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GENETIC AND HORMONAL CONTROL OF A SERUM PROTEIN VARIANT ASSOCIATED WITH THE H-2 REGION OF THE MOUSE

by
Howard Clinton Passmore, Jr.

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of the requirements for the degree of
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ABSTRACT

GENETIC AND HORMONAL CONTROL OF A SERUM PROTEIN VARIANT ASSOCIATED WITH THE H-2 REGION OF THE MOUSE

By Howard Clinton Passmore, Jr.

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The Ss protein is a serologically detected serum protein in the house mouse. Quantitative variation in this protein is controlled by a gene (Ss) which maps within the complex histocompatibility-2 (H-2) locus or chromosome region. This quantitative variation could be due to a difference either in a "structural" gene, which determines the amino acid sequence of the Ss protein, or in a "regulatory" gene, which in some unknown way controls serum levels of the protein. The objective of the present study was to distinguish between these two alternatives, i.e., Is the Ss locus a structural or a regulatory locus for the Ss protein?

Structural variation in the Ss protein was sought by immunizing inbred mice of one strain with a partially purified serum fraction from another strain. This alloimmunization produced an antiserum (RF/J♂anti-DBA/2J♂) which on microslide immunodiffusion detected an antigen present only in serum from males of certain inbred strains.

Genetic tests revealed that the presence of this antigen is controlled by a dominant autosomal gene which is expressed only in males of the proper genotype. Since this protein is

normally limited to males, it was designated the sex-limited protein (Slp). The controlling locus is called Slp. Test-crosses and analyses of congenic resistant strains indicated close linkage between the Slp locus and the H-2 region. Analyses of Slp types in fourteen intra-H-2 recombinant strains places the genetic determinant for Slp within the H-2 region, in the same position as the Ss determinant.

The similar electrophoretic mobility of Ss and Slp antigens on immunoelectrophoresis, and their partial cross-reactivity in absorption tests and on immunodiffusion, indicated that Slp antigenic sites are carried on the Ss protein. No functional or antigenic relationship could be demonstrated between Slp antigen and the H-2 cellular antigens. Alloimmunizations involving various strain combinations failed to produce antithetical specificities, subtype specificities, or specificities defining other alleles of the Slp system. No dosage effect could be clearly demonstrated in heterozygous animals. Slp has a molecular weight of approximately 125,000 to 155,000, heat stability to only 52°C for 20 minutes, and pH stability from 3.2 to 11.0.

Slp antigen is first detected in serum of Slp-positive males at five to six weeks of age and reaches full adult level by ten weeks. Males of Slp-positive strains castrated at 3 1/2 weeks failed to develop Slp antigen, while females treated with testosterone propionate starting at 3 1/2 weeks developed normal adult Slp levels. Similar hormone-influenced effects were demonstrated in mature males and females of a genotypically positive strain. The possibility that testosterone acts directly

in the serum to expose Slp antigenic sites on the Ss protein was ruled out. Hormone treatment of both males and females of a genotypically negative strain failed to initiate the appearance of Slp antigen.

Despite the complication of sex-limitation, the genetic and immunological evidence supports the conclusion that the Ss locus is the structural locus for Ss protein. This conclusion and the evidence that Slp is functionally and antigenically unrelated to H-2 antigen favor the hypothesis that the H-2 region consists of more than one functional locus, i.e., at least two loci controlling cellular antigens separated by an Ss structural locus.

Although the mechanism of sex-limitation is not precisely known, it is clear that male hormone is required for development and maintenance of Slp in genotypically positive animals. Two models involving hormonal control either at the gene level, through the activation of an additional genetic locus, or at the protein level, through modification of the Ss protein by an intermediate mechanism, are presented as working hypotheses.

To my wife, Irene

and

To my daughter, Lisa

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INTRODUCTION

The *H-2* Region

The histocompatibility-2 locus, or chromosome region, controls a set of cellular antigens which play the predominant role in allograft and tumor rejection in the house mouse (Snell, 1963). Its importance lies both in the strength of the immune rejection that its antigens elicit, and in its genetic and immunological complexity. The H-2 system was discovered by Peter Gorer, who initially described an antigen II, detected by hemagglutination with a rabbit antiserum (Gorer, 1936). Gorer later presented evidence for single gene inheritance of this antigen (1937, 1938), and for its association with transplant rejection (1938, 1942). The *H-2* locus was shown to control both histocompatibility and blood group antigens by Gorer *et al.* (1948). As a result of advances during the past two decades (most recently reviewed by Snell and Stimpfling, 1966, and by Lengerova, 1969), this system has become an important experimental model for the study of immunological phenomena involved in transplantation rejection, of genetic control of transplantation antigens, and of genetic fine structure in mammals.

An important milestone in the characterization of the H-2 system was achieved by Gorer and Mikulska (1954) with the

development of a sensitive serological technique involving hemagglutination in a dextran solution containing normal human serum. This method permitted the first detailed serological definition of H-2 antigenic differences among inbred strains of mice (Hoecker et al., 1954; Amos et al., 1955). These initial studies and more recent contributions from a number of laboratories defined more than 25 antigenic specificities controlled by the H-2 chromosome region, each detected by a distinct antiserum (see Snell et al., 1964; Shreffler and Snell, 1969). Investigations on the distribution of these specificities among inbred strains and the development of many congenic resistant strains* of mice (Snell, 1958) led to the definition of numerous alleles in the H-2 system. Each allele determines a series of multiple antigenic specificities which segregate together as a single gene trait, with the rare exception of intra-H-2 recombination. At least 18 of these alleles have been investigated in detail (see Snell, 1964; Shreffler and Snell, 1969).

A number of questions concerning the genetic organization and the mechanism of gene action of the H-2 region are raised by this multiplicity of antigenic specificities which segregate as a single genetic unit. One key question is whether the H-2 region consists of one cistron coding for a single

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*Congenic resistant strains are genetically identical, except for the introduction of a different histocompatibility locus into one of the strains. These strains have been produced by backcrossing different H-2 alleles onto certain inbred backgrounds for at least seven generations.

gene product or of multiple, closely linked cistrons producing multiple, independent gene products. Another relates to the chemical nature of the cellular antigens controlled by the H-2 region: the antigens could be either protein or carbohydrate. The distinction between protein and carbohydrate is critical to the interpretation of the mechanism of gene action, since a gene can code directly for a protein antigen, but can determine a carbohydrate antigen only indirectly through intermediate steps. Conclusive answers to the latter question must await the purification and chemical characterization of H-2 antigenic determinants. Such work is now underway in a number of laboratories (Davies, 1969; Shimada and Nathenson, 1969). Some information on the former question has come out of genetic fine structure analysis of the H-2 chromosomal region, based on recombination which occurs within this region.

Crossingover within the H-2 region was first reported by Allen in 1955 and by Amos et al., also in 1955. Since these initial discoveries, more than 40 cases of intra-H-2 recombination have been reported (Shreffler, 1970, a review of 35 cases; Pizarro and Dunn, 1970; Stimpfling and Reichert, 1970). Fifteen of these recombinants have been characterized in enough detail to make them useful for mapping of the genetic determinants of H-2 antigenic specificities (Gorer and Mikulska, 1959; Stimpfling and Richardson, 1965; Shreffler, 1965; Shreffler et al., 1966; David et al., 1969; Klein et al., 1969; Shreffler and Snell, 1969; Shreffler, 1970). Multipoint mapping with these well-defined intra-H-2 recombinants

has partitioned the genetic determinants for H-2 antigenic specificities among a minimum of five linearly arranged chromosome segments within the H-2 region. The genetic determinants of many specificities have been precisely positioned in one of these segments; others have been partially, but less precisely, positioned because of a lack of the proper recombinants to define their exact positions.

Some clues to the genetic organization of the H-2 region emerge from this analysis of intra-H-2 recombination. The genetic determinants for some antigenic specificities can be physically separated and linearly positioned by crossingover, indicating that these determinants represent separate, independent mutational sites. Since separation of these genetic determinants could possibly occur by intragenic recombination, such separation does not require that they are determined by multiple genes, but it does require multiple mutational sites. Furthermore, in recent years an additional dimension of complexity has been added with the discovery of a number of traits genetically associated with the H-2 region.

The genetic determinants for two such associated traits have been precisely mapped with respect to H-2. The Tla locus (Boyse et al., 1965; Boyse and Old, 1969), which determines cellular antigens present only in thymus and some leukemic tissues, is genetically distinct from H-2 and is located approximately 1.5 recombination units away from the H-2 region. The Ss locus (Shreffler and Owen, 1963) controls the quantitative level of a serologically detected serum

protein and has been mapped within the H-2 region. A number of additional traits are known to be closely linked to H-2, but have not been precisely mapped. These include the hybrid histocompatibility factor (Cudkowicz, 1968), variation in the immune response to synthetic antigens (McDevitt and Tyan, 1968; Rathbun and Hildemann, 1969), and variation in susceptibility to leukemia viruses (Lilly, 1966, 1968; Tennant and Snell, 1968) and to Bittner mammary tumor virus (Nandi, 1967). No direct functional or antigenic relationship with the H-2 cellular antigens has been defined for any of these associated traits.

Genetically, the most interesting of the numerous traits associated with the H-2 region is the Ss locus (Shreffler and Owen, 1963; Shreffler, 1964). This locus controls the quantitative level of a serum protein which is detected by an antiserum made in the rabbit against mouse serum. The function of this "serologically detected serum protein" or Ss protein is as yet unknown. Two alleles segregate at this locus. They have been designated Ss^{l}, determining low quantitative levels, and Ss^{h}, determining high levels of the protein. The Ss^{h}/Ss^{h} genotype (Ss-H phenotype) has approximately 20 times the level of Ss protein as the Ss^{l}/Ss^{l} genotype (Ss-L phenotype). The heterozygote, Ss^{h}/Ss^{l} (Ss-HL phenotype), has an intermediate level. An analysis of four intra-H-2 recombinants revealed that the Ss locus maps within the H-2 region, with determinants of H-2 antigenic specificities lying on both sides of the Ss determinant (Shreffler, 1965, 1967). This trait, and its

position within the H-2 region, have particular relevance to the question of how many genes there may be in this region.

Dissertation Problem

The quantitative variation in the Ss protein could be due to a difference either in a "structural" gene, i.e., a gene which carries in its DNA base sequence the code that specifies the amino acid sequence of the Ss protein and thus determines its primary structure, or in a "regulatory" gene, i.e., a gene which by some undefined mechanism controls the expression of the structural gene for Ss protein, thus regulating serum levels of the protein. In the former case, the quantitative variation in Ss protein would be a consequence of a mutational change in the structure of the Ss protein affecting such property as its rate of synthesis, rate of secretion into the serum, or stability. In the latter case, the primary structure of the Ss protein would be determined by another locus, probably located elsewhere in the genome, and no structural difference between the Ss proteins of Ss-H and Ss-L sera would be required. Structural variation in Ss protein would not be excluded; however, it would not be controlled by the H-2-associated Ss determinant. The objective of this dissertation research was to distinguish between these two alternatives, i.e., Is the Ss locus a structural or a regulatory locus?

Because of the location of the Ss determinant within the H-2 region, a distinction between these two alternatives is critical to any interpretation of the genetic organization of

the H-2 region. For example, it would be difficult to envision the H-2 region as a single cistron if a gene which determines the structure of an unrelated serum protein is positioned within this region. Therefore, if Ss is the structural gene, the H-2 region must be divided into at least three cistrons, one controlling Ss protein structure, and at least one on either side controlling H-2 antigens. On the other hand, it is less difficult to envision how a cellular antigen determined by H-2 could in some way regulate the concentration of a serum protein (e.g., via cell permeability). Thus, if Ss is a regulatory gene, its position within H-2 does not require that this region be multi-cistronic. In addition to its importance to the interpretation of the genetic organization of the H-2 region, the Ss locus is of interest because of its potential for the study of specific gene regulation.

The planned steps in an experimental approach to this question were as follows: 1) to identify structural differences in the Ss protein among inbred strains; 2) to determine whether such differences are controlled by a single gene; 3) to determine by linkage analysis whether the genetic locus (or loci) controlling this variation maps in the Ss segment within the H-2 region or elsewhere in the genome.

The most direct and conclusive manner in which to define structural variation in a protein is purification and chemical structural analysis. In the case of the Ss protein, chemical purification has not progressed to the point where such direct

structural analysis can be attempted. For this reason, a less direct approach, that of alloimmunization, was used to screen for structural variation in the Ss protein. Alloimmunization entails immunization using genetically different members of the same species as donor and recipient. This method is especially suitable with inbred mice because of the unlimited supply of animals of the same genotype. Therefore, immunizations were made between inbred strains, using a partially purified preparation of Ss protein. The use of alloimmunization to define structural variants (allotypes) in other serum protein systems will be discussed in the next section.

The research done for this dissertation defined a qualitative serum protein variant which was revealed by alloimmunization and which is characterized by: 1) autosomal dominant genetic control; 2) genetic association with the H-2 region; 3) molecular association with the Ss protein; and 4) sex-limitation. The unexpected finding that this qualitative trait is sex-limited, i.e., expressed only in males, raised a number of interesting additional questions not envisioned at the inception of the research. Because of this unusual property of sex-limitation, the variant was designated the sex-limited protein (Slp), and the controlling locus--Slp.

Allotypes

Allotypes are inherited serum protein variants defined by specific antigenic differences between individuals of the same species. The first report of well-defined allotypic differences,

as well as the first use of the term "allotype," was by Oudin (1956) with the description of intra-species differences in rabbit immunoglobulin. In this work, Oudin used immune precipitates containing rabbit antibody (anti-ovalbumin) from one rabbit for alloimmunization of other individual rabbits. In several instances, anti-allotype antibodies were produced, detectable by immunodiffusion. Following this initial discovery, extensive work on rabbit immunoglobulins in a number of laboratories resulted in the definition of two genetic loci controlling heavy and light chain allotypes of γG_1 immunoglobulins (Dray and Young, 1959, 1960; Oudin, 1960a, 1960b; Dubiski et al., 1961; Dray et al., 1963). More recently, genetically controlled allotypes of γM immunoglobulin (Kelus and Gell, 1965), γA immunoglobulin (Conway et al., 1968) and γG_2 immunoglobulin (Hamers et al., 1966) have also been defined in the rabbit.

Shortly after their discovery in the rabbit, immunoglobulin allotypes were also reported in man (Grubb, 1956) and in the mouse (Kelus and Moor-Jankowski, 1961). As with the rabbit system, subsequent investigation of human and mouse immunoglobulin allotypes uncovered a complex system of multiple allotypic specificities controlled by at least five loci in man (Gm and Inv loci reviewed by Steinberg, 1969; Am locus reported by Vyas and Fudenberg, 1969), and three loci in the mouse (reviewed by Herzenberg et al., 1968).

From the beginning, it was generally assumed that immunoglobulin allotypes reflected structural variation in

immunoglobulins within a species (see Oudin, 1960b). However, it was not until more recently that peptide analysis and amino acid sequencing of allelic allotypes confirmed the structural basis for this variation. Baglioni et al. (1966) showed that the human light chains which carry the allotypes Inv(1) and Inv(3) differ only by the substitution of valine in Inv(3) for leucine at amino acid residue number 191. Thus, allotypic differences can result from a single amino acid difference. Also in 1966, Frangione et al. demonstrated that Gm(1) and Gm(-1) allotypes on human γG_1 heavy chains reflect a difference of two amino acids in the same tryptic peptide. Allotypes of both the γG light (Reisfeld et al., 1965) and heavy chain (Koshland, 1968) loci in rabbits have also been shown to reflect differences in amino acid composition and sequence. Thus, in cases where they have been examined, allotypic differences have been shown to be based on primary structural differences in the protein molecules. These findings indicate the utility of alloimmunization as a means for defining structural differences in serum proteins.

In addition to the immunoglobulins, allotypes have also been reported for a number of other serum proteins. For example, genetically controlled allotypes of low-density lipoprotein (Albers and Dray, 1968), α_2-macroglobulin (Knight and Dray, 1968), and haptoglobin (Chiao and Dray, 1969) have been defined in the rabbit. In man, two immunologically detected serum protein variant systems of low-density lipoproteins (Ag--Allison and Blumberg, 1961; and Lp--Berg, 1963)

and three systems associated with the α_2-macroglobulin fraction of human serum (Xm--Berg, 1966; Xh--Bundschuh, 1966; and Pa--MacLaren *et al.*, 1966) have been reported.

Sex-limitation

If a genetically determined trait occurs only in one sex, it is defined as *sex-limited*. This term should be clearly distinguished from sex-influenced and sex-linked. *Sex-influenced* traits occur in both sexes, but with different penetrance or expressivity, *e.g.*, pattern baldness in man. Sex-limited and sex-influenced traits are usually autosomally inherited, with their expression controlled or influenced by developmental or physiological differences between the sexes. *Sex-linked* refers only to genes located on the X-chromosome. Recessive sex-linked genes such as hemophilia in man occur more often in males, while dominant sex-linked genes such as the Xg blood group in man are more frequent in females. The differences in frequencies in this case are only a function of the genetic segregation of genes on the X-chromosome.

Sex-limitation of a trait can occur in three general ways: 1) Y-linkage of a gene, restricting the presence of a trait to males; 2) the restriction of a trait to an organ or tissue present in only one sex; and 3) the expression of a gene only under the influence of a sex hormone. An example of each of these can be cited from work in inbred mice. The *H-Y* histocompatibility gene (Eichwald and Silmer, 1955) is thought to be Y-linked and thus expressed only in males. *Syp*

or seminal vesicle protein is an autosomal locus controlling isozyme variants of a specific seminal vesicle protein (Platz and Wolfe, 1969). Finlayson et al. (1963) demonstrated that males and females gave different electrophoretic patterns of the major urinary protein complex controlled by the Mup-1 locus. These patterns could be reversed by castrating males and treating ovariectomized females with testosterone.

Endocrine Control of Gene Expression

In 1961, Jacob and Monod presented an elegant model for metabolic regulation based on the induction or repression of genes involved in the synthesis of specific enzymes. In this model, the formation of messenger RNA by a specific structural gene is inhibited by the attachment of a "repressor" molecule to a specific chromosomal site (operon) which controls the transcription of the structural gene. If a specific "inducer" is then added to this system (the inducer is often a substrate in the biochemical pathway being regulated), it interacts or binds with the repressor, causing it to detach from the chromosomal regulation site, thus allowing for the normal transcription of the structural gene.

The subsequent experimental proof and verification of this hypothesis in microorganisms has led to its general acceptance as a model for gene and metabolic regulation in all living organisms. Thus, hormone regulation of gene expression in higher organisms has commonly been interpreted in terms of the Jacob-Monod model of gene regulation. It is assumed that

hormone activates genes by serving as an inducer. Despite its frequent use in this context, no rigorous or direct proof of this model of regulation exists in mammals or other higher organisms, and the precise mechanism of hormone action at the gene level remains largely unknown. It is clear, however, that hormone does increase specific RNA and protein synthesis, which is at least consistent with expectations under the Jacob-Monod model.

The giant chromosomes of insects provide a unique tool for the study of chromosomal changes involved in gene activation (Beerman, 1962). Specific local swellings or "puffs" can be observed cytologically in these giant chromosomes after the injection of third instar larvae with the steroid molting hormone, ecdysone (Clever and Karlson, 1960). These puffs occur in single specific chromosome bands or chromomeres as rapidly as 15 minutes after the *in vivo* administration of ecdysone (Clever, 1961) and have been shown to be the sites of active RNA synthesis (Beerman, 1963; Pelling, 1959, 1964), and of acidic, non-histone protein accumulation (Swift, 1962). The ecdysone induction of a specific enzyme (dopa decarboxylase) involved in the process of sclerotization* in the blowfly has been reported by Karlson and Sekeris (1962, 1964). Thus, it seems that the effect of ecdysone is to activate specific

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* The process of sclerotization involves the deposition of products of tyrosine metabolism into the larval cuticle as it is transformed into a puparium.

chromosomal regions (and presumably specific gene loci) as evidenced by physical changes in the chromosome and by increased RNA and protein synthesis.

The induction of specific liver enzymes, tyrosine aminotransferase and tryptophan pyrrolase, by cortisol has long been recognized (Lin and Knox, 1958; Civen and Knox, 1959). This adrenal hormone has been shown to cause, in addition to a general increase in the synthesis of all major classes of RNA (Kenney, 1965), the induction of specific RNA synthesis in the rat liver (Drews and Brawerman, 1967; Lang et al., 1968). Hybridization studies have indicated that this specific RNA is a new species of messenger RNA produced only in hormone-treated animals. Cortisol-initiated increases in RNA polymerase activity (the enzyme catalyzing the synthesis of mRNA) have been demonstrated in the rat liver and in isolated liver nuclei (Lang and Sekeris, 1964; Lukacs and Sekeris, 1968). Similar studies with estradiol on the rat uterus (Trachewsky and Segal, 1967; Hamilton et al., 1968) and with testosterone on the rat prostate gland (Liao et al., 1966; Liao and Lin, 1967) also demonstrate a selective increase in the synthesis of specific RNA, as well as increases in RNA polymerase activity.

Although the action of hormone is not known, it is clear that the stimulation of the genetic chain of events from DNA to protein via transcription and translation occurs in response to various steroid hormones. It is not known whether this stimulation is a result of activation at the DNA level (Jacob and Monod, 1961), or post-transcriptionally at the level of messenger RNA (see Tomkins et al., 1969). Actually, both of

these mechanisms may be operating, but in different systems. For example, specific doses of actinomycin, an inhibitor of transcription, do not block the induction of tyrosine aminotransferase in rat liver, indicating that already existing mRNA is activated to produce enzyme (Thompson et al., 1966).

It is not clear whether hormone acts directly to derepress genetic information or whether the hormone interacts with an intermediate repressor molecule. In this regard, histones, the basic proteins associated with chromosomes, have been implicated as repressors of DNA-dependent RNA synthesis (Allfrey and Mirsky, 1964; Bonner and Huang, 1965) and are at present the best candidates for the intermediate repressor molecules.

Although a substantial amount of effort has gone into the investigation of hormonal regulation of gene expression, the precise mechanisms are not yet understood for any system. A distinct asset in such studies would be the availability of clearly defined genetic variant systems which are subject to hormonal control. The system described in this dissertation may be of some value in this regard.

MATERIALS AND METHODS

Animals

Inbred strains of mice were obtained either from the laboratory stocks of Dr. Donald C. Shreffler, Department of Human Genetics, University of Michigan, or from the Jackson Laboratory, Bar Harbor, Maine, with the exception of YBR/a, obtained from Dr. Morris Foster of the Department of Zoology, University of Michigan. The congenic resistant lines were all from the laboratory stocks of Dr. D. C. Shreffler. A list of abbreviated designations or synonyms for congenic resistant lines and for frequently used inbred strains, which will be used throughout this dissertation, is given in Table 1. The backgrounds and sources of 14 intra-H-2 crossover stocks used during the course of this investigation are summarized in Table 2.

Breeding

The inheritance of the Slp trait was examined in crosses between strains C57BL/6J and DBA/2J and between C57BL/6J and A/J. (C57BL/6J♀ x DBA/2J♂)F_1 hybrids (hereafter shortened to B6D2) and (C57BL/6J ♀ x A/J♂)F_1 hybrids (shortened to B6A) were purchased from the Jackson Laboratory, while the reciprocal F_1-hybrids (D2B6 and AB6) as well as reciprocal backcross and F_2 generations were produced in this laboratory.

Table 1

List of Commonly Used Synonyms for Inbred Strains and Congenic Resistant Lines

Full Designation	Synonym
Inbred strains	
A/J	A
A/Sn	A
DBA/1JSf	D1
DBA/2J	D2
DBA/2JSf	D2
C57BL/6J	B6
C57BL/10JSf	B10
RF/J	RF
Congenic resistant lines	
A/Sn-H-2^b	A.BY
A/Sn-H-2^f	A.CA
A/Sn-H-2^s	A.SW
AKR/JSn-H-2^m	AKR.M
C57BL/10ScSn-H-2^d new	B10.D2
C57BL/10JSf-H-2^k	B10.K
C57BL/10ScSn-H-2^{pa}	B10.Y
C3H/JSf-H-2^b	C3H.B10
C3H/JSf-H-2^d	C3H.D
C3H/JSf-H-2^q	C3H.Q
C3H/JSf-H-2^r	C3H.RIII
DBA/1JSn-H-2^c	D1.C

Table 2

Intra-H-2 Recombinants Employed in These Studies

Line	Recombinant H-2 Allele	Hetero-zygote of Origin	Originally Reported by	Source
HTG	g	b/d	Gorer and Mikulska (1959)	Shreffler
HTH	h	a/b	"	"
HTI	i	a/b	"	"
BIO.A(1R)	h-Sg	a/b	Stimpfling and Richardson (1965)	Stimpfling
BIO.A(2R)	h-2Sg	a/b	"	"
BIO.A(3R)	i-Sg	a/b	"	"
BIO.A(4R)	h-3Sg	a/b	"	"
BIO.A(5R)	i-2Sg	a/b	"	"
C3H.OL	ol	d/k	Shreffler (1965)	Shreffler
C3H.OH	oh	d/k	"	"
A.AL	al	d/k	"	"
A.TL	tl	al/s	"	"
A.TA	ta	th/f	Shreffler (1970)	"
AQR	y	a/q	Klein et.al. (1969)	Klein

Antibody Preparation

Ss protein was partially purified for immunizations by applying 5.0 to 6.0 ml of DBA/2J or A/J whole serum to a 1.5 cm x 90 cm Sephadex G-200 column, and eluting with phosphate buffered saline, pH 7.0. The Ss-positive fractions were pooled and concentrated two to three times the original concentration. In one case Ss protein precipitated with anti-Ss was used for immunization.

Initially, alloimmunizations for the production of anti-Slp were made by subcutaneous injections at days 0, 14, 21 and 28, with 0.2 ml of the partially purified Ss preparation emulsified in an equal volume of complete Freund's adjuvant, followed by an intraperitoneal injection at day 42 without adjuvant. In most later immunizations, the number of injections was reduced by eliminating the day 21 subcutaneous injection. This modified immunization schedule yielded strong typing sera as effectively as the original schedule. Animals were bled seven days after the last injection and every second day thereafter for approximately one week. Pools of RF/Jσ anti-A/Jσ or RF/Jσ anti-DBA/2Jσ were used for routine Slp typing.

Antiserum for Ss typing was prepared according to Shreffler and Owen (1963).

Serum Typing - Microslide Immunodiffusion

Blood for serum typing was drawn from the retro-orbital sinus and allowed to clot. The serum was drawn off and typed

immediately, with the excess being stored at -80°C. Animals were tested only after ten weeks of age, since the Slp antigen does not appear in the serum until approximately six to eight weeks of age. Individual animals were typed at least twice, using separate bleedings. In cases of discrepancy, repeat typings were done with separate bleedings. In most cases, this involved animals that were classified as negative on the first typing and positive on a second typing because they were late in developing the Slp antigen.

Serum Slp typing was done by microslide immunodiffusion using a modification of the method of Ridgway et al. (1962), in which the diffusion medium consisted of 1.5 gm Bacto-agar (Difco Laboratories) or 0.9 gm No. 2 Ionagar (Colab Laboratories), 0.6 gm sodium citrate, and 0.72 gm sodium chloride in 100 ml of distilled water, with a final pH of 7.3. The antibody and antigen wells were 4 mm in diameter and spaced 7 mm apart (center to center). The immunodiffusion slides were read for the Slp reaction after 18 hours of incubation at 37°C. The method of Ss typing was the microslide technique previously described by Shreffler (1964) and was essentially identical to that used for Slp typing.

Estimation of Slp Quantities on Immunodiffusion Plates

Immunodiffusion for the estimation of the quantitative level of Slp antigen was done by the method described in the previous paragraph, with the exception that 8 x 10 cm glass

plates, each with 12 ml of agar gel, were used instead of 2.5 x 7.5 cm microscope slides. This allowed for more typing wells per plate and provided a flatter gel surface. Twenty µl of antigen or antibody were used in all wells. The quantity of Slp antigen in individual serum samples was estimated on immunodiffusion plates by measuring the distance from the antigen well to the precipitin line and expressing this distance as a percentage of the total distance between the antigen and antibody wells, and then comparing these Rf values with values obtained by standard dilutions of the Slp antigen. Since a standard pool of antiserum (RF/J♂ anti-DBA/2J♂) was used in all tests, precipitin band position was solely a function of antigen concentration. A pool of adult DBA/2J male serum (four to eight months of age) was used as the 100% antigen standard. This technique reliably distinguished between two-fold serial dilutions, i.e., between undiluted antigen and a 1/2 dilution, between 1/2 and 1/4 dilutions, etc. Similar quantitative plate techniques have been described previously by Gell (1957) and Hayward and Augustin (1957).

Quantitative Tube Immunodiffusion

Quantitation of the Ss protein was performed by double immunodiffusion in tubes, according to the method of Preer (1956), as modified by Shreffler and Steinberg (1967). In this method, 6 x 50 mm glass culture tubes which had previously been coated with 0.5% Bacto-agar were used. Fifty µl of a mixture of one part anti-Ss and one part 1.0% No. 2 Ionagar

in 0.85% saline were pipetted into the bottom of the tube. A standard pool of anti-Ss was used in all tubes. Both antiserum and the agar solution were maintained at 50°C before mixing. After the first layer had gelled, 250 µl of 0.5% Ionagar in 0.85% saline at 50°C were pipetted onto the first layer. Upon solidification of this layer, 10 or 20 µl of sample containing Ss protein were applied to the top of the agar gel column. Finally, the tubes were sealed with paraffin and vertically incubated for 72 hours at 37°C.

Tubes were read by measuring the position of the precipitin line in the agar gel column. This position was expressed as the ratio of the distance from the antigen-agar interface to the center of the precipitin band to the total length of the 250 µl agar section. The relative quantity of Ss protein in each sample was calculated by comparing these ratios with values obtained by standard dilutions of Ss protein. Adult DBA/2J male serum was used as the 100% standard for Ss protein.

Immunoelectrophoresis

Agar immunoelectrophoresis was carried out on 8 x 10 cm glass plates using a 1% concentration of No. 2 Ionagar in a pH 8.6 veronal-calcium lactate buffer, modified from Hirschfeld (1959) by a 25% reduction in molarity of the buffer components. The cell buffer was the same as that used by Hirschfeld (1959). Electrophoresis was run for 90 minutes at 5.0 v/cm on an Immunobox immunoelectrophoresis cell (Model EC 640, E-C Aparatus Corp.).

Hemagglutination

H-2 typing was carried out using the polyvinylpyrrolidine (PVP) method essentially as described by Stimpfling (1961), with minor modifications (Shreffler *et al.*, 1966). The basic method was modified by the addition of 0.3% bovine serum albumin to enhance agglutination. Typing sera for specificity H-2.4 were prepared as previously described (Shreffler *et al.*, 1966), using the following strain combinations: (C3H/JSF x STOLI/Lw)F_1 anti-DBA/2J and (C57BL/10JSf x AKR.M)F_1 anti-A/Sn.

In Vivo Absorption

In vivo absorptions were done essentially as described by Amos (1962) by injecting 0.3 ml of anti-H-2.4 intraperitoneally, and bleeding the animals after two hours. Serum was then titrated by hemagglutination, starting at a 1/5 dilution.

Castration and Hormone Treatments

DBA/2J males were castrated at 3 1/2 or 12 weeks of age by making a dorsal incision into the peritoneal cavity, after anesthetizing with sodium pentabarbital. The testes were pulled from the body cavity, ligated at the ductuli efferentes, and excised. The sham control consisted of anesthesia, incision, pulling the testes out of the body cavity, mild probing, and then replacing the testes into the peritoneal cavity.

Testosterone propionate (Calbiochem) was injected subcutaneously, using sesame oil as a carrier. The dosages used

were as follows: 1) Animals treated from 3 1/2 to 10 weeks of age received 1.5 mg of testosterone propionate per week, in two injections for the first ten days, and then 2.25 mg of hormone per week in three injectiors for the remainder of the treatment period. 2) Animals in which treatment was started at one week of age received gradually increasing doses of testosterone propionate, equivalent to 0.1 mg/week/gm of body weight in two or three injections per week. This dose is equivalent to the adult dose of 2.25 mg per week. 3) Females in which treatment was started at 12 weeks of age received 2.25 mg of the hormone per week in three injections. 4) An eight-fold increased dose of testosterone propionate was used in one experiment, consisting of 18.0 mg of hormone per week.

RESULTS

Inheritance

The Slp variant

The immunization of RF/J male mice with a partially purified preparation of Ss protein from DBA/2J male serum produced an antiserum which gave a well-defined precipitin line when tested on microslide immunodiffusion against sera from males of strains DBA/2JSf and A/J. However, the alloantiserum gave no reaction with sera from females of these same strains, and no reaction with the sera of animals of either sex from C57BL/6J, C3H/JSf, and RF/J. The immunodiffusion plate in Figure 1 illustrates the reaction of RF/J♂ anti-DBA/2J♂ serum with sera from strains DBA/2JSf, A/J, and C57BL/6J. A number of other alloantisera, including RF/J♂ anti-A/J♂, C3H/JSf♂ anti-A/J♂, and C3H/JSf♀ anti-A/J♂, gave identical reaction patterns and produced lines of complete fusion when compared with RF/J♂ anti-DBA/2J♂ in adjacent wells (Figure 2). Since no reaction was observed with female sera of any of the strains tested, a positive reaction with male sera of a given strain was the criterion for designating the strain as positive for this antigen, which has been referred to as the Slp (sex-limited protein) antigen. The reactive or positive phenotype will be designated Slp-a, the non-reactive or negative phenotype Slp-o.

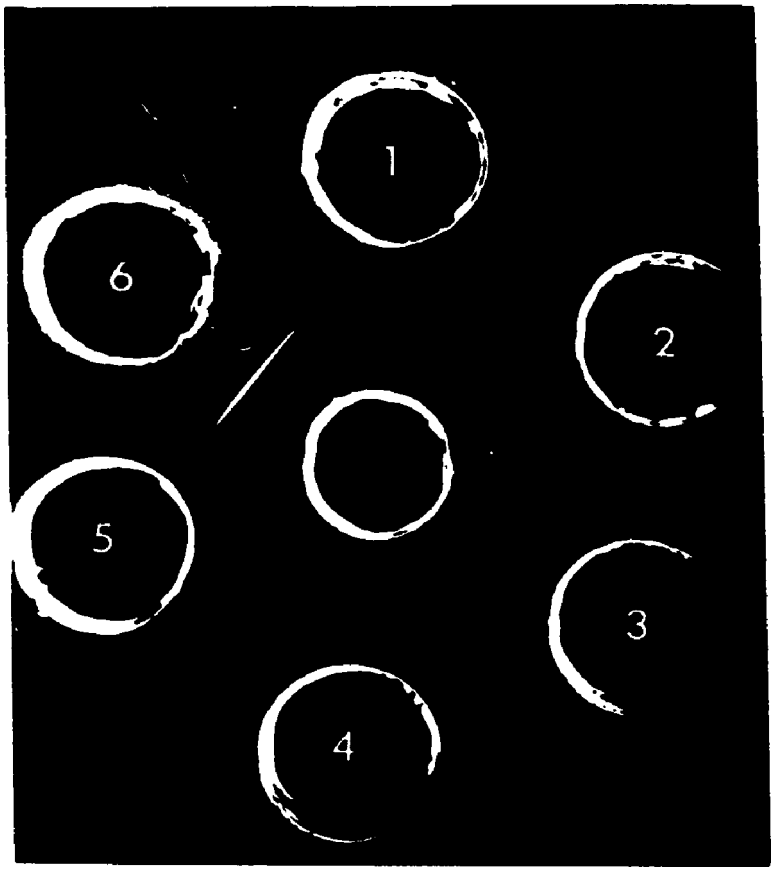


Figure 1. Immunodiffusion plate illustrating the typing of inbred mouse sera for Slp. Anti-Slp (RF♂ anti-DBA/2J♂) is in the center well. Well 1. A/J♂ serum, Well 2. A/J♀ serum, Well 3. C57BL/6J♂ serum, Well 4. C57BL/6J♀ serum, Well 5. DBA/2J♀ serum, Well 6. DBA/2J♂ serum. Undiluted adult sera were used in all wells.

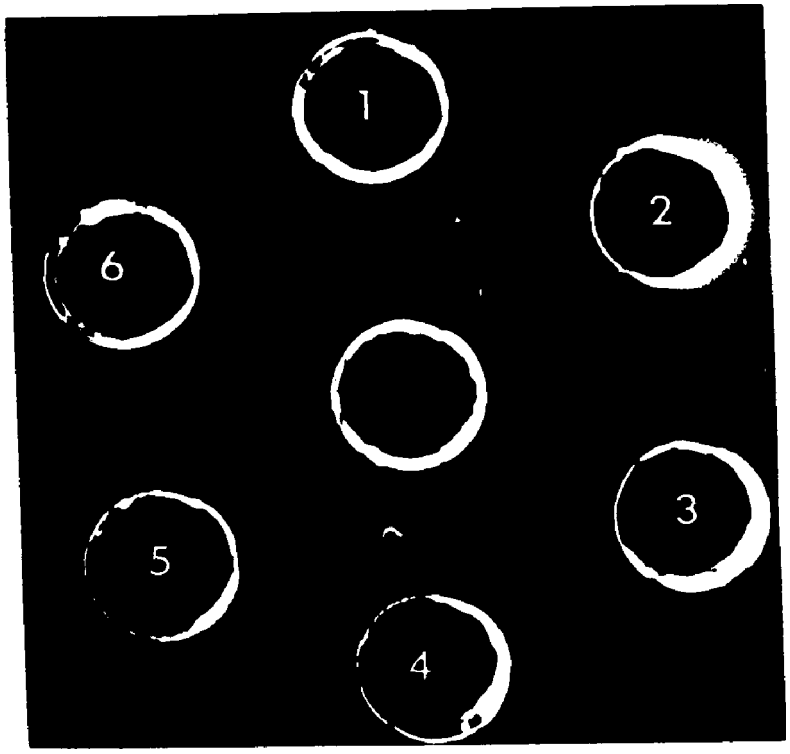


Figure 2. Immunodiffusion reactions of various antisera prepared against partially purified Ss preparations. DBA/2J♂ serum is in the center well. Well 1. C3H/JSf♂ anti-A/J♂, Well 2. RF/J♂ anti-A/J♂, Well 3. C3H/JSf♀ anti-A/J♂, Well 4. Blank, Well 5. RF/J♂ anti-DBA/2J♂, Well 6. RF/J♂ anti-A/J♂. Occasionally, splitting of the Ssp band occurs as seen in the reaction with well 5. The splitting is not regularly reproducible, and it is not known whether its occurrence is due to heterogeneity of the antigen or to artifact.

Segregation analysis

Crosses were made between strains DBA/2J (Slp-a) and C57BL/6J (Slp-o) and F_1, F_2 and backcross generations produced to determine the inheritance of this serum antigen. The segregation of the Slp trait in the progeny of these crosses is shown in Table 3. F_1 hybrid males from reciprocal crosses were all Slp-positive. This result ruled out simple X- or Y-linkage and suggested dominant, autosomal inheritance. This was confirmed in the backcross and F_2 generations. All male progeny from the backcross to DBA/2J were positive, regardless of the direction of the cross. Among male progeny from the backcrosses to the negative parent, the observed ratio of Slp-a to Slp-o was close to the expected 1:1 ratio, and the F_2 showed a close approximation to a 3:1 ratio. Note that, as in both parental strains, all females in these crosses were Slp-o, yet both DBA/2J and F_1 females produced Slp-a male offspring when mated to C57BL/6J Slp-o males. This indicated that these females carry the gene for Slp-positivity, but do not express the antigen. The trait is therefore sex-limited, i.e., confined to expression in males, but controlled by a single, dominant, autosomal gene. The allele which determines presence of the antigen in males is designated Slp^a; the allele determining absence of the antigen is denoted Slp^o. Similar crosses between C57BL/6J and A/J (Table 4) also support these conclusions.

Table 3

Slp Types of Progeny from the Cross: DBA/2 x C57BL/6J

	Males		Females[a]		X^2	P
	Slp-a	Slp-o	Slp-a	Slp-o	Males	Males
Parental Generation						
DBA/2	18	0	0	10		
C57BL/6J	0	9	0	5		
F_1 Generation[b]						
B6D2	6	0	0	5		
D2B6	11	0	0	6		
Total	17	0	0	11		
Backcross to DBA/2						
B6D2 x DBA/2	31	0	0	12		
DBA/2 x B6D2	17	0	0	13		
Total	48	0	0	25		
Backcross to C57BL/6J						
B6D2 x C57BL/6J	22	19	0	19	0.22	0.6-0.7
C57BL/6J x B6D2	5	6	0	3		
C57BL/6J x D2B6	7	8	0	12		
Total	34	33	0	34	0.02	0.9
F_2 Generation						
B6D2 x B6D2	39	12	0	30	0.06	0.8
D2B6 x D2B6	55	22	0	18	0.35	0.5-0.6
Total	94	34	0	48	0.17	0.6-0.7

[a] In each cross, only a sample of the total female progeny produced was tested, so the totals for females deviate from the totals for males.

[b] B6D2 = shortened form for (C57BL/6J♀ x DBA/2♂)F_1

D2B6 = shortened form for (DBA/2♀ x C57BL/6J♂)F_1

Table 4

Slp Types of Progeny from the Cross C57BL/6J x A/J
(Reciprocal crosses pooled)

	Males		Females*		X^2	P
	Slp-a	Slp-o	Slp-a	Slp-o	Males	Males
A/J	7	0	0	4		
C57BL/6J	0	9	0	5		
F_1 Generation	12	0	0	8		
Backcross to A/J	20	0	0	10		
Backcross to C57BL/6J	21	16	0	10	0.66	0.4
F_2 Generation	37	17	0	22	1.21	0.2-0.3

*In each cross, only a sample of females was tested, so female totals deviate from male totals.

Linkage analysis

Since the initial purpose of the alloimmunization was a search for structural variation in the H-2-linked Ss protein, the progeny from the backcrosses to C57BL/6J (Tables 3 and 4) were classified for H-2 type to test for linkage of Slp with H-2. Specificity H-2.4 was utilized as the marker specificity for both the H-2d type of strain DBA/2J and the H-2a type of strain A. Table 5 lists the four equally probable classes of male animals expected from these backcrosses under the hypothesis of independent assortment, and the number of progeny obtained from each class. As can be seen, however, with one exception, only the two parental types (with respect to H-2.4 and Slp) were observed in the 104 testcross progeny. These data, therefore, are indicative of very close linkage between H-2 and Slp. The (C57BL/6J x DBA/2J)F_2 generation was tested for linkage in the same way (Table 6) and was again found to be consistent with very close linkage between H-2 and Slp. One further apparent recombinant was detected in this series.

The one exception in the testcross (♂486) and the single exception in the F_2 generation (♂300) were both repeatedly typed as H-2.4-negative and Slp-a. These two animals were then progeny tested by crossing to C57BL/10J females. In both cases, H-2.4-negative Slp-a male offspring were produced, confirming the exceptional types of these two animals. In vivo absorption tests (Table 7) indicating inability of the recombinant animals or their progeny to absorb out anti-H-2.4

Table 5

Testcrosses for Determination of Linkage Between the H-2 Locus (Specificity 4) and Slp Type*

Phenotype	Cross: (C57BL/6J x DBA/2)F_1 x C57BL/6	Cross: (C57BL/6J x A/J)F_1 x C57BL/6
+ 4, Slp-a	33	16
- 4, Slp-o	33	21
+ 4, Slp-o	0	0
- 4, Slp-a	1	0

* Only males are included

Table 6

Linkage Relationship Between the H-2 Locus (Specificity 4) and Slp Type in the (C57BL/6J x DBA/2J)F_2 Generation*

Phenotype	Number in F_2 Generation
+ 4, Slp-a	93
- 4, Slp-o	34
+ 4, Slp-o	0
- 4, Slp-a	1

* Only males are included

Table 7

In Vivo Absorption Analysis of Anti-H-2.4 [(B10 x AKR.M) F_1 Anti-A/Sn]

Test Cells	Result of Absorption in					
	σ300	σ562[a]	σ486	σ569[b]	σ B10[c]	σA[d]
B10	-	-	-	-	-	-
A	+	+	+	+	+	-
B10.D2	+	+	+	+	+	-

[a] Slp-a progeny of ♂300 x B10

[b] Slp-a progeny of ♂486 x B10

[c] Strain B10 lacks specificity H-2.4

[d] Strain A carries specificity H-2.4

established that these animals were, in fact, H-2.4-negative. These exceptional animals could be either simple recombinants between H-2 and Slp, or could involve recombination within the H-2 region. On the basis of further results, presented later, it seems reasonable to suspect that both of these animals resulted from intra-H-2 crossovers which carry the "D end" from the $H\text{-}2^b$ chromosome and the "K end" from $H\text{-}2^d$. Tests of additional H-2 specificities will be needed before final confirmation of crossing over can be made.

To further confirm that females are capable of carrying the gene for Slp-positivity, several females from the backcross to C57BL/6J and the F_2 generation were typed for specificity H-2.4 and then progeny tested by crossing to C57BL/6J males. Since the genetic determinant for H-2.4 is linked to the Slp^a allele in this case, females that are +4 should bear some Slp-a male offspring, while -4 females should bear no Slp-a male offspring. The results of these progeny tests (Table 8) demonstrate that +4 females do produce Slp-a male offspring and -4 females produce only Slp-o male offspring.

Strain distribution

The association of Slp with H-2 can also be seen in a very striking way in the distribution of Slp and H-2 types in inbred strains. Table 9 gives the Slp types of 40 inbred strains of mice, including some congenic resistant strains, grouped according to H-2 type to emphasize the association between Slp and H-2. All males were tested with both RF/J ♂

Table 8

Progeny Tests of Three (F_1 x C57BL/6J) Females and Four F_2 Females

Female Parent*			Male Offspring		
Code Number	Cross	H-2.4 Type	No. of Litters	Slp-a	Slp-o
487	Backcross	+ 4	2	4	7
497	F_2	+ 4	5	7	12
535	F_2	+ 4	2	2	3
491	Backcross	- 4	2	0	9
492	Backcross	- 4	3	0	11
496	F_2	- 4	1	0	4
536	F_2	- 4	2	0	7

*Each female was crossed to C57BL/6J.

Table 9

Distribution of Slp Type in Inbred Lines of Mice Arranged to Reflect the Association with H-2 Type

Line	Slp Males	Slp Females	H-2 Type	Line	Slp Males	Slp Females	H-2 Type
A/HeJ	+	-	a	C58/J	-	-	k
A/J	+	-	a	MA/J	-	-	k
A/SnSf	+	-	a	RF/J	-	-	k
B10.A/SnSf	+	-	a				
				P/JSf	+	-	p
C57BL/10JSf	-	-	b	BDP/J	+	-	p
C57BL/6J	-	-	b				
C57L/J	-	-	b	DBA/1JSf	-	-	q
C3H.B10/Sf	-	-	b	C3H.Q/Sf	-	-	q
A.BY/SnSf	-	-	b				
129/J	-	-	b	C3H.RIII/Sf	-	-	r
				CR/Sf[a]	-	-	r
DBA/2JSf	+	-	d				
BALB/cAnM	+	-	d	D1.C/SnSf	+	-	c
YBR/a	+	-	d	A.CA/SnSf	-	-	f
B10.D2/SnSf	+	-	d	CJ/Sf[b]	+	-	j
C3H.D/Sf	+	-	d	I/AoSf	+	-	l
				AKR.M/SnSf	-	-	m
AKR/J	-	-	k	BF/Sf[c]	+	-	n
CBA/J	-	-	k	A.SW/SnSf	+	-	s
C3H/JSf	-	-	k	PL/JSf	+	-	v
B10.K/Sf	-	-	k	WB/ReSf	-	-	w
C57BR/cdJ	-	-	k	FL/2ReSf	-	-	?
				B10.Y/SnSf	-	-	pa

[a] Stock derived from C3H/JSf x RIII/Wy cross, inbred 18 generations.
[b] Stock derived from C3H/JSf x JK/St cross, inbred 13 generations.
[c] Stock derived from C57BL/10JSf x F/St cross, inbred 14 generations.

anti-DBA/2J♂ and RF/J♂ anti-A/J♂ with identical results. In confirmation of the earlier indication of sex-limitation, females of all 40 strains were negative.

Notice that all H-2a and H-2d strains were positive (Slp-a) while all H-2b and H-2k strains were negative (Slp-o). This close relationship is even more apparent when the congenic resistant strains are considered. Congenic resistant strains are essentially identical genetically, except for the introduction of a different allele at a histocompatibility locus, in this case the H-2 locus. These strains have been produced by backcrossing different H-2 alleles onto certain inbred backgrounds for at least seven generations. For example, C57BL/10 (synonym B10) and C57BL/10-H-2^a (synonym B10.A) are congenic strains that are different only at the H-2 locus; their genetic background is the same. Notice that strain B10.A has the Slp type corresponding to the strain of origin of its H-2 type (Strain A), not the Slp type of the background strain (C57BL/10). This relationship would be expected if H-2 and Slp are closely linked. Five additional congenic resistant strains were informative in the same way: A/Sn-H-2^b (synonym A.BY), DBA/1JSn-H-2^c (D1.C), C57BL/10ScSn-H-2^d new (B10.D2), A/Sn-H-2^f (A.CA), and C3H/JSf-H-2^d (C3H.D).

Map position of Slp; analysis of recombinant lines

The map position of Slp in relation to H-2 was more precisely determined by analysis of a number of strains derived from crossovers within the H-2 region. In Figure 3,

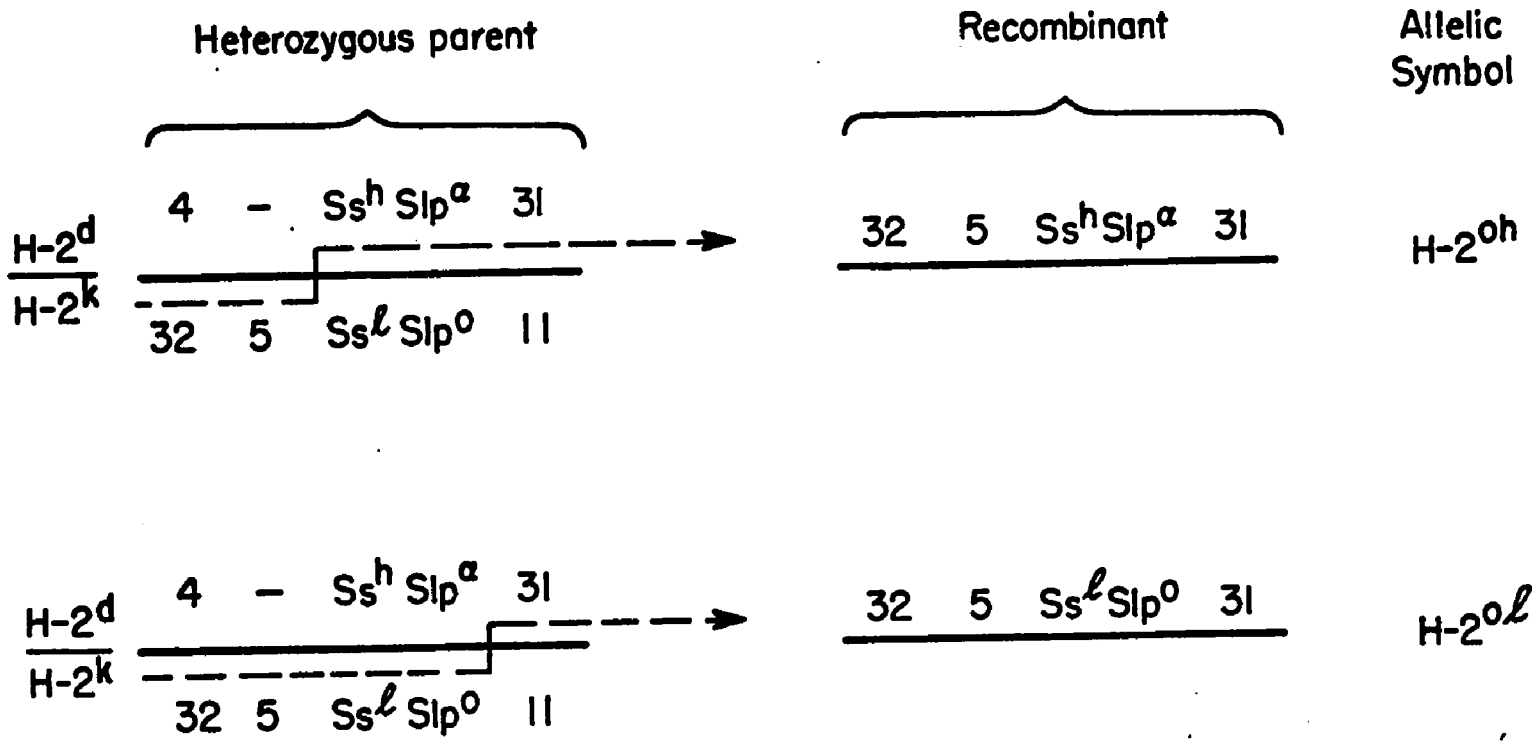


Figure 3. A pair of intra-H-2 crossovers which position the Slp determinant within the H-2 region (see text for description). Only selected specificities have been included.

a pair of intra-H-2 recombinant chromosomes is diagrammed. These are designated H-2^{oh} and H-2^{ol} (see Shreffler, 1965; Shreffler et al., 1966; David and Shreffler, in preparation). On the left side of the figure is the parental heterozygote combination in which the crossovers took place. Both crossovers occurred between alleles H-2^{d} and H-2^{k}. At the right of the figure are the configurations of the two recombinant chromosomes. Only selected H-2 specificities are included. In both cases, the recombinant chromosome contains specificities 32 and 5 from one chromosome and specificity 31 from the other parental chromosome, indicating that a crossover has occurred between the locations controlling these specificities. The recombinant chromosomes, as shown, have the same H-2 specificities but differ with respect to Ss type. Therefore, assuming that both represent single crossover events, in one case the crossover occurred to the left of the Ss segment and in the other case to the right of this segment. When males of these crossover types were tested for Slp type, the first was found to be Slp-a, while the other was Slp-o. Since these two recombinants differ only with respect to a small chromosomal segment within the H-2 region, the Slp determinant must also reside within the H-2 region and in the same segment as the Ss determinant. Analyses of twelve additional crossovers indicated a position for Slp to the right of the intra-H-2 crossover point in six of them, while the remaining six indicated a position to the left of the intra-H-2 crossover point. All of these were consistent with a localization of Slp within the H-2 region.

Table 10

Fourteen Intra-H-2 Recombinants Used to Map the Genetic Determinant for Slp

	Parental Heterozygote Type		Recombinant Type		
	Alleles	H-2 Specificities*	H-2 Specificities	Allele	Slp
1.	$H\text{-}2^a$ Slp^a	4,13 3 - 5 Ss^h 8,11	2 3 - 5 Ss^h 8,11	h	a
2.	$H\text{-}2^b$ Slp^o	2 - 22 5 Ss^h 33		h-Sg	a
3.				h-2Sg	a
4.	$H\text{-}2^a$ Slp^a / $H\text{-}2^b$ Slp^o	4,13 3 - 5 Ss^h 8,11 / 2 - 22 5 Ss^h 33	4,13 3 22 5 Ss^h 33	i	o
5.	$H\text{-}2^d$ Slp^a / $H\text{-}2^b$ Slp^o	4,13 3 - - Ss^h 8,31 / 2 - 22 5 Ss^h 33	2 - 22 - Ss^h 8,31	g	a

*Crossover points and direction of crossovers are indicated by thick bars.

Table 10 (Continued)

Fourteen Intra-H-2 Recombinants Used to Map the Genetic Determinant for Slp

	Parental Heterozygote Type		Recombinant Type		
	Alleles	H-2 Specificities	H-2 Specificities	Allele	Slp
6.	$\frac{H\text{-}2^d\ Slp^a}{H\text{-}2^k\ Slp^o}$	4,13 3 - - Ss^h 8,31 / - 3 - 5 Ss^l 8,11	4,13 3 - 5 Ss^l 8,11	a1	o
7.	$\frac{H\text{-}2^d\ Slp^a}{H\text{-}2^k\ Slp^o}$	4,13 3 - - Ss^h 8,31 / - 3 - 5 Ss^l 8,11	- 3 - 5 Ss^h 8,31	oh	o
8.	$\frac{H\text{-}2^a\ Slp^a}{H\text{-}2^b\ Slp^o}$	4,13 3 - 5 Ss^h 8,11 / 2 - 22 5 Ss^h 33	-(2) 3* (22) 5 Ss^h 8,11	h-3Sg	o
9.	$\frac{H\text{-}2^a\ Slp^a}{H\text{-}2^b\ Slp^o}$	4,13 3 - 5 Ss^h 8,11 / 2 - 22 5 Ss^l 33	4,13 3 (-) 5 Ss^h 33	i-Sg	a
10.				i-2Sg	a

( ) = Predicted, but not tested

* Inconsistent - see text

Table 10 (continued)

Fourteen Intra-H-2 Recombinants Used to Map the Genetic Determinant for Slp

	Parental Heterozygote Type		Recombinant Type		
	Alleles	H-2 Specificities	H-2 Specificities	Allele	Slp
11.	$H\text{-}2^{a1}$ Slp^{o} / $H\text{-}2^{s}$ Slp^{a}	4,13 3 - 5 Ss^{l} 8,11 / - 3 - 5 Ss^{h} 19	4,13 3 - 5 Ss^{l} 19	t1	o
12.	$H\text{-}2^{a}$ Slp^{a} / $H\text{-}2^{q}$ Slp^{o}	4,13 3 - 5 Ss^{h} 8,11 / - 3 - 5 Ss^{h} 17	4,13 3 - 5 Ss^{h} 17	y	a
13.	$H\text{-}2^{th}$ Slp^{a} / $H\text{-}2^{f}$ Slp^{o}	4,13 3 - 5 Ss^{h} 19 / - - - - Ss^{h} 8,9	4,13 3 - 5 Ss^{h} 8,9	ta	a
14.	$H\text{-}2^{d}$ Slp^{a} / $H\text{-}2^{k}$ Slp^{o}	4,13 3 - - Ss^{h} 8,31 / - 3 - 5 Ss^{l} 8,11	- 3 - 5 Ss^{l} 8,31	o1	o

These 14 informative recombinants are listed in Table 10. The linear order of the H-2 specificities is based on the recombination map of Shreffler (1967). References to the H-2 serology of each of these recombinants can be found in Shreffler (1970). Each recombinant chromosome can be derived from the parental heterozygote by a single crossover event, and in fact, this is the basis for the given linear order. It is apparent from an examination of these recombinants that the only possible position for the Slp determinant which is common to all recombinants is the Ss segment within the H-2 region.

In one recombinant, h-3g (Table 10, number 8), the presence of H-2 specificity 3 appears to be inconsistent with its given map position. Shreffler (1970) has discussed this apparent exception in detail. Despite the possible discrepancy of one H-2 specificity, this recombinant is not inconsistent with the positioning of Slp within the H-2 region.

Additional crossovers, which should be informative for the positioning of the Slp determinant, have been discovered more recently, but extensive serological analyses of the H-2 specificities of these recombinants have not yet been done. These recombinants include three from the heterozygote combination $H\text{-}2^{th}/H\text{-}2^{f}$ (Shreffler, unpublished), two derived from $H\text{-}2^{b}/H\text{-}2^{d}$ (see segregation analysis in this dissertation), two from $H\text{-}2^{a}/H\text{-}2^{f}$ (B10.M-11R and B10.M-17R of Stimpfling and Reichert, 1970), one from $H\text{-}2^{n}/H\text{-}2^{b}$ (B10.F-13R of Stimpfling and Reichert, 1970), and two from $H\text{-}2^{a}/H\text{-}2^{b}$ (Pizarro and Dunn, 1970).

Immunological Characterization

Relationship between Slp and Ss

A number of experiments were carried out to determine whether the Slp variation was physically and chemically associated with the quantitatively variant Ss protein. For this purpose, the immunological relationship between the Ss and Slp antigens was investigated. In Figure 4 an agar immunodiffusion pattern is presented in which the antigen (normal mouse serum from strain DBA/2) was in one well, with anti-Slp and anti-Ss placed in adjacent wells. A spur is visible at the point of the junction of the two reaction lines with anti-Slp and anti-Ss. Such a reaction is best explained by a heterogeneity of antigen, in which some molecules contain both Ss sites and Slp sites, while some molecules contain only Ss sites.

A number of modifications were made in the immunodiffusion procedure, to eliminate the possibility that the precipitin band, formed between anti-Ss and the antigen well, might in fact be two independent precipitin bands which only fortuitously lie one on top of the other, giving the false impression of spur formation. By replacing the A/J male serum with a 50:50 mixture of A/J male (Slp-positive) and C57BL/10JSf (Slp-negative) male serum, the concentration of Slp antigen was diluted one-half, while the concentration of Ss protein remained unchanged. Thus, if two independent bands exist, the one reacting with the Slp antigen should have been displaced toward the antigen well, since band position is dependent on antigen

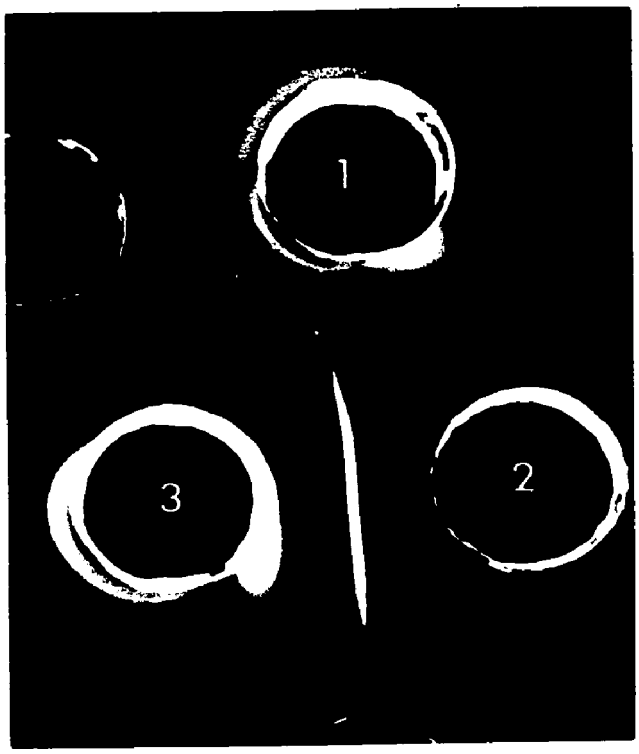


Figure 4. Immunodiffusion plate showing the precipitin reactions of anti-Slp (Well 1) and anti-Ss (Well 2) with DBA/2J ♂ serum (Well 3).

concentration. However, this dilution procedure had no effect on the spur pattern observed in Figure 4, i.e., two bands were not observed. Thus the conclusion of partial identity (partial fusion) between the reactions of anti-Ss and anti-Slp is valid.

This apparent crossreactivity between Ss and Slp antigens was confirmed in absorption studies designed to determine the effect of anti-Ss on Slp activity and conversely the effect of anti-Slp on Ss activity. Unlike conventional absorption tests, in which antiserum is absorbed with antigen and then tested against a second antigen, antibody of one type was used to absorb antigen, which was then tested for its ability to react with antibody of the other type. In the first series, DBA/2J male serum was absorbed with rabbit anti-Ss in tubes for one hour at 37°C and for eight additional hours at 4°C. The tubes were then centrifuged and the supernatant was tested for Slp reactivity with RF/J♂ anti-DBA/2J♂. The absorption was done in a number of antigen-antibody ratios from 2:1 to 1:32. As controls which should have no effect (other than dilution) on Slp reactivity, normal rabbit serum or phosphate buffered saline were similarly mixed with DBA/2J male serum.

As seen in Table 11, one part DBA/2J male serum diluted with 16 parts normal rabbit serum or phosphate buffered saline still gave a positive reaction for Slp antigen (lines 1 and 2, Table 11). However, when the same serum was mixed with two parts anti-Ss, all Slp activity was

Table 11

The Effect of Anti-Ss on Slp Reactivity

	Absorbed with	Tested with	Ratio - Antigen : Absorbing Material 2:1	1:1	1:2	1:4	1:8	1:16	1:32
1. DBA/2JSfσ serum	NRS[a]	Anti-Slp	+	+	+	+	+	+	-
2. DBA/2JSfσ serum	PBS[b]	Anti-Slp	+	+	+	+	+	+	-
3. DBA/2JSfσ serum	Anti-Ss	Anti-Slp	+	+	-	-	-	-	-
4. DBA/2JSfσ serum	Anti-Ss	Anti-Ss	+	trace	-	-	-	-	-

[a]NRS = normal rabbit serum [b]PBS = phosphate buffered saline

removed (line 3). Note that the Slp activity was completely absorbed at the same level of anti-Ss as that at which all Ss activity was lost (line 4). It appears, therefore, that anti-Ss is reactive with all molecules carrying the Slp antigenic determinant and is effective in absorbing out all Slp activity. This result is consistent with the hypothesis that all molecules carrying Slp sites also carry Ss sites.

In a second series of tests, shown in Table 12, DBA/2J male serum was absorbed with anti-Slp (RF/J ♂ anti-DBA/2J♂) for one hour at 37°C, and overnight at 4°C. It was then centrifuged and tested for Ss reactivity with anti-Ss. In this case, the Ss activity was tested quantitatively in immunodiffusion tubes. Quantitation was necessary because the serum containing the anti-Slp (from strain RF/J) also contains Ss protein. This Ss protein will, of course, be Slp-negative. Fortunately RF/J is Ss-L and has only approximately 1/20th as much Ss protein as does DBA/2J which is Ss-H. However, since a small amount of this Slp-negative Ss protein was present in each of the reaction tubes, all would be scored positive in a qualitative test. In the quantitative immunodiffusion tube method, the percentage reduction in antigen activity could be more reliably estimated.

The effect of anti-Slp (RF/J♂ anti-DBA/2J♂) on the Ss activity of DBA/2J♂ serum is shown in Table 12. In line 1, DBA/2♂ serum was diluted with phosphate buffered saline. These dilutions produced a linear calibration curve when the

Table 12

The Effect of Anti-Slp on Ss Reactivity. Reactions Expressed in Units of Activity with Undiluted DBA/2JSf Male Serum = 100 Units

Antigen	Absorbed with	Tested with	Ratio - Antigen : Absorbing Material 2:1	1:1	1:2	1:4	1:8	1:16
1. DBA/2JSfσ serum	PBS[a]	Anti-Ss	67	50	33	20	11	No reaction
2. DBA/2JSfσ serum	Ss-L NMS[b]	Anti-Ss	69	57	37	21	NT[c]	NT
3. DBA/2JSfσ serum	Anti-Slp	Anti-Slp	+	trace	-	-	-	-
4. DBA/2JSfσ serum	Anti-Slp	Anti-Ss	25	<11, >6	< 11	< 11	< 11	
5. Calculated units of non-reactive Ss from RF/J(Ss-L) antiserum			2	3	3	4	5	
6. Net units of possibly reactive Ss protein (line 4 - line 5)			23	<8, >3	<8	<7	<6	
7. Loss of activity			66%	84-94%	76-100%	65-100%	46-100%	

[a]PBS = Phosphate [b]NMS = Normal mouse serum [c]NT = Not tested

precipitin line position was plotted against the $\log_2$ of the dilution. Units of Ss activity were assigned to each dilution based on a standard of 100 units for undiluted DBA/2J♂ serum. When DBA/2J♂ serum was absorbed with anti-Slp (line 4) at a ratio of two parts antigen to one part anti-Slp, Ss activity was reduced from 67 units to 25 units. Since the addition of anti-Slp adds a small amount of presumably non-reactive Ss in the form of Ss-L mouse serum, it was necessary to subtract this activity (estimated in line 5) from 25 units of activity to obtain the net remaining activity of 23 units (line 6). Ss activity at this absorption ratio was therefore reduced approximately 66% (line 7). In a similar way, percent loss of Ss activity can be calculated at each absorption ratio. The value <11 (line 4) is based on the last dilution standard (line 1) which will give a reaction on this immunodiffusion tube method (1:8 dilution or 11 units) and indicates the maximum score that can be assigned to a negative reaction. At the 1:1 ratio, absorption was almost complete, as indicated by the trace reaction with anti-Slp (line 3). At this ratio approximately 84 to 94 percent of the Ss reactivity was lost. This seems to be the best minimum estimate that can be derived from this table, since the calculations made from the three higher dilutions are increasingly less sensitive.

In any case, substantial reduction of Ss level by anti-Slp has been demonstrated by this absorption series. It is not possible to determine whether this crossreactivity is

complete because of technical limitations of the absorption method; however, it seems that a substantial fraction, if not all, of the Ss protein molecules in Slp-a serum carry Slp antigenic sites.

On immunoelectrophoresis, Ss protein and the sex-limited protein gave very similar migration patterns (Figure 5), with the only difference being that a portion of the band formed by the Ss protein extended slightly more in the direction of the anode. This difference is consistent with the interpretation, presented earlier in relation to spur formation on immunodiffusion, that not all Ss molecules carry Slp sites.

Relationship between Slp and H-2

Shreffler and Owen (1963) were unable to demonstrate any functional or antigenic relationship between H-2 antigens and the Ss protein. This lack of functional or antigenic association is extremely important for the interpretation of the genetic fine structure of the H-2 region. The following work on the Slp antigen provides evidence in favor of the working hypothesis that Slp is also antigenically unrelated to H-2.

1) Anti-Slp made in three strain combinations (RF/J♂ anti-DBA/2J♂ , RF/J♂ anti-A/J♂ , C3H.Q♂ anti-DBA/2J♂) failed to hemagglutinate erythrocytes from Slp-a males (DBA/2, B10.D2) or Slp-o males (B10, RF, C3H.Q).

2) Absorption of anti-Slp with erythrocytes and washed cell stroma from liver and spleen homogenates for 30 minutes

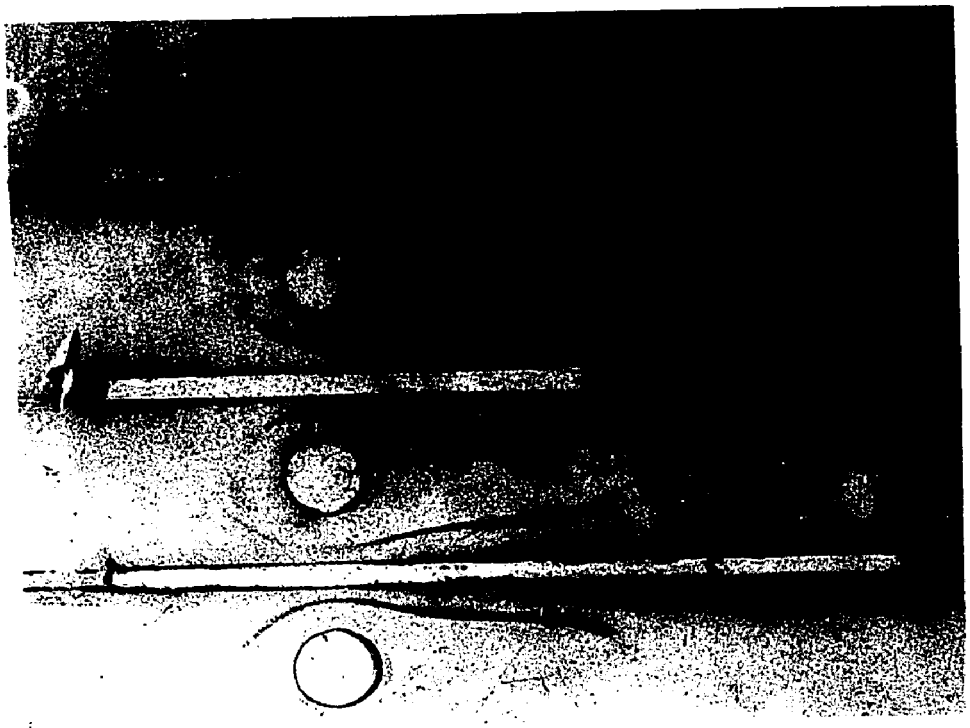


Figure 5. Immunoelectrophoretic plate showing the precipitin reactions of anti-Ss (Slots A and C) and anti-Slp (Slot B) with DBA/2J male serum. The anode was at the right.

at 25° and 37°C failed to inhibit the antibody reaction with Slp antigen (Table 13).

3) An examination of intra-H-2 recombinants (based on Shreffler, 1970) reveals that many of the H-2 specificities can be separated from the Slp determinant by crossing over, and that all the known specificities have allele distributions which differ significantly from that of the Slp trait. Thus, genetic identity of the Slp determinant and the determinant for any known H-2 specificity is highly unlikely.

Immunizations

Anti-Slp has now been produced in five different strain combinations (Table 14, combinations 1 to 4, 16, 17), all of which involve the immunization of an animal from an Slp-negative strain with an Ss preparation from a male of an Slp-positive strain. All of these antisera showed reactions of complete identity (complete fusion) in immunodiffusion and, where tested, gave identical reactions against a panel of serum from different inbred strains. Note that anti-Slp has been produced both in Ss-L strains (C3H/Sf and RF/J) and in an Ss-H strain (C3H.Q).

Since the strain combination C57BL/6J♂ anti-DBA/2J♂ (number 5) is of the same class of immunizations as combinations 1 through 4 (Slp-a♂ into Slp-o♂), we would expect anti-Slp to be produced in this combination also. The one animal which completed such an immunization schedule failed to produce anti-Slp (two died during the immunization procedure).

Table 13

Reaction of absorbed antisera with Slp antigen (DBA/2J♂ whole serum)

Tissue used for absorption	Antisera (undiluted)			Antisera (diluted 1/3)		
	anti-Slp RF♂ anti-D2♂	anti-Slp C3H.Q♂anti-D2 ♂	anti-Ss	anti-Slp RF♂anti-D2 ♂	anti-Slp C3H.Q♂anti-D 2♂	anti-Ss
D2 erythrocytes	++	++	++	+	++	+
spleen	++	++	nt*	+	++	+
liver	nt	nt	nt	+	++	+
B10 erythrocytes	++	++	++	+	++	+
spleen	++	++	nt	+	++	+
liver	nt	nt	nt	+	++	+
Unabsorbed	++	++	++	+	++	+

*nt - not tested

Table 14

Alloimmunization using partially purified Ss protein

Strain combination		Number immunized	Number producing anti-Slp
Recipient	Donor		
Slp-o recipients, Slp-a donors			
1. RF/J ♂	A/J ♂	18	17
2. RF/J ♂	DBA/2J ♂	24	24
3. C3H/Sf♂	A/J ♂	2	2
4. C3H.Q♂	DBA/2J ♂	3	3
5. C57BL/6J ♂	DBA/2J ♂	1	0
Slp-a recipients, Slp-o donors			
6. A/Sn ♂	C57BL/6J♂	3	0
7. B10.D2 ♂	C57BL/10JSf♂ *	2	0
8. DBA/2J ♂	C57BL/10JSf ♂	2	0
9. BALB/c♂	DBA/1J♂	5	0
10. PL/JSf♂	C57BL/6J♂	5	0
Slp-o recipients, Slp-o donors			
11. C3H/Sf♂	DBA/1J ♂	2	0
12. RF/J♂	DBA/1J ♂	4	0
13. C3H/Sf♂	C57BL/6J♂	2	0
14. RF/J♂	C57BL/6J♂	4	0
Slp-a recipient, Slp-a donor			
15. BDP/J♂	DBA/2J♂	5	0
Female recipients or donors			
16. C3H/Sf♀	A/J♂	2	2
17. C3H/Sf♀	DBA/2J♂	2	2
18. RF/J♀	DBA/2J♀	3	0
19. RF/J♂	DBA/2J♀	3	0
20. A/Sn♀	A/Sn♂	3	0

* Ss protein precipitated with anti-Ss was used for immunization

If this failure to produce Slp is confirmed after further alloimmunizations using the strain combination C57BL/6J♂ anti-DBA/2J♂, an interesting problem involving the immune response to the Slp antigen could emerge. If there are differences in the immune response to Slp antigen among inbred strains of mice, the failure of this combination to make anti-Slp could be either a property of the H-2^b allele, or a property of the C57BL/6J background.

A number of immunizations were performed to determine the existence (if any) and the nature of the gene product of the Slp^o allele. Slp-a male animals were injected with Ss protein preparations from Slp-o males in an attempt to demonstrate an allotypic specificity controlled by the negative allele. Alloimmunizations in five strain combinations (Table 14, combinations 6 to 10) produced no antisera defining a product of the Slp^o allele.

In addition to these alloimmunizations, a rabbit antiserum was made against an Slp preparation from DBA/2♂ serum, and no specific rabbit anti-Slp could be found when the antiserum was absorbed with Slp-o serum, although a good anti-Ss was produced. This would be expected if the Slp^o allele produced a very similar gene product indistinguishable by the rabbit antiserum or if the rabbit failed to respond to the Slp specificity. With only negative results thus far, very little can be said from these approaches as to whether or not the Slp^o allele produces an immunologically distinguishable product.

A series of alloimmunizations was also done for the purpose of searching for allotypic differences among Slp-o males of different strains (combinations 11 through 14, Table 14) where the donor and recipient differed in Ss type. In this work, the antigen donors were from Ss-H, Slp-o strains and the recipients from Ss-L, Slp-o strains. These immunizations failed to define any additional allotypic specificities in the Slp system.

The immunization of BDP/J males with an Ss preparation from DBA/2J male serum revealed no differences in specificity between these two Slp-a strains. This particular combination was employed because the reactions of BDP/J male sera with anti-Slp are weak and variable, and it seemed possible that this might reflect a structural difference.

The extensive alloimmunizations described in this section failed to produce: 1) subtype specificities, 2) antithetical specificities, or 3) specificities defining other independent alleles. Thus, the Slp allotypic specificity first defined by the antiserum RF/J♂ anti-A/J♂ is to date the only allotypic specificity that has been detected.

Results of a series of alloimmunizations involving either female recipients or donors are also summarized in Table 15. Females from the Slp-negative strain, C3H/Sf, were capable of producing anti-Slp when immunized with partially purified A/Sn male or DBA/2J male serum. However, the immunization of A/Sn females with a serum preparation from A/Sn males produced no antibody, despite the fact that the

donor was phenotypically positive and the recipient was phenotypically negative. This result would suggest that females from positive strains are tolerant to Slp antigen. It is, of course, possible that these females have very low levels of the Slp antigen or that the antigen is present, but masked in some way. Partially purified Ss protein from DBA/2J females (phenotypically negative, but genotypically positive) did not produce anti-Slp in animals of the Slp-negative strain, RF/J. If, in fact, DBA/2J females have low levels of Slp or masked Slp, it is not of sufficient quantity to elicit an immune response in these RF/J animals.

Quantitation of heterozygotes

In order to determine if dosage compensation occurred in Slp heterozygotes, the quantity of Slp antigen in $\underline{Slp}^{a}/\underline{Slp}^{o}$ heterozygotes was estimated by the quantitative immunodiffusion method. All animals used for these experiments were on standard C57BL/10JSf (B10) background, to eliminate background effect. The results of three replicate quantitations on each of three different kinds of $\underline{Slp}^{a}/\underline{Slp}^{o}$ heterozygotes are presented in Table 15. In Groups I and II, the Slp heterozygotes were homozygous for the Ss determinant (Ss-H) and in Group III they were heterozygous for Ss level (Ss-HL). The quantity of Slp antigen in heterozygous $\underline{Slp}^{a}/\underline{Slp}^{o}$ animals is expressed as the ratio of the quantity of Slp found in $\underline{Slp}^{a}/\underline{Slp}^{o}$ heterozygotes to the quantity of Slp in $\underline{Slp}^{a}/\underline{Slp}^{a}$ homozygotes. Groups I and II showed a wide range of results between .67 and

Table 15

Estimates of Slp activity in Slp^a/Slp^o heterozygotes

Homozygotes (no. used)	Heterozygotes (no. used)	Ss type of heterozygote	Age at time of testing	Slp activity Het/Homo
Group I				
B10.A(2)	B10.A x B10(4)	H	35 weeks	.94
B10.A(2)	B10.A x B10(3)	H	36 weeks	.67
B10.A(2)	B10.A x B10(4)	H	37 weeks	.94
Group II				
B10.D2(3)	B10.D2 x B10(3)	H	14 weeks	.95
B10.D2(3)	B10.D2 x B10(4)	H	15 weeks	.87
B10.D2(3)	B10.D2 x B10(4)	H	16 weeks	.74
Group III				
B10.D2(3)	B10.D2 x B10.K(1)	HL	14 weeks	.42
B10.D2(3)	B10.D2 x B10.K(3)	HL	15 weeks	.50
B10.D2(3)	B10.D2 x B10.K(3)	HL	16 weeks	.48

.95. Heterozygotes clearly have more than 50% of the Slp activity found in $\underline{Slp}^a/\underline{Slp}^a$ homozygotes, but complete dosage compensation is apparently lacking. However, considering the error involved in the method of estimation and the small number of animals used in each experiment, the values of .94, .94, and .95 are certainly not significantly different from 1.00. Interpretation of these data is further confused by evidence that the Ss protein molecule consists of polymers or aggregates of many subunits (Shreffler, unpublished). Thus, even in a case where only 50% of the subunits in a heterozygote carry Slp antigenic sites, the random association of these subunits with subunits lacking Slp sites would result in more than 50% of molecules or aggregates carrying Slp antigenic sites. There are, of course, other possibilities that could account for results intermediate between 50 and 100%, such as different rates of synthesis or of turnover of the products of the $\underline{Slp}^a$ and $\underline{Slp}^o$ alleles.

Ss-HL animals produce approximately 50% as much Ss protein as do homozygous Ss-H animals. Most of the Ss production in an Ss-HL animal is attributable to the $\underline{Ss}^h$ allele. If the Ss protein originating from the $\underline{Ss}^h$ allele also carries Slp antigenic sites, then one would expect Slp activity in such a heterozygote for Ss and Slp to also be 50% of the activity in an animal homozygous for both Ss and Slp. Levels of Slp in Group III of Table 15 did in fact approximate 50%.

Characterization of Slp antigen

On Sephadex G-200 gel filtration, the peak concentration of Slp antigen is detected in those fractions with a molecular weight in the range of approximately 125,000 to 155,000, based upon comparison with hemoglobin, albumin, and Blue Dextran 2000 (Pharmacia). Although these fractions are also positive for the Ss protein, the peak of Ss activity occurs in somewhat earlier fractions. The Slp antigen in whole serum is heat stable only to 50-52°C for 20 minutes, and substantial reduction of antigenic activity occurs after three days' storage at 4°C. This stability is significantly less than that of the Ss protein, which is heat stable to 70°C for 20 minutes. Both antigens, however, have identical pH stability ranges of pH 3.2 to 11.0. The Slp antigen is not sensitive to exposure to 0.1M 2-mercaptoethanol for 18 hours at 30°C. However, antigen activity is lost in controls not treated with mercaptoethanol, due to the exposure to heat during the treatment period.

In addition to the serum, the Slp antigen is also present in ascites fluid of A/He males. Seminal fluid, testis homogenate, and urine from DBA/2JSf males have been tested and found to be Slp negative. A number of pregnant females from positive strains were tested and also found to be negative.

Although no precise determinations have been done, it appears that Slp-a strains differ somewhat in quantitative levels of Slp antigen, as determined by both the intensity and the position of precipitin bands on immunodiffusion. All

of the positive strains, however, give moderate to strong reactions with the exception of strain BDP/J. Eight BDP/J males that were repeatedly typed gave the following reactions: two moderately positive, four very weakly positive, and two negative.

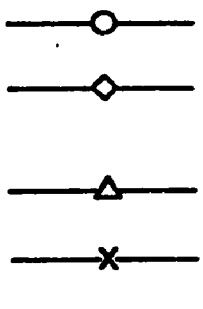
Hormone Studies

The Slp antigen is first detected in trace amounts in normal DBA/2J male serum at approximately five to six weeks of age, a time which roughly corresponds to the onset of sexual maturity. The level of antigen then begins to increase until the adult level is reached at about ten weeks of age. However, immature DBA/2J males which were castrated at 3 1/2 weeks of age failed to develop Slp antigen and were still negative for the antigen when tested after 25 weeks of age (Figure 6). When these castrated males were then treated with testosterone propionate, beginning at 25 weeks, Slp antigen was detected seven days after the start of treatment. In untreated DBA/2J males, the Slp development was normal. Animals which were castrated, but then received replacement therapy with testosterone propionate, developed normal levels of Slp at a rate very similar to untreated males.

To test further the effect of male hormone on expression of the Slp variant, females were treated with testosterone propionate from 3 1/2 to 10 weeks of age. As illustrated in Figure 7, these animals developed normal male levels of Slp

Figure 6. Effect of castration on immature DBA/2J males.

Legend:



Sham control males (four animals)

Castrated males + testosterone beginning at 3 1/2 weeks of age (four animals)

Castrated males (eight animals)

Males + testosterone beginning at one week of age (four animals)

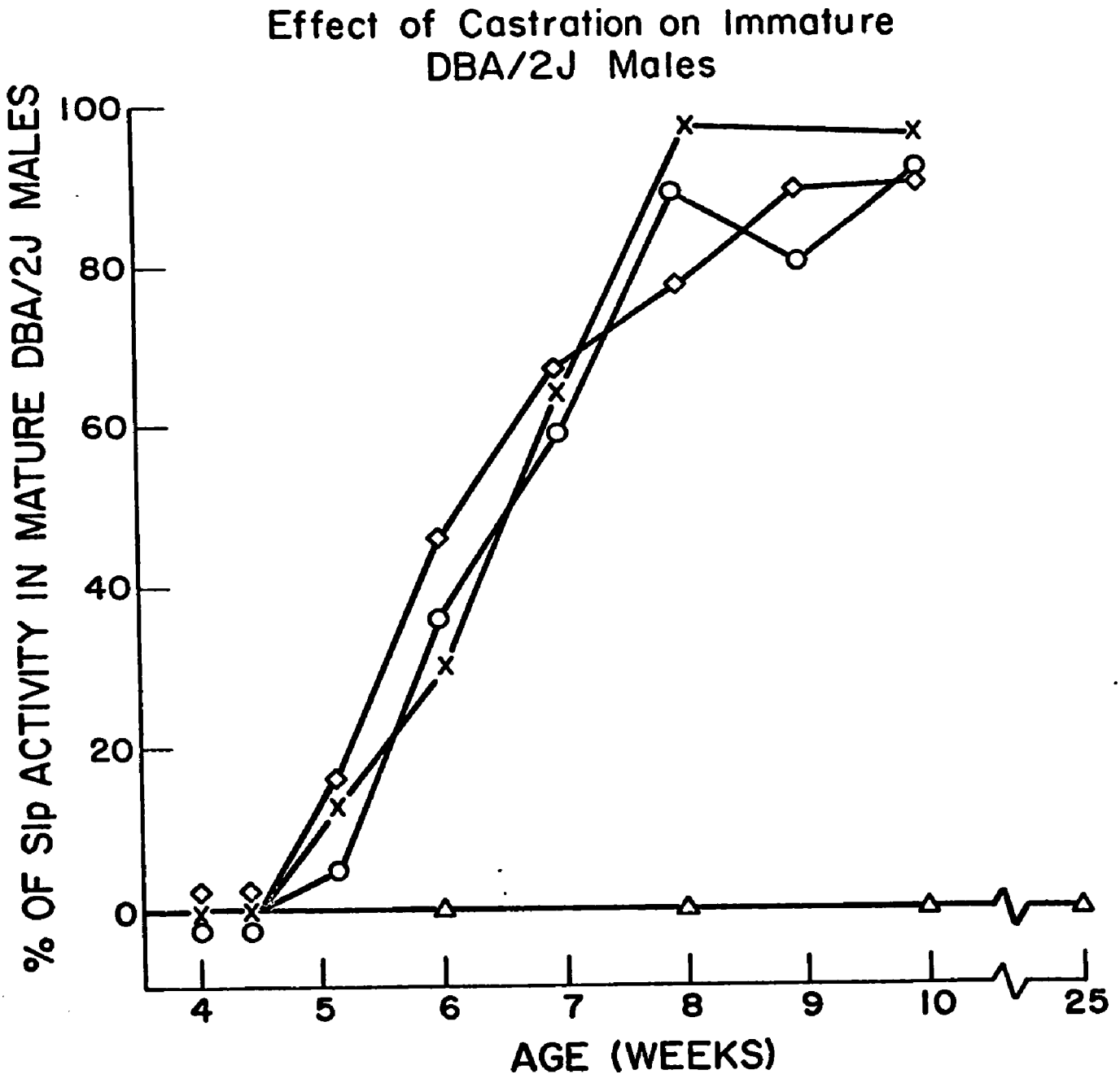
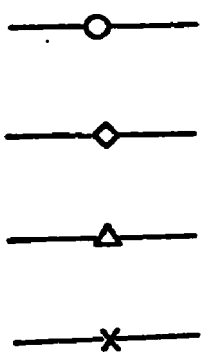

Effect of Castration on Immature
DBA/2J Males
% OF Slp ACTIVITY IN MATURE DBA/2J MALES
100
80
60
40
20
0
4
5
6
7
8
9
10
25
AGE (WEEKS)

Figure 7. Effect of testosterone propionate on immature DBA/2J females.

Legend:

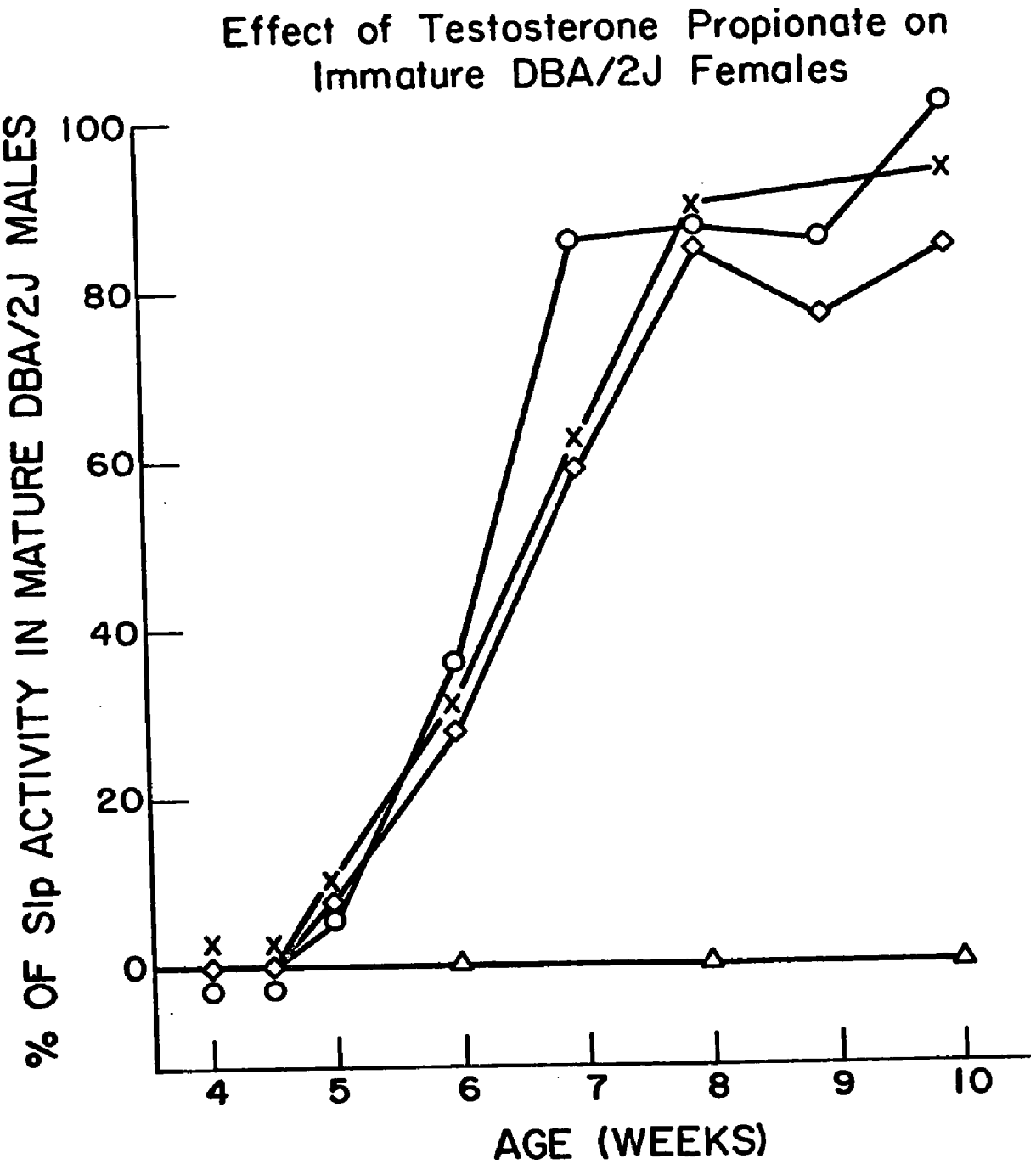


Ovariectomized females + testosterone beginning at 3 1/2 weeks of age (eight animals)

Females + testosterone beginning at 3 1/2 weeks (eight animals)

Ovariectomized females + sesame oil beginning at 3 1/2 weeks (four animals)

Females + testosterone beginning at one week of age (four animals)


Effect of Testosterone Propionate on
Immature DBA/2J Females
% OF Slp ACTIVITY IN MATURE DBA/2J MALES
100
80
60
40
20
0
4
5
6
7
8
9
10
AGE (WEEKS)

by ten weeks of age. Females that were first ovariectomized and then treated with testosterone show a similar appearance of Slp. The slope and shape of these two curves are almost identical to that of untreated males in Figure 6. Control animals which were ovariectomized and treated with sesame oil (the carrier for the testosterone propionate) did not develop Slp antigen.

In the preceding experiments, in which treatment of females or castrated males was started at 3 1/2 weeks of age, the Slp antigen began to appear at about five weeks of age. It was of interest to know whether it was possible to make the Slp antigen appear earlier in post-natal development by beginning the hormone treatment earlier. In an experiment in which hormone treatment was started at one week rather than 3 1/2 weeks of age, in two litters containing both males and females, the Slp antigen appeared again at five weeks of age, i.e., no earlier than in the previous experiments (see Figures 6 and 7). These animals developed the antigen at the same rate as animals started at 3 1/2 weeks of age.

The failure of castrated males to develop Slp, and the ability of females treated with testosterone propionate to develop the antigen, strongly suggest that the male hormone, testosterone, acts to trigger the appearance of Slp antigen in genotypically positive strains such as DBA/2J. Treatment of both males and females of a genetically negative strain such as C57BL/10Sf with testosterone propionate did not induce the appearance of the Slp antigen.

Similar studies of the effects of castration and hormone treatment on Slp activity were carried out with mature 12-week-old animals. At 12 weeks of age, DBA/2J males have already developed full levels of Slp antigen. Males castrated at this age showed a sharp reduction in the quantitative level of Slp to about 30% of the normal adult level by 21 days post-castration (Figure 8). After this rapid drop the level of antigen decreased at a much slower rate, reaching 8-10% by 150 days post-castration, and by 200 days falling to a level below the smallest quantity detectable by the immunodiffusion technique (less than 5%). Untreated sham controls maintained 100% Slp levels throughout the experiment. Castration of DBA/2J adult males, therefore, results in the loss of all detectable amounts of Slp antigen. Two of these castrated males showed a return to full adult male levels when treated with testosterone propionate after 60 days.

The effect of treating mature, 12-week-old DBA/2J females with testosterone propionate is shown in Figure 9. As was the case with immature females, the treatment results in the development of full adult levels of Slp. When the hormone treatment was suspended after 39 days, the level of Slp antigen declined, and was not detectable 80 days after suspension of treatment. Control females, not treated with hormone, did not develop Slp antigen. Females from the genotypically negative strain C57BL/10JSf failed to develop Slp antigen when treated with hormone.

To determine whether increasing the dose of testosterone propionate would affect the time or rate of Slp appearance

Figure 8. Effect of castration on mature DBA/2J males (12 weeks of age).

Legend:

——x—— Castrated males (nine animals)

——◇—— Sham control males (four animals)

— —○— — Males treated with testosterone 60 days after castration (two animals)

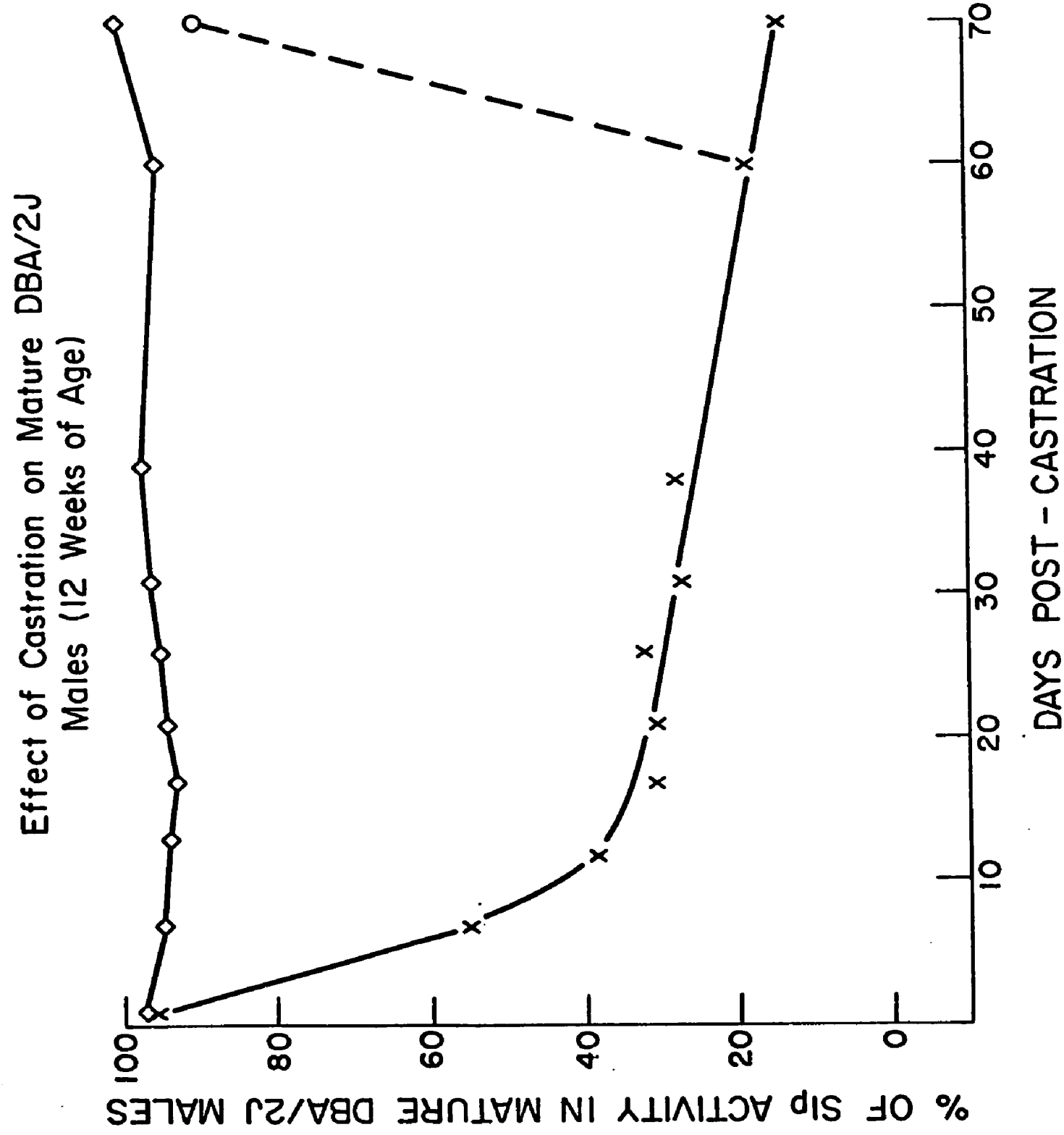
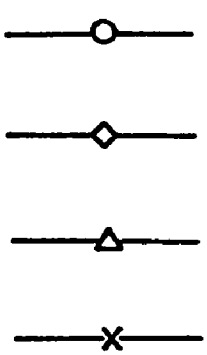

Effect of Castration on Mature DBA/2J
Males (12 Weeks of Age)
% OF SIp ACTIVITY IN MATURE DBA/2J MALES
100
80
60
40
20
0
10
20
30
40
50
60
70
DAYS POST - CASTRATION

Figure 9. Effect of testosterone propionate on mature DBA/2J females (12 weeks of age)

Legend:

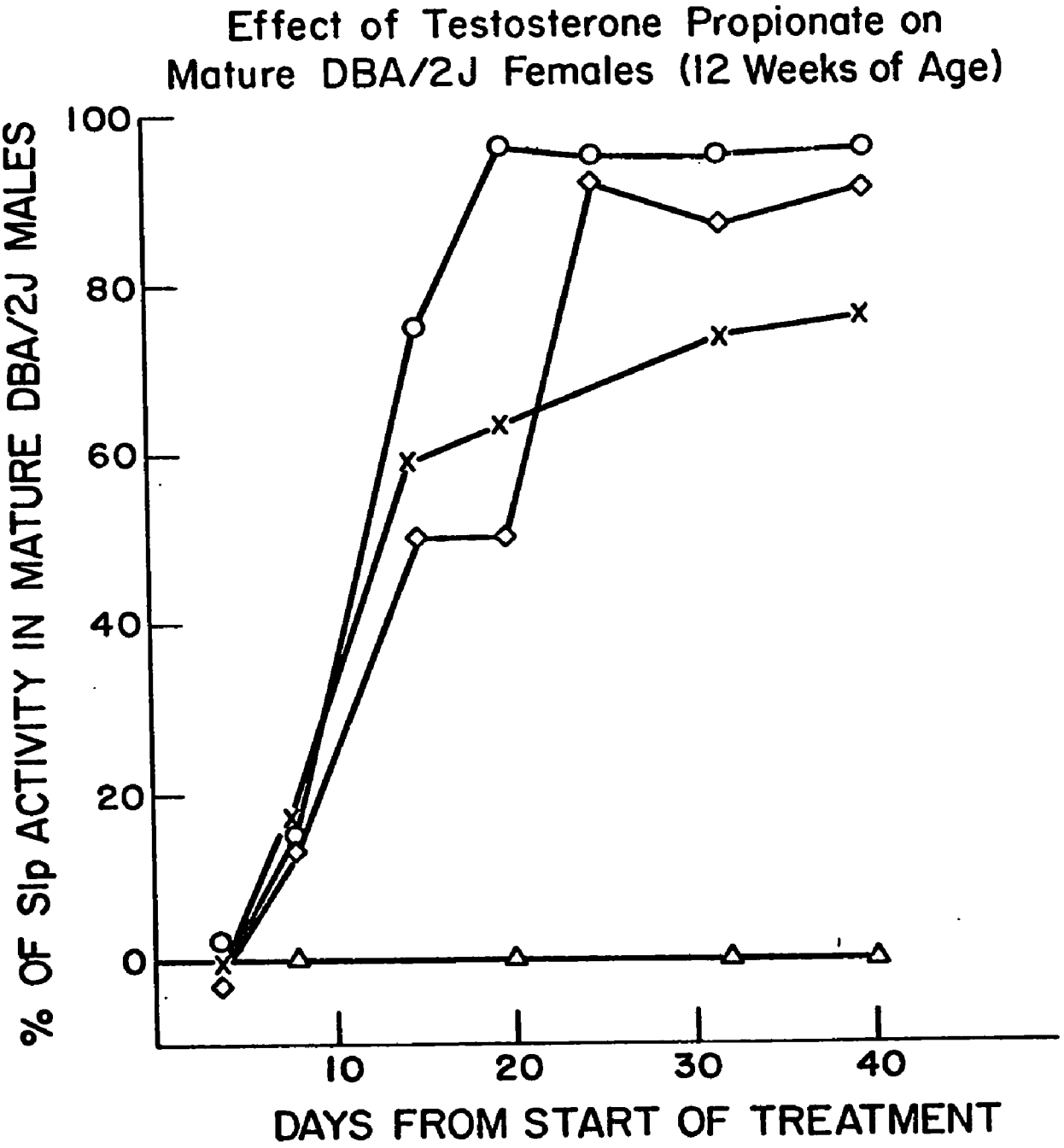


Ovariectomized females + testosterone treatment (nine animals)

Females + testosterone treatment--normal dose (six animals)

Ovariectomized females + sesame oil (five animals)

Females + testosterone treatment--eightfold increased dose (four animals)


Effect of Testosterone Propionate on
Mature DBA/2J Females (12 Weeks of Age)
% OF SIp ACTIVITY IN MATURE DBA/2J MALES
100
80
60
40
20
0
10
20
30
40
DAYS FROM START OF TREATMENT

or the final level of the antigen, a group of adult females was treated (starting at 12 weeks of age) with a dose of hormone eightfold greater than that used in the previous experiments with adult females. The results, illustrated in Figure 9, clearly showed that the eightfold dose did not increase the rate of Slp appearance or the final level of the antigen. On the contrary, the final level of antigen may have actually been reduced.

The quantitative level of the Ss protein was followed in the experiments on immature DBA/2J animals (Figures 6 and 7), to determine whether the development of Slp was correlated with any quantitative changes in Ss protein levels. This is of particular interest, because the Slp antigenic sites are believed to reflect structural differences in the Ss protein of different strains. The quantitative level of Ss was approximately the same at five weeks of age in all groups of animals. At this age, the level was 50 to 60% of the adult DBA/2J male standard. At ten weeks of age, Ss levels had increased to 80 to 100% in DBA/2J males, castrated DBA/2J males given hormone replacement, and DBA/2J females treated with testosterone. Ss levels remained unchanged at 50 to 60% in castrated DBA/2J males and normal females. Although the three groups showing increased Ss levels all developed Slp antigen, while the two groups showing no increase did not develop Slp antigen, similar quantitations of an Slp-negative strain,

C57BL/10JSf, showed the same pattern of increase in Ss levels in normal males and treated females, with no increase in normal females. None of these C57BL/10JSf animals developed Slp antigen. Therefore, the increase in Ss levels is correlated only with the male hormonal environment and appears to occur in both Slp-positive and Slp-negative strains.

A number of experiments were performed to determine whether the hormone acts directly on the DBA/2J female serum to modify the Ss protein so that Slp activity is expressed, or whether the hormone triggers some intermediate step which allows for the appearance of Slp, e.g., the induction of an enzyme which in turn modifies the Ss protein. When DBA/2J female serum was mixed in vitro with testosterone propionate or with male serum from the Slp-negative strain, C57BL/10JSf, no Slp appeared after 24 and 48 hours of incubation at 37°C and 25°C for both experiments. Therefore, no direct modification of protein by the hormone could be demonstrated.

When 0.4 ml of DBA/2J male whole serum was injected intraperitoneally into DBA/2J females or into C57BL/10JSf males daily for eight days, Slp could be detected in the serum of the recipient animals for as long as 24 hours after the last injection. This would indicate that the Slp antigen is absorbed from the peritoneal cavity into the circulation of the recipient animals. Presumably, if testosterone acted directly on the Ss protein from Slp-positive strains to produce Slp antigen, the injection of female serum from an Slp-positive strain into an Slp-negative male would provide

the proper environment for modification of the Ss protein, and therefore for the appearance of Slp antigen. However, when DBA/2J female whole serum was injected into C57BL/10JSf males using the same eight-day injection schedule, no Slp antigen could be detected 24 hours after the last injection.

DISCUSSION

Inheritance

The results demonstrate the existence of a serum protein variant in the house mouse detected by a specific alloantiserum on agar immunodiffusion. The genetic data are consistent with the hypothesis of control by a single, autosomal dominant gene. Two of the characteristics of this variant system are particularly interesting. First, an analysis of 14 intra-H-2 recombinants clearly demonstrates that the genetic determinant for Slp maps within the H-2 region of linkage group IX, in the same segment as the Ss determinant. Secondly, the antigen is normally expressed only in males of the proper genotype and therefore presents an interesting problem relating to the endocrine control of gene expression.

Relationship to the Ss Protein

One of the most important questions to be answered from the results is: Does the Slp trait represent the structural variation in the Ss protein which was originally sought? Both agar immunodiffusion and absorption studies show significant crossreactivity between Ss protein and the Slp antigen, and it seems rather clearly established that all molecules which carry Slp antigenic sites also carry the antigenic determinants

for Ss. Whether there are Ss molecules without Slp sites is not yet clear. In any case, the immunological evidence indicates that the Slp antigenic sites are on the quantitatively variant Ss protein. The migration pattern of Ss and Slp antigens on agar immunoelectrophoresis and their identical genetic map position within the H-2 region are also consistent with this interpretation.

Since the Slp variant is defined by an alloantiserum, it can be assumed that the distinction between Slp-a and Slp-o is based on a structural difference. The genetic data map this structural difference in the Ss subdivision within the H-2 region. If the Slp antigenic site is on the Ss protein, the anti-Slp alloantiserum must be defining structural differences in the Ss protein. One might then postulate that the Ss locus is the structural locus for this protein, rather than a regulatory locus. However, such a conclusion is complicated by the fact that the Slp trait is sex-limited, with the structural variation only expressed in males. This complication could be accounted for if some basic structural variation exists in the Ss protein which is exposed, modified, or activated only in the male environment.

While models in which the Ss determinant functions as a regulatory locus cannot be ruled out, the structural locus model appears far less complex at present. The discovery of an Slp specificity antithetical, or allelic, to Slp-a would strengthen the argument that Ss is a structural locus, since it is highly unlikely that different alleles at a regulatory

locus could each control or turn on a series of different antigenic specificities determined by another locus. A regulator gene would be expected to affect a structural gene in a quantitative manner, not in a qualitative manner. Antithetical or allelic specificities could not be demonstrated in the extensive series of alloimmunizations which were performed (see Table 15). However, the failure to find such specificities does not mean they do not exist, nor would their absence particularly weaken the argument that Slp reflects structural differences in the Ss protein, since the absence of additional specificities can be easily accounted for on the basis of models for control of Slp which are presented later.

Contribution to H-2 Genetic Fine Structure

The Slp variant is a useful marker for the genetic analysis of both the Ss determinant and the complex H-2 region. Slp types subdivide the large number of Ss-H inbred strains into two groups: Ss-H, Slp-a and Ss-H, Slp-o. This distinction is useful in the analysis of a large number of intra-H-2 recombinants which do not differ with respect to Ss. Thus Slp provides an intra-H-2 marker useful in the analysis and positioning of H-2 specificities in recombinants originating from heterozygote combinations such as $H\text{-}2^a/H\text{-}2^b$ and $H\text{-}2^d/H\text{-}2^b$, e.g., those which have been reported by Gorer and Mikulska, 1959, and by Stimpfling and Richardson, 1965. Assuming that the Slp antigenic sites do in fact represent structural differences in the Ss protein, the location of the Slp

determinant within the H-2 region confirms the previously indicated map position of the Ss determinant (Shreffler, 1967).

Snell et al., 1964, and Shreffler, 1965, 1967, have discussed the influence of the location of the Ss locus on the interpretation of the genetic organization of the H-2 region. In these discussions it was suggested that if the Ss locus maps within the H-2 region, and if it determines a protein functionally or antigenically unrelated to H-2, then one is forced to view the H-2 region as being made up of more than one functional locus, i.e., at least two histocompatibility loci, separated by a locus for the Ss protein. Shreffler and Klein (1970) have extensively reviewed a number of lines of evidence, exclusive of the positioning of the Ss determinant, which strongly suggest that the H-2 region consists of a minimum of three distinct genetic loci. The work presented here on the Slp variant bears on this suggestion in three ways. First, the Slp variant adds support for location of the Ss determinant within the H-2 region. Secondly, genetic and immunological evidence suggest that the Slp locus may be the structural locus for the Ss protein. And lastly, experiments dealing with the relationship between Slp and H-2 antigens have failed to demonstrate any antigenic or functional association. This information on the Slp variant thus adds additional support for the idea of a multicistronic H-2 region.

Sex-limitation

The castration and hormone treatment experiments with both immature and adult animals reveal that the proper genotype, as well as the presence of male hormone, is required for the appearance of the Slp antigen. Thus, the development of the Slp antigen clearly is a result of both genetic and hormonal factors. The disappearance of Slp antigen from serum of castrated males, and from treated females after suspension of treatment, demonstrates that hormone is also required for the maintenance of the antigen.

Since the genetic studies show the Slp trait to be autosomally inherited, females from genotypically Slp-positive strains should have the genetic information necessary to code for the variant Ss protein strucutre. Therefore, in view of the fact that DBA/2J females can produce Slp antigen after treatment with male hormone, the absence of Slp antigen from the serum of normal untreated females must result from absence of the male hormone environment, not from inability to code for the variant protein.

Although it is evident that male hormone plays a primary role in the control of the phenotypic expression of the Slp variant, the precise mechanism for this control is unclear. Some preliminary clues to this mechanism can be discerned from the experimental results. The discussion which follows will be based on the assumption that the Slp variant reflects structural differences in the Ss protein among inbred strains of mice.

A number of general mechanisms could, *a priori*, account for the role of hormone in the phenotypic expression of the Slp antigen. Hormonal control could be exerted at the *gene* level or at the *protein* level. The hormone could act *directly* on the gene or protein, or *indirectly* through the induction of some intermediate such as an enzyme or derepressor. A number of points pertinent to these alternatives are discussed in the following paragraphs.

Since hormone treatment of DBA/2J animals beginning at one week, rather than 3 1/2 weeks of age, does not alter the time (approximately five weeks of age) or the rate of appearance of Slp antigen, it seems likely that a differentiation step, independent of the presence of hormone, must occur during post-natal development before hormone stimulation can be effective. Thus, this phenomenon represents an example of developmental competence on a genetic and biochemical level. Assuming that mature DBA/2J females are completely competent, the minimum lag time necessary to reach detectable levels of Slp antigen is the period of approximately one week from start of treatment until appearance of Slp antigen in the serum of these adult females. Treated immature females develop the antigen at five weeks of age. If the lag time is one week, then the hormone-independent differentiation step must be complete no earlier than four weeks of age, and treatment before this time is ineffective.

The failure to produce Slp upon direct exposure, *in vivo* or *in vitro*, of DBA/2J female serum to hormone, and the lag

period of six to eight days between administration of hormone and appearance of Slp antigen in adult females, argue against any direct modification of Ss protein by male hormone. The improbability of direct modification and the apparent existence of a hormone-independent differentiation step at four weeks of age, argue in favor of the hypothesis that hormone triggers some intermediate step, e.g., induction of an enzyme or a derepressor, which in turn determines the appearance of Slp antigen. The hormone-independent differentiation step might, for example, represent the normal developmental maturation of the synthetic apparatus necessary to produce the intermediate enzyme or derepressor. Moreover, the failure to recover Slp antigen in the *in vivo* experiments involving the injection of DBA/2J female serum into C57BL/10JSf males indicates that this intermediate step probably does not operate on the already-synthesized Ss protein. If it did, the C57BL/10JSf male would be expected to have the testosterone-induced intermediate necessary for the development of Slp antigen. It is also possible that the intermediate might act on the variant Ss protein only under restricted conditions, or only at specific sites, e.g., intra-cellularly prior to release of newly synthesized Ss protein. In this case, it could not be modified after it enters the serum.

The castration and hormone treatment experiments reveal several facts about the hormonal control of Slp antigen: 1) Male hormone is required for the development and maintenance of Slp antigen in genotypically positive animals. 2) Females

of Slp-positive strains have the genetic information necessary to code for and produce Slp antigen. 3) There is a differentiation step independent of male hormone which must be completed before Slp antigen can develop. 4) Male hormone probably does not act directly on Ss protein in the serum to modify it or to expose masked Slp antigen sites. The models considered in the next section are based on these facts, correlated with the immunological, chemical, and genetic data which have also been presented in this dissertation.

Models for the Genetic and Hormonal Control of Slp

The work on the Slp variant has provided sufficient information to permit the construction of working hypotheses concerning the genetic and hormonal roles in Slp antigen expression. In this section two possible models will be presented. Numerous modifications on the general themes of these two models are possible. Furthermore, it is not implied that these are necessarily the only models which can account for the genetic and hormonal control of the Slp antigen. However, they are the two which best fit the presently available data. Although quite different, both are based on the underlying assumption that the Slp antigen reflects structural differences in the Ss protein.

The first model, which will be referred to as the "gene duplication model," invokes hormonal control at the gene level. In this model the hormone induces an intermediate, such as a

derepressor, which in turn activates the genetic determinant for a variant Ss protein which carries the Slp antigenic site. This hypothesis is entirely consistent with the several conclusions from hormonal studies, outlined at the end of the last section. The properties of this model are as follows:

1. The Ss determinant within the H-2 region consists of two duplicated Ss loci, possibly resulting from unequal crossingover. The possibility that the H-2 region or a portion of the region may have evolved by gene duplication has been suggested by a number of authors (Stimpfling and Richardson, 1965, Shreffler et al., 1966, Shreffler and Klein, 1970).

2. In chromosomes carrying the Slp^a allele, one of these two Ss loci has mutated after duplication, such that its product carries a structural difference responsible for the Slp antigenic trait. Furthermore, some mutational change, either the same change responsible for Slp antigenicity or another, located elsewhere in the locus, must have occurred, such that the locus is now susceptible to control by male hormone. It is also possible that the evolutionary process could have been the reverse, with the original Ss locus under hormonal control and the mutated, duplicated locus escaping hormonal control. In any case, an animal carrying the Slp^a allele would have two linked loci for the production of two structurally different kinds of Ss molecules, with only one of the loci under hormonal control. Anti-Slp then detects a primary structural alteration in the product of the mutated locus.

The production in Slp-a animals of two populations of Ss molecules, one class with Slp antigenic sites and a second class without allotypic sites, is consistent with the observation and interpretation of spur formation between anti-Ss and anti-Slp on immunodiffusion (see Figure 4).

3. The $\underline{Slp}^{o}$ allele or chromosome consists either of the duplicated pair of loci, without the mutation at the second locus (i.e., with both loci identical), or of a single, unduplicated locus. The presence of this unaltered or unmutated Ss locus in both $\underline{Slp}^{a}$ and $\underline{Slp}^{o}$ chromosomes accounts for the observation that Ss from an $\underline{Slp}^{o}/\underline{Slp}^{o}$ male is not recognized as foreign in an Slp-a male and therefore does not induce an antithetical, allotypic antibody.

4. Male hormone induces an intermediate mechanism, such as a derepressor, which in turn directly activates the mutant Ss locus, allowing for the production of the structurally variant Ss protein. Derepression could occur either at the transcriptional level or post-transcriptionally. The one-week lag time from the start of hormone treatment in genotypically positive adult females represents the time needed for this newly synthesized class of Ss molecules to reach detectable levels in the serum.

At least two pieces of data are not completely consistent with this model as outlined thus far. First, under this model as proposed, one might expect the Ss protein of an $\underline{Slp}^{a}/\underline{Slp}^{a}$ male to consist of an approximately equal mixture of Ss molecules with Slp sites and Ss molecules lacking Slp sites.

However, the absorption data in Table 12 indicate that, in DBA/2J male serum, significantly more than 50% of the Ss molecules carry Slp antigenic sites--in fact, the value appears nearer 90%. Secondly, $\underline{Slp}^a/\underline{Slp}^o$ heterozygotes contain significantly more than 50% as much Slp antigen as do $\underline{Slp}^a/\underline{Slp}^a$ homozygotes. A possible explanation for both of these observations is that the subunits produced by the two duplicated loci combine at random to form mixed polymers or aggregates, thereby increasing the number of molecules with at least one Slp site. Unpublished data (Shreffler) indicating that Ss protein consists of aggregates or polymers of smaller subunits makes this supposition more valid.

The second model, the "protein modification model," is based on hormonal control at the protein level. This model is also consistent with the conclusions listed at the end of the last section, if one assumes that modification does not take place in the serum. Its principal features are:

1. The Ss determinant within the H-2 region consists of a single locus.

2. A mutation at this locus results in a structurally altered Ss protein which is susceptible to modification. The $\underline{Slp}^a$ allele codes for this variant Ss protein, while the $\underline{Slp}^o$ allele codes for the unaltered Ss protein which is not susceptible to modification.

3. The structurally variant Ss protein is modified by an intracellular enzyme prior to secretion into the serum. This modification could, for example, represent the enzymatic

addition of a carbohydrate or the cleavage of a small peptide. In any case, this modification results in sufficient additional structural alteration to make this modified molecule antigenic in animals lacking an Slp^a allele. Injection of Slp-a animals with Ss protein from Slp^o/Slp^o males would not result in the formation of an antithetical antibody if the structural difference between the product of the Slp^o allele and the unmodified product of the Slp^a allele could not be antigenically distinguished. Thus, only the modified variant Ss protein is antigenic, and the product of the Slp^o allele does not act as a substrate for this enzyme.

4. The enzyme responsible for the modification of the variant Ss protein is induced by male hormone. This enzyme would quite likely be controlled by a locus at a position in the genome unrelated to the H-2 locus, and it is assumed that this locus is operative in males of all mouse strains.

The principal value of the models presented in this section is to allow for the design of future experimentation. For example, future research should be extended in the direction of attempting to demonstrate crossingover between the genetic determinant for the quantitative Ss trait (Ss^l and Ss^h) and the determinant for the allotypic Ss trait (Slp^a and Slp^o). The discovery of such crossovers would strengthen, but not prove, the gene duplication model. The use of cell extracts to isolate or demonstrate a modifying enzyme responsive to testosterone would support the protein modification model. The *in vitro* culture of cells producing Slp antigen would

permit the use of inhibitors for elucidating the level of regulation. Chemical and physical characterization of the gene products of the various alleles of the Ss-Slp system would also be extremely valuable in understanding the nature of the structural differences of these gene products.

This dissertation has led to a basic understanding of the genetic and hormonal control of a serum protein variant in the mouse. Such knowledge, amplified by future research, should prove valuable in at least two major ways. First, it provides a model system for the study of hormonal control of gene expression, and of developmental problems such as genetic and biochemical competence, and sexual differentiation. Secondly, the Slp determinant will provide a most useful tool in the analysis and interpretation of the genetic fine structure of the complex H-2 locus.

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