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The mode of action of intersex in terminal sexual differentiation in Drosophila.

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The Mode of Action of *Intersex* in Terminal Sexual Differentiation in *Drosophila*

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by

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THESIS ACCEPTANCE

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ABSTRACT

The *intersex* gene (2-60.5) lies at the terminus of the regulatory pathway that determines sex-type in *Drosophila*. Its product functions with the female-specific product of *doublesex*, another gene in the sex-determination regulatory pathway, to regulate female-specific differentiation. However, the mechanism of this regulation has not been clearly demonstrated. Using a temperature-sensitive allele at *intersex* to eliminate its function at time points both during development and in the adult stage, the mode by which *intersex* regulates female determination was addressed.

When chromosomal females bearing a temperature-sensitive *intersex* allele are raised at a permissive temperature, they develop as phenotypic females. Animals raised at a restrictive temperature until the mid-pupal stage of development and then shifted to the permissive temperature also develop as normal females. In contrast, animals kept at a restrictive temperature past the mid-pupal stage, or animals raised at a permissive temperature and then shifted to a restrictive temperature before the mid-pupal stage develop as sterile females. Therefore, for fertility to be retained, *intersex* function must be present at least until the mid-pupal stage.

To determine if the function of *intersex* is also required in the adult to maintain the female differentiated state, as well as to address its mode of action, *intersex* function was eliminated in the adult female. To this end, diplo-X females bearing a temperature-

sensitive *intersex* allele were place at a restrictive temperature as adults and used to analyze whether *intersex* exerts transcriptional control over the female-specific expression of the yolk protein gene, yp1. Even after placing animals bearing the temperature-sensitive allele at a restrictive temperature for up to twenty days, yp1 transcription persisted at levels equivalent to sibling controls having normal *intersex* function. These data suggest that *intersex* does not function to positively regulate female-specific gene expression, but does not rule out that *intersex* functions to repress male-type gene expression in females.

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INTRODUCTION

Investigations into sexual differentiation in *Drosophila* have defined a regulatory pathway for sexual differentiation in which a set of genes acts hierarchically to control all aspects of sex type (Figure 1). The primary determinant for sex-type is the X-chromosome to autosome ratio (Baker et al., 1987; Bridges, 1921). Haplo-X flies with one X chromosome and two sets of autosomes, a 1:2 X:A ratio, are male. On the other hand, diplo-X flies with two X-chromosomes and two sets of autosomes or a 2:2 ratio of X:A are female.

The mechanism by which sex-type is determined can be summarized as follows: Sex- type in *Drosophila* is initially determined by a choice of whether or not to activate an early promoter for the *Sex-lethal* (*Sxl*) gene. Activation of the early promoter initiates a female-type developmental pathway in diplo-X individuals. Failure to activate this early promoter results in a male-type pathway. It is the early *Sxl* promoter that is sensitive to the X-chromosome to autosome ratio (X : A ratio). This ratio is transmitted to *Sxl* via the products of "numerator" signal genes, *sisterless-a* (*sis-a*) and *sisterless-b* (*sis-b*) (Cline 1983), and denominator maternally expressed genes, *daughterless* (*da*) and *deadpan* (*dpn*) (Cline 1980; Young-Shepard et al., 1992). The products of the signal genes transcriptionally activate the *Sxl* early promoter in diplo-X individuals. Once a functional *Sxl* product is made via this early promoter, a female-specific pathway is maintained is

a cell autonomous manner: A *Sxl* late promoter is activated in a non-sex specific manner, but the pre-mRNAs deriving from it are processed in a sex-specific manner, and dependent on functional *Sxl* product for a functional mRNA to be produced. This product of *Sxl* autoregulates *Sxl* pre-mRNA splicing and directs female-specific development. This autoregulation action of *Sxl* protein in females initiates a cascade of female-specific splicing of downstream genes that ultimately directs female development. An absence of functional *Sxl* product, obtained if the early *Sxl* promoter is not activated, leads to a male-specific pathway.

To consider the details of how the early *Sxl* promoter is activated, consider that for an organism to initially distinguish between male and female developmental pathways, there must exist a mechanism that responds differentially in diplo-X animals (females) and haplo-X animals (males): one that is sensitive to the X : A ratio. This mechanism uses X-linked "x/a numerators" or signal genes, whose relative dose is directly linked to the number of X-chromosomes. The *sis* genes have been identified as two of the many possible signal genes which are distributed uniformly on the X-chromosome (Cline 1988). These genes become activated prior to cellularization in the syncitial embryo at nuclear cycle 9 and cease transcription at the blastoderm stage of development (Cline and Erickson, 1991). Genetic analyses have shown that these numerator elements act in conjunction with the maternal products of the *da* and *dpn* loci to activate *Sxl* early

in development. *da* and *dpn* are maternal effect gene that function during oogenesis to produce materials required for normal development of progeny. Consequently, the maternal genotype, and not the progeny genotype, determines the progeny phenotype. As a result, the *da* and *dpn* maternal contribution is the same in both haplo-X and diplo-X animals, and itself is unable to distinguish different X:A ratios. *da* is a lethal mutant: Its lethal to both males and females at a restrictive temperature, and lethal only to females at a semi-permissive temperature. Early *Sxl* transcripts needed to initiate a female-type pathway fail to accumulate in progeny of *da* mothers at the semi-permissive and restrictive temperature.

Because the signal genes are X-linked, their dosage will depend on the number of X-chromosomes. Males will have one dose of *sis-a*$^{+}$ and *sis-b*$^{+}$, whereas females will have two doses of *sis-a*$^{+}$ and *sis-b*$^{+}$. Their dose is critical to sex-type determination. Decreasing the dose of *sis* genes from one to two in diplo-X animals results in female lethality, as functional *Sxl* gene product is not produced, and genes used in dosage compensation are not inactivated. Furthermore, increasing the dose from one to two in haplo-X animals results in male lethality. This result from the activation of *Sxl* which is not tolerated in haplo-X animals (Cline, 1984). Increasing the dose of *sis* genes also suppresses the diplo-X lethal effect of *da* at the semi-permissive temperature (Cline 1988). This interaction suggests that the maternal *da* product is needed only to control

the effectiveness of the signal genes.

Taken together, these data support a model in which the signal elements, the *sis* genes, along with maternal gene products *da* and *dpn* transduce the X:A ratio to the target, *Sxl*. A female-specific pathway will be initiated if a functional *Sxl* product is produced and a male-specific pathway will be initiated if a non-functional product is produced.

Once activated in a diplo-X individual, *Sxl* has two functions. First, *Sxl* acts to positively autoregulate itself, serving as a memory mechanism for sex-type in each of the cells (Cline 1985). Second, it controls the expression of other genes in three distinct regulatory pathways: somatic sex-determination, germline sex-determination, and dosage compensation (Cline 1985; Gergen 1987; cline 1983; Maine et al., 1985).

The function that *Sxl* has in somatic sex determination is controlled by sex-specific RNA-splicing. In haplo-X flies, *Sxl* has no function (Salz et al. 1987), as the pre-mRNA transcript is alternatively spliced to include an exon containing a termination codon resulting in a truncated non-functional protein (Bell et al. 1988). In diplo-X animals, a female-specific alternative splicing pattern of the *Sxl* pre-mRNA marks the initiation of a cascade of sex-specific alternative RNA splicing events. In haplo-X animals, this cascade is absent, suggesting that the male-type pathway for sexual differentiation is a default pathway in this regulatory hierarchy.

Initial activation of *Sxl* occurs only in diplo-X animals, where early *Sxl* mRNAs begin to accumulate. These early *Sxl* mRNAs use an alternate early promoter which is within the first intron of later produced transcripts (Keyes 1992)(Figure 2). These early transcripts are not detected in mutant *da* mothers. Furthermore, early transcripts are abnormally expressed in *sis-a* and *sis-b* embryos. *sis-b, dpn,* and *da* proteins are members of the helix-loop-helix family, which bind DNA as heterodimers and function as transcriptional regulators (Murres et al., 1989). Therefore, it would appear that these signal elements activate *Sxl* activating transcription at *Sxl*'s early promoter (P_E).

The mRNAs produced using this early promoter begin to accumulate at approximately the same time the protein products of these *sis* genes are accumulating, two to four hours after fertilization. They are of three different size classes: large (4.0 kb), medium (3.1 kb), and small (1.7kb) (Figure 2). After this early sex-specific transcriptional activation, this promoter is silent and a late promoter is activated in a non-sex-specific manner. mRNAs about 300 base pairs larger than the early transcripts are produced in females and mRNAs about 500 base pairs larger are produced in males.

Upon processing, both the early and late female-specific pre-mRNAs yield two similar protein products that differ in only the first 24-26 N-terminal amino acids. Male-specific splicing of the pre-mRNA retains a male-specific exon, #3. This exon contains an inframe stop codon which results in a nonfunctional, truncated protein. Transcripts

in females use an alternative splicing pattern which excludes this exon, allowing them to produce a functional *Sxl* product. It is this *Sxl* product that functions to autoregulate the female-specific splicing at later produced pre-mRNAs. *Sxl* protein acts by blocking the 3' splice site of the male exon of the *Sxl* mRNA produced from the late promoter (P_L) thereby generating female-specific spliced transcripts. In contrast to the late mRNAs, the early transcripts do not appear to be subject to regulation by sex-specific processing. Transcripts produced from the early promoter have a default splicing pattern that skips both exon #2 and the male-specific exon #3 (FIG #2). Therefore, mRNAs produced from the early promoter have a default splicing pattern that excludes the male-specific exon and produces a functional *Sxl* product. This exon skipping may be a result either of some special feature of the E1 exon, such as secondary structure or incompatibilities between splice donor and splice acceptor sites, or of a maternally derived splicing factor that is required for this exon skipping event.

To control female somatic sex-type in diplo-X animals, the *Sxl* locus acts solely through the *transformer* (*tra*) locus (McKeown et al., 1988). Like *Sxl, tra* gene also functions only in diplo-X animals (Baker et al., 1987) and is regulated at the level of alternative RNA splicing (Boggs et al., 1987). The first exon in *tra* can be spliced at either a sex-non-specific or female-specific site. In females an alternative 3' splice site is used to generate an mRNA with a long open reading frame encoding a functional *tra*

gene product (McKeown, 1992). A default site is used in males and about half of the time in females. Like the *Sxl* male transcripts, this default splice site results in an mRNA with a stop codon that does not produce a functional *tra* gene product. Alternative splicing is achieved by female-specific inhibition of the splice site used in males and is dependent on thirty-five nucleotides nearby the regulated splice-site (McKoewn, 1992). Sequence comparisons between *tra* and *Sxl* reveal this to be a conserved sequence, suggesting that *Sxl* regulates *tra* and *Sxl* directly by binding and competing with factors necessary for male-specific binding.

Unlike *Sxl* and *tra*, *transformer-2* (*tra-2*) produces identical transcripts in the somatic cells of both female and male flies, suggesting that *tra-2* may be expressed constitutively. However, like *tra*, *tra-2* is required for female differentiation, as an absence of *tra*-2 function leads to diplo-X animals developing as males. *tra*-2 appears, with *tra*, to regulate a third locus, *dsx*. The female specific product of *tra* works in unison with the product of the *tra-2* gene to control female-specific RNA splicing at the *dsx* locus, a locus which is functional in both males and females (Baker and Ridge, 1980).

Two *dsx* functions are achieved in the two sexes by sex-specific splicing of its pre-mRNAs. This ultimately leads to sex-specific proteins, dsx^{male} and dsx^{female}. In the absence of female-specific regulators, *Sxl*, *tra*, and *tra*-2, male-specific 3' splice sites and

polyadenylation sites are used. With the regulatory factors, a female-specific 3' splice site and polyadenylation site are used. This results is the production of two different proteins with a common sequence of 397 amino acids coupled to sex-specific C-terminal regions of 152 (male) or 30 (female) amino acids (Burtis and Baker 1987).

The female-specific splice site choice at *dsx* depends on the function of the *tra* and *tra*-2 gene products. In their absence, a male-specific pattern of processing occurs. Thus, all of the components necessary for female-specific splicing are present in males, except for *tra* or unknown factors that are induced by *tra*. In the presence of *tra* and *tra*-2, the otherwise inefficient dsx^{female} 3' splice is activated to produce a female-specific mRNA. The regulation of the splicing of the *dsx* pre-mRNA could result from activation of the female-specific splice and polyadenylation site or repression of the male-specific splice and polyadenylation sites, or a combination of these two mechanisms. However, considering that the *dsx* female-specific 3' splice site appears to have a poor consensus sequence, and deletions or mutations in a set of 6, 13-nucleotide repeats renders *dsx* incapable of responding to the regulatory effects of *tra* and *tra*-2, it has been suggested that regulation occurs by splice site activation (McKoewn 1992).

Genetic studies have shown that the products of the *dsx* locus are required for correct somatic sexual differentiation of both sex types. These same studies have also suggested that the sex-specific *dsx* product functions by repressing the expression of

terminal differentiated genes specific to the opposite sex. However, it has not been conclusively demonstrated whether *dsx* gene products function directly to regulate target genes or indirectly through other regulatory genes.

Regulation of the yolk protein (yp) genes provides an ideal system in which to look for direct interactions between regulatory and target genes. Yolk protein gene transcription is controlled by temporal, sex-specific and tissue-specific factors (Salz et al. 1989). The yp genes are expressed only in adult females, and are not detected in the larval or pupal stages. These genes also show expression limited to two tissues, the non sex-specific fat bodies, and the sex-specific ovarian follicle cells (Garabedian et al. 1985).

The yp transcription in the follicle cells is limited to one temporal stage of the follicle cell development. In the follicle cell, transcription of the yp genes first appears at stage 8, they are abundant through stages 9 and 10, and are scarcely detected at stage 11 (Brennen et al. 1982). Although the initial development of the ovaries is dependent on the presence of the female-specific regulatory genes, once the ovaries have formed, synthesis of the yp genes in the follicle cells are no longer under the control of the female-specific pathway (Bownes, 1990). Therefore, once the yps have been synthesized they are directly transported into the egg.

The yp synthesized in the fat bodies are detected approximately forty-eight hours after eclosion. After eclosion, the yp begin to be secreted into the hemolyph, where their

concentration rises from undetectable to approximately one third of the total hemolyph protein (Barnett et al., 1980). The yolk proteins are then selectively transported into the egg.

Previous studies have shown that proper sex-specific expression of the yp genes in the fat body requires continued action of the sex determination regulatory hierarchy (Belote, 1985). Furthermore, a 122 base pair fat body enhancer (FBE) of the yolk protein genes 1 and 2 (yp1 and yp2) is likely to be the target site for sex -specific regulation because this enhancer is sufficient to direct female-specific transcription of the yp genes in adult fat bodies (Shepherd et al., 1985). The dsx^{male} and dsx^{female} proteins appear to act as sequence specific DNA binding proteins, and interact with this FBE sequence in gel mobility shift assays (Burtis 1991). Because the two *dsx* proteins have similar binding specificities, it may be that the FBE binding domain exists within the amino acid sequence common to their amino-termini. Thus, either their C-termini, or a factor that specifically interacts with their C-termini, must provide sex-specificity to their action.

Genetic evidence suggests that, in diplo-X animals the dsx^{female} protein functions with the product of the *intersex* (*ix*) gene to repress genes involved in terminal male-type differentiation (Baker and Ridge, 1980). In haplo-X animals however, the dsx^{male} protein functions alone, without *ix* function to repress terminal female differentiation function. The mechanism by which *ix* and *dsx* act together to control terminal

differentiation is not well understood. Thus, while it is known that the *dsx*^{male} and *dsx*^{female} proteins can bind *in vitro* to the FBE of the yp gene, a gene specifically transcribed in females that functions in terminal sexual differentiation (Burtis et al., 1991), it is unclear how *ix* acts within this context to repress male-specific gene expression in chromosomal females. In particular, it is not known if *ix* has any role in sex-specific yp gene expression.

One hypothesis addressing this issue (Burtis et al., 1991) is that the male-specific C-terminal domain on *dsx*^{male} interferes with the function of an activator protein bound to female-specific enhancers and the female-specific C-terminal domain of *dsx*^{female} interferes with the function of an activator protein bound to male-specific enhancers. Therefore, the binding of *dsx*^{female} *in vivo* to the FBE would not, in itself, necessarily result in the repression of the yp genes. A second hypothesis (Burtis et al., 1991) is that *dsx*^{female} binds to the FBE *in vitro* but not *in vivo*. In this model, the unique domain of *dsx*^{female} interacts with another protein (perhaps *ix*) or is modified to alter its binding specificity and change its set of target genes. Experiments using P-element transformation of a cDNA for *dsx*^{female} (to express small quantities of the female-specific gene product) demonstrated a significant derepression of yp1 mRNA expression in haplo-X animals (Burtis and Baker, 1989). Since *ix* is not thought to be required in haplo-X animals, these experiments may be explained by supposing that the *dsx*^{female} protein can interfere with the formation of the

dsx^{male} protein. Alternatively, the dsx^{female} protein may act to regulate *ix*. In any event, it remains an open question whether *ix* functions together with dsx^{female} or acts independently of *dsx* to control female development.

As ix is the most terminally positioned gene in the sex-differentiation regulatory hierarchy, it should serve to link the hierarchy with the implementation of terminal sexual-differentiation. An understanding of *ix* function should therefore provide significant insight into how terminal differentiation is implemented. Unfortunately, *ix* is the least well- characterized gene in the sexual differentiation pathway in Drosophila. Although *ix* function is required for normal female development, and it is thought to act with *dsx* to repress male-specific functions, the regulatory factors produced by *ix* have not been identified, and the temporal requirements for its function have not been determined. For other regulatory loci, such studies have provided insight as to where in the regulatory pathway they are active and with additional genetic analyses, suggested possible functional roles.

To further address the mechanism by which *ix* functions to control terminal sexual-differentiation, I have determined the times during development when *ix* function is required and assessed whether *ix* function is required in the adult to maintain female-specific gene expression. These studies have provided insight into how *ix* functions within the regulatory sex-determination hierarchy.

MATERIALS AND METHODS

Temperature shift assay

Diplo-X animals bearing a non-functional *ix* allele develop into adults that display both female and male characteristics. One of the most sensitive measures of "intersexuality" is infertility, as intersexual individuals cannot produce offspring because of deformed genitalia and analia. Using a temperature-sensitive allele for *ix,* the gene can be turned "off" or "on" during development by placing animals at restrictive or permissive temperatures, respectively. Thus, the temporal requirement for *ix* function can be determined by assessing the fertility of flies with temperature-sensitive *ix* function that have been raised for defined developmental periods at a restrictive temperature. The ability of such temperature-shifted flies to produce offspring was used to indicate whether *intersex* function was disrupted at a developmental period during which *intersex* was normally required. Temperature-sensitive *intersex* diplo-X flies were obtained by crossing ix^{ph}/ SM5, Cy with $Df(2R)en^{B}$ /CyO where ix^{ph} is a temperature sensitive *ix* allele on a multiply inverted chromosome that is recessive lethal, $Df(2R)en^{B}$ is a deletion of cytological region 47E3-6;48A that removes *ix* function, and SM5 and CyO are balancer

chromosomes, bearing the dominant Cy (Curly wing) mutation. The straight wing progeny that are ix^{ph}/ Df(2R)en^{B} raised throughout development at a permissive temperature of 18°C produce diplo-X adults that are normal females. When raised at a restrictive temperature of 29° C, diplo-X adults are sterile and intersexual, displaying both male and female characteristics.

To assess when *intersex* is required for fertile female fertility then, female ix^{ph}/ SM5, Cy flies were crossed with Df(2R)en^{b} /CyO males, and the reciprocal of this cross, ix^{ph}/ SM5, Cy males with Df(2R)en^{b} /CyO females, was performed. Virgin female animals were collected to carryout this cross. Virgin female flies were collected by screening for newly eclosed adults three or four times a day. This allowed for female flies to be collected shortly after eclosion, reducing the chances of their being inseminated. Females were kept in vials with food at room temperature for three to four days and the food examined for production of larvae to assure the females collected were virgin. Approximately 125 - 150 females and 150 - 175 males were mated in bottles containing standard *Drosophila* media (molasses / agar / cornmeal / sucrose). To be able to restrict *ix* function at different developmental periods, a series of timed egg lays was performed. To this end, females were allowed to oviposit in fresh bottles for 24 hours at the permissive temperature of 18^{0}C, and then transferred to new bottles. This procedure was repeated until progeny from the first egg lay

began to eclose. At this time, the remaining bottles were shifted to the restrictive temperature of 29°C. This produced a set of experimental treatments that differed by increments of 24 hours at the restrictive temperature and spanned from fertilization to eclosion. A parallel set of experiments in which animals was shifted from 29°C to 18°C in 12 hour intervals were also performed. After eclosion, single straight-wing diplo-X adults (ix^{ph}/ Df(2R)en^{b}) were collected, allowed to mate with Canton-S (wild-type) males, and placed in individual vials with media. Individual female siblings were also placed in vials with Canton-S males as a control. These crosses were performed at room temperature (22-25°C). Fertility was assessed by examining the media for larval activity.

Assessment of ix control over yp1 transcription

Some regulatory genes are only necessary for initially determining a developmental pathway, while others are essential throughout the life of an organism to both set and maintain a pathway. If *ix* is required to maintain female-specific gene expression in diplo-X animals, one would expect a decrease in female-specific gene expression in the absence of *ix* function. One abundant gene product nearly exclusively seen in adult females is the product of the yolk protein gene, yp1. This

gene is expressed in the fat bodies (a non-sex-specific tissue) and the follicle cells (associated with the ovaries and a sex-specific tissue). To test whether *ix* regulates the female-specific expression of this gene in adults, temperature-sensitive *ix* function was again employed. First, diplo-X adults bearing temperature-sensitive *ix* function were obtained (as just described) that were normal, phenotypic females having been raised at the permissive temperature. These flies were then shifted to the restrictive temperature for defined periods of time to eliminate their ix^+ function and allow assessment of *ix* function on yp expression. Cy siblings bearing ix^+ function exposed to the same conditions were used as controls.

Isolation of RNA

RNases were removed from glassware by baking at 180°C for 24 hours, and from plasticware by washing with detergent, rinsing with dH_2O, drying with ethanol, soaking in 3% H_2O_2 for 10 minutes at room temperature, and rinsing with 0.1% DEPC (Diethylpyrocarbonate) treated H_2O.

Total RNA was isolated by a hot phenol extraction method (W. Mattox, personal communication). Flies were homogenized in a solution of 10 mM Tris-HCl pH 7.6 equilibrated phenol and 2x NETS (200 mM NaCl, 2mM EDTA, 20 mM Tris-

HCl pH 7.5, 0.1 % SDS) at a ratio of 2:1 heated to 65°C. A total volume of 800 ul was used for 20-30 flies. The homogenate was centrifuged for 10 minutes at 12,000g and the aqueous phase was aspirated from the organic phase. One back extraction of the organic phase was performed by adding 300 ul 2x NETS, mild vortexing and centrifuging as above. The aqueous phases were combined and two more extractions performed using a volume of phenol equal to that of aqueous solution. The RNA was precipitated from the aqueous solution by adding two and a half volumes of ethanol at -20°C for at least one hour. The RNA was pelleted by centrifugation (10 minutes at 12,000 g) and the ethanol was removed by aspiration. The pellet was washed with RNase free 70% ethanol (100% ethanol : 0.1% DEPC H_2O) dried in a Speed-vac centrifuge. The pellet was resuspended in 50 ul Elution buffer (10 mM Tris-HCl pH 7.6, 1mM EDTA, Ph 8.0, 0.05% SDS). 10 ul of the suspension was removed and quantified by spectrophotometry. The remaining RNA was stored at -70°C.

Northern Analysis

Total RNA was separated on a denaturing formaldehyde MOPS 1.5% agarose gel (as described in Sambrook et al., 1989) at 50 volts for approximately 7 hours. The time was determined empirically by visual inspection of a tracking dye (80%

formamide, 10mM EDTA at pH 8.0, 1mg/ml xylene cyanol FF, 1 mg/ml bromophenol) loaded with each sample. The RNA was then transferred to a MSI Magna NT nylon membrane. Prior to transfer, the gel was treated by soaking in two changes of a 10X solution of SSPE to remove the formaldehyde, then in a denaturing solution of 0.05 N NaOH and 0.15 M NaCl for 30 minutes, and finally in a solution containing 0.1 M Tris pH 7.5 and 0.15 NaCl for 30 minutes. This treatment of the gel is in accordance with the manufacture's instructions accompanying the Possiblot^{tm} pressure blotter for probing transcripts the size of yp1 (approximately 1.7 Kb). The RNA was transferred to the membrane by using the Possiblot^{tm} pressure blotter at 75 mm Hg overnight. The RNA was cross-linked to the membrane with ultraviolet light (254 nm) for thirty seconds at 12,000 microjoules/cm^2 in the Stratalinker UV crosslinker 1800, and/or by baking at 80^{0}C under a vacuum for one hour.

DNA probes were labeled with ^{32}P by random oligonucleotide priming using the Ambion Decaprimer^{tm} DNA labeling kit. Reactions were performed as described in the manufacture's instructions using 25ng of linearized yp1 (Garabedian et al., 1986) template DNA and 25 ng of linearized rp49 DNA (O'Connell andRobash, 1984) (both isolated from a plasmid) .

Hybridizations with radioisotopically labeled probes were carried out at 42°C in a 0.25 ml/cm^2 (of blot) volume of hybridization buffer containing 50% formamide, 5X

SSPE, 0.1% SDS, 1X Denhardt's Reagent (0.02% Ficoll, 0.02% Polyvinylpyrollidone, 0.02% Bovine Serum Albumin), and 100 mg/ml denatured, sheared fish-sperm DNA. Washes were carried out in 5 ml/cm^2 0.1X SSPE,0.1% SDS at 50°C with one change of wash solution after 10 minutes and three changes of wash solution after successive 20 minute intervals.

The blots were then scanned at the University of Nebraska Medical Center on the Phosphor-Imager to quantitate the level of yp1 expression. The blots were also exposed to X-ray film at -70°C.

RESULTS

To determine when during development *ix* function was required in diplo-X flies to produce fertile females, the progeny of the cross ix^{ph}/ SM5, Cy with w; Df(2R)en^{b} /CyO were shifted between restrictive and permissive temperatures at defined stages of development. As expected, Df(2R)en^{b} / ix^{ph} diplo-X animals raised at a permissive-temperature throughout their development developed as phenotypic females. When this genotype was raised at a restrictive-temperature until the mid-pupal period and then shifted to a permissive temperature, phenotypic females also developed. In contrast, *ix* temperature-sensitive diplo-X animals kept at a restrictive-temperature past the mid-pupal period, or raised at a permissive-temperature and shifted to the restrictive-temperature before the mid-pupal period were sterile (Figure #3 and #4). Sibling controls showed normal fertility in all treatments. Therefore, for female fertility to be retained, *ix* function must be present during the mid-pupal period (Figure #5).

From these data, *intersex* temperature-sensitive diplo-X flies raised at the permissive-temperature until eclosion develop as fertile, phenotypic females in the adult. To understand how ix might control sex-specific transcription in the adult, female-specific gene expression was assessed such animals were raised at the permissive temperature until eclosion, and then either kept at the permissive-

temperature or shifted to a restrictive temperature.

The effect of *ix* function on yp1 transcription was addressed through Northern blot analysis. Total RNA was extracted from flies kept at the permissive temperature after eclosion and shifted to the restrictive temperature after eclosion from diplo-X animals containing the temperature-sensitive allele for *ix* ($Df(2R)en^{b}/ix^{Ph}$) and their sibling controls. After the RNA was size-separated and blotted, and the resulting Northern blot probed with a ^{32}P-cDNA of the yp1 gene (Figure #6), the relative amount of yp1 transcripts as assessed within the RNA samples. The amount of yp1 transcription was quantified by scanning the blots with a phospho-imager and performing a volume integration of the pixel density of bands resulting from the hybridization of ^{32}P-labeled cDNA with yp1 RNA transcripts (Figure #7).

To assess the change in levels of transcription between the experimental and control groups, the amount of RNA present within each lane was normalized to the level of transcripts from the gene rp49 (Figure #6). The amount of rp49 transcripts should be the same in flies of the same experimental treatment (Robash,$^%%). Hence, a profile of the level of yp1 normalized to the level of RP49 for experimentally treated flies will show how steady state yp1 transcription varies with experimental treatment. In turn, a dramatic drop in yp1 expression in the absence of *ix* function will indicate that *ix* normally positively regulates (indirectly or directly) the

transcription of yp1.

To asses whether the genotype, [Df(2R)en^{b}/*ix*^{Ph}], had any effect on the transcription of the yp1 gene, the ratios of the volume integration values , yp1/ RP49, were plotted for animals kept at the permissive temperature for 1, 4, 8, 12, 16, and 20 days after eclosion (Figure #8). This graph provided a reference to the transcriptional level of yp1 this genotype, and compared to the sibling controls, when *ix* function was present. A comparison of the range of yp 1 expression in these groups with ranges found in previous experiments (Bownes 1983), indicates that this genotype itself does not alter steady state yp1 expression.

The effects of transcription on the female-specific gene, yp1, in diplo-X animals lacking *ix* function was determined by assaying relative yp1 transcription in Df(2R)en^{b}/*ix*^{Ph} female and sibling controls after they were placed at the restrictive temperature for 4, 8, 12, 16, and 20 days after eclosion. Even after 20 days at the restrictive temperature, substantial amounts of yp1 transcripts were seen in animals lacking *ix* function (Figure #9). Since the half-life of the yp1 transcripts is only approximately 12 hours (Bownes and Williams, 1985) and that yp1 expression dropped to levels barely detectable in *tra*^{ts} mutants after only 12 days at a restrictive temperature (Belote et al., 1985), an absence of *ix* function appears to have no effect on transcription of the yp1 gene in adult diplo-X animals.

DISCUSSION

In somatic tissues, sexual differentiation appears to be under the control of a small number of regulatory genes arranged in a regulatory hierarchy fashion (reviews: Baker and Belote, 1883, Baker, 1989; Cline, 1993). At the apex of the hierarchy lies *Sxl,* which acts in diplo-X animals to initiate a cascade of female-specific splicing of downstream regulatory genes. This culminates in female-specific transcripts produced at *dsx*. *Sxl*, *tra*, and *tra*-2 act within this cascade and must be active to obtain dsx^{female} protein. If any of these genes is inactivated by mutation, *dsx* is spliced using a default pattern and produces a male-specific protein. A fifth gene, *ix*, acts at the same level as *dsx*. While the molecular regulation of *ix* has not yet been characterized, it is thought to function like dsx^{female}, as the absence of a functional *ix* allele produces an intersexual phenotype identical to the absence of a functional *dsx* allele in diplo-X animals.

To better understand how *ix* functions in this pathway, it is essential to know when during development *ix* is required for normal female development. This information, with genetic analysis of genes acting upstream of *ix* allows for the development of testable models that address the means by which regulatory genes control *ix* expression as well as the function of *ix*.

The transcriptional profiles for the genes that function upstream of *ix* in the

sex-determination regulatory cascade have been determined and mirror when these genes are known to be required to function. Keyes and Cline (1992) showed that early female-specific *Sxl* transcripts produced briefly during the syncitial blastoderm stage were required for later female-specific transcription profiles at times in development when a constitutive promoter is used. Transcripts at *tra*-2 were detected starting in the second larval instar, and found throughout adult life, times when *tra*-2 has been shown to be needed to function. Sex-specific *tra* and *dsx* transcripts are seen in diplo-X third instar and continue throughout adult life, when *tra* and *dsx* function is known to be required. In each of these cases, requirements for gene function parallel the transcriptional profiles observed.

Not only do individual gene transcriptional profiles parallel their functional requirements, the interdependence of one gene's transcriptional profile on another's reflects the epistatic interactions between them. Female-specific *Sxl* mRNA splicing is required for female-specific *tra* mRNA splicing as well as the autoregulatory *Sxl* female-specific splicing. Female-specific splicing of *tra* is then required for the female-specific *dsx* splicing.

Results presented in this thesis demonstrate that *ix* is required to function at least between the embryo and mid-pupal period. as *ix* function is required at this time to produce fertile females. Thus, it may be predicted that *ix* transcription will be

required during this time as well. Previous studies have shown that *ix* mutations do not alter *dsx* transcriptional profiles. Taken with the results presented here, this suggests that either (1) *dsx* controls *ix* function (directly or indirectly) or (2) *dsx* functions in parallel with *ix*.

Given the temporal requirements for *ix* function that have been demonstrated here, which overlap when sex-specific splicing occurs at *dsx* pre-mRNAs, one can propose a number of hypotheses as to how *dsx* and *ix* may function in parallel. However, before considering potential molecular mechanisms for their interactions, it will be useful to review prior experiments on the control of yp transcription by the sex-determination regulatory hierarchy and, more specifically, consider implications of experiments presented here in regards to *ix* control over yp expression.

Belote (1985) demonstrated that fat body yolk protein expression is under the control of the sexual regulatory hierarchy pathway. Using a temperature-sensitive mutation (*tra*-2^{ts}) of the *tra-2* gene, synthesis was shown to be dependent on a functional *tra-2* gene product. Diplo-X *tra*-2^{ts} heterozygotes reared at the permissive temperature develop as normal fertile females and display female-type yp expression. However, diplo-X *tra*-2^{ts} homozygotes reared at the restrictive temperature develop as pseudomales and display male-type yp expression (i.e., none). In this experiment, diplo-X animals were allowed to develop as normal females at a permissive

temperature and shifted to a restrictive-temperature two days after eclosion. The steady state level of yp1 transcription in these flies persisted for several days; however, after three days the levels of the yps began to drop off significantly. After thirteen days the yps were only present at very low levels relative to wild-type controls. Hence, the decrease in yps was a result of eliminating the function of *tra*-2, and is a result of transcriptional control.

The role of *dsx* is controlling yp expression has also been examined. Diplo-X animals that are dsx^{D}/dsx^{+} develop as intersexual animals which often have only partially developed ovaries. Comparison of yp hemolymph levels, (which reflect fat body yp expression) between dsx^{D}/dsx^{+} and wild-type flies showed that yp levels are greatly reduced in dsx^{D}/dsx^{+} animals (Bownes, 1983). Using diplo-X animals heterzygous for the dsx^{D}, $dsx^{D}/+$, the transcription and translation of the yp genes were analyzed from animals up to ten days after eclosion. The level of transcription in these intersexual animals was not significantly different from wild-type controls, although one population of dsx^{D} animals did produce transcripts several fold higher then the same population of wild type-controls. However, the level of yp present in the hemolymph was dramatically reduced in all the dsx^{D} populations compared to their wild-type controls. Further analysis of the fat body tissue showed that the lack of yp in the hemolyph was not due to a secretion deficiency but rather to a decreased rate of

translation. A mechanism for decreased efficiency in translation is not known; however, it is not related to structural aspects of the mRNA population, for the mRNA was equivalent to wild-type mRNA in a cell-free translation system. It is important to keep in mind that these results may not reflect the direct control by *dsx* of yp transcription, as yp transcription in dsx^{D}/dsx^{+} animals may also be impacted by a feedback mechanism in which the amount of ovarian tissue indirectly regulates the amount of yp expression. Therefore, because of the intersexuality of the dsx^{D}/dsx^{+} animals it is difficult to make inferences that *dsx* directly controls yp transcription from these data alone.

Indeed, all previous experiments used to assess whether *dsx* or *ix* directly regulates sex-specific regulation in somatic cells have used diplo-X adults flies which have developed as phenotypically intersexuals. While an earlier experiment (Bownes and Nothiger, 1981) on the effects of *ix* function on yp expression in diplo-X animals demonstrated that diplo-X flies lacking a functional *ix* allele had a reduced level of yolk protein circulating in the hemolymph. These *ix* flies exhibit a wide range of intersexual phenotypes. Since the synthesis of yolk proteins may be related to the extent of intersexuality (for example, intersexes which have a more male phenotype may show a greater reduction of yolk protein synthesis than intersexes having a more female phenotype), these experiments are difficult to interpret. Furthermore, in these

experiments the effects of *ix* were assessed on yp protein levels, by determining the concentration of yp secreted into the hemolymph, and *ix* effect on yp transcription was not assessed. The decreased amount of yps found in the hemolyph in these intersexes could also be due to a decreased rate in translation, as was seen in the dsx^{D}/+ flies performed by Bownes (1983). Therefor, the effects that the functional product of *ix* exerts on the transcriptional regulation of the yp genes has not been addressed directly.

To address more directly the control by *ix* on yp transcription, the experiments presented here were performed using a temperature-sensitive allele for *ix* and diplo-X adults that developed as phenotypic females (identical to their sibling controls). The function of *ix* was turned off by shifting the mutants to the restrictive temperature after eclosion. The role ix^{+} plays in regulating the control of yp transcription in adult females was assessed through analysis of the level of yolk protein transcripts (yp1) present in these flies compared to sibling controls exposed to the same conditions. The results suggest that *ix* does not function to regulate sex-specific transcriptional control of the yp1 gene. Thus, previously reported results of reduced yp levels in the hemolymph in *ix* flies appear to be the result of assaying an intersexual phenotype. However, the possibility remains that the earlier results might reflect translational control of yp, similar to that seen in dsx^{D} animals.

More solid support for *dsx* control over yp transcription comes from molecular

analysis. A 127 base pair enhancer element of the yp1 and yp2 genes is likely to be target of sex-specific regulation as this enhancer is sufficient to direct the female-specific transcription of the *yp* genes in adult fat bodies (Garabedian et al., 1986). Burtis and Baker (1991) have used DNA footprinting experiments with in *vitro* expressed dsx^{male} and dsx^{female} protein to demonstrate that these bind specifically to the fat body enhancer (FBE). While this provides a molecular mechanism by which sex-specific products of the sex-determining regulatory hierarchy control the target genes involved in terminal somatic differentiation, it does not, *per se*, provide a mechanism for sex-specific control; both dsx^{male} and dsx^{female} products bind to the FBE.

Genetic analysis of the sex-determining regulatory pathway has suggested that it acts through negative regulation. Each sex-specific *dsx* product is thought to repress the expression of gene products needed for terminal differentiation in the opposite sex. This view stems from the fact that in the absence of either *dsx* product, both diplo-X and haplo-X animals, display identical intersexual characteristics.

Given the sex-specific carboxy-termini of the *dsx* proteins, a number of models may be formulated as to how *dsx* negatively regulates the FBE. A hypothesis presented by Burtis and Baker (1989) is that in males, dsx^{male} binds to the FBE *in vivo* (inhibiting transcription of the yp genes), while in females, dsx^{female} does not bind the FBE in *vivo*, even though it binds in *vitro*. This change in specificity for the FBE

results from the interaction of dsx^{female} protein with that of another female-specific regulatory gene. One candidate gene product for this interaction is thought to be that of *ix*.

The results presented here show that a lack of *ix* function in phenotypic females has no dramatic effect on yp1 expression. These results therefore suggest that *ix* function is not required to positively control female-specific gene expression, consistent with the idea that *ix* functions as a negative regulator.

If dsx^{female} protein fails to interact with the FBE due to its interaction with *ix* protein, and *ix* protein is not positively regulating yp1 expression, then one would expect that in a simple model, *ix* blocks *dsx* from negatively regulating yp1 expression. The presence of *ix* protein in diplo-X animals blocks *dsx* from binding to the FBE, and consequently, yp transcription proceeds. It would seem to follow that in the absence of *ix* function then, dsx^{female} would bind the FBE and yp expression would be limited. This is not seen however. Therefore, at this simplistic level, the proposal by Burtis and Baker is untenable.

Could *ix* protein still interact with dsx, but not be a negative regulator? Suppose *ix* protein acted with dsx^{female} so that it would still bind to the FBE, but this binding then results in stimulation of yp expression, and not inhibition in its absence. This model too would seem untenable, as in the absence of *ix* function, one would

expect decreased stimulation. Logically then, these simple models seem equivalent, and neither fully explains the data here.

How might these data and models be reconciled? One simple, and untested possibility is that *ix* does not act in the adult. An absence of *ix* function due to a temperature-sensitive product would not appear any different than a "natural" absence of *ix* function. Under this scenario, dsx^{female} might bind to the FBE, and because of its female-specific carboxy-terminal, stimulate yp expression independent of any other regulatory gene. The function of *ix* would therefore be required only up to the mid-pupal period for female-type cellular differentiation, as shown by the temperature-shift/fertility experiment presented here.

A second model would propose that *ix* function is required in the adult, but only to repress male-specific functions. It may or may not interact with *dsx*, but if it does, only at the loci normally expressed in males. Experiments to test this hypothesis are currently underway by other workers in the laboratory in which this work was performed. Clearly, detailed knowledge of the transcriptional profile and protein product of *ix* would aid in the resolution of these issues.

A third model intended to explain the need for *ix* and dsx^{female} function to produce a female phenotype in diplo-X animals may be generated by considering the newly obtained data. In this model, dsx^{female} acts as a positive regulator for terminal

female-specific gene expression. Furthermore, *dsx*^{female} acts in conjunction with *ix* to repress male-specific gene expression. This cooperative interaction could proceed through a heterodimer between the two gene products or they could act independently of one another to recognize cis-acting DNA elements that repress transcription. *dsx*^{male} would then act as only a negative regulator of female-specific gene expression in haplo-X animals.

Previous evidence (Burtis et al., 1991) has shown *dsx*^{female} can activate female-specific gene expression in haplo-X adults. Using P-element transformation (Burtis and Baker, 1989) a cDNA copy of *dsx*^{female} was transformed into haplo-X flies. Expression of this female-specific regulatory cDNA gene or a gene controlled by it significantly derepressed the expression of the yp 1 gene. However, the mechanism for this derepression is unknown at the present time. Based on the observations that the FBE is able to direct the sex-specific expression of the yp genes, and *ix* is not required in haplo-X individuals, this model would predict that *dsx*^{female} functions by positively regulating yp1 gene expression by binding to the FBE and inducing transcription.

The function of *dsx*^{female} and *ix*, in this model, would be to coordinately repress male-specific terminal differentiation. Since, diplo-X flies lacking either *ix* or *dsx*^{female} produce similar phenotypes which possess both male and female characteristics, these

two female-specific regulatory genes are thought to repress male differentiation in diplo-X animals. However, in this model the specificity for male-specific gene repression is generated through direct interactions of the functional gene products of both *ix* and dsx^{female}. This interaction emerges from the fact that neither *ix* nor dsx^{female} is able to repress male gene expression by itself.

Figure #1. Model for the Sex Determination Regulatory Hierarchy

In this figure, arrows represent steps of positive control, while a bar at the end of a line indicates negative control. In males, the regulatory pathway is relatively simple, and a single gene, *doublesex*, acts in a male-specific mode to repress female differentiation. In females, an X:Autosome ratio of 1:1 leads to the sex-specific transcriptional activation of *Sex-lethal*, which in turn leads to a cascade of sex-specific RNA splicing events that ultimately cause the *doublesex* locus to be expressed in a female-specific manner. The *doublesex* female product acts with that produced by *intersex* to repress male differentiation, thereby allowing female differentiation to ensue.

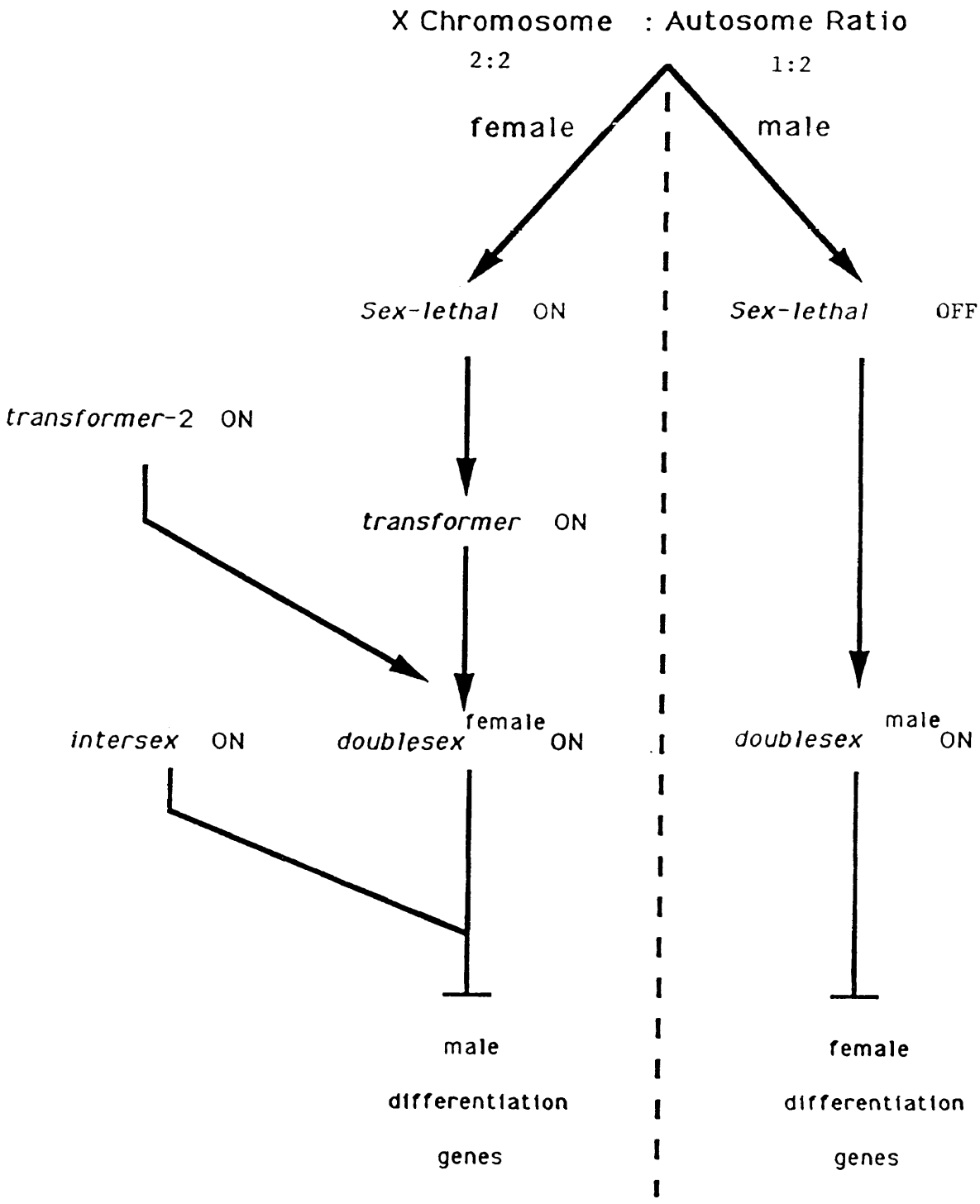

X Chromosome : Autosome Ratio
2:2
1:2
female
male
Sex-lethal ON
Sex-lethal OFF
transformer-2 ON
transformer ON
intersex ON
doublesex female ON
doublesex male ON
male differentiation genes
female differentiation genes

Figure 2. Model for Regulation of *Sxl*

This figure illustrates a simplified model (Keyes, 1992) for the pathway initiated through transcriptional regulation of *Sxl*. The actual details of the activation process are likely to be more complex than shown here. For example, there are many additional maternal and zygotic regulatory proteins that could be involved in promoter activation.

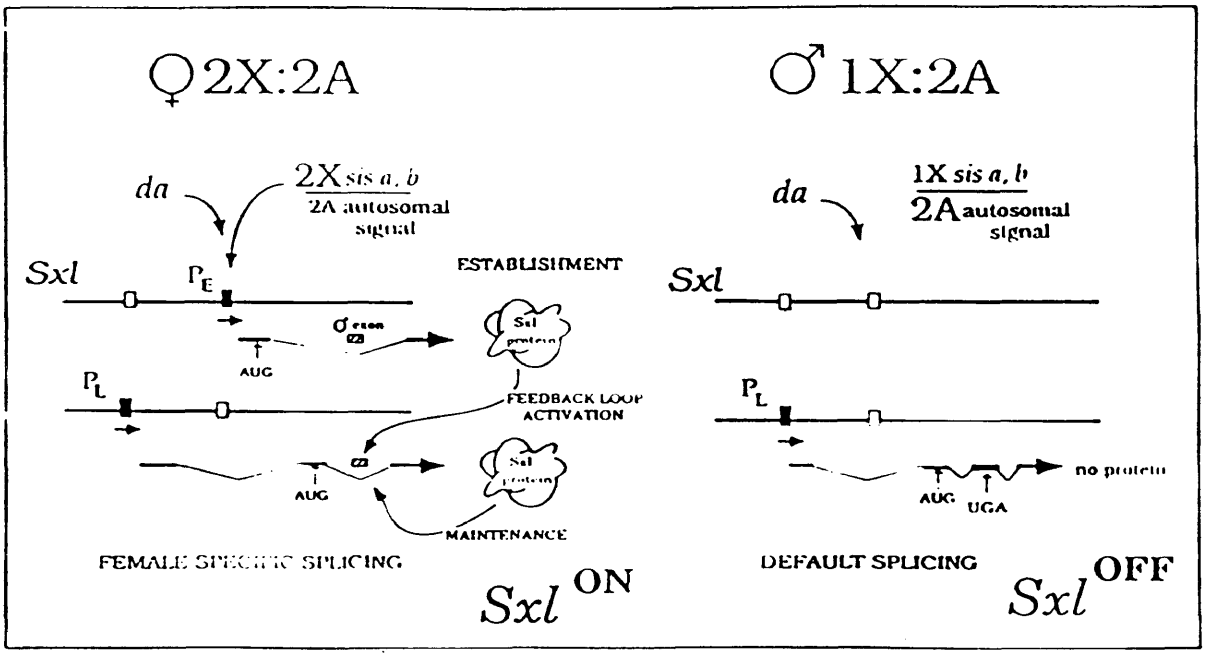

♀2X:2A
♂1X:2A
da
2X sis a, b
2A autosomal signal
Sxl
P_E
ESTABLISHMENT
AUG
Sxl protein
P_L
FEEDBACK LOOP ACTIVATION
AUG
Sxl protein
MAINTENANCE
FEMALE SPECIFIC SPLICING
Sxl ON
da
1X sis a, b
2A autosomal signal
Sxl
P_L
AUG
UGA
no protein
DEFAULT SPLICING
Sxl OFF

Figure 3. Temporal Requiremenof *ix* Function for Fertility.

At 18°C the fertilized egg is able to develop into a mature adult in approximately seventeen days. The pupal stage of development is reached after approximately 10 days. When *ix* function is limited before the mid-pupal stage (13-14 days), diplo-X animals develop as sterile females. On the other hand, if *ix* function is present up to or at this stage, these individuals develop as fertile females.

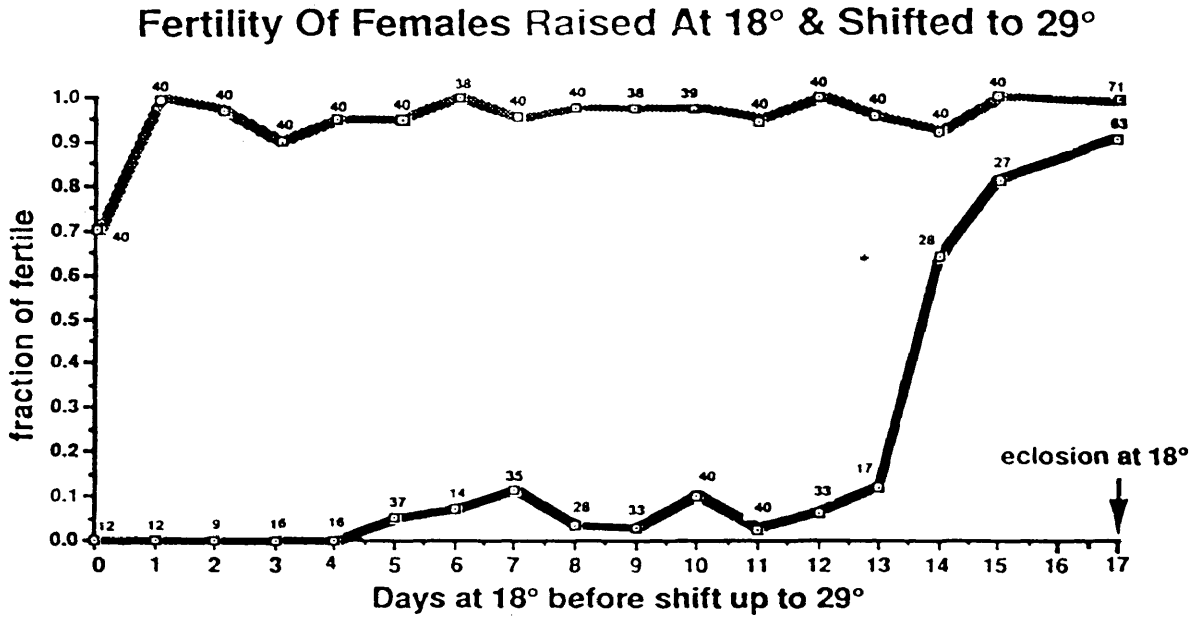

Fertility Of Females Raised At 18° & Shifted to 29°
fraction of fertile
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Days at 18° before shift up to 29°
eclosion at 18°
40 40 40 40 40 40 38 40 40 38 39 40 40 40 40 40 71
40 12 12 9 16 16 37 14 35 28 33 40 40 33 17 28 27 63

Figure 4. Restoration of Fertility by Providing *ix* Function.

At 29^0C the fertilized egg develops into a mature adult in about sixteen half-days (8 days). The pupal stage of development at this temperature is reached at about 9 half-days (4 1/2 days) after fertilization. If *ix* function is not restored before the mid-pupal stage (11 half-days) the flies develop as sterile females. However, if *ix* function is restored before the mid-pupal stage, they develop as fertile females.

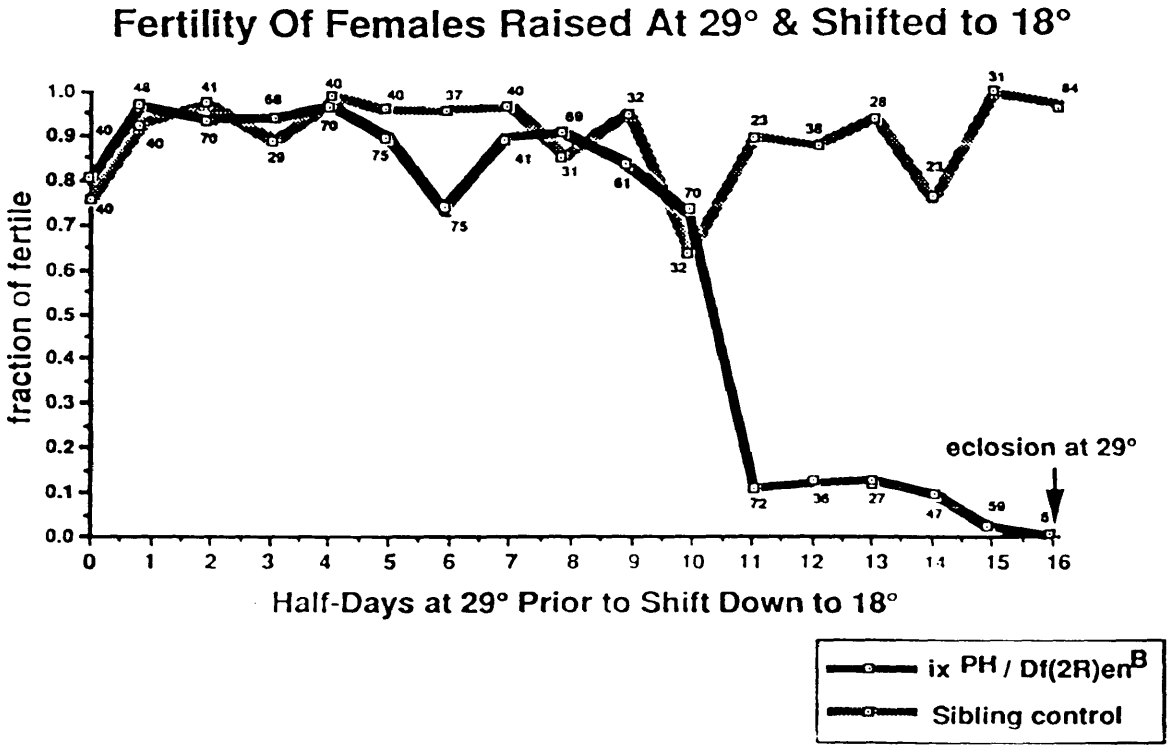

Fertility Of Females Raised At 29° & Shifted to 18°
fraction of fertile
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Half-Days at 29° Prior to Shift Down to 18°
eclosion at 29°
ix PH / Df(2R)enB
Sibling control

Figure 5. Transcriptional Activity of the Sex-Determination Regulatory Hierarchy.

This developmental time plot is based on observations of flies developing at 25°C. The segments for *sis*, *Sxl*, *tra*, *tra*-2, and *dsx* indicate the time during development that transcripts are present in diplo-X flies. The segment for *ix* spans the time period that this gene is required to produce fertile females. Arrows at the end of the lines indicate that these genes are required through the adult stage.

**tra* is expressed at a very low rate in early development but, increases to a much higher level that corresponds to the temporal need for *tra* in the sexual regulatory pathway.

**While I have shown that *ix function is* necessary during the mid-pupal stage, my data does not clearly demonstrate its requirement in the adult female. Therefore, the terminal part of the line is indicated by a dashed line.

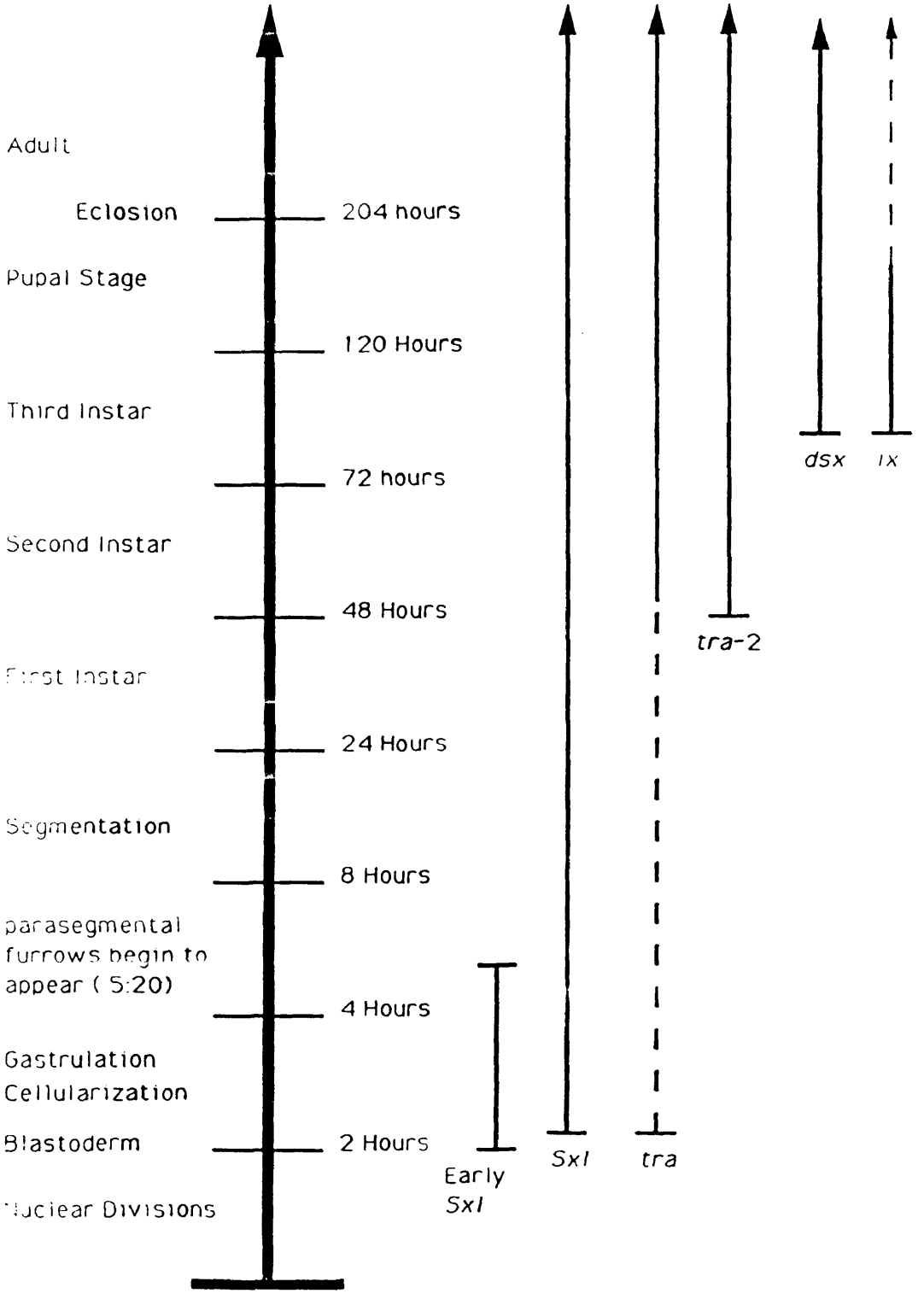

Adult
Eclosion
204 hours
Pupal Stage
120 Hours
Third Instar
72 hours
Second Instar
48 Hours
First Instar
24 Hours
Segmentation
8 Hours
parasegmental furrows begin to appear (5:20)
4 Hours
Gastrulation
Cellularization
Blastoderm
2 Hours
Nuclear Divisions
Early Sxl
Sxl
tra
tra-2
dsx
ix

Figure #6. yp1 and rp49 Expression in Females Having ix^{ts} Function.

Diplo-X Df(2R)en^{b}/ix^{Ph} (*ix*) and sibling controls (+) kept at the permissive temperature or shifted to the restrictive temperature for various periods of time after eclosion. The numbers above each pair of *ix* and + represent the number of days after eclosion transcription was assayed. Total RNA was extracted, size-separated by denaturing electrophoresis, and blotted. The Northern blot was sequentially probed with ^{32}p-labeled yp1 and rp49, and a autoradiograph obtained. A composite of the two autoradiographs is shown here.

There is considerable expression of yp1, relative to sibling controls, even after females spent 20 days at the restrictive temperature.

YP1 Transcription in Females Having Temperature-Sensitive *intersex* Function

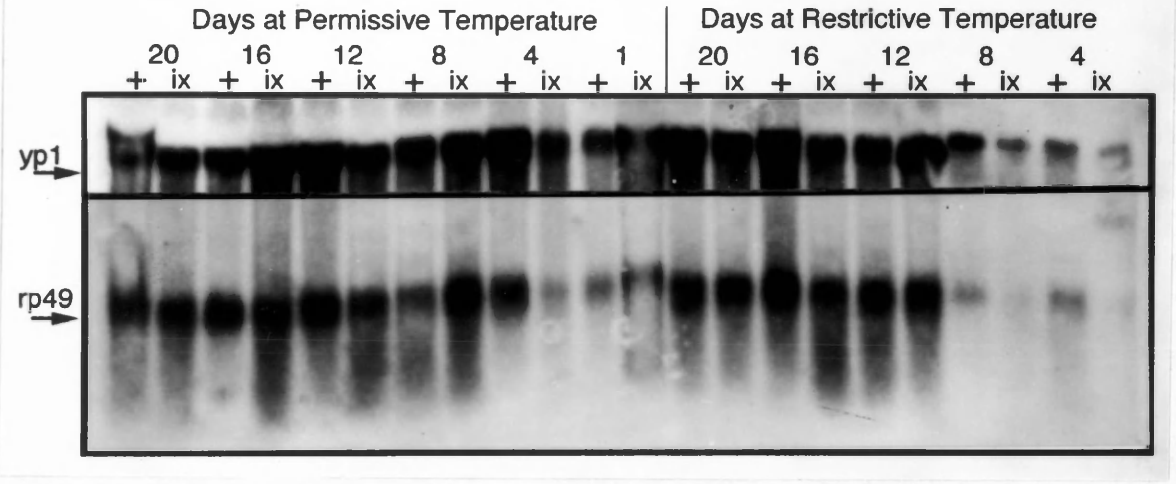


Figure #7. Quantitative Analysis of yp1 Transcription in *ix*^{ts} Females.

The blot described in figure 6 was quantified by a volume integration (using the Image Quant program), that summed all pixels minus the background within a set area specified on the blot. This was performed to give the indicated values which correspond to samples kept at the permissive-temperature, samples shifted to the restrictive temperature. (ix) indicates samples that were diplo-X Df(2R)en^{b}/ix^{Ph}, and (+) indicates samples that were sibling controls. (Time) refers to the number of days after eclosion transcription was assessed. The yp1 value divided by the rp49 value gives the ratio, which will be used to compare transcriptional level between the *ix*^{ts} and the sibling controls.

SAMPLES KEPT AT THE PERMISSIVE TEMPERATUE

INTERSEX

Time	yp1	rp49	Ratio
1	255927	29585	8.65
4	2122501	98220	24.61
8	795683	43617	18.24
12	3064281	122792	24.96
16	1385535	96262	14.39
20	517569	95919	5.4

SIBLING CONTROL

Time	yp1	rp49	Ratio
1	401703	51918	7.74
4	349811	19042	18.37
8	1304343	154233	8.46
12	753748	76649	9.83
16	24909990	85565	29.11
20	882934	75470	11.70

SAMPLES SHIFTED TO THE RESTRICTIVE TEMPERATURE

INTERSEX

Time	yp1	rp49	Ratio
4	71519	20108	3.56
8	398003	11387	34.95
12	509170	72811	6.99
16	2579165	103264	8.04
20	968584	71564	13.53

SIBLING CONTROL

Time	yp1	rp49	Ratio
4		6642	
8	72063	4680	15.4
12	1361038	72717	18.72
16	476223	59221	8.04
20	657059	71954	9.13

Figure #8. Level of yp1 Expression in the ix^{ts} and Sibling Controls Kept at the Permissive Temperature.

In this figure are depicted the level of yp1 expression relative to rp49 for diplo-X Df(2R)en^{b}/ix^{Ph} and their sibling controls at the permissive-temperature (data analyzed as described in figure 7).

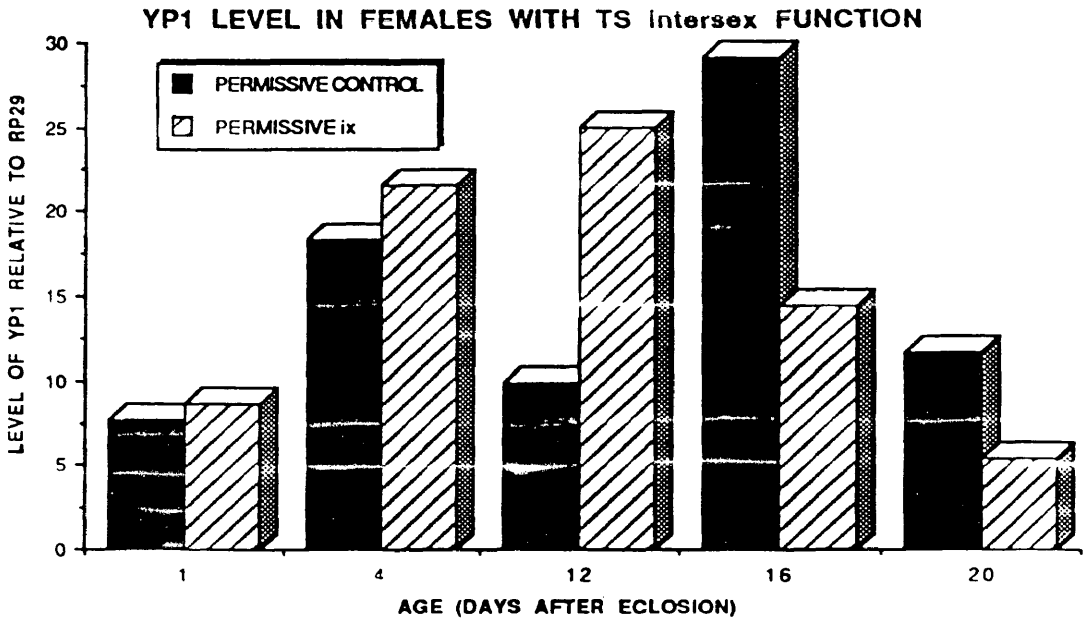
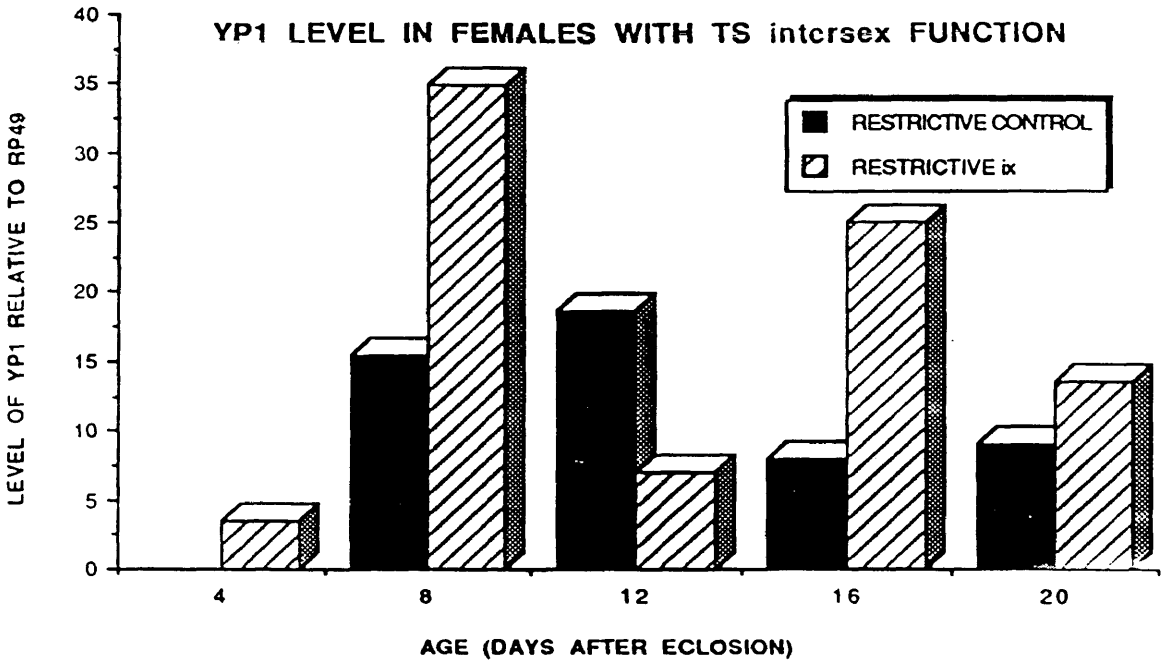

YP1 LEVEL IN FEMALES WITH TS Intersex FUNCTION
PERMISSIVE CONTROL
PERMISSIVE ix
LEVEL OF YP1 RELATIVE TO RP29
30
25
20
15
10
5
0
1
4
12
16
20
AGE (DAYS AFTER ECLOSION)

Figure #9. The Level of yp1 Expression ix^{ts} and Sibling Controls Kept at the Restrictive Temperature.

In this figure are depicted the level of yp1 expression relative to that of rp49 for Diplo-X Df(2R)en^{b}/ix^{Ph} and their sibling controls shifted to the restrictive-temperature after eclosion (data analyzed as described in figure 7).


YP1 LEVEL IN FEMALES WITH TS intersex FUNCTION
RESTRICTIVE CONTROL
RESTRICTIVE ix
LEVEL OF YP1 RELATIVE TO RP49
40
35
30
25
20
15
10
5
0
4
8
12
16
20
AGE (DAYS AFTER ECLOSION)

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