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Localization of the mouse gene releasing sex-limited expression of *Slp*

(sexual dimorphism/gene regulation/*mhc*/complement)

PETER P. JIANG, KATHY FREDERICK, TED H. HANSEN, AND RAYMOND D. MILLER*

Department of Genetics, Washington University School of Medicine, St. Louis, MO 63110

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ABSTRACT **To probe genetic variation in the regulation of sexual dimorphism, we have characterized the mouse protein Slp, coded by the gene sex-limited protein (*Slp*). Slp expression in many strains is limited to males and is androgen-dependent. However, female expression is also observed in rare strains, due to nonlinked gene(s) termed regulator of sex-limitation (*rsl*). In this report we demonstrate that female expression of Slp results from homozygous recessive allele(s) at a single autosomal locus that maps to a 2.2-centimorgan interval on chromosome 13. This conclusion was supported by extensive genetic analyses including the use of polymorphic microsatellites to type numerous backcross progeny and a recombinant inbred series and to identify the congenic interval in three independently derived congenic strains. Four attractive candidate genes were identified by the localization of *rsl*. Interestingly, *rsl* was found not only to enable expression in females but to also increase expression in males. The findings suggest that the expression of Slp and perhaps other sexually dimorphic proteins is regulated by two pathways, one that is dependent upon *rsl* but not androgens and another that is *rsl*-independent but requires androgens.**

As in other mammals, the tissue-specific levels of expression of many proteins differ between male and female mice, and the differences are frequently dependent upon the expression of gonadal hormones. A few of many examples of sexually dimorphic protein expression in mice are aromatase in brain, acidic epididymal glycoprotein and nerve growth factor in submandibular gland, 11β-hydroxysteroid dehydrogenase in kidney, steroid 16α-hydroxylase P450$_{16\alpha}$ and steroid 15α-hydroxylase P450$_{15\alpha}$ in liver, urinary proteins, and complement proteins C5, C6, and C7 in sera (1–7). In mammalian development, it is believed that the product of a single Y chromosome-borne gene (*Sry* in the mouse) is the switch that changes the gonad development from the default female pathway to the male pathway. In its simplest form, this one-switch model suggests that other differences between the sexes result directly from the subsequent sex-specific production of gonadal hormones (8, 9). However, not all sexual dimorphisms can be explained solely by this simple model. For example, in mice the serum C4 protein (coded in major histocompatibility complex *H-2* by the gene complement component 4, *C4*) is expressed higher in males than females in strains B10.Q ($C4^q$) and A.BY ($C4^b$); however, it is expressed lower in males than females in strains 020.Q ($C4^q$) and B10 ($C4^b$) (allelic designation of *Slp* and *C4* is by original *H-2* haplotype) (10). This and similar examples suggest that in addition to a Y chromosome-borne gene, other genetic variation affects sexual dimorphism.

The sex-limited protein (Slp), a serum glycoprotein, provides a dramatic example of sexual dimorphism in mice, and a potential model system for studying differential expression in males and females. Slp is the product of the gene *Slp*, located in the *H-2* (11). The name Slp was derived from the original observation that the protein was found only in males of the investigated strains (12). Slp was found in several independent haplotypes (Slp^d, Slp^u, Slp^s, Slp^p) (13, 14), and it has recently been shown to have activity in a putative complement pathway (15). In the liver, the major source of Slp, the difference in expression levels of the protein in males and females is due to nuclear factors acting at the transcriptional stage (16). Full expression of Slp in males occurs only after puberty and is dependent upon the presence of testosterone, androgen receptors, and an adult male pattern of growth hormone release (16, 17).

Due to an apparent tandem duplication, *Slp* and *C4* form a small multigene family (18, 19). At the cDNA level, *Slp* is 95% similar to *C4*, and the genomic organization indicates each has 41 exons, with boundaries at comparable locations (20). A transposable element, which contains an androgen response site, is located upstream from *Slp* but not *C4* (21–23). The transposable element is present in all standard laboratory and all tested wild-derived *H-2* haplotypes (24). Each haplotype contains one full-length *Slp* and one full-length *C4* gene; also, three wild-derived haplotypes contain two or three additional hybrid genes that are regulated like *C4* but express a protein recognized by antibodies to Slp (24, 25).

The fascinating observation was made by Brown and Shreffler (26) that in four strains that do not contain hybrid *Slp* genes (FM, LG, NZB, and PL), the "sex-limitation" is removed in that females also express Slp (26). Expression of Slp was characterized in detail in the strain FM (Slp^d); Slp is expressed only after 4 weeks of age in either sex and is expressed in males at higher levels than in females. When FM was crossed to C57BL/10-based (B10) congenic strains, Slp was not expressed in female progeny. In the next generation of these crosses, expression of Slp in females required at least one copy of a permissive allele for *Slp* and, additionally, permissive alleles at one or two hypothesized genes not linked to *Slp*. These putative gene(s) were called regulator of sex-limitation (*rsl*).

In this paper, we demonstrate that expression of Slp in females of strains FM, NZB, and PL is due to a single *rsl* gene. The gene was localized by analyses of microsatellite (MS) loci in congenic strains, backcross progeny, and a recombinant inbred series. Based on the observation that *rsl* was also found to increase expression of Slp in males, a model for the regulation of Slp and other sexual dimorphisms is discussed.

MATERIALS AND METHODS

Mice. All mice were maintained in the Animal Facility of the Department of Genetics. The strains used in crosses were B10.D2/nSnSf, PL/J, NZB/B1NJ, and the Shreffler stock of FM, originally from Japan. The *rsl* congenic strains were constructed by the cross–intercross method (27), with selection

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Abbreviations: MS, microsatellite; cM, centimorgan(s).
*To whom reprint requests should be addressed.

among female intercross progeny for Slp expression. The background strain was B10.D2/nSnSf, and the donor strains were PL/J, NZB/B1NJ, or the Shreffler stock of FM. Slp expression was determined by radial immune diffusion from the serum of mice that were at least 8 weeks old (13). Serum samples from the NXSM recombinant inbred series were collected by Donald Shreffler in the laboratory of Eva Eicher, and DNA from these strains was purchased from The Jackson Laboratory.

Analysis of MS Loci. DNA was isolated by ethanol precipitation after homogenization of tissues in guanidine hydrochloride (28). For localization of *rsl*, 160 MS loci approximately 10 cM apart were selected throughout the genome; loci were chosen that were demonstrated to be polymorphic within the subspecies (29, 30), and primers were purchased (Research Genetics, Huntsville, AL). For initial localization of *rsl*, PCR was conducted with internal radiolabeling and analysis on sequencing gels, as described (31, 32). For segregation analysis, PCR products were analyzed on horizontal agarose gels (3.5% MetaPhor, FMC BioProducts) with 0.5× TBE buffer, and a multichannel pipette was used for loading the gels (33).

RESULTS

Genetic Crosses Indicate that *rsl* Is a Single Autosomal Gene. To clarify the genetics of the putative *rsl* gene(s), we analyzed Slp expression in adult female progeny from an extensive backcross, (PL × B10.D2) × PL. In this cross, each of the parental strains is permissive for Slp expression in males (Slp^u for PL and Slp^d for B10.D2), and consequently, segregation of whether or not Slp is expressed in each female progeny is determined solely by the *rsl* gene(s). The resulting frequency of female progeny in which Slp was expressed was approximately 1/2, regardless of whether the heterozygous parent was male or female (Table 1). The results are well explained by the model of a single autosomal *rsl* gene with complete penetrance and the recessive allele in strain PL. Consistent with this explanation, in the cross PL × B10.D2, the frequency of F_2 females in which Slp was expressed was approximately 1/4 (Table 1).

The number and allelic relationship of *rsl* genes was further investigated in strains FM and NZB, in which Slp is expressed in females. Each strain crossed to PL yielded F_1 and F_2 females in which Slp was expressed in all (Table 1). Strain FM or NZB crossed to B10.D2 yielded F_1 females in which Slp was expressed in none and F_2 females in which Slp was expressed in approximately 1/4 of animals (Table 1). These results demonstrate that there is a single autosomal gene, with a dominant allele *Rsl* in B10.D2 and a recessive allele(s) *rsl* in FM, NZB, and PL, and that *rsl/rsl* is required for expression of Slp in females.

***rsl* Was Localized to Chromosome 13 by Searching the Genome of Congenic Strains.** To further study expression of Slp, three *rsl* congenic strains were constructed by the cross–intercross method over a period of 10 years by using background strain B10.D2 and donors FM, NZB, and PL (Table 2). The new congenic strain names are derived from the names of the background and the *rsl* donor strains (34). The strain based on *rsl* donor PL has two major sublines: the tyrosinase gene *c* for albinism, on chromosome 7, segregated in early generations, and albino females in which Slp was expressed were still observed after several breeding cycles, suggesting *rsl* might be linked to *c*. Although this association was later shown to be spurious, an albino stock was separated as a subline after seven cycles of breeding and maintained by sib mating as the strain B10.D2.PL(1)-*rsl*. Strain construction was continued for three additional cross–intercross breeding cycles and the nonalbino stock was designated B10.D2.PL(2)-*rsl*.

To search for the *rsl* gene, we used an extensive collection of polymorphic MS loci to identify the new *rsl* congenic interval in strain B10.D2.PL(1)-*rsl*. This method was feasible because of two factors: (*i*) about half of all MS loci are expected to be informative in any comparison of inbred strains (30) and (*ii*) a congenic interval should be identified by a modest-sized genome-wide framework of loci because the expected length of the congenic interval after seven cross–intercross cycles is about 35 cM (31). For analysis, some 160 MS loci were chosen approximately 10 cM apart throughout the genome. For each locus, the *rsl* congenic strain was compared with the background strain and the *rsl* donor. Excluding chromosome 7 and the proximal portions of chromosome 12, which were previously investigated, 57% of the loci were visualized as informative in this comparison. Except for the known congenic interval on chromosome 7 containing *c*, the only analyzed informative locus in the *rsl* congenic strain that was derived from the *rsl* donor was *D13Mit13*, associated with interleukin 9 (*Il9*) on chromosome 13.

To determine whether a congenic interval was present on chromosome 13 in each of the four *rsl* congenic strains, four informative MS loci were analyzed, each 3–8 cM apart (30). In

Table 1. *rsl* maps as a single gene

Cross	Gen.	*N*	Frequency of Slp$^+$ Observed	Predicted
(PL × B10.D2) × PL	BC	179	0.55 (0.47–0.62)	1/2
PL × B10.D2	F_2	106	0.19 (0.12–0.28)	1/4
NZB × B10.D2	F_2	105	0.22 (0.14–0.31)	1/4
FM × B10.D2	F_2	100	0.34 (0.25–0.44)	1/4
PL × NZB	F_2	63	1.00 (0.94–1.00)	1
PL × FM	F_2	60	1.00 (0.94–1.00)	1

Generation (Gen.) assayed for Slp (BC for backcross or F_2 for cross and sib mating) and the number (*N*) of adult females assayed are shown. The frequency of Slp$^+$ females observed (95% confidence interval in parentheses) and predicted, based upon a single autosomal gene, is shown. In the first cross, the resulting frequency of female progeny expressing Slp was approximately the same regardless of whether the heterozygous parent was male or female. For the F_1 generation (cross), in the first four crosses, none of the females expressed Slp, and in the last two crosses, all females expressed Slp. All results fit a single gene model (respective probabilities by χ^2 test for segregating crosses are 0.20, 0.14, 0.46, and 0.04). The last probability indicates a slightly rare sampling event in the cross FM × B10.D2, not an additional gene, because the cross PL × FM demonstrated that no additional *rsl* genes are segregating. Models ruled out include those involving two or more unlinked *rsl* genes with complete penetrance, whether all *rsl* genes are required to be homozygous for expression or only one homozygous *rsl* is sufficient for expression. For these models in the backcross, the expected frequency of females expressing Slp and the χ^2 test results are $\leq 1/4$ with $P < 0.0001$ and $\geq 3/4$ with $P < 0.0001$, respectively.

Table 2. Strains congenic for *rsl*

Strain	Donor	Generation
B10.D2.PL(1)-*rsl*	PL	$G_{13}F_{15}$
B10.D2.PL(2)-*rsl*	PL	$G_{19}F_5$
B10.D2.NZB-*rsl*	NZB	$G_{21}F_2$
B10.D2.FM-*rsl*	FM	G_{14}

Generations of cross–intercross breeding (G), where odd and even numbers, respectively, represent progeny of crosses and intercrosses, and current generations of sib mating (F) are listed. Each strain has two congenic intervals, one containing *rsl* from the donor and one containing the *H-2* (originally from DBA/2) from the background strain B10.D2. Additionally, B10.D2.PL(1)-*rsl* has a congenic interval on chromosome 7 that contains *c* from PL.

order from the centromere, these loci were *D13Mit139, D13Mit13, D13Mit26*, and *D13Mit144*. Congenic intervals in each strain contained loci *D13Mit139* and *D13Mit13*; additionally the NZB-derived congenic interval contained *D13Mit26* and the FM-derived congenic interval contained all four loci (data not shown). The probability that each of three strains in addition to B10.D2.PL(1)-*rsl* would contain an unselected congenic interval containing *D13Mit13* is $(1/2)^{-19}$. We conclude that *rsl* is located on chromosome 13 and, because of the staggered boundaries of congenic intervals, is proximal to the centromere from *D13Mit26*.

Backcross Progeny and a Recombinant Inbred Series Further Localize *rsl* to a 2.2-cM Interval Containing *D13Mit66*. To further localize *rsl*, seven informative MS loci spanning chromosome 13 and including *D13Mit13* and *D13Mit26* were mapped in 83 female progeny from the portion of the previously described backcross PL × (PL × B10.D2) in which the heterozygote parents were female (Fig. 1 *Left*). The seven loci were chosen to be spaced 5–10 cM apart (30). The genetic map order deduced from this data is consistent with others (30); however, there is variation in the observed genetic distances, ranging from 4 cM between *D13Mit3* and *D13Mit63* to 20 cM between *D13Mit63* and *D13Mit13*. This map is longer than previously observed, probably because it was derived solely from recombination in females which on average is greater than recombination in males (30). The *rsl* gene maps between *D13Mit13* and *D13Mit26*, and both genotypes *Rsl/rsl* and *rsl/rsl* are completely penetrant.

To further localize *rsl* in the interval between *D13Mit13* and *D13Mit26*, a total of 179 female backcross progeny were analyzed for these loci. Eight recombinants were identified, five between *D13Mit13* and *rsl*, and three between *rsl* and *D13Mit26*. These recombinants and B10.D2.PL(1)-*rsl* were analyzed for five additional nearby informative loci, enabling local ordering of the loci (Fig. 2*A*). No recombinants were observed between *rsl* and *D13Mit66*, and one and three recombinants were, respectively, observed between *rsl* and the nearest proximal or distal locus, localizing *rsl* to a 2.2-cM interval (95% confidence interval, 0.6–5.6 cM, Fig. 1 *Right*).

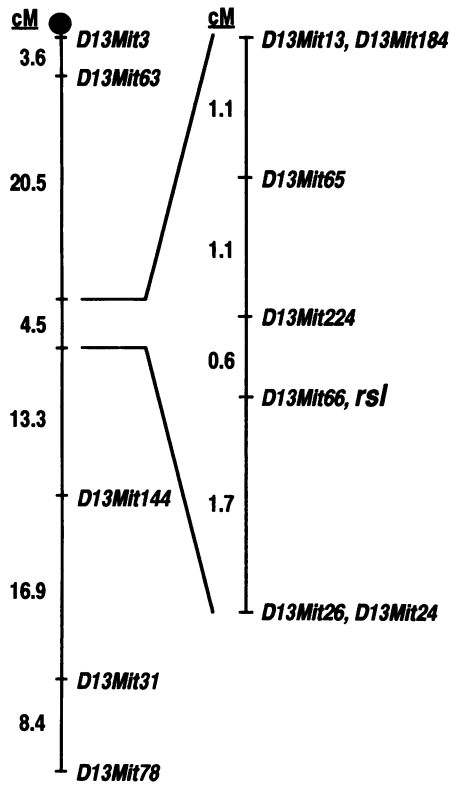

FIG. 1. *rsl* maps to chromosome 13. On the left is shown the genetic map of chromosome 13 derived from 83 female progeny of a backcross [PL males × (PL × B10.D2) females]. The expanded portion of the map shown on the right is derived from a total of 179 female progeny of the PL × (PL × B10.D2) backcross as shown in Fig. 2. The total map length is 67.2 cM, and map distances are presented as derived from unadjusted recombination frequencies.

Further confirmation of the location of *rsl* was obtained by analysis of the recombinant inbred series NXSM, constructed from strain NZB, containing *rsl* and Slp^d, and strain SM, containing *Rsl* and the nonexpressing Slp^v (35). Slp was expressed in males of six available lines, each of which inherited Slp^d, and females from three of these lines also express Slp, due to the segregation of *Rsl* and *rsl*. The six Slp^d lines were analyzed for informative MS loci between *D13Mit13* and *D13Mit26*, and the results further demonstrated that *rsl* is near *D13Mit66* (Fig. 2*B*). Results from congenic strains, a backcross, and a recombinant inbred series are consistent: *rsl* maps near *D13Mit66*.

***rsl* Increases Expression of *Slp* in Males.** To determine whether or not *rsl/rsl* affects the levels of Slp in males in addition to enabling Slp expression in females, we compared expression in both sexes in B10.D2.PL(1)-*rsl* and B10.D2 (Fig. 3). Males from the *rsl* congenic strain express significantly more Slp than males from the background strain, and the additional increment of expression is approximately equal to

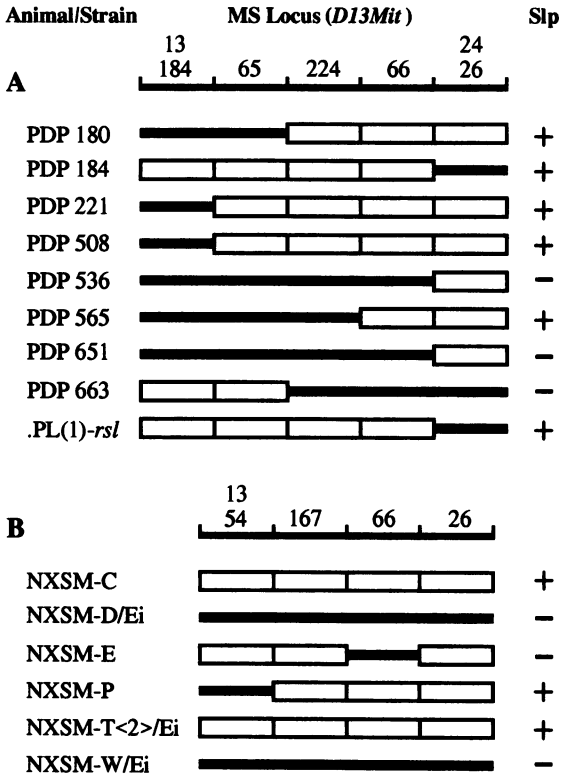

FIG. 2. *rsl* maps near *D13Mit66*. (*A*) Backcross. A diagram of the segregating chromosome in female progeny recombinant between *D13Mit13* and *D13Mit26* and from the backcross (PL × B10.D2) × PL is shown. The representation of a solid bar and an open bar are, respectively, used for loci scoring like B10.D2 and PL, and the Slp phenotype is shown. The first three recombinants were derived from recombination in females and the last five were derived from recombination in males. Also shown is the congenic interval boundary in B10.D2.PL(1)-*rsl*. (*B*) Recombinant inbred. A diagram of the genetic composition for loci between *D13Mit13* and *D13Mit26* in strains containing Slp^d from the NXSM recombinant inbred series. The representation of a solid bar and an open bar are, respectively, used for loci scoring like SM/J and NZB/B1NJ, and the Slp phenotype in adult females is shown.

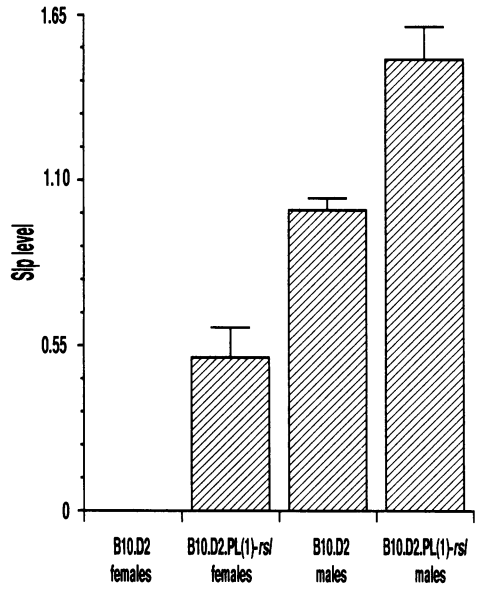

FIG. 3. *rsl* affects Slp expression in males as well as females. Levels of Slp in sera of 8-week-old mice are shown with bars for the SEM. The values are scaled to levels in B10.D2 males.

the quantity of Slp expression in females from the *rsl* congenic strain. Thus, the *rsl/rsl* genotype has a sex-independent role, causing Slp expression in females and increasing Slp expression in males by a similar increment.

DISCUSSION

Analysis of the genetic crosses and congenic stains constructed and used in this study demonstrated that expression of Slp in female mice from strains FM, NZB, and PL is caused by recessive allele(s) of the single gene *rsl*, and this gene is located on chromosome 13 near *D13Mit66*. An application of the localization of *rsl*, a gene with expression determined only in homozygote recessives, is that breeding of other congenic strains or additional generations, such as for B10.D2.FM-*rsl*, can be accomplished by using a backcross scheme and following nearby MS loci rather than the slower cross–intercross breeding scheme.

Homozygosity mapping is a technique that has been proposed and used to localize genes in humans; the pooled genomes of several individuals having some phenotype determined by recessive alleles at a locus are systematically searched to find a location in which most of the individuals are homozygous (36, 37). Heterozygosity mapping is a similar technique that has been used in three-generation congenic strains of mice to map the autosomal dominant *Whl*, for which homozygous mutants are lethal, by searching for a location at which most individuals are heterozygous (38). In using a late-generation congenic strain to search the genome for the donor congenic interval containing *rsl*, the process of general localization was even more experimentally efficient than homozygosity or heterozygosity mapping because the DNA from only one mouse of the congenic strain and controls was required for mapping.

The localization of *rsl* establishes four candidate genes for which DNA sequence information is available from rodents (39). The diversity of the known functions of these genes reflects the range of possibilities for *rsl*. The four genes and the known functions of their products are growth arrest specific 1 (*Gas1*), tumor suppressor; steroid 5α-reductase (*Srd5a1*), conversion of testosterone into dihydroxytestosterone; Fanconi anemia group C (*Facc*), DNA repair; and neurotrophic tyrosine kinase receptor type 2 (*Ntrk2*, formerly *Trkb*), protooncogene. It will be of interest to test whether any of these candidates is *rsl*.

Recent studies have begun to resolve several intriguing features of *Slp* regulation that may impact on our understanding of the *rsl* product. One molecular mechanism affecting gene expression involves the sequence TTCCGGGC in the 5′-flanking region of *Slp*. This sequence is necessary for high levels of promoter activity in human HepG2 cells, and mutation at the CpG in this sequence (−121 bp relative to the transcription initiation site) greatly reduced the promoter activity. Further, in DNA from the livers of DBA/2 mice, the CpG (−121 bp) is methylated to a much higher degree in females (67%), which do not express Slp, than in males (30%) (40). The authors point out that hepatocytes, which express Slp, account for about half of the DNA from the liver and that DNA unmethylated at CpG (−121 bp) in females may be derived from other cell types that do not express Slp. It will be interesting to determine whether methylation at CpG (−121 bp) is reduced in both male and female *rsl/rsl* mice or whether the androgen-independent pathway affects Slp expression through other means.

Expression of Slp in females occurs only in homozygous recessive *rsl/rsl* mice. One possible molecular model to account for this expression pattern is that the product of the *Rsl* allele may bind more strongly to a target molecule, either protein or nucleic acid, than the product of the *rsl* allele, and as a consequence of the binding of the *Rsl* product, Slp is not expressed; then in *Rsl/rsl* heterozygotes, the product of *Rsl* will out-compete the product of *rsl* for the binding sites. Similar arguments would follow if no product were produced from the *rsl* allele.

The normal expression of Slp in males of strains that contain *Rsl*, such as B10.D2, is dependent upon the presence of androgen, androgen receptors, and an adult male pattern of growth hormone release (16, 17). We have shown that the *rsl/rsl* genotype increases expression of Slp by approximately the same amount in both females and males of the congenic strain. When FM (*rsl/rsl*) was crossed to a strain containing the androgen receptor mutation *ar* (also called *tfm*), male expression of Slp was no longer dependent upon the receptor (26). Thus, the broad outline of the regulation of Slp is clear: there are two pathways to activate Slp expression, an androgen-dependent pathway leading only to expression in males and an androgen-independent pathway, equally active in males and females, that requires recessive alleles at *rsl*. Either pathway can be activated independently, and consequently, the pathways demand separate promoters, enhancers, repressors, signaling mechanisms, or other differences. It will be of interest to determine whether *rsl* regulates other sexually dimorphic traits, but regardless, the finding that expression is controlled by two pathways provides a basis for understanding genetic variation for sexual dimorphism.

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