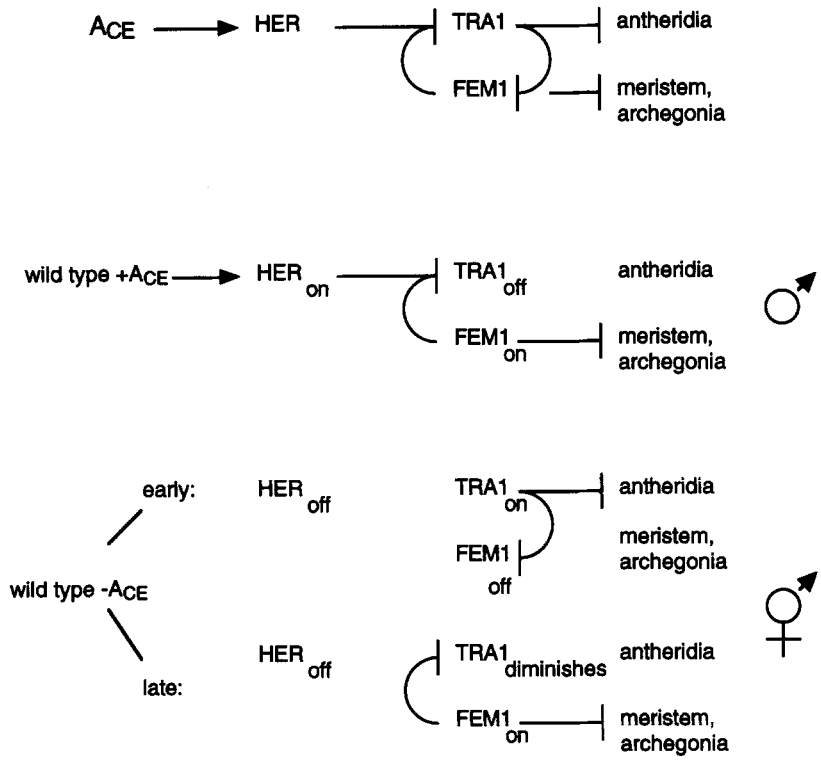

FIGURE 5.—A diagrammatic representation of the negative model of sex determination. The regulatory interations among A_{CE} and the major sex-determination genes are shown at the top. The effect of exogenous A_{CE} on the activity of these genes and the sex of the wild-type gametophyte is shown in the middle of the diagram. The effect of no exogenous A_{CE} on gametophyte development, shown at the bottom of the diagram, is divided into two parts, early and late. Early refers to the events that occur early in gametophyte development as the meristem is initiated and the archegonia begin to form. These same events also apply to dividing cells of the meristem of older gametophytes and the archegonia initials that develop throughout the life of the gametophyte, which ends when the egg inside the archegonia is fertilized. Late refers specifically to the events that occur in some cells at the border of the meristem where antheridia initials develop. In these cells, *TRA1* activity diminishes (as indicated) to the point where *TRA1* no longer represses *FEM1* and *FEM1* consequently represses *TRA1* activity. "Antheridia" indicates genes required for antheridia development and "meristem, archegonia" indicates genes necessary for meristem and archegonia formation. "On" indicates a requirement for the wild-type gene product, and "off" indicates that the wild-type gene product is not required. The lines ending in arrows indicate positive interactions whereas those ending in bars indicate repressing interactions. HER includes at least three *HER* loci defined by *her1*, *her11* and *her13* whose order in the pathway has not been established.

sex determination, the hermaphrodite is viewed as an essentially female gametophyte that later produces antheridia in only a few cells. How the genes are regulated during normal male and hermaphrodite and mutant intersex development according to this model is described.

The development of the male gametophyte: The developing gametophyte is initially competent to respond to A_{CE} early in development as the spore wall cracks and division of the spore nucleus begins (BANKS *et al.* 1993). If exogenous A_{CE} is present from this point in time onward, the gametophyte develops as an ameristic male. According to the negative model, A_{CE} activates the *HER* genes, which, together with *FEM1*, repress *TRA1* activity. Because *TRA1* is not expressed, the genes required for antheridia differentiation are expressed and *FEM1* is not repressed. The expression of *FEM1* leads to repression of the genes required for meristem and archegonia formation. The gametophyte thus develops as an ameristic male and will continue that program of expression as long as exogenous A_{CE} is present. If A_{CE} is withdrawn from the supporting medium, the *HER* genes, no longer active, cannot repress *TRA1*. Consequently, *TRA1* is expressed resulting in repression of antheridia differentiation and *FEM1* activity. The repression of *FEM1* activity in newly dividing cells allows the expression of genes required for meristem and archegonia differentiation. New growth of the A_{CE}-dependent male thus switches from ameristic male to meristic hermaphrodite after exogenous A_{CE} is withdrawn from the gametophyte.

The evidence indicating that both the *HER* genes and *FEM1* are required to repress *TRA1* is the phenotype of the *fem1* mutant, which does not form antheridia in either the presence or absence of A_{CE}. If A_{CE}-mediated activation of *HER* genes was sufficient for *TRA1* repression, the *fem1* gametophyte would be expected to be intersexual in the presence of A_{CE} because under these conditions *FEM1* would be inactive by mutation and *TRA1* would be repressed by the A_{CE}-activated *HER*

genes. Because the *fem1* gametophyte is not intersexual but female, it is likely that the *FEM1* and the *HER* genes are both necessary for the repression of *TRA1*. This interpretation of male development is also consistent with the *tra1* and *her tra1* mutant phenotypes. If the *TRA1* gene is irreversibly inactivated by mutation, neither *FEM1* nor antheridia differentiation can be repressed. In this case, antheridia differentiate because *TRA1* is inactive, meristem and archegonia differentiation is repressed because *FEM1* is active, and the *tra1* gametophyte develops as a male even in the absence of A_{CE}. Because *tra1* is epistatic to the *her* mutations, the phenotype of *her tra1* double mutants can be seen in the same way.

The development of the hermaphrodite: The Ceratopteris gametophyte develops as a meristic hermaphrodite if never exposed to A_{CE} or if exposed to A_{CE} during a period of development when it is not competent to respond to A_{CE} (BANKS *et al.* 1993). The events that occur during the early stages of gametophyte development, when the gametophyte is competent to respond to exogenous A_{CE} but A_{CE} is absent, are considered first. According to the negative model, in the absence of A_{CE} the *HER* genes are not active and *TRA1* is not repressed in the developing gametophyte. The expression of *TRA1* has two consequences: it represses differentiation of antheridia in the region of the gametophyte occupied by the actively dividing meristem and it represses *FEM1*, which promotes differentiation of a meristem and archegonia. This interpretation is consistent with the *fem1* and *her fem1* mutant phenotypes, which are meristic females. In both cases, the irreversible inactivation of *FEM1* by mutation has two consequences. One is that the genes required for meristem and archegonia differentiation are not repressed, and the other is that *TRA1* cannot be repressed. Consequently, genes required for antheridia differentiation are repressed while genes required for meristem and archegonia differentiation are not repressed in the *fem1* gametophyte, regardless of the absence or presence of A_{CE}.

In both *her* and wild-type gametophytes grown in the absence of exogenous A_{CE}, antheridia develop after the establishment of the meristem and are first observed at the perimeter of the meristem, distal to the meristem notch and archegonia. As the cell passes from an undifferentiated state in the meristem and becomes committed to differentiating as an antheridium, the *TRA1* gene (the repressor of antheridia differentiation genes) should become inactive and the *FEM1* gene (the repressor of meristem/archegonia differentiation genes) should become active if the negative model is correct. Furthermore, these changes in *TRA1* and *FEM1* activity that accompany the change in fate from undifferentiated meristematic to antheridial are independent of the *HER* genes, because *her* mutant gametophytes produce antheridia, and dependent on *FEM1* gene activity since *fem1* mutant gametophytes do not produce antheridia. Understanding how *FEM1* and *TRA1* activities are modulated to allow differentiation of antheridia in some cells surrounding the meristem is key to understanding how antheridia form on an essentially female gametophyte. One possible explanation is that *TRA1* encodes a diffusable factor that is only expressed in actively dividing cells and whose activity diminishes as cells exit the meristem. This could establish a gradient of *TRA1* activity that is highest in the center of the meristem and progressively lower in surrounding cells. If *TRA1* is a negative regulator of *FEM1*, as proposed, diminished *TRA1* activity in cells at the periphery of the meristem would allow some expression of *FEM1* in these cells. Assuming that *FEM1* by itself is a weak negative regulator of *TRA1*, the regulatory interactions between *FEM1* and *TRA1* could, in these cells, favor a state where *TRA1* is repressed and *FEM1* is expressed. Such cells would differentiate as antheridia. If *FEM1* is a weak negative regulator of *TRA1*, this repression of *TRA1* should be relieved in *fem1* mutants and *TRA1* maintained at a level high enough to completely suppress antheridia differentiation in *fem1* mutants, thus explaining why antheridia fail to differentiate in the *fem1* female. The negative regulatory interactions between *FEM1* and *TRA1* can be tested by isolating gain-of-function alleles of *FEM1* and *TRA1*. According to this model, gain-of-function alleles of *FEM1* and *TRA1* are predicted to exhibit phenotypes similar to that for *TRA1* and *FEM1* loss-of-function alleles, respectively. At this time, the possibility that each gene acts in both a positive and negative fashion, as with the *doublesex* (*dsx*) gene of *D. melanogaster* (BURTIS *et al.* 1991; JURSNICH and BURTIS 1993), cannot be excluded.

The development of the intersexual gametophyte: The phenotypes of the *tra1 fem1* and *tra1 fem1 her1* mutant gametophytes are intersexual, as previously described. If the negative model of sex determination is correct, the irreversible inactivation of both *TRA1* and *FEM1* should lead to the simultaneous expression of genes required for meristem, archegonia and antheridia in the developing gametophyte. The absence of *TRA1* and *FEM1* activity should also eliminate the negative regulatory interactions between *TRA1* and *FEM1*. Although the intersexual gametophyte initiates antheridia, archegonia and a meristem, the archegonia and meristem are either nonfunctional or poorly developed, indicating that these structures cannot be maintained as they are in the wild-type hermaphrodite. This could be due to the inappropriate expression of male-specific genes in these female structures. To determine whether sex cells (or their precursors) of the intersexual gametophyte simultaneously express genes that are normally repressed by *TRA1* and *FEM1* can be determined using molecular markers specific to cells of each sex type.

The proposed models of sex determination in Ceratopteris are preliminary and likely to be modified as more genes that are involved in sex determination are

identified. However, the negative model of sex determination does resemble in many ways a model put forth by NÄF (1961, 1979) to explain how sex is regulated by antheridiogen in other species of homosporous ferns. NÄF proposed that there exists a block to antheridium formation which is a function of the meristem. The block is reversible by antheridiogen in juvenile gametophytes that have not yet formed a meristem but is not reversible in older meristic gametophytes. NÄF based this model primarily on his observations (1961) and the observations of others (CZAJA 1921; PARÉS 1958; DÖPP 1959) that if the meristem is removed from the gametophytes of other homosporous ferns, the fragments lacking the meristem form antheridia in the absence of exogenous antheridiogen indicating that the meristem produces an inhibitor of antheridia formation in the gametophyte. The results of the genetic analysis of sex determination in Ceratopteris indicate that, like other ferns, there exists a block to antheridia formation, a consequence of *TRA1* activity. The block (*TRA1*) can be reversed (repressed) by A_{CE} as long as the gametophyte is competent to respond to A_{CE} (*HER* genes can be activated) and a meristem has not formed (*FEM1* is also active). The parallels between the NÄF model and the negative model of gene interaction in Ceratopteris indicate that the mechanism underlying sex determination may be conserved among homosporous ferns with an antheridiogen response. However, the sex-determining genes and their interactions may vary among different species of ferns as there is considerable variation in the spatial and temporal expression of meristem, archegonia and antheridia during gametophyte development among species. If the sex-determining mechanism is conserved, it will be of interest to understand the fate of the sex-determining genes as plants evolved from homospory, where sex is determined after sporogenesis during the gametophyte generation, to heterospory, where sex is determined during the sporophyte generation such that the sex of each spore is determined prior to its formation (SUSSEX 1966).

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