THE IDENTIFICATION OF MALE-SPECIFIC TRANSCRIPTS FROM

D.MELANOGASTER

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The research reported within this thesis is my own work, except if otherwise indicated. This work has not been submitted for any other degree.

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CONTENTS

ABBREVIATIONS

ATP - adenosine triphosphate
bp - base pairs
cDNA - complementary DNA
Ci - Curies
cm - centimetres
cpm - counts per minute
^{o}C - degrees centigrade
DNA - deoxyribonucleic acid
dNTP - deoxynucleotide triphosphate
DTT - dithiothreitol
EDTA - ethylenediamine tetra-acetic acid
EtBr - ethidium bromide
g - grammes
g - centrifugal force equal to gravitational acceleration
hr - hours
kb - kilobases/kilobasepairs
kd - kilodaltons
l - litres
L1,2,3 - 1st, 2nd, 3rd larval instars
M - Molar
min - minutes
ml - millilitres
mM - milliMolar
mMol - milliMoles
mRNA - messenger RNA
NaPPi - sodium pyrophosphate
ng - nanogrammes
nt - nucleotides
OD - optical density
pfu - plaque forming units
pH - acidity [$-\log_{10}$(Molar concentration of H^{+} ions)]
RNA - ribonucleic acid
rpm - revolutions per minute
rRNA - ribosomal RNA
SDS - sodium dodecylsulphate
Tris - tris(hydroxymethyl) amino ethane
u - units
uCi - microCuries
ug - microgrammes
ul - microlitres
vol - volume

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SUMMARY

A primary Lambda genomic library was screened with cDNA probes derived from male and female 3rd instar larval mRNA. Twenty clones which show male-specific or male-elevated expression were identified. Ten of these clones have been characterised in some detail. Two of the clones, gK14 and gK21, appear to be re-isolates of the previously identified male-specific genes mst349 and mst336 (DiBenedetto et al., 1987). These, along with five new isolates (gS4, gS8, gS9, gS10, gK33) show expression patterns on northern blots consistent with germline-specific expression. The transcripts are expressed in male larvae, pupae and adults but not in agametic males or XX individuals transformed to somatic males by the tra2 mutation (pseudomales).

Two of these clones, gS8 and gS9, are non-overlapping but cross hybridise at high stringency indicating that they contain the same or very similar sequences. Partial cDNA clones identify a single band in female DNA but multiple bands in male DNA, suggesting the transcribed sequence is Y associated. Many of these male-specific bands are conserved in at least 3 different laboratory strains. Northern blots using RNA from XO and XY males show similar levels of the transcript suggesting that the non sex-specific locus can be transcribed, at least in the absence of a Y chromosome. In-situ hybridisation to polytene chromosomes with either of the genomic clones give no signal suggesting that the non sex-specific genomic location is under replicated or heterochromatic.

Another of these germline-specific clones, gS4, appears to be heterochromatic. Southern blots of genomic DNA indicate the presence of several different repeats. In addition in-situ hybridisation to polytene chromosomes indicate that these repeats reside at a single genomic location: at the base of the left arm of chromosome 2.

Of the three remaining clones, gS1 identifies a weakly expressed 3.0kb male-specific transcript. Although this transcript is not detected in agametic males low levels of expression during the pupal stage suggests that it may not be germline specific.

The other two clones gS2 and gK15, are overlapping and

map uniquely to region 61F on the polytene chromosome map. These clones identify at least 3 male-specific transcripts on northern blots as well as a non sex-specific transcript which is elevated during the embryonic and pupal stages. The pattern of expression appears to be complex, all of the transcripts are developmentally regulated. One of the male-specific transcripts is expressed in agametic males and possibly in pseudomales. This transcript is also present in the embryo although it is not known at present if it is sex-specific at this stage.

CHAPTER 1

INTRODUCTION

The end result of the pathways which regulate the different aspects of sexual dimorphism in the fly is the expression of the structural genes responsible for sex-specific phenotypes. It is here that we can see the similarity between the control of the sexual phenotype and the way in which homeotic selector genes specify structural features of the body segments. As with the homeotic genes the sex determination genes make binary decisions which result in a cell or tissue choosing a particular pathway of development. Thus by studying the way in which the sex determination hierarchy co-ordinately regulates sets of genes we may gain some insight into the way in which developmental processes are regulated. Furthermore sex-specific gene expression is not regulated in isolation from the rest of the organism: temporal and positional information must be co-ordinated along with the specification of sex to ensure that genes are expressed in the correct place at the correct time. Thus the study of sexual differentiation may give us some insight into the way in which different regulatory hierarchies interact to specify the appropriate expression of a particular gene.

In this chapter I shall review our current understanding of the regulation of sexual differentiation as a prelude to a discussion of a set of male-specific genes which I have isolated. As we shall see the extensive knowledge of the control of sex-specific gene expression offers a unique opportunity to investigate the molecular mechanisms which underlie complex problems in development.

Before discussing the genetic and molecular aspects of sex-specific gene expression I will briefly discuss the biology of some of the aspects of sexual differentiation.

Development Of The Sexes

Pole cell nuclei, the precursors of the germline, separate from the somatic nuclei prior to the blastoderm stage. They proliferate at the posterior end of the embryo until there are approximately 50 cells. These cells are then internalised and migrate, during gastrulation, to the level of the 5th abdominal segment. Genetic evidence, which will be

discussed later, suggests that the pole cells have already determined their sex by this time. At approximately the 10th. hour of embryogenesis a few of the pole cells begin to associate with mesodermal cells and during the next hour the pole cells become completely encapsulated by somatic cells and the gonad is formed (Campos-Ortega and Hartenstein, 1985).

Sonnenblick (1950) reports that there is a bimodal distribution in the number of pole cells encapsulated. In one population an average of 5-7 pole cells are incorporated into the gonad, in the other a larger gonad contains an average of 9-13 pole cells. He proposed that this difference reflects the first manifestation of sexual dimorphism in the fly; the larger gonad being male and the smaller female. Although definitive confirmation of this hypothesis awaits, it is supported by studies of gonad development during the larval stage (Keris, 1931). These show that upon hatching a size difference in the gonads is apparent and can be reliably used to sex 1st instar larvae. The gonads of 1st instar larvae are also sexually dimorphic in terms of the position they occupy relative to the fat body. While the smaller female gonads are embedded in the fat body the larger male gonads are merely bordered by this tissue.

Ablation of the pole cells by UV irradiation prior to their migration does not appear to affect the sexual dimorphism of the somatic component of the gonad (Aboim, 1945). Similarly the effect of grandchildless mutations (eg.gS(1)N26 and gS(1)N441, Niki and Okada, 1981; agametic, Engstrom et al., 1982; grandchildless of D.subobscura, Fielding, 1967), which result in the loss of pole cells either prior to or after they reach the gonad, appear not to affect the sexual phenotype of the somatic component of the gonad. However in the absence of germ cells the gonads do not develop to full maturity, though they are recognisable as male or female and appear to contain all the somatic components of the wild type gonad. Taken together these observations suggest that the somatic component of the gonad is sexually determined independently of the germline. This suggestion is further supported by observations on the development of the gonad in individuals mutant in somatic sex determination genes. As will be discussed later the somatic sex determination hierarchy controls the sexual

differentiation of the somatic component of the gonad but plays no role in the sexual differentiation of the germline. Thus it is probable that in at least one tissue, the gonad, the sexually determined state is set early in development.

Apart from the gonads, which increase in size, overt sexual dimorphism is not apparent through most of the larval stages. Lauge (1982) however has reported that sexually dimorphic differentiation of the genital imaginal disk becomes apparent during the second larval instar. Major sexual differentiation occurs during metamorphosis at the pupal stage. During this stage the gonads and the genital disk differentiate to form the adult reproductive tract. The following outline, which is by no means complete, has been drawn from Lauge (1982), Mahawold and Kambysellis (1980) and Lindsley and Tokuyasu (1980). A diagram of the male and female internal reproductive tracts is presented in Figure 1.1.

Early investigators believed that the single genital disk contained indifferent cells capable of forming either male or female derivatives depending on the chromosomal constitution of these cells. However clonal analysis (Nothiger et al., 1977; Schupbach et al., 1978) demonstrates that the single genital disc contains three distinct primordia. One specifies male structures, one specifies female structures and the third is an indifferent primordium of the analia. The genital disk primordia specify the formation of the internal genitalia and the external structure of the 8th and successive abdominal segments.

The male reproductive system consists of a pair of coiled testes joined to the seminal vesicle by a short testicular duct or vas deferens. From the seminal vesicles the sperm exit, via the anterior ejaculatory duct, to the sperm pump and then via the posterior ejaculatory duct to the penis apparatus. The accessory glands, which produce secretions such as pheremonal peptides, join the anterior ejaculatory duct near the seminal vesicles. During the early stages of metamorphosis the testes develop apart from the disc derivatives, joining the vas deferens mid way through the pupal stage. Attachment to the vas deferens induces the testes to elongate and coil. Later in development the testes assume their characteristic yellow colour which spreads to the vas deferens.

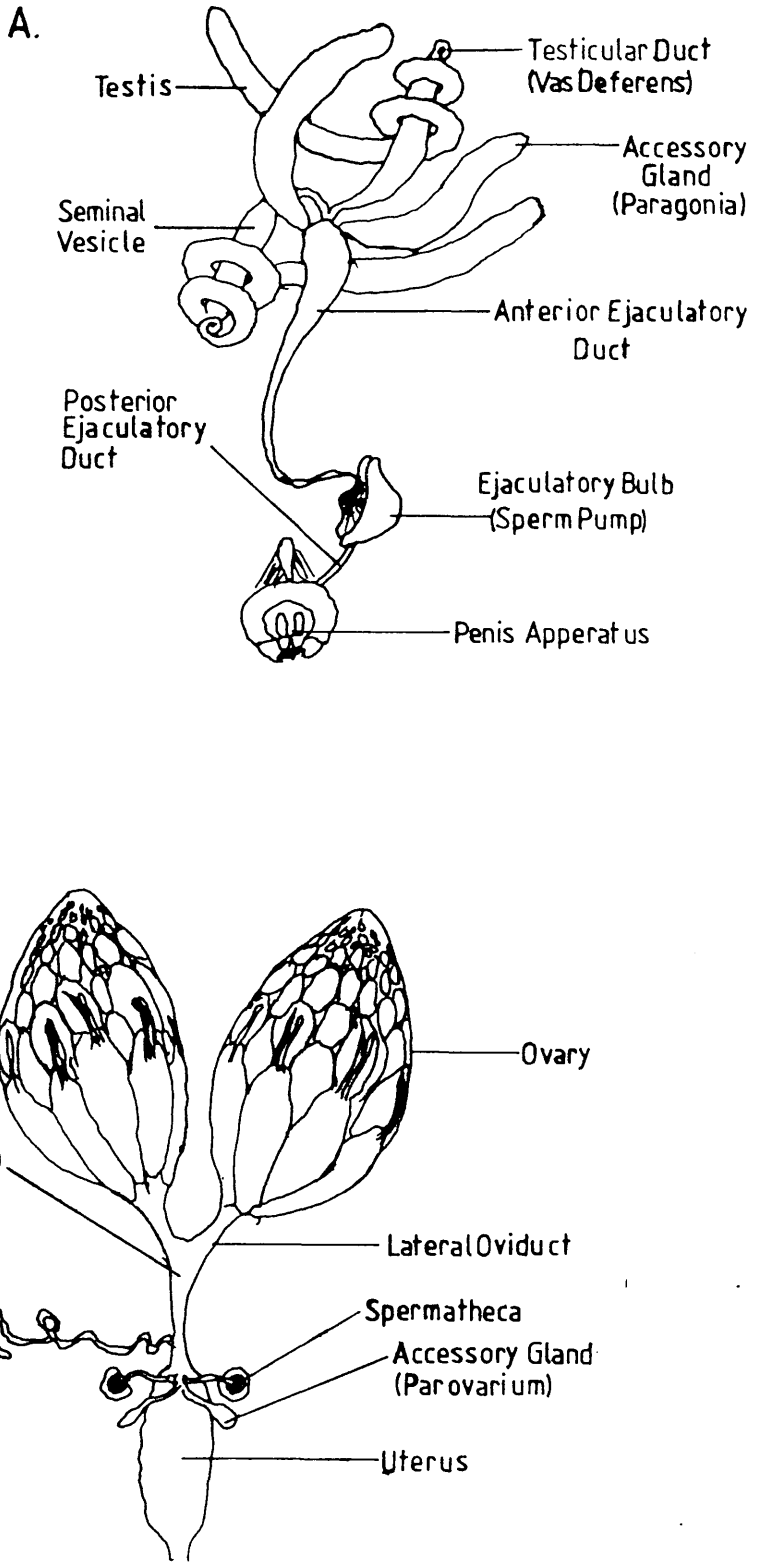


Figure 1.1a: Diagram of the male internal reproductive tract from D.melanogaster. After Lindsley and Tokuyasu (1980).

b: Diagram of the female internal reproductive tract fromD.melanogaster. After Mahowald and Kambysellis (1980).

External male characteristics include dark pigmentation over all of the posterior tergites, fusion of the 6th and 7th segments and the genital arch which forms the claspers and supports for the penis apparatus.

The female portion of the genital disk produces a pair of laterel oviducts which extend anteriorly to meet the developing ovaries. Posteriorly the oviducts fuse to form a common oviduct leading to the uterus. A pair of spermatheaca and a single sperm receptacle open into the uterus. These function as sperm storage organs. A pair of accessory glands or parovaria, which produce secretion for coating the egg, also open into the uterus. Externally the female differs from the male in abdominal pigmentation, which is reduced and restricted to the posterior margin of the tergites. In addition the 6th and 7th segments remain separate and the genital arch forms the vaginal plate or ovipositor.

Clonal analysis and gynandromorph studies indicate that only one of the genital disk primordia develops in males or females and that the choice is determined by the somatic sex determination hierarchy. In true intersex flies, such as those produced by mutations in the gene doublesex, both primordia develop and structures derived from both are visible in the adult (Hildreth, 1965).

The other major site of external sexual dimorphism is the basitarsus of the fore-leg. In males there are generally 6 rows of transverse bristles and 1 row of longditudinally arranged thick heavily pigmented bristles known as the sex comb. Females have on average 8 rows of transverse bristles but no longditudinal bristles (Tokunaga, 1962). Intersexual individuals tend to have 6 rows of transverse bristles and a row of bristles lying in a position intermediate between transverse and longditudinal rows. These bristles are also intermediate in size and pigmentation when compared with the sex comb and normal bristles. The third obvious sexual dimorpism is that of body size, females are larger than males. It is not clear how this dimorphism is controled but sex transforming mutations in Sex-lethal can transform body size (Cline, 1984). Mutations in the somatic sex determination hierarchy do not affect this particular dimorphism.

As well as the above dimorphisms internal differences are also apparent in biochemistry (eg yolk protein synthesis) and

behaviour. These observations suggest that the sexually determined state may extend to most, if not all, somatic tissues. This hypothesis is supported by the finding that embryonically derived cell lines (Kc and Schnieder-2) maintain either male or female expression of sex determination genes but not both (M.McKeown, pers.comm.).

Spermatogenesis in Drosophila

This brief account of spermatogenesis is drawn from the descriptions of several authors (Lindsley and Tokuyasu, 1980; Hennig, 1985; Lifschytz, 1987), due to constraints of space oogenesis will not be discussed and interested readers are referred to the review by Mahawold and Kambysellis (1980).

During spermatogenesis in Drosophila a group of 64 haploid sperm are produced from a single gonial cell. The production of mature spermatozoa takes approximately 10 days. Gonial cells are generated at the apex of the testis by the unequal division of a stem cell. The stem cells, which are in fact the pole cells which populated the embryonic gonad, remain anchored at the apex. The gonial cell is invested by two apical somatic cells to form a cyst. The cyst will contain the descendants of the gonial cell during the entire period of sperm development. The gonial cell within the cyst undergoes four rapid mitotic divisions to produce 16 primary spermatocytes. Cytoplasmic bridges continue to link the daughter cells. Thus the gametes within a cyst develop as a syncytium. Primary spermatocytes are transcriptionaly active and produce all the mRNA required for subsequent sperm development. Autoradiographic studied on tritiated uridine uptake have failed to detect transcription in post-meiotic stages of spermatogenesis (Olivieri and Olivieri, 1965). The spermatocytes grow for approximately four days, increasing in volume some 25 fold. During this stage the nuclei are arrested at meiotic prophase. It is also during this stage that the Y chromosome becomes active. At the end of the spermatocyte stage meiotic divisions produce the 64 spermatids which again remain interconnected by cytoplasmic bridges. As noted above it is generally accepted that there

is little or no postmeiotic transcription, spermatids are however translationally active.

The spermatids develop synchronously within the cyst, in what can only be described as a remarkable morphological differentiation, to produce the 1.8mm long sperm (the sperm of D.hydei are even longer, attaining a length of over 10mm !). The major features of this differentiation are: **1)** The condensation and organisation of the mitochondria into the nebenkern structure. **2)** The elongation of the centriole to form the axoneme and its subsequent association with the nebenkern. **3)** The elongation of the axoneme and surrounding nebenkern derivatives to form the tail of the sperm. **4)** The condensation and elongation of the spermatid nucleus. **5)** Separation of each sperm into a membrane bound individual by the passage of a "cystic bulge", which also rids the sperm of excess cytoplasm. **6)** The coiling and final release of the sperm from the cyst into the seminal vesicle. To put this morphological change into some perspective, if we consider the size of a pre-elongation spermatid to be equivalent to the size of a standard postage stamp, then the resulting sperm would be equivalent to a piece of sewing thread approximately 6 feet long! This is all the more remarkable when we consider that this differentiation occurs in the absence of any on-going transcription. In a sense it can be viewed as a gigantic piece of self assembly.

The testis of the newly hatched larva contains approximately 38 gonial cells (Sonnenblick, 1950; Keris, 1931) but no primary spermatocytes. According to Garcia-Bellido (1964) the first spermatocytes become apparent during the 1st larval instar. Unfortunately there are no descriptions of spermatocyte numbers during the larval stages. During the pupal stage there may be a maximum of approximately 100 spermatocyte cysts per testis (Garcia-Bellido, 1964). This author reports a reduction to around 50 cysts per testis post-eclosion. A genetic analysis estimates 30 cysts per testis during adult life (Lindsley and Tokuyasu, 1980), it is not clear if these differences are significant. No spermatids are detected during the larval stages, the first meiotic divisions occur around the time of pupariation.

As one may suspect such a complex process is susceptible to disruption by a large number of mutations (see Lifschytz 1987 for an extensive review). Apart from autosomal and X-

linked point mutations various chromosomal aberrations, including X to autosome translocations and certain proximal X deficiencies, also disrupt spermatogenesis. The Y chromosome, dispensable for the rest of development, also has a vital role in spermatogenesis. some of these unusual chromosomal mutations will be considered in chapter 6.

The Genetics of Sex Determination

Sex in Drosophila melanogaster is determined by the ratio of X chromosomes to sets of autosomes (X:A ratio). Bridges (1921) demonstrated, by studying individuals with aneuploid and polyploid chromosomal constitutions, that an X:A ratio of > 1 results in female differentiation while a ratio of < 0.5 results in male differentiation. Individuals with an intermediate X:A ratio of 0.67, as with the genotype XX:AAA (triploid intersexes), develop as mosaics composed of both male and female tissue. The observation that haplo-X tissues still differentiate as phenotypically male when lacking a Y chromosome, as in XX/XO mosaics or in XO individuals, demonstrates that the Y chromosome plays no part in sex determination. It also suggests that the sex determining mechanism operates at the level of the individual cell. This latter suggestion is strongly supported by the results of somatic recombination experiments which generate clones of cells homozygous for sex transforming mutations (Baker and Ridge, 1980; Sanches and Nothiger, 1982).

The choice of a particular pathway of sexual development has three major consequences for the fly: **1)** Somatic tissues differentiate in a sexually dimorphic way. **2)** The cells of the germline are directed to the appropriate developmental pathway. **3)** The level of transcription of X-linked genes is regulated in order to compensate for the imbalance in X-linked gene dosage between males and females. This is achieved by transcribing most genes on the single X chromosome of males at twice the rate of those same genes in the diplo-X female (Lucchesi and Manning, 1987). This is in contrast to the mammalian X inactivation mechanism of dosage compensation (Cattanach, 1975).

The use of genetic, and latterly molecular, techniques to

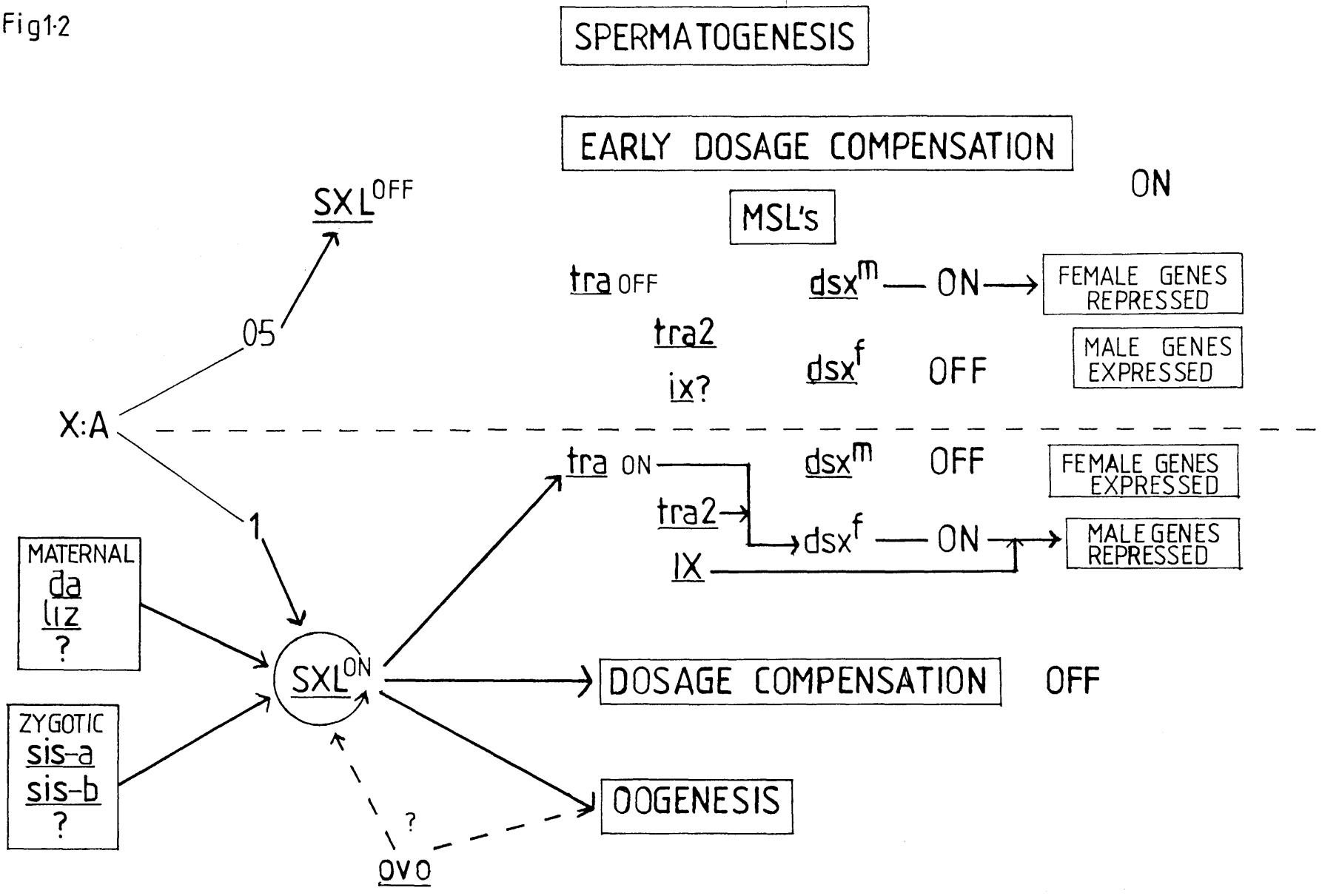
study mutations which perturb these functions has led to an enormous increase in our understanding of the control of sexual differentiation. This has been particularly fruitful in the case of those genes regulating somatic sex determination, with the result that the somatic sex determination hierarchy is one of the best understood examples of the molecular control of gene expression in any complex eukaryote. Rapid advances have been possible for a number of reasons: 1) The wealth of genetic and molecular techniques available to the Drosophila investigator. 2) The non-lethal nature of many of the mutations which perturb sexual differentiation, allowing the examination of mutant phenotypes in adult individuals where sexually dimorphic features are most easily observed. 3) The ability to study those mutations which are lethal in mosaic individuals without experiencing interference from neighbouring non-mutant tissue.

I shall overveiw our current understanding of the control of sexual differentiation and follow this with a more detailed description of the individual genes involved. The somatic sex determination hierarchy, being the easiest to study, is understood in the greatest detail. Dosage compensation and germline sex are more poorly understood. Dosage compensation has recently been the subject of an extensive review (Lucchesi and Manning, 1987) and will only be dealt with briefly in this discussion. A diagrammatic representation of the genetic interactions is presented in Figure 1.2.

As mentioned above the primary determinant of sex is the X:A ratio. Early in development individual cells, in response to this ratio, set the state of activity of the key gene Sex-lethal(Sxl) such that it is "on" in XX zygotes and "off" in XY zygotes (Cline, 1988). Sxl requires the activity of both maternal (eg daughterless) and zygotic (eg sisterless-A) genes to initiate or maintain the appropriate expression state. After assessment of the X:A ratio Sxl maintains the sexually differentiated state independently of the initiating signal. Somatic sex, germline sex and dosage compensation all appear to be regulated by Sxl. Downstream of this gene their control is for the most part mediated by independent pathways.

Sex-specific gene expression in the soma is regulated by

Fig1·2


SPERMATOGENESIS
EARLY DOSAGE COMPENSATION
ON
MSL's
SXL OFF
05
X:A
1
tra OFF
tra2
ix?
dsx m — ON → FEMALE GENES REPRESSED
dsx f OFF
MALE GENES EXPRESSED
tra ON
tra2 →
IX
dsx m OFF
FEMALE GENES EXPRESSED
dsx f — ON → MALE GENES REPRESSED
MATERNAL
da
liz
?
ZYGOTIC
sis-a
sis-b
?
SXL ON
DOSAGE COMPENSATION
OFF
OOGENESIS
?
ovo

a hierarchy of four known genes: transformer (tra), transformer2 (tra2), doublesex (dsx) and intersex (ix). No other regulatory genes have been identified in this pathway, although their existence cannot be discounted. Baker and Ridge (1980) proposed a model for the interactions of the above loci based on genetic evidence. Subsequent molecular studies (Nagoshi et al., 1988; Goralski et al., 1989) strongly support and refine this model. The female-specific activity of Sxl is required for the production of a functional tra mRNA. The tra gene product is in turn required, in conjunction with the product of the tra2 gene, to direct the production of female-specific dsx mRNA. The female-specific dsx gene product acts, in conjunction with the ix gene product, to repress male terminal differentiation functions. Female terminal differentiation functions are not repressed and hence female somatic development ensues. In the absence of Sxl function, as in XY individuals, tra female-specific mRNA is not produced. tra2 alone is not sufficient to direct the production of the female-specific dsx mRNA and male-specific dsx mRNA is produced by default. The dsx male product has the opposite effect to the female product, female terminal differentiation functions are repressed and male functions are expressed. The molecular mechanisms by which dsx acts to control terminal differentiation are as yet unknown.

Dosage compensation functions necessary for the hyperexpression of X-linked genes in males are negatively regulated by Sxl. In males, where Sxl is absent, the products of the male-specific-lethal 1 and 2 and male less (collectively referred to here as msls) genes are able to direct X chromosome hypertranscription, at least in the later stages of development. In the presence of Sxl these functions are repressed. There is substantial evidence that other, as yet unidentified, functions are required for correct dosage compensation in the early embryo (Cline, 1984; Gergen, 1987; Gergen and Wieschaus, 1988).

Less is known about the correct development of the germline. It has been established that the correct X:A ratio and the appropriate activity of Sxl are required (Schupbach, 1985; Steinman-Zwicky et al., 1989). The regulatory genes involved in somatic sex and dosage compensation are not required in the germline for sexual differentiation (Marsh

and Wieschaus, 1978; Schupbach, 1982), however tra2 and mle gene products are required for the successful completion of spermatogenesis (Belote and Baker, 1983; Bachiller and Sanchez, 1986). The product of the Ovo locus is necessary for the establishment of the female germline (Oliver et al., 1987). Undoubtedly more genes remain to be uncovered which are necessary for germline sex. Interestingly recent experiments (Steinmann-Zwicky et al., 1989) have shown that in addition to the cell autonomous control of germline sex an inductive interaction between germline and soma can be demonstrated for XX germ cells. Thus, in this case at least, sex determination is not cell autonomous.

Sex-lethal(Sxl)

Sxl is a central gene which regulates all aspects of sex determination in Drosophila. The gene maps to salivary gland chromosome region 6F on the X chromosome. Much of our understanding of the role Sxl plays in sexual differentiation comes from the study of various mutations which perturb the function of this gene in a sex-specific fashion. Simply stated loss of function mutations are female-specific lethals whereas gain of function mutations are male-specific lethals. The study of more subtle mutations in Sxl and its positive regulators, principally in the laboratory of T.W. Cline, has allowed the separation of different Sxl functions and the elucidation of a genetic model for its action. Subsequent molecular cloning of the gene has provided some insight into how sexual differentiation is controlled at the molecular level.

Genetics of Sxl

Both null alleles (eg Sxl^{f1}) and deletions are homozygous lethal in diplo-X (female) individuals but have no discernible effect on the viability or development of haplo-X (male) individuals. Gain of function mutations (eg Sxl^{M1}) are dominant male lethal. They appear to have no effect on

otherwise wild type females, rather these alleles are able to rescue females from mutations in positive regulators of Sxl (eg daughterless, da) which would otherwise be female lethal (Cline, 1978, 1979). Due to the lethal nature of these mutations much of the early work focused on clonal analysis either in gynandromorphs or in homozygous clones generated by somatic recombination. These experiments indicated that: **a)** Sxl mutations are sex-transforming. Sxl^{f1}/Sxl^{f1} clones undergo male somatic differentiation in a diplo-X background (Cline, 1983) and Sxl^{M1} somatic clones undergo female differentiation in a haplo-X background. **b)** The lethality associated with these mutations may be due to perturbations in dosage compensation as the clones were both under-represented and poorly developed, indicating disruption of a vital function. These conclusions are supported by studies which examined the effect of these alleles in triploid intersexes (Cline, 1983). Here gain of function alleles are feminising and result in reduced expression of the X-linked genes Bar and Hairy wing. On the other hand loss of function alleles are masculinising and appear to some extent to increase the expression of X-linked genes. Lucchesi and Skirpsky (1981) have also provided evidence from transcriptional autoradiography studies which implicates Sxl in the regulation of dosage compensation.

By using a variety of selection procedures various hypomorphic Sxl alleles have been isolated. The study of these alleles in a variety of genetic backgrounds has allowed the dissection of Sxl function. The most informative of these will be discussed.

$Sxl^{fm7,M1}$ is a derivative of Sxl^{M1} which is hemizygous viable in males and homozygous viable in females (Cline, 1984). It retains the capability of the parent allele to rescue chromosomal females from maternal da mutations. Females carrying this allele are somatically sex-transformed, indicating that female somatic sex determining functions have been lost. Somewhat surprisingly these females have male body size. Sxl is the only sex determining gene which affects this pronounced sexual dimorphism.

The viability in both sexes observed with this allele suggest that if Sxl controls dosage compensation then appropriate functions are being provided in both sexes. Two copies of this allele allow the repression of (female-lethal)

X chromosome hyperactivation genes whereas one copy is not sufficient for this purpose (ie males survive). Evidence for the involvement of Sxl in establishing the correct level of X chromosome transcription comes from an examination of this allele in hemizygous females. $Sxl^{fm7,M1}/Df(Sxl)$ (XX) individuals fail to survive beyond the late larval stage, however when these individuals are simultaneously homozygous for any of the male-specific-lethal (msl) loci the lethality is partially rescued. This indicates that it is the inappropriate expression of msls which is ,at least in part, responsible for the lethal effect. However this rescue is only observed with this particular Sxl allele. Other female-lethal alleles, used both in the above study and others (Cline, 1983; Skripsky and Lucchesi, 1982), are not rescued by msl mutations. This observation can be explained if we assume that there are two dosage compensation pathways, one operating early in development and the other, regulated by msls, operating later. If this is correct we can reconcile the msl rescue of hemizygous $Sxl^{fm7,M1}$ individuals by assuming that this allele is defective primarily in its interaction with late dosage compensation functions. Consistent with this view is the late lethal period observed in hemizygous females. That this allele is hemizygous male viable but still retains the constitutive male-lethal mutation reflects the selection for a male viable derivative. This must have leaky interactions with dosage compensation functions to allow males to survive (Cline, 1984).

Other lines of evidence support the contention that there are two dosage compensation pathways: 1) There is a discrepancy between the lethal phases of Sxl and msl mutations, the latter cause male lethality during the late larval or early pupal stages while the former are lethal considerably earlier (early larval for Sxl^{M1} and late embryo for Sxl^{f1}). 2) Experiments on hypomorphic runt alleles indicate that this gene is dosage compensated in the early embryo during the blastoderm stage (Gergen, 1987; Gergen and Wieschaus, 1987). When individuals carry mutations in any, or all, of the msls there is little or no effect on runt expression. In contrast the mutation Sxl^{f1} appears to increase runt expression in XX individuals, this is consistent with a failure to repress early dosage compensation functions which do not involve msls. The effect

of the $\underline{Sxl}^{M1}$ allele was also examined in these experiments, it did not show the expected repression of runt. Subsequent experiments (Steinmann-Zwicky, 1989) suggest that this allele is not constitutive for all Sxl functions when the X:A ratio is inappropriate for normal Sxl expression.

Genes involved in an early pathway for dosage compensation would be expected to show an embryonic male lethal phenotype when mutated. Two such loci have been reported, killer of male A and killer of male B (Pierre, 1972). Disappointingly further reports on these mutations have not been published nor are they listed in any of the stock centres.

The studies described above allow us to separate genetically two distinct Sxl functions; that regulating somatic sex and that which regulates dosage compensation. In a further series of experiments Cline was able to establish a third function for Sxl, that of a positive autoregulator. If a fly of the genotype $\underline{Sxl}^{fm7,M1}/\underline{Sxl}^{+}$ (XX) is produced from da/da mothers these individuals develop with large patches of female soma. Since the wild type allele in this stock cannot be activated in the absence of maternal da product and the mutant allele specifies male soma, the appearance of female somatic tissue must result from activation of the wild type copy by the partially constitutive $\underline{Sxl}^{fm7,M1}$ allele. Thus an autoregulatory function for Sxl is uncovered (Cline, 1984).

Studies involving other partial loss-of-function alleles ($\underline{Sxl}^{f9}$, $\underline{Sxl}^{fLS}$, Maine et al., 1985a; Salz et al., 1987) have allowed further dissection of Sxl function. Somatic recombination experiments show that homozygous $\underline{Sxl}^{fLS}$(XX) clones develop as male tissue. In similar experiments homozygous $\underline{Sxl}^{f9}$(XX) clones have a female phenotype. However when these alleles are examined in $\underline{da}^{-}$ backgrounds $\underline{Sxl}^{fLS}$ appears more wild type than $\underline{Sxl}^{f9}$: ie. it is more able to tolerate reductions in the amount of da gene product. The above observations can be reconciled if we assume that $\underline{Sxl}^{f9}$ is defective in initiating stable expression (da sensitive) but once started it can maintain appropriate expression (female soma). The opposite would hold for $\underline{Sxl}^{fLS}$: it can initiate expression but cannot maintain it.

A requirement for correct Sxl activity in the female germline has been demonstrated by Schupbach (1985). Pole cell transplantation experiments show that $\underline{Sxl}^{f1}/\underline{Sxl}^{f1}$(XX) germ

cells in wild type soma develop as undifferentiated multicellular cysts. The allele $\underline{Sxl}^{fs1}$ is homozygous female viable and wild type with respect to somatic function but it shows the same germline phenotype as the $\underline{Sxl}^{f1}$ null allele. Interestingly a heteroallelic combination $\underline{Sxl}^{fm7,M1}/\underline{Sxl}^{M1,fm3}$, which in somatic tissue produces male development, permits normal oogenesis with wild type eggs being produced. Again different Sxl functions can be separated by genetic means.

The picture which emerges from these studies is of a complex gene involved in assessing the primary sex determining signal, establishing the appropriate response to the signal, retaining a memory of the signal via an autoregulatory mechanism and finally transmitting that assessment to the regulatory genes which control somatic sex, germline sex and dosage compensation (Cline, 1984).

Molecular Analysis of Sxl

The Sxl locus was cloned via a P-element insertion allele $\underline{Sxl}^{fPb}$ (Maine et al., 1985a; 1985b; Salz et al., 1987). Subsequently molecular studies have been used to investigate the organisation of the gene (Salz et al., 1987,1989; Bell et al., 1988). The results of these investigations can be summarised as follows: The Sxl transcription unit spans over 23kb of DNA and encodes multiple overlapping transcripts which are regulated in a sex-specific, tissue-specific and temporal way. Ten transcripts have so far been distinguished on northern blots.

Three of these are embryo specific and are transcribed only during the earliest stages of development (2-5 hr). They may be transcribed from an embryo-specific promoter located downstream from the start site used during the rest of development. The presence of Sxl alleles believed to be defective in pathway initiation and evidence from P-element mobilisation experiments are consistent with the presence of a regulatory element involved in establishing Sxl expression (Salz et al., 1987).

The embryo-specific transcripts are replaced by a set of seven transcripts which persist throughout the remainder of

the life cycle. Three of these transcripts are male specific and four are female-specific. Two of the female-specific transcripts depend on the presence of a germline. Sequence analysis of cDNA clones corresponding to some of the Sxl transcripts indicates that all of the male-specific mRNA species contain a 190bp exon which is not present in female or embryonic mRNA's. It is not clear at present if the embryo-specific primary transcripts contain this exon. The exon contains an in-frame termination codon which severely truncates the conceptual Sxl polypeptide. As male and female transcripts are believed to share the same start site it would appear, therefore, that sex-specific regulation of Sxl throughout the majority of the life cycle is not at the level of transcription: rather it is at the mRNA processing stage.

The four female transcripts appear to differ at their 3'ends by the use of alternative exons. At present it is not known if these sequence differences reflect functional differences. However given that two of these transcripts are germline specific it is highly probable that the transcripts encode different functions. All of the female-specific transcripts are found in unfertilised eggs. They appear to be rapidly degraded when the zygote-specific transcripts first appear. It is unclear what role these maternally derived transcripts play in early development but since Sxl autoregulates the simplest view would be that these transcripts have no function. Rather they are present as a consequence of their transcription in the female germline. In support of this it should be noted that Sxl dependent maternal effects cannot be detected (Cline, 1980; Oliver et al., 1988).

Five dominant male-lethal alleles all contain transposable element insertions within 1kb of the male-specific exon (Maine et al., 1985b). These all appear to be downstream of the 3' acceptor site for this exon. Two of these insertions are B104 elements which are transcribed in the opposite direction to Sxl, two are 297 elements which are transcribed in the same direction, the fifth is of unknown origin. The dominant gain of function phenotype associated with these insertions could, in principal, be explained in several ways. The three dimensional structure of the primary transcript could be disrupted by these insertions such that splicing at the terminating exon is impaired (Baker, 1989).

Another possibility is that antisense transcripts from the B104 elements could base pair with the Sxl primary transcript and again disrupt splicing. These two suggestions would mean that the terminating exon cannot be included in the Sxl mRNA and hence an active (male-lethal) Sxl polypeptide is always produced. These mechanisms however do not readily explain the ability of these alleles to rescue mutations in positive regulators. In light of the finding that both the da and sis-b products may be transcriptional activators (see below), an alternative explanation is needed. Perhaps the transposable elements provide alternative transcriptional start sites which allow for the production of primary transcripts devoid of the male-specific exon (Maine et al., 1985b). In this context it should be noted that the B104 element was isolated by virtue of its high level of expression in the early embryo, at the same time (2-5 hr) as zygotic Sxl expression begins (Sherer et al., 1982). Schupbach (1985) has suggested that da may be required to allow Sxl expression at this early stage in development when there is little transcriptional activity in the rest of the genome. This suggests the possibility that these mutations are male lethal not because of any disruption of splicing but because of the inappropriate expression of Sxl in the male embryo. In other words differential splicing is the mechanism Sxl uses to maintain the sexually determined state but sex-specific transcription may be the mechanism used to establish it. Two lines of preliminary evidence support this conjecture: 1) in-situ hybridisation experiments to blastoderm embryos with Sxl probes identify two populations of embryo, one expressing high levels of Sxl and one not (D.Coulter cited in Salz et al., 1989). 2) Maine et al. (1985a) report that two of the three embryo-specific Sxl transcripts are absent or severely reduced in the progeny of homozygous da mothers. Further experiments on the embryonic transcripts will be required to confirm this hypothesis. Particularly enlightening would be the identification of the 5' ends of these transcripts.

Sequence analysis of female-specific cDNA clones reveals a long open reading frame with the potential to produce a 39kd polypeptide. This protein would contain two domains which have sequence similarity to the RNA binding domains of several Ribonucleoproteins (RNP) from yeast, rat and man (see

Bell et al.,1988 for details). Many of the female-lethal loss of function mutations are DNA rearrangements which disrupt the coding sequence of this polypeptide. The finding that the Sxl gene product may be an RNA binding protein provides an attractive way to explain the autoregulatory function of Sxl. In this model a Sxl gene product can prevent the inclusion of the translational terminating exon in the processed mRNA by binding to the 3' acceptor site of this exon. When the Sxl polypeptide is absent the acceptor is not blocked and the exon included in the mRNA. Indirect evidence in support of this model for Sxl autoregulation has been obtained from studies on the tra gene and will be discussed below. (Bell et al., 1988; Sosnowski et al., 1989).

The molecular cloning of Sxl has given some working hypotheses as to how the gene functions. The presence of a male-specific translation terminating exon is consistent with the genetic evidence that no Sxl function is required in males. The presence of zygotic transcripts at the earliest stages of development is consistent with clonal analysis (Cline, 1984) and dosage compensation data (Gergen, 1987) which suggest that Sxl activity is required at or before the blastoderm stage. The presence of multiple female-specific transcripts is consistent with the different roles Sxl has to play in regulating sexual dimorphism in the fly. One major question remains unanswered; how is Sxl able to respond to the primary sex determining signal? Progress in this area is being made by isolating and characterising genes which regulate Sxl, some of which will be briefly considered below.

Regulators of Sex-lethal

Several loci have been reported which have effects suggesting an interaction with Sxl. The most thoroughly characterised of these is daughterless (da).

da, which maps to salivary gland chromosome region 31E (2L), was first isolated as a recessive, temperature sensitive, maternal effect mutation resulting in the production of all male progeny (Bell, 1954). Cline (1978) demonstrated that this gene interacts with Sxl by isolating a da suppressor, which turned out to be the constitutive Sxl^{M1}

allele described above. Subsequent studies confirmed that da is required in XX zygotes to activate Sxl (Cline, 1979,1980). These initial experiments were carried out using the hypomorphic da^1 allele. This allele is a zygotic lethal in both sexes either when hemizygous or at 29°C when homozygous (Mange and Sandler, 1973; Cline, 1976). Several null alleles have now been isolated (Cronmiller and Cline, 1987; Caudy et al., 1988) and these show that da is required zygotically in both sexes for the formation of the peripheral nervous system. The phenotype of da nulls is similar to that observed with Achaete-scute (AS-C) mutations with regard to their effect on neural cell development. Caudy et al. (1988) suggest that the products of both genes may interact in neural development. The maternal effect of da appears to be strictly sex-specific.

The gene has been cloned (Cronmiller et al., 1988; Caudy et al., 1988), and shown to produce two transcripts present throughout the life cycle. These encode a conceptual polypeptide of 74kd, regions of which show striking sequence similarity with the myc oncogene, products of the AS-C complex (a startling finding in light of the similar phenotypes mentioned above) and more recently a 75% sequence identity with the DNA binding domain of Immunoglobulin Enhancer Binding protein. (Murre et al., 1989a). In addition the da polypeptide also contains a helix-loop-helix motif which has been implicated in protein dimerisation. This finding suggests a mechanism by which da may interact with Sxl. The da gene product, either alone or in conjunction with other polypeptides, could act as a transcriptional activator at the regulatory sequences for the embryo-specific transcripts discussed above. Several lines of evidence support this hypothesis: 1) temperature shift experiments with embryos from da/da mothers indicate that the maternal da product is required within the first three hours post oviposition (Cline, 1978). 2) Sxl activity is required in XX zygotes at approximately this time. 3) Sxl embryo-specific transcripts appear at approximately this time. 4) It has been reported that at least two of the embryo-specific transcripts are absent or severely reduced in embryos which lack da gene product (Maine et al., 1985a). An alternative explanation for da function, which is also consistent with these observations, is that it acts indirectly on Sxl by activating

another gene(s) which is required for Sxl expression.

Since the da gene product is maternal and appears to be active in all eggs irrespective of their chromosomal constitution it is unlikely to transmit information regarding the X:A ratio. It is more likely to be a factor without discriminatory ability required to permit the activation of Sxl. Loci which assess and respond to the X:A ratio are expected to be zygotic. Some are expected to be X-linked, so called numerator elements, and others autosomal denominator elements. They are expected to behave in a genetically predictable way. For numerator elements an increase in dosage should be detrimental to males as the apparent X:A ratio is increased. Decreasing the dosage should have the opposite effect and be detrimental to females. At least two loci have been identified which fulfil these and other genetic requirements for such factors: namely sisterless-a (sis-a) and sisterless-b (sis-b).

sis-a is an X-linked recessive female-specific lethal, it shows no maternal effect (Cline, 1986). Homozygous females can be rescued by Sxl^{M1}. The lethal period is similar to that observed for Sxl nulls and clonal analysis indicates that sis-a activity is only required early in development. sis-b is also X-linked and has similar properties to sis-a (Cline, 1988; Torres and Sanchez, 1989). Several observations support the view that these genes are part of the X:A counting mechanism which activates Sxl. Increasing the dosage of either gene can be male-lethal and is feminising in triploids; females are rescued from reductions in Sxl or maternal da levels. Conversely reductions in dosage are masculinising in triploids and decrease the viability of females with reduced Sxl or da levels. The interaction of the sis genes with the Sxl alleles Sxl^{f9} and Sxl^{fLS} are especially informative. As previously discussed these alleles are believed to be defective in pathway initiation (Sxl^{f9}) and pathway maintenance (Sxl^{fLS}), both da and sis-a show very similar phenotypes in combination with these Sxl alleles when tested for dominant synergistic effects; sis-a Sxl^{f9} (XX) double heterozygotes from da/+ mothers are not viable whereas sis-a Sxl^{fLS} females from the same mothers are as viable as individuals wild type for Sxl. This seems to indicate that reductions in sis-a levels are only detrimental when coupled with Sxl alleles defective in pathway initiation. No effect

is detected when sis-a is coupled with Sxl alleles which can initiate Sxl expression normally but which are defective in maintaining expression. This result was previously shown for da (Maine et al., 1986a).

The molecular nature of sis-a is not known but sis-b has recently been shown to be a product of the Achaete-scute complex, at least in part a product of the T4 transcription unit of this complex (Torres and Sanchez, 1989). The product of this locus appears to be a DNA binding protein with the same DNA binding and helix-loop-helix motifs as the putative da polypeptide. Murre et al. (1989b) have shown that da and AS-C T3 gene products form heterodimers in-vitro. The AS-C T3 gene product appears to have a similar structure to the T4 product. These heterodimers can bind specifically to DNA fragments containing promoter elements, whereas the individual polypeptides cannot. This remarkable finding suggests that da and AS-C products interact by forming heterodimers required for transcriptional activation of both Sxl and genes involved in neurogenesis. This is compatible with the previous suggestion that da acts as a transcription factor directly at the putative Sxl embryo specific regulatory elements. An alternative explanation is that da product is required to activate the expression of AS-C transcription units, which are then required early in development to activate Sxl (sis-b function) and later in development for neurogenesis (šcute function). It will be particularly informative to examine the effects of maternal da mutations on zygotic sis-b expression.

Whether the sis-a gene is a conventional protein coding gene remains to be determined. It is possible that it could be a binding site for a regulator which counts the number of X chromosomes by a titration effect. This suggestion echoes, in some respects, a model proposed by Chandra (1985) to account for the assessment of the X:A ratio. He proposes a maternally activated zygotic repressor which is titrated by binding sites on the X chromosome. While diplo-X individuals would have enough sites to mop up the repressor and hence allow Sxl expression, in Haplo-X individuals there would be an insufficient number of sites and hence Sxl would be repressed. The proposed repressor need not act on Sxl directly but instead on an X-linked positive regulator of Sxl such as the sis-b gene. Such a model is appealing in its

simplicity but is rather unsatisfactory when one considers that the level of repressor would have to be exquisitely balanced. Minor fluctuations either way would be lethal to one sex or the other. A similar model with a number of repressor-site interactions could perhaps buffer the system against changes in the level or activity of a single component. As to the existence of other numerator elements Cline suggests that more are present but that their number is limited. He proposes selecting for the dominant synergism discussed above to isolate such genes (Cline, 1988).

Several other genes which appear to interact with Sxl have been reported but are at present rather poorly characterised.

Female sterile(1)^{1621} also known as liz and snf (Steinmann-Zwicky, 1988; Oliver et al., 1988) is an X linked gene which has both maternal and zygotic functions required for Sxl activity. It is required in both the germ line and the soma. Some lethal effect is apparent which can be enhanced by reductions in Sxl or da levels and rescued by Sxl^{M1}.

Daughterkiller (Dk) is a dominant maternal effect female lethal, it can be rescued by Sxl^{M1}. (Steinmann-Zwicky at al., cited by Lucchesi and Manning, 1987).

female lethal(2)d a temperature sensitive homozygous female lethal. It is reported that this allele can rescue males from the lethal effects of Sxl^{M1}. (San Juan and Sanchez, cited by Lucchesi and Manning, 1987). This suggests that it either acts downstream of Sxl or is an activation function which Sxl^{M1} is not constitutive for.

In summary genetic and molecular evidence suggest a model for the regulation of the sex determination gene sex-lethal. Zygotic functions (eg sis-a, sis-b) cell autonomously assess the X:A ratio early in development. When the X:A ratio is >1 these functions, in conjunction with maternal and zygotic functions (eg da, liz), activate Sxl. This initial activation may take the form of sex-specific transcription. Thereafter Sxl, by virtue of its autoregulatory capacity, is able to maintain the sexually differentiated state independent of the X:A ratio. This maintenance function probably operates at the

level of sex-specific RNA processing mediated by the Sxl gene product(s). The product(s) of the Sxl gene control dosage compensation, germline sex and somatic sex. This latter pathway is probably regulated by the same sex-specific splicing mechanism which maintains Sxl. Baker (1989) suggests that germline sex and dosage compensation may also be regulated by differential splicing. Functional studies of the different Sxl transcripts will be required to address this question.

Somatic Sex Determination Hierarchy

As discussed previously the genes which control somatic sex have been well characterised at the genetic and molecular level. As with Sxl a detailed genetic analysis of tra, tra2, dsx and ix has provided a framework for subsequent molecular studies. Baker and Ridge (1980) carried out a clonal analysis of these genes which demonstrated cell autonomy of gene activity. They also constructed flies simultaneously homozygous for mutations in more than one of these genes. In this way they were able to demonstrate a clear epistatic relationship with dsx at the end of the pathway and tra and tra2 regulating dsx. Due to the similarity in phenotype the interaction between tra and tra2 could not be investigated. It was also unclear where ix acted in view of its similarity with dsx. Molecular cloning of three of these genes has helped to resolve these ambiguities and has provided strong support for Baker and Ridge's model. Each of the genes involved in the hierarchy will be discussed below.

transformer(tra)

tra resides at 73A on the polytene chromosome map. The gene was first discovered by Sturtevant (1945) as a recessive mutation which transforms chromosomally female individuals into phenotypic males, there is no discernible effect on chromosomal males. In homozygous females all somatic tissue is phenotypically male but these individuals have a female

body size. In addition these individuals exhibit male courtship behaviour (McRobert and Tompkins, 1985). The gonads of homozygous tra females develop as rudimentary testes (Brown and King, 1960) however the status of the germ cells within these underdeveloped gonads is unclear. The appearance, at a low frequency, of oogenic and spermatogenic germ cells is reported but in the majority of testes examined only a mass of degenerating tissue is found (Brown and King, 1960; Seidel, 1963; Steinmann-Zwicky et al., 1989). Pole cell transplantation experiments with tra/tra germ cells and wild type hosts indicate that tra is not required in the female germline for normal oogenesis (Marsh and Wieschaus, 1978; Schupbach, 1982). There is no apparent effect on dosage compensation as X-linked genes appear to be expressed at wild type levels (Brown and King, 1960). Thus it would appear that tra is required solely for female somatic differentiation. Somatic recombination experiments indicate that tra activity is cell autonomous and that it is required at least until pupariation in some tissues (Baker and Ridge, 1980).

The gene has been cloned (Butler et al., 1986; McKeown et al., 1987) and localised to a 3.5kb EcoRI fragment which is able to rescue tra/tra females in P-element transformation experiments. This fragment hybridises to four transcripts on northern blots, two of these correspond to unrelated genes which lie very close to the tra gene (the 3' gene actually overlaps with tra). The other two transcripts, which are expressed throughout development, are from the tra gene. Only the smaller of these two transcripts is female specific.

Sequence analysis of cDNA clones led to the surprising finding that tra was regulated by differential splicing (Boggs et al., 1987). Both tra transcripts use the same start site but differ by the use of alternative 3' splice acceptor sites within the first intron of the gene. The non sex-specific transcript is spliced in such a way that translational terminating codons are included in all reading frames. The female specific transcript on the other hand uses an acceptor approximately 200bp downstream of that used by the non sex-specific transcript. The female-specific transcript encodes a long open reading frame capable of producing a 22kd polypeptide. This conceptual polypeptide shows no homology with known sequences but it is extremely basic suggesting that it could possibly bind to nucleic

acids. Sex-specific splicing of the wild-type gene has been used to monitor gene interactions within the sex determination hierarchy (Nagoshi et al., 1988). As expected only the activity of Sxl was required to produce the female-specific transcript.

P-element transformation experiments with cDNA clones under the control of the heat shock promoter show that the non sex-specific transcript is without detectable function. Only the female-specific cDNA can rescue tra/tra females, moreover this cDNA alone is sufficient to transform XY individuals into somatic females. This surprising finding indicates that the tra gene is sufficient to direct all other genes downstream to female somatic development (McKeown et al., 1988). Expression of this construct in various mutant backgrounds has been used to verify the genetic model of somatic sex determination. In agreement with the model ectopic expression of tra is able to rescue sex-transforming Sxl mutations but not mutations in tra2, dsx or ix. The observation that tra2 mutations are epistatic to tra indicates that tra2 does not act upstream of tra.

A more detailed examination of the splicing reaction has provided considerable insight into the regulation of tra (Sosnowski et al., 1989; Belote et al., 1989). When the whole intron is inserted into another gene, sex-specific splicing can be detected suggesting that the specificity resides entirely within the intron. Support for this comes also from the analysis of deletions within the intron when in its normal environment (Sosnowski et al., 1989). These experiments suggest that the non sex-specific splice site is the target for regulation such that in the presence of Sxl this splice site is only used 50% of the time. A comparison of the sequences at the regulated tra splice-site and the 3' splice site which proceeds the male-specific exon in the Sxl gene reveals a striking homology. The homology occurs at the polypyrimidine tract upstream of the 3' acceptor. A region of this type is believed to be necessary for successful splicing in all eukaryotic introns (Krainer and Maniatis, 1988). In both of the regulated introns this tract is composed almost exclusively of T residues. This is highly unusual and not a feature found in other introns. It has been suggested that a Sxl gene product binds at these splice sites to prevent their use. This would result, in the case of tra, in the

preferential use of the downstream female specific acceptor. A similar proposal has been put forward to explain the autoregulatory capacity of Sxl (Bell et al., 1988).

Transformer 2 (tra2)

This gene maps cytologically to region 51B of the salivary gland chromosome map. Phenotypically it is similar to tra, homozygous females being transformed into somatic males (Watanabe, 1975). However in contrast to tra, tra2 homozygous males are sterile. This appears to reflect a requirement for tra2 in the male germline to allow the completion of spermatogenesis. The defect in spermatogenesis, a failure to elongate sperm heads, suggests that tra2 may be involved in X chromosome condensation (Belote and Baker, 1983). tra2 is not required in the female germline (Schupbach, 1982) or for dosage compensation (Fujihara et al., 1978).

The availability of hypomorphic and temperature sensitive tra2 alleles has been an invaluable aid in the investigation of somatic sex determination, particularly in the study of terminal differentiation genes. The temperature sensitive alleles $tra2^{ts1}$ and $tra2^{ts2}$ (Belote and Baker, 1982) have been used to estimate the temporal requirements for the action of the sex determination hierarchy in different somatic tissues. Temperature shift experiments indicate that tra2 is required from at least the mid 2nd larval instar through to the pupal stage to permit female differentiation. Different tissues however require tra2 activity at different times; for example female genital disk derivatives require the product throughout the entire period. However expression is only required from just before to just after pupariation to prevent the formation of the male sex comb. The $tra2^{ts1}$ allele has also been used to provide evidence for the involvement of the sex determining hierarchy in regulating male courtship behaviour. Hemizygous $tra2^{ts1}$ chromosomal females develop as female like intersexes at the permissive temperature. Under these conditions they do not appear to exhibit male courtship behaviour. If these individuals are shifted to the restrictive temperature post eclosion a

substantial proportion show male courtship behaviour after a delay of several days (Belote and Baker, 1987). The authors suggest that this behavioural change is indicative of a certain amount of plasticity in the regulation of sexual behaviour. It is not clear however if this plasticity is primarily a reflection of the intersexual nature of the females when they are raised at the permissive temperature.

The hypomorphic allele tra2^{OFT} (Fujihara et al., 1978) is homozygous male fertile and homozygous females are partially transformed. Interestingly some of these individuals have apparently normal ovaries containing well developed eggs. Hemizygous females have more testis like rudimentary gonads which are frequently associated with ovarian-like tissue. This allele could prove to be very useful in studying the proposed interaction between the germline and somatic component of the gonad uncovered by Steinmann-Zwicky et al. (1989).

tra2 has recently been cloned (Amrein et al., 1988; Goralski et al., 1989). Transcripts from the gene (it is not yet clear how many) are detected at all stages of development in both germline and soma. It appears that tra2 is not expressed sex-specifically or regulated at the RNA level, no difference having been detected between males and females with respect to tra2 transcript levels. This is compatible with the observation that expression of female-specific tra cDNA is sufficient to promote female somatic differentiation in XY individuals. A conceptual tra2 polypeptide of approximately 250 amino acids has extensive similarity to RNA binding proteins found in hn-RNPs and sn-RNPs, both of these particles have been implicated in mRNA splicing. This finding suggests that tra2 may act directly to regulate the splicing of dsx mRNA. tra may act in conjunction with tra2 in this role or tra may be required for the translation of the tra2 mRNA. These models are being actively investigated (Goralski et al., 1989; Burtis and Baker, 1989). The expression of tra2 in the female germline and male soma is somewhat puzzling given that no function for tra2 has been detected in these tissues. The expression of tra2 in the female germline is at very high levels, much higher than in the soma where the function is apparent. High levels of tra2 transcript are also detected in the 0-3hr embryo (Amrein et al., 1988; Goralski et al., 1989). Taken together these observations suggest that

there may be a maternal contribution of tra2 to the zygote. If this is the case any function for maternally derived tra2 transcripts must be redundant: recall that pole cell transplantation experiments indicate that tra2 is not required in the female germline. It is possible, then, that tra2 expression in the female germline merely reflects the presence of a regulatory element which is needed for high expression in the male germline but which is also active in the female germline. This could also be an explanation for tra2 expression in the male soma.

doublesex (dsx)

First discovered by Hildreth (1965) as a recessive mutation affecting both males and females dsx maps to salivary gland chromosome region 84E. Both XX and XY individuals homozygous for null dsx alleles develop as morphologically identical intersexes apart from the sexual dimorphism in body size which is maintained. In contrast to triploid intersexes which are mosaics of male and female tissue, dsx intersexuallity is evident at the level of the individual cell. Here it would appear that cells are expressing both male and female characteristics simultaneously. This is particularly striking in the genital disc derivatives where both male and female structures are apparent in adults. The gonads of dsx individuals are, in most cases, irregular spherical masses resembling neither the male or female gonad, more than two gonads have never been observed. dsx is not required in the female germline (Schupbach, 1982).

Two alleles of the dsx gene have been particularly informative in the dissection of the role this gene plays in sexual differentiation.

dsx^{D} (dominant): this allele has no effect on chromosomally male individuals but transforms females to intersexes when they carry a wild type copy of the gene. Hemizygous females or $dsx^{D}/+$ females simultaneously homozygous for tra or tra2 develop as somatic males.

dsx^{136}: when homozygous or heterozygous with a null allele chromosomal males are transformed to intersexes. There

is no effect on chromosomal females. $\underline{dsx}^{D}$ is dominant to $\underline{dsx}^{136}$. $\underline{dsx}^{D}/\underline{dsx}^{136}$ chromosomal males are normal whereas chromosomal females are intersexual. Finally, when examined in a homozygous tra2 background, $\underline{dsx}^{136}$ females are transformed to intersexes.

Baker and Ridge (1980) were able to reconcile these observations by proposing that dsx is a bifunctional gene required in one of two different states for the correct sexual differentiation of either males or females. Furthermore they proposed that the products of the tra, tra2 and ix genes are required only for expression of the dsx female state. Their model predicts that $\underline{dsx}^{D}$ represents constitutive expression of the male state. $\underline{dsx}^{136}$, by their model, has lost the ability to express the male state but still requires the activity of tra, tra2 and ix for expression of the female state. They go on to suggest that dsx acts as a repressor of terminal differentiation functions such that $\underline{dsx}^{M}$ represses female differentiation genes and $\underline{dsx}^{F}$ represses male functions. In the absence of the dsx gene both sets of terminal differentiation genes are expressed hence the observed intersexual phenotype. Surprisingly it would appear that simultaneous expression of $\underline{dsx}^{M}$ and $\underline{dsx}^{F}$ has the same phenotype (eg (XX) $\underline{dsx}^{D}/+$), suggesting that the products are mutually antagonistic. The model is consistent with $\underline{dsx}^{M}$ being a default state of expression and with the tra, tra2 and ix gene products being required to override the default state to bring about female differentiation.

The molecular cloning of dsx sheds some light on the bifunctional nature of this gene (Baker and Wolfner, 1987; Burtis and Baker, 1989). It appears that the gene produces two major transcripts, one male-specific the other female-specific. These result from the sex-specific splicing and alternative polyadenylation of a primary transcript. These transcripts initiate at the same start site. Other non sex-specific transcripts are also present, it is not yet clear if they have any functional role. The sex-specific transcripts are detected in 3rd instar larvae, pupae (where their expression is highest) and adults. Genetic evidence predicts that they be present much earlier for differentiation of the gonad during the embryonic stage (Hildreth, 1965) and the genital disk during the 2nd larval instar (Wieschaus and Nothiger, 1982). Whether failure to

detect the adult sex-specific transcripts during these earlier stages is due to a lack of sensitivity in the experiments or a requirement for other, as yet undetected, dsx transcripts early in development remains to be determined. Strong evidence that the sex-specific transcripts represent the functional dsx product comes from an examination of four dsx^D alleles. They all contain disruptions of the female specific exon. Burtis and Baker (1989) propose that tra and tra2 regulate the sex-specific splicing of the dsx transcript, they suggest that regulation is mediated at the 3' acceptor site of the female-specific exon by the action of tra and tra products. In support of this they note that the sequence at this particular splice junction is a poor match to consensus Drosophila acceptor sequences. Further support comes from an examination of the same splice site in D.virilis. While the intron sequences upstream of the female specific exon are highly diverged the poor acceptor is well conserved. The model proposed by Burtis and Baker is a splice site activation mechanism where tra and tra2 act at this splice site to allow the use of the otherwise poor acceptor. This is in contrast to the splice site blockage model proposed for Sxl and tra genes. A molecular analysis of the sex-specific splicing shows that Sxl, tra and tra2 but not ix are required for the production of the female-specific transcript (Nagoshi et al., 1988). This is consistent with the genetic model and the idea of a male default state.

Although these data are encouraging other experiments have been less successful. P-element transformation studies have been carried out using sex-specific cDNA clones controlled by an actin promoter, these constructs show very little effect on homozygous dsx individuals. Similarly there are only minor effects on wild type individuals carrying a cDNA from the opposite sex. In both of these experiments it is mainly tergite pigmentation which is noticeably affected. There is however some elevation of yolk protein mRNA in males carrying dsx^F. It is not yet clear whether or not the weak phenotype associated with these constructs is due to inappropriate expression from the actin promoter or whether it reflects the lack of another essential dsx function. Unfortunately the sequence of the dsx polypeptides has been less informative than one would have hoped. Male dsx encodes

a putative 57kd polypeptide and the female transcript one of 45kd. These polypeptides differ at their carboxy termini. No significant homology between the dsx polypeptides and known protein sequences has been detected. The polypeptides do, however, contain strings of repeated amino acids found in other Drosophila regulatory proteins, although it is not known if these regions are significant (see Burtis and Baker, 1989 for a full discussion). Clearly further studies of the dsx gene and the targets which it regulates are required before we know how the gene is acting.

intersex (ix)

Comparatively little is known about ix compared with other genes in the somatic sex determination hierarchy. Homozygous ix females are transformed to intersexes but there is no effect on males (Morgan et al., 1943; Baker and Belote, 1983). There is no information regarding the autonomy of ix but it seems likely, by analogy with the other sex determining genes, to act cell autonomously. In the double mutant analyses of Baker and Ridge (1980) dsx, tra and tra2 were all epistatic to ix. This would suggest that it is at the beginning of the pathway. The analysis was hampered, however, by the similarity of dsx and ix phenotypes. Molecular analysis of tra and dsx sex-specific splicing show that ix is not required for the production of female-specific transcripts from either of these genes (Nagoshi et al., 1988). This would place ix above dsx in the hierarchy. These observations are compatible if ix is required to act in conjunction with the dsx^F product(s) for its activity. Thus tra and tra2 would appear to be epistatic because they fail to produce dsx^F (Baker and Ridge, 1980, Baker et al., 1987). A molecular analysis of this gene may help to clarify its role in sexual differentiation. One attractive idea is that the ix product provides an opposite function to that encoded in the carboxy terminal of the dsx^M polypeptide (B.Baker pers.com).

As we have seen correct activity of Sxl is required for germline sex determination. Several other genes affecting the development of the germline have been identified (eg. agametic, tudor, grandchildless) however these genes are not sex-specific, rather they disrupt pole cell development in both sexes. In addition most of these genes have effects on somatic development.

One gene, ovo (Busson et al., 1983), has been isolated which is required only for female germline development. Mutant males are not affected and develop as normal and fertile individuals. Ovo, which maps to region 4E1 on the X chromosome, was initially identified by dominant alleles, the most severe of which, Ovo^{D1}, results in female sterility. The ovaries of Ovo^{D1}/+ females are atrophied and almost completely devoid of germ cells. Clonal analysis indicates that ovo may be required continuously in the female germline (Perrimon, 1984). Loss of function alleles have been isolated and used to determine ovo requirement during development (Oliver et al., 1987). Here pole cell death is observed from late blastoderm onwards. This finding suggests that germ cells determine their sex at around the same time as somatic tissue. If this is the case it is before they come into contact with the gonadal soma. Sxl has been shown to be active in the soma at this time but it is not yet known if it is active in the germline this early. The germline phenotype of mutations in Sxl and ovo are radically different. Sxl mutations result in undifferentiated growth of germ cells whereas ovo mutations cause germ cell death. It is not clear as yet if, or how, ovo and Sxl interact but as mutations in these genes show a different phenotype an epistatic analysis may be possible. In this respect the allele Sxl^{fs1} may prove to be of some use as it allows normal female somatic development. It would also be informative to assess the effect of ovo mutations on the pattern of Sxl transcription. Clearly it is important to establish any interaction if we are to understand germline sex determination. The region containing the ovo gene has recently been cloned (Mevel-Ninio et al., 1989) and is under investigation, it would be of some interest to investigate the ectopic expression of the gene in males, perhaps by P

element transformation experiments.

The recent study of Steinmann-Zwicky et al. (1989) indicates that control of germline sex is not entirely cell autonomous: an interaction between germline and gonadal soma is apparent. In these studies pole cells were transplanted into agametic host embryos and their development assessed at the adult stage. In ovaries germ cells develop according to their X:A ratio; XX germ cells undergo oogenesis with fertile eggs being produced. XY germ cells initiate spermatogenesis, however they do not progress beyond the spermatocyte stage. In testes both XY and XX germ cells enter spermatogenesis, XY cells produce functional sperm while XX cells degenerate after the spermatocyte stage. These observations led the authors to conclude that germ cells have an inherent ability to determine their sex but that compatible soma is required to support full development. However in the case of the XX germ cells an inductive interaction with the gonadal soma in the determination of germline sex also seems to be apparent. This can be interpreted as either a signal from ovarian tissue necessary to initiate the female pathway of development or a testicular signal capable of imposing spermatogenic development irrespective of chromosomal sex. Arguing against the latter hypothesis; XY cells enter spermatogenesis irrespective of the sex of the soma, a somatic signal from the male gonad imposing spermatogenesis would appear to be redundant.

Steinmann-Zwicky et al. also studied pole cells carrying various Sxl mutations, again transplanted into agametic hosts. XX pole cells homozygous for Sxl^{f1} enter the spermatogenic pathway in both ovaries and testes. This observation supports the previous studies of Schupbach (1985) on Sxl requirement in the female germline. XX pole cells carrying a copy of the semi-constitutive Sxl^{M1} allele initiate oogenesis irrespective of somatic sex. Thus this allele is able to over-ride the somatic induction previously observed. This would seem to suggest that the somatic induction involves an interaction between the soma and Sxl or one of its positive regulators. Female soma may be required to activate and/or maintain Sxl expression or male soma may repress Sxl. Again the latter explanation would appear less likely; if Sxl is never expressed in the male germline why have a mechanism to repress it? The Sxl^{M1} allele has no

effect on haplo-X germ cells. This supports previous suggestions that this allele is not constitutive for all functions in situations where the X:A ratio is inappropriate for Sxl activation (Cline, 1988; Steinmann-Zwicky, 1989).

Identification of other genes involved in germline sex determination is clearly a high priority if we are to unravel the regulation of this process. Two routes are available at present, one is to examine sex-specific sterile mutations in the hope of identifying those in which germ cell development is arrested at an early stage. This is how the ovo gene was identified. Such an undertaking is however fairly daunting given the large number of sterile mutants so far isolated (for example over 600 genes are estimated to be mutable to male sterility, Lindsley and Lifschyts 1972). An alternative approach may be to isolate genes expressed sex-specifically early in development either by +/- screening or by using subtracted cDNA probes. Single sex embryo populations can be obtained easily using fly stocks which contain the Responder element of Segregation Distorter translocated on to the sex chromosomes (Lyttle, 1989; Walker et al., 1989). These strains allow the production of embryo populations which are greater than 95% single sex. By examining the expression of, and making mutants in, genes isolated by the above method one would hope to identify candidates for genes involved in germline sex determination.

Sex-specific Gene Expression In The Soma

Several genes have been isolated which show sexually dimorphic expression in somatic tissues. Two basic types of regulation are apparent; direct regulation where the somatic sex determining hierarchy is required continuously to ensure the appropriate expression of a gene, and indirect regulation where the hierarchy specifies the formation of a sexually dimorphic tissue which in turn determines the expression of a gene.

Direct Regulation

The yolk protein (YP's) genes are the only known examples

of genes subject to this form of regulation. These three X-linked genes are expressed in the female fat body and the follicle cells associated with vitellogenic oocytes. It is only in the fat body that direct regulation is apparent, expression in the follicle cells is by virtue of tissue specificity. YP1 and YP2 are closely linked separated by around 1.2kb. They are transcribed divergently. YP3 is located about 1000kb away from the other two genes (Shepherd et al., 1985)

Several investigators have studied the expression of these genes. Of particular relevance are studies on the effects of mutations in the sex determination hierarchy (Bownes and Nothiger, 1981; Ota et al., 1981; Brennan et al., 1982). The results of these investigations may be summarised by stating that YP expression in the female fat body requires the expression of the female specific dsx product. Belote et al. (1985) conducted temperature shift experiments with the $tra2^{ts1}$ allele to further demonstrate that tra2, and by inference dsx, is required continuously for YP expression in the adult. If an XX individual homozygous for $tra2^{ts1}$ is raised at the restrictive temperature it develops as a somatic male and does not synthesis YP's. If it is then shifted down to the permissive temperature YP synthesis is initiated. Similarly YP synthesis declines dramatically if a similar individual is raised at the permissive temperature and then shifted up to the restrictive temperature. in contrast YP synthesis in the follicle cells of the ovary is not directly affected by these temperature shifts (M. Bownes pers. comm.). Molecular cloning of the YP1 gene and P-element transformation experiments with upstream sequences have identified a 125bp enhancer-like element which is sufficient to direct fat body specific and sex-specific expression to a heterologous gene (Garabedian et al., 1986). An additional element which confers follicle cell specific expression has also been identified (Shepherd et al., 1985). At present it is unclear how the enhancer element is regulated by the sex determining hierarchy. The dsx product may act directly at this element, or indirectly by regulating the expression of some other factor required for YP expression. The direct regulation of these genes is probably a special feature in the control of sex-specific gene expression as the fat body is not a sex-specific tissue. The majority of sex-specific

genes are expressed in a tissue specific manner. Nevertheless the YP genes offer a unique opportunity to investigate directly the molecular mechanisms by which the sex determination hierarchy operates.

Indirect Regulation

All of the genes so far isolated which are indirectly regulated by the somatic sex determination hierarchy are expressed in the reproductive apparatus. In the female the chorion genes are expressed in the somatic follicle cells of the ovary. Expression is dependent on the presence of an ovary and a functional germline (DiBenedetto et al., 1987). In males, genes expressed specifically in the accessory glands and the ejaculatory duct have been identified. These include esterase-6 (Oakshott et al., 1987) and glucose dehydrogenase (Cavener et al., 1985), pheremone peptides 70A (Chen et al., 1988) and mst355 (Monsma and Wolfner, 1988) and several accessory gland transcripts of unknown function: mst-1, 2, 3, 35 (Schafer, 1986a) and mst316 (DiBenedetto et al., 1987). Expression of these accessory gland transcripts has been examined in chromosomal females carrying mutations in various sex determining genes. As expected their expression was dependent upon the presence of an accessory gland. Chapman and Wolfner (1988) examined the expression of mst316 and mst355 in $\underline{tra2}^{ts1}$ temperature shift experiments. They found that the expression of these genes was irreversibly determined at approximately the same time as accessory gland formation is irreversibly determined. By further examination of these genes it should be possible to identify the regulatory elements which confer tissue specificity. The key questions will then be what factors act at these elements and how are these factors regulated.

One interesting example of sex-specific gene expression is the glucose dehydrogenase gene (gld). This gene is expressed in both sexes during the pupal stage where it appears to be required for normal eclosion. However in adults expression is restricted to the male ejaculatory duct where the gene product is believed to have a role in reproductive behaviour. It would be of some interest to examine the regulatory elements of this gene to try and separate the different modes of expression.

Before leaving sex-specific genes in the soma it is worth noting that many genes may not be regulated by simple all or nothing expression. For example many genes may show sexual dimorphism merely in terms of the level or the site of expression (eg pigment distribution on the adult cuticle). The study of these genes may permit a more subtle understanding of how gene expression is regulated. However methods to isolate this class of genes are at present limited.

All of the somatic sex-specific genes so far isolated are expressed during the adult stage. However phenotypic differences between the sexes are apparent at earlier stages, most notably in the gonad. Identification of genes expressed specifically in the soma of the developing gonad, particularly those genes expressed early in development, would be most useful in attempting to elucidate regulatory mechanisms (see below).

Germline-Specific Transcripts

Rather more progress has been made in the study of the regulatory sequences controlling genes expressed in the germline. Here however we are hampered because our understanding of germline sex determination is rather poor. Of the genes that have been studied mst(3)gl9 and B2-tubulin have been the subject of particularly extensive examination. Both genes are only expressed in the male germline. Kuhn et al. (1988) have shown that the 102bp upstream of the mst(3)gl9 start site is sufficient to confer spermatocyte-specific expression on a heterologous gene. In addition they demonstrate that 110nt of 5' untranslated sequence and 10nt of coding sequence are sufficient to allow appropriate germline translational control of a lacZ fusion construct. The B2-tubulin gene has been dissected by Michiels et al. (1989), they find that the 52bp upstream of the start site is sufficient to direct testis specific expression. Within this region they identified a 14bp element (BUE1) which is conserved in sequence and position in the homologous gene from D.hydei. If this sequence is mutated expression is abolished. However in contrast to the element found at the

YP1 gene, BUE1 does not act as an enhancer but as a conventional promoter element. The BUE1 sequence is not found upstream of the mst(3)gl9 gene. Whether this reflects a requirement for these genes at different times in spermatogenesis or that a variety of sequences confer sperm specific transcription remains to be determined.

The isolation and characterisation of more genes showing sexually dimorphic expression, both in the soma and the germline, will be important in our attempts to define regulatory elements. The identification of regulatory factors acting at these elements is dependent upon the ease with which reliable nuclear extracts can be isolated for DNA binding studies. In this respect sex-specific transcripts expressed early in development will be particularly useful as nuclear extracts can be prepared with ease from the Drosophila embryo. In chapter 3 I will outline the experimental approach I have chosen to try and identify genes in this class.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The reagents used during the course of this work were supplied by the following manufacturers.

a)Enzymes: Restriction endonucleases, T4 Polynucleotide kinase, T4 DNA ligase, Ecoli DNA polymeraseI, E.coli RNaseH, (BRL). Klenow fragment E.coli DNA polymeraseI, calf intestinal phosphatase, (Boeringer Mannheim). AMV reverse transcriptase, (Pharmacia). T4 DNA polymerase, (Anglian Biotechnology). EcoRI methylase, (New England Biolabs).

b)Specific reagents: Oligo(dT)-cellulose type 7, oligo$(dT)_{12-18}$, dNTP's, RNAGUARD [human placental ribonuclease inhibitor], pd(N_6), Sephadex G50, (Pharmacia). Biotin-16-dUTP, (Boeringer Mannheim). Streptavidin-biotinylated horseradish peroxidase complex, Hybond-N membrane, Lambda in-vitro packaging kit (Amersham). EcoRI linkers, (BRL). Ultragel AcA34, (LKB).

c)Others: All other chemicals and reagents were purchased from generally available sources with no preference for any one manufacturer. Wherever possible AnAlar or Biochemical grade chemicals were used.

Buffers and Media: Most common buffers and media recipes were obtained from the Cold Spring Harbour Molecular Cloning manual (Maniatis et al., 1982). Buffers for restriction and modifying enzymes were prepared according to the manufacturers recommendations.

2.2 METHODS

2.2i Nucleic Acid Isolation:

a)Plasmids: Large scale plasmid isolation was carried out by the alkaline lysis method (Birnboim and Doly, 1979) as described in Maniatis et al.(1982) or on a small scale by the boiling method (Holmes and Quigley, 1981).

b)Bacteriophage lambda: Large scale isolation of lambda DNA was performed by a modification to the protocol of R.Buckland (EMBL Heidelberg). A high titre stock was prepared from a plate lysate using a fresh single plaque as starting material. This stock was used to infect appropriate host cells (OD_{660} approx. 0.3) in a 2l flask containing 200ml of L-broth, 10mM $MgSO_4$. The culture was incubated at 37°C with vigorous shaking until lysis was apparent (4-5hrs.), 1ml of $CHCl_3$ and 8g of NaCl was added and shaking continued for a further 10-15min. The debris was pelleted (15,000g, 15min) and 20g of PEG 8000 was added to the recovered supernatant. The PEG was dissolved at room temperature and the samples were left at 4°C overnight to precipitate the phage. The precipitate was pelleted (15,000g, 15min) all of the supernatant carefully removed and the pellets resuspended in 11ml of phage buffer. The phage suspension was vortexed with an equal volume of $CHCl_3$, the phases separated (27,000g, 10min) and 10ml of the aqueous phase added to 7.2g of CsCl. Once the CsCl had dissolved the samples were decanted into quickseal tubes and spun for 12hr at 49,000rpm in a Beckman Ti70 rotor. The phage band was removed from the side with an 18G syringe needle and dialysed against TE (3 changes 1l each). DNA was purified by sequential extractions with phenol, 1:1 phenol/$CHCl_3$ and $CHCl_3$. The DNA was then dialysed against TE as above and stored at 4°C. Yields for EMBL3 and NM1149 recombinants were generally 100-200ug.

c)Lambda minipreps: Small scale isolation of lambda DNA for the analysis of recombinants was performed by a plate lysate method. (Maniatis et al., 1982).

d)Drosophila DNA: Drosophila high molecular weight chromosomal DNA was prepared by modifications to the procedure of Kaiser and Murray (1985). One gram of flies was ground in liquid N_2 with a precooled mortar and pestle and homogenised with 10ml of freshly prepared ice cold buffer (10mM Tris-HCl pH7.6, 60mM NaCl, 10mM EDTA, 0.15mM spermine, 0.15mM spermidine, 0.5%(v/v) Triton X-100) in a Wheaton 15ml homogeniser (A pestle).The solution was decanted into a 30ml corex tube on ice and the nuclei pelleted (6,000g, 10min) at $4^{o}C$. The supernatant was carefully removed and the nuclei resuspended in 10ml of homogenisation buffer by gentle pipetting. The nuclei were pelleted and resuspended a further 3 times and then finally transferred to a 50ml Falcon tube where they were lysed by the addition of 1ml of 20% (w/v) sarcosine followed by gentle inversion of the tube. **RNaseA** was added to a final concentration of 100ug/ml and the lysate incubated at $37^{o}C$ for 30-60min. Proteinase K was added to a final concentration of 100ug/ml and incubation continued for 2-12hr. The lysate was extracted, once with phenol, three times with 1:1 phenol/$CHCl_3$, twice with $CHCl_3$ and the aqueous phase was concentrated with iso-butanol to a volume of 1-2ml. This solution was dialysed for 24-48hr against several changes of TE and stored at $4^{o}C$. Yields of DNA were generally 200ug/g of starting material.

e)Drosophila RNA: RNA for Northern blot analysis was isolated by a modification of the method of Chomzynski and Sacchi (1987) that is described in the laboratory manual of K.Stanley (EMBL Heidelberg), followed by batch purification of poly(A)$^{+}$ mRNA using oligo(dT)-cellulose. One gram of tissue was homogenised at full speed in a Silverstone homogeniser for 2-3min in 10ml of denaturing solution (4M guanidinium isothiocyanate, 0.1M Tris-HCl pH8, 0.1M B-mercaptoethanol, 0.1%(v/v) antifoam-A). To the homogenate the following were added sequentially, 1ml 2M NaOAc pH4, 10ml phenol and 2ml $CHCl_3$. The mixture was vortexed, left to stand on ice for 15-30min and centrifuged (12,000g, 10min). The aqueous phase was removed to a fresh tube, 1vol of isopropanol added and the mixture left at $-20^{o}C$ for 1hr to allow the RNA to precipitate. The precipitate was pelleted (12,000g, 10min) and resuspended in 5ml of denaturing solution. The RNA was again precipitated as described above,

washed in 70% EtOH, and finally resuspended in 1ml of oligo(dT)-cellulose binding buffer (0.5M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA, 0.1% (w/v) sarcosine). Oligo(dT)-cellulose (50mg/g tissue) was hydrated overnight at 4°C in H_2O, treated for 15 min at room temperature with 0.1M NaOH and washed (5x 10ml sterile H_2O, 5x 10ml binding buffer). The RNA was heated to 68°C for 5min, chilled on ice, thoroughly mixed with the oligo(dT)-cellulose in a 15ml Falcon tube and incubated at room temperature for 30min with gentle agitation. The oligo(dT)-cellulose was pelleted by centrifugation (1500g bench-top centrifuge) and washed 5 times using 10ml of binding buffer for each wash. The poly(A)$^+$ mRNA was eluted by resuspending the resin in 0.5ml of sterile H_2O and incubating at 58°C for 15-30min with occasional shaking. The oligo(dT)-cellulose was pelleted (3000g bench-top centrifuge) and the supernatant transferred to a 1.5ml microfuge tube. The RNA was precipitated by the addition of 50ul 3M NaOAc pH5, 1ml EtOH, stored at -20°C for 1-12hr and collected by centrifugation in a microfuge for 30min. The RNA was resuspended in 100ul of sterile H_2O and stored at -70°C. Yields were generally 50ug of RNA/g of tissue.

2.2ii:In-vitro DNA manipulation

Restriction endonuclease digestion of DNA, subcloning of DNA into plasmid vectors and introduction of recombinant plasmids into E.coli were performed using minor modifications of standard procedures (Maniatis et al., 1982).

2.2iii:Electrophoresis and blotting

Electrophoresis of DNA was carried out in TBE agarose gels. Fragment sizes were calculated according to the method of Helling et al. (1974) using restriction digested λcI857 s7 DNA as a standard (Sanger et al., 1982). For hybridisation gels were blotted to Hybond-N membranes using recommended procedures (Maniatis et al., 1982). Hybridisations were carried out either in 6X SSC, 1% SDS, 10X Denhardts, 100ug/ml denatured herring testis DNA, 15ug/ml polyA, 0.005% NaPPi at 68°C or in 50% formamide, 5X SSPE, 1% SDS, 10X Denhardts,

100ug/ml denatured herring testis DNA, 15ug/ml polyA, 0.005% NaPPi at 42°C. Blots were washed in 2X SSC, 1% SDS, 0.1% NaPPi at 68°C where low stringency was required or with additional washes in 0.1X SSC, 1% SDS, 0.1% NaPPi at 68°C for high stringency. Blots were exposed to either Kodak Xomat-S or XAR-5 film at -70°C with intensifying screens. RNA analysis was carried out by electrophoresis in agarose formaldehyde gels as described by Maniatis et al. (1982) followed by transfer to Hybond-N using the conditions recommended by the manufacturer. Blots were hybridised in the 50% formamide solution and washed as described above for DNA at high stringency. Transcript sizes were estimated by comparison with an RNA size ladder (BRL).

2.2iv:Radiolabeled nucleic acid

For blotting experiments ^{32}P labelled DNA was prepared either by nick translation of plasmid and phage DNA (Rigby et al., 1977) or by random priming of gel purified restriction fragments (Feinberg and Vogelstein, 1983). All probes were purified by Sephadex G50 chromatography in columns prepared from disposable 1ml syringes and were generally used at 10^5-10^6cpm/ml of hybridisation solution. First strand cDNA was labelled to high specific activity in the following way: 100-150uCi of a^{32}PdCTP (800Ci/mMol) was dried down in a siliconised microfuge tube, to this the following was added in order, 4ul 5X 1st strand buffer (250mM Tris-HCl pH8.3, 700mM KCl, 50mM $MgCl_2$, 50mM DTT), 1ul 80mM NaPPi, 30u RNAGUARD, 2ul dNTP mix (10mM dAGT), 2ug oligo$(dT)_{12-18}$, 1ug poly$(A)^+$ mRNA, 20u AMV reverse transcriptase and sterile H_2O to a final volume of 20ul . The reaction was incubated at 42°C for 30min. Then 1ul of 10mM dCTP was added and incubation continued for a further 30min. The RNA was hydrolysed by the addition of 1 volume of 0.6M NaOH, 20mM EDTA and incubation at 65°C for 30min. Radiolabelled cDNA was purified on Sephadex G50 columns and incorporation estimated by Cherenkov counting a small aliquot (Maniatis et al, 1982). Specific activities of >1x10^8 cpm/ug mRNA were obtained for the best probes, lower incorporation appeared to be associated with impure mRNA template.

2.2v:Construction of Drosophila cDNA libraries

Single sex 3rd instar cDNA libraries were constructed as follows:

a)RNA preparation. RNA was prepared by modifications to the procedure of Chirgwin et al. (1979). Hand sexed late 3rd instar larvae were homogenised, 30 at a time, in 1ml of 4M guanidinium isothiocyanate, 50mM Tris-HCl pH7.6, 10mM EDTA, 2% (v/v) sarcosine, 140mM B-mercaptoethanol, 25mM NaCitrate, 0.1% Antifoam-A. 30ml of pooled homogenate was layered onto a 15ml cushion of 5.7M CsCl, 0.1M EDTA in a Beckman polyallomer SW28 centrifuge tube which was spun at 25,000rpm for 40hr. After centrifugation the supernatant and cushion were removed by aspiration and the pellet resuspended in 3ml of the above denaturing solution. This solution was in turn layered onto a 1.5ml CsCl cushion in a polyallomer SW50 tube and spun for 20hr at 35,000rpm. The supernatant and cushion were removed with care, the pellets allowed to drain and the RNA resuspended in 1ml of sterile H_2O. Poly(A)$^+$mRNA was isolated by chromatography on three sequential oligo(dT)-cellulose columns (Aviv and Leder, 1972) following the manufacturers instructions except that sarcosine replaced SDS in all buffers (sarcosine is less susceptible to precipitation in high salt than SDS) and the poly(A)$^+$ was eluted with sterile water.

b)First and second strand cDNA synthesis: This was carried out by a modification to the procedure of Gubler and Hoffman (1983) as described in the Amersham cDNA synthesis kit but using reagents from different manufacturers. Reaction products were sized on 1.5% alkaline agarose gels (Maniatis et al., 1982) and yields estimated by determining radioactivity incorporated into acid precipitable material (Berger, 1987). The cDNA was extracted twice with a 1:1 mixture of phenol/$CHCl_3$ (in this and all subsequent extractions organic phases were combined and reextracted with TE), and the combined aqueous phases were extracted 3 times with ether. The cDNA was precipitated (in siliconised microfuge tubes) by the addition of 1vol 4M NH_4OAc and 2vol EtOH, incubated on ice for 30min and pelleted (12,000g, 30min). The pellet was resuspended in 50ul of H_2O, subjected

to a second precipitation as described above, rinsed with 70% EtOH, air dried briefly and resuspended in 10ul of H_2O. To the cDNA 4ul 5X R1 methylase buffer (0.5M NaCl, 0.5M Tris-HCl pH8, 5mM EDTA), 2ul 1.3mM S-Adenosyl Methionine, 20u EcoRI methylase was added and the reaction incubated at 37°C for 60min. The cDNA was purified by two phenol/$CHCl_3$ and two ether extractions, precipitated with 0.1vol 3M NaOAc pH5, 2vol EtOH and resuspended in 10ul H_2O.

c)Addition of Linkers and Ligation To Vector: EcoRI linkers were kinased and added to the cDNA as described by Huynh et al, (1985). It is imperative that the free linker molecules are all removed prior to ligation with the vector. This was achieved in the following way: the linkerd cDNA was extracted, once with phenol/$CHCl_3$ and once with ether, precipitated with 0.1vol 3M NaOAc pH5 , 0.6vol Isopropanol at room temperature for 30min and recovered by centrifugation (30min, 12,000g). This is a selective precipitation in which small molecules are left in the supernatant. The pellet was resuspended in 20ul of STE (0.4M NaCl, 10mM Tris-HCl pH8, 1mM EDTA) and applied to a column of Ultragel AcA34. The matrix had previously been extensively washed with STE and poured into a siliconised disposable 1ml pipette. The column was developed with STE and 50ul fractions were collected. The latter were assayed for radioactivity and the peak fractions containing cDNA were pooled and precipitated with 2vol EtOH. The cDNA was resuspended in 20ul H_2O and the concentration determined by spotting 1ul onto an ethidum bromide plate along with standards. The cDNA was ligated into the λ insertion vector, NM1149 (Murray, 1983) using a vector:cDNA molar ratio of 2:1. In practice 10ug of EcoR1 cut NM1149 was precipitated with 100ng of cDNA, resuspended in 7ul 10mM $MgCl_2$ and incubated at 42°C for 15min to allow the cohesive ends of lambda to anneal. To this tube 1ul 10X ligase buffer (600mM Tris-HCl pH7.5, 100mM $MgCl_2$, 100mM B-mercaptoethanol, 10mM EDTA), 1ul 10mM ATP, 1u T4 DNA ligase was added and the reaction was incubated overnight at 15°C. The ligation mix was packaged into infectious phage particles using a commercial kit and the resultant phage suspension titered on L87 and NM514(hfl) E.coli strains.

2.2vi:Screening Recombinant Lambda Libraries

Plaque hybridisations were carried out by a modification to the method of Benton and Davis (1977). Five to ten thousand recombinant phage were plated per 10cm x 10cm L-agar plate with 10^8 host cells and 0.6% top L-agarose, 10mM $MgSO_4$. The plates were incubated at 37°C for 6-8hr (or until the plaques were just visible) and cooled at 4°C for at least 1hr. Hybond-N filter replicas were taken from plates (up to 6 filters/plate), denatured (0.5M NaOH, 1.5M NaCl) for 3-5min, neutralised (0.5M Tris-HCl pH7.5, 1.5M NaCl) for 5min and washed in 2X SSC for 15min. The filters were air dried and then baked at 80°C for 1hr. They were then immersed in 1% Triton X-100, blotted briefly on 3MM paper and soaked in 2X SSC for 15min. For optimal results filters were prehybridised in individual plastic bags for 2-12hr in 10ml of aqueous hybridisation solution (see section on DNA blotting above) at 68°C in a shaking water bath. The prehybridisation solution was removed and the probe added with 3ml of fresh hybridisation solution. Levels of background were found to be greatly reduced when hybridisation solution and probes were filtered through 0.22um filters. The filters were incubated for 8hr (recombinant probes) or 48hr (cDNA probes) at 68°C with shaking. After hybridisation the filters were treated as described above for DNA blots.

2.2Vii:In-Situ Hybridisation to Polytene Chromosomes

Isolation and treatment of *Drosophila* salivary glands was performed as described by Pardue (1986). Probes were EMBL3 or NM1149 recombinants labelled with Bio-16-UTP by nick translation (Rigby et al., 1977). Hybrids were detected with Streptavidin-Biotinylated Horseradish Peroxidase complex and diaminobenzidine/H_2O_2 as described by Pardue (opp.cit). The slides were stained with Giemsa and the coverslips were mounted with DPX. Chromosomes were photographed using a Leitz Vario-orthomat camera system and Kodak Ectachrome 160 Tungsten film.

Plasmid and Lambda vectors

PLASMID	GENOTYPE	REFERENCE
pUC19	*amp*^{r}	Yanisch-Perron et al (1985)
pEMBL18	*amp*^{r} f1(G)	Dente et al (1985)
NM1149	λb538 *srl*λ3^o *imm*434 *srl*λ4^o *shnd*111λ6^o *srl*λ5^o	Murray (1983)
aDM2023	SalI fragment of sgs3 in pBR322	Kaiser et al (1986)
aDM1523	HindIII fragment of sgs4 in pBR322	Muskavitch and Hogness (1980)
Actin A2	EcoRI fragment of Actin 5C in pBR322	Fyrberg et al (1981)
YP-1	BamHI-BglII fragment of yolk protein 1 in pGEM-1	Hung and Wensink (1981)

TABLE 2.1

E.coli strains

STRAIN	GENOTYPE	SOURCE/REFERENCE
DS941	(*rec*F143 *pro*A7 *str*31 *thr*1 *leu*6 *tsx*33 *mlt*12 *his*4 *arg*E3 *lac*Iq *lacZ*Δm15)	D. Sherratt
L87	(*sup*E *sup*F *hsd*R *trp*E *met*D *ton*A)	Amersham
NM514	(*hsd*R *arg*H *gal*E *gal*X *str*A *lyc*B7)	Murray (1983)
NM621	(*hsd*R *mcr*A *mcr*B *sup*E44 *rec*D1009)	Whittaker et al (1988)

TABLE 2.2

2.3 DROSOPHILA

A description of the drosophila stocks used in this work can be found in table 2.3. All designations are as described by Lindsley and Grell (1968) or Lindsley and Zimm (1987). Flies were kept at 18°C or 25°C depending on genotype and reared on Glasgow fly food (10g agar, 15g sucrose, 30g glucose, 35g yeast, 15g maize meal, 10g wheatgerm, 30g treacle, 10g soya flour, 0.1% Nipagen, 0.5% propionic acid per litre of water).

Flies carrying homozygous mutations in sex transforming loci were generated as follows.

tra2: $tra2^{ts1}$ bw/CyO;BsY males were crossed to virgin Df(2L)trix/CyO females, mated for 3 days at 18°C and transferred to 29°C. Straight winged progeny were homozygotes and XY flies were identified by their Bar eye phenotype.

Germline deficient flies were produced in two ways, both of which utilise maternal effect mutations.

Tudor (wc^8): Virgin F1 homozygotes were collected from a wc^8 bw sp/CyO stock and self crossed, the F2 generation fail to form pole cells. To assess the penetrance of the phenoype 150 F2 males were mated to virgin wild type females. No larvae were detected in a two week period.

Oskar: A similar crossing scheme was used for osk^{301} flies but due to the temperature sensitive nature of this mutation the F2 generation was produced at 18°C

XXY females were produced from a C(1)M3/FM6/Y stock.

XO males were produced from a C(1)RM/In(1)E Y/O stock.

In the later stages of this work single sex non-mutant flies for the preparation of mRNA for northern blots were generated with the segregation distorter stocks constructed by T.W. Lyttle and kindly provided by J. Lucchesi (Lyttle, 1989; Walker et al., 1989), Table 2.4 lists the genotypes of the strains used. The following is a description of the crossing scheme used.

Drosophila stocks

STRAIN GENOTYPE	REFERENCE
Oregon R (WT)	Lindsley and Grell (1968)
Canton S (WT)	Lindsley and Grell (1968)
m56i (WT)	Schalet and Lefevre (1973)
$tra2^{ts1}$ bw / CyO ; BsY	Belote and Lucchesi (1980)
Df(2R)trix / CyO	Belote and Baker (1987)
wc^{8} bw sp / CyO	Boswell and Mahawold (1985)
p^{p} osk^{301} e / TM3	Lehmann et al (1986)
C(1)RM, y w / In(1)E $Y^{L}.Y^{S}$, y w f / O	Lindsley and Grell (1968)
C(1)M3 / FM6 / Y ; bw st	Lindsley and Grell (1968)

TABLE 2.3

SD STRAINS

SD No1 C(1)DX, y f / T(X;Y)22-3, y v f. Y^{L} Rsp^{s} B^{s} / Y ; In(2L)Cy, Cy E(SD) Rsp^{i} bw / cn bw

SD No2 C(1)DX, y f /+/ Y ; SD-ARM 28 / In(2L)Cy In(2R)Cy, S^{2} Cy cn bw

SD No3 +/+/Dp(2;Y)CB25-4, y^{+} $Y^{S}.Y^{L}$ Rsp^{s} B^{s} ; In(2L)Cy, Cy E(SD) Rsp^{i} bw / cn bw

SD No4 +/+/Y ; SD-ARM 28 / In(2L)Cy In(2R)Cy, S^{2} Cy cn bw

TABLE 2.4

XY flies: Curly-winged, brown-eyed males from SD No1 were mated to curly winged, star-eyed virgin females from SD No2. Curly winged vermilion-eyed male progeny were mated with virgin OregonR females. The resultant progeny were >96% male.

XX flies: Curly-winged, brown-eyed males from SD No3 were crossed with curly-winged star-eyed virgin females from SD No4. Curly-winged male progeny with wild type eyes were mated with OregonR virgin females. The progeny from this cross were >97% female.

Drosophila stocks

STRAIN GENOTYPE	REFERENCE
Oregon R (WT)	Lindsley and Grell (1968)
Canton S (WT)	Lindsley and Grell (1968)
m56i (WT)	Schalet and Lefevre (1973)
$\underline{tra2}^{ts1}$ $\underline{bw}$ / CyO ; BsY	Belote and Lucchesi (1980)
Df(2R)trix / CyO	Belote and Baker (1987)
$\underline{wc}^{8}$ $\underline{bw}$ $\underline{sp}$ / CyO	Boswell and Mahawold (1985)
$\underline{p}^{p}$ $\underline{osk}^{301}$ $\underline{e}$ / TM3	Lehmann et al (1986)
C(1)RM, $\underline{y}$ $\underline{w}$ / In(1)E $Y^{L}.Y^{S}$, $\underline{y}$ $\underline{w}$ $\underline{f}$ / O	Lindsley and Grell (1968)
C(1)M3 / FM6 / Y ; $\underline{bw}$ $\underline{st}$	Lindsley and Grell (1968)

TABLE 2.3

SD STRAINS

SD No1 C(1)DX, $\underline{y}$ $\underline{f}$ / T(X;Y)22-3, $\underline{y}$ $\underline{v}$ $\underline{f}$. Y^{L} $\underline{Rsp}^{s}$ $\underline{B}^{s}$ / Y ; In(2L)Cy, $\underline{Cy}$ $\underline{E(SD)}$ $\underline{Rsp}^{i}$ $\underline{bw}$ / $\underline{cn}$ $\underline{bw}$

SD No2 C(1)DX, $\underline{y}$ $\underline{f}$ /+/ Y ; SD-ARM 28 / In(2L)Cy In(2R)Cy, $\underline{S}^{2}$ $\underline{Cy}$ $\underline{cn}$ $\underline{bw}$

SD No3 +/+/Dp(2;Y)CB25-4, $\underline{y}^{+}$ $Y^{S}.Y^{L}$ $\underline{Rsp}^{s}$ $\underline{B}^{s}$; In(2L)Cy, $\underline{Cy}$ $\underline{E(SD)}$ $\underline{Rsp}^{i}$ $\underline{bw}$ / $\underline{cn}$ $\underline{bw}$

SD No4 +/+/Y ; SD-ARM 28 / In(2L)Cy In(2R)Cy, $\underline{S}^{2}$ $\underline{Cy}$ $\underline{cn}$ $\underline{bw}$

TABLE 2.4

CHAPTER 3

ISOLATION OF SEX-SPECIFIC GENES FROM D.MELANOGASTER

3.1 INTRODUCTION

To help further our understanding of the sex specific control of gene expression I set out to isolate Drosophila genes expressed only in one sex. By studying such genes it is to be hoped that some insight can be gained into the molecular mechanisms that act to regulate genes in a tissue specific and temporal way. Sexual dimorphic gene expression in D.melanogaster is a convenient system in which to study this problem for several reasons. Firstly this is because the phenotype, either male or female, is specific to the whole organism (with the caveat mentioned at the end of chapter 1 regarding spatial differences in gene expression). This facilitates isolation of large quantities of RNA populations which differ only in the transcripts of interest. Secondly the regulatory hierarchy which leads to somatic sexual differentiation, and to a lesser extent dosage compensation and germline sex determination, is well characterised at the genetic and molecular level. This allows the initial characterisation of sex-specific genes by straightforward genetic tests. Thirdly the wealth of genetic and molecular techniques available to the Drosophila investigator allows identification of DNA sequences that confer specific regulation in-vivo. This can be routinely accomplished by reintroducing cloned regulatory sequences, together with appropriate reporter genes, into the fly and studying the expression of the reporter (Spradling, 1986).

There are several ways in which one could envisage isolating genes expressed sex-specifically. The classical way would be to mutagenise and screen for a sex-specific phenotype. Such an approach has been very successful in identifying new alleles of the genes involved in the control of somatic sex determination as well as the male-specific-lethal genes. Such screens are relatively straightforward to carry out when the phenotype is sex-specific lethal or sex transforming. However the majority of genes expressed sex-specifically may be expected to have a less dramatic phenotype (e.g. sterility) when mutated . In such cases it is far more difficult to determine the molecular nature of the phenotype or indeed the tissue in which the gene is normally expressed. Each mutant line generated would require a laborious examination of individuals in order to establish a

morphological defect associated with the mutation. Since it has been estimated that approximately 10% of the mutable loci in the Drosophila genome can be mutated to give a male sterile phenotype (Lindsley and Lifschytz, 1972; Schafer, 1986a) this approach would involve screening a prohibitively large number of individuals (>600). A molecular analysis of any mutation isolated in this way would still require localisation and isolation of the gene which in itself can be a difficult and time consuming process.

More recently an elegant approach has been developed by O'Kane and Gehring (1987) utilising a P-element vector containing the E.coli B-galactosidase gene (LacZ). The lacZ gene is under the control of the weakly constitutive P-element promoter which permits only low levels of lacZ transcription. If this vector is induced to transpose to a new chromosomal location where it comes under the influence of a genomic enhancer element then elevated lacZ expression can readily be detected by staining for B-galactosidase activity. The pattern of staining, to a first approximation, reflects the developmental specificity of the particular enhancer element. By using a vector containing an antibiotic resistance gene and an E.coli origin of replication the genomic DNA flanking the insertion site can be easily isolated. This approach is already being widely used to identify early markers of neural cell development (for example Bellen et al., 1989; Ethan et al., 1989; Wilson et al., 1989) and will undoubtedly be more widely used in the study of sexual differentiation. Indeed some insertions which appear to have staining localised to the embryonic germ cells have already been identified (Bellen et al., 1989). It must be born in mind however that insertions may be influenced by more than one regulatory element and hence give misleading patterns of expression. Also the enhancer elements detected by this technique may be located some distance away from the genes they control making it rather more difficult to isolate these genes. A third difficulty may also arise in detecting "naked enhancers" which are not associated with any particular gene.

An alternative approach to the isolation of sex-specific genes is differential screening of a cDNA or genomic DNA library; so called +/- screening. With this method duplicate filter replicas of plaques are taken from a library and

screened with probes derived from two different mRNA populations, one population containing the transcripts of interest and the other deficient or containing reduced levels of these sequences. Clones which hybridise with one probe but show no or reduced hybridisation with the other may then be examined further as candidates for differentially expressed genes. Such an approach has been successful in the isolation of stage-specific transcripts from developing Xenopus embryos (Dworkin and Dawid, 1980) and transcripts regulated during Dicyostelium development (Williams and Lloyd, 1978). The major advantage of the differential screening approach is that clones identified in this way correspond directly to the transcript of the differentially expressed gene and relatively little subsequent manipulation is required to obtain the purified sequence. On the other hand a severe disadvantage is that such a sequence is isolated with no knowledge of it's biological function other than the fact that it is differentially expressed.

This technique would appear to be well suited to the isolation of sex-specific transcripts from Drosophila since the main requirement is two RNA populations which differ significantly in the abundance of the transcripts of interest. The method has been successfully employed by other workers in this field (Schafer, 1986a; Di Benedetto et al., 1987). However these groups did not identify an important class of genes, namely sex-specific transcripts expressed in early development. In one case the screen was deliberately biased against detecting such transcripts; Schafer, being primarily interested in spermatogenesis, prepared probes from size selected adult mRNA of low molecular weight (6-9S) and screened out any clones which also hybridised with embryonic mRNA. In the other Di Benedetto et al screened with sexed adult and pupal probes and identified 7 new sex-specific genes. However none of these showed detectable expression in the embryo. As the somatic and germline components of the gonad are almost certainly sexually differentiated during the embryonic stage, isolation of transcripts from these tissues would be a considerable asset to molecular studies of sex-specific gene expression. With this in mind I set out to isolate sex-specific transcripts using mRNA from male and female third instar larvae, this being the earliest developmental stage which could be reliably sexed in large

enough quantities.

Before embarking upon such a screening procedure there are several important considerations.

1) As discussed above the primary requirement is the identification of suitably different mRNA populations to use in the preparation of cDNA probes. In this case male and female 3rd. instar larvae.

2) The relative abundance of the transcripts one wishes to identify is important when considering this experimental approach. Generally speaking +/- screening will allow the isolation of sequences present as 0.1% of the mRNA population (Sargent, 1987). If careful attention is paid when preparing the probes and hybridisation conditions are optimised this limit can be reduced by an order of magnitude. If one is interested in isolating genes expressed at a lower level than 0.01% then subtracted probes must be employed (Sargent, 1987). As the production, of subtracted probes is more technically demanding than +/- screening I decided to opt initially for the simpler approach.

3) Finally the type of library to be screened, either cDNA or genomic, must be decided upon and several considerations are relevant to this matter.

a) Number of clones to be screened: for a genomic library this parameter (N) is determined by the insert size and may be estimated from the equation,

$$N = \frac{\ln(1-p)}{\ln(1-x/y)}$$

(Kaiser and Murray, 1985) where p is the probability of finding a particular clone in a library with inserts of x kb, from an organism with a haploid genome size of y kb. Using a Drosophila library containing 20kb inserts one would need to screen approximately 30,000 primary recombinants to have a 99% probability of finding a particular sequence. The use of an amplified library would require screening of a larger number of clones in order to overcome misrepresentation bias.

For a cDNA library the number of clones to be screened (N) depends on the abundance of the required transcript in the mRNA population and is estimated from the equation

$$N = \frac{\ln(p)}{\ln(1-z)}$$

(Sargent, 1987) where p is again the probability of finding the sequence and z is the fractional abundance of the sequence in the mRNA population. Using the limit of 0.01% of the mRNA population detectable in a +/- screening then we would need to screen 50,000 cDNA clones to have a 99% probability of finding a transcript in this abundance class; almost twice as many as for a Drosophila genomic library. If however the subject of investigation was an organism with a significantly larger genome than Drosophila then screening a cDNA library would require fewer clones than a genomic library.

b) Insert characteristics; each individual clone in a cDNA library should contain sequences representing only a single gene (or gene family). The insert of a clone from a genomic library however can potentially contain sequences which represent more than one gene. In practice this means that if a differentially expressed gene is linked to another non-differential gene on the same clone the hybridisation signal of the latter may obscure the differential signal of the former. Although this problem may be avoided by using a subtracted probe to screen a genomic library this consideration would favour the use of a cDNA library in +/- screens.

c) Screening considerations: When a complex mRNA population is used to generate a probe then the intensity of the hybridisation signal generated by a given transcript is generally directly proportional to the abundance of that sequence in the mRNA population (Williams, 1981). This means that clones which show strong hybridisation code for abundant transcripts. With a genomic library, clones which code for abundant mRNAs are generally present at the same frequency as clones which code for transcripts in the low abundance class. This is not the case for a cDNA library where the frequency of clones containing a particular sequence is dependent on the abundance of that sequence in the mRNA population. In practise this means that the clones which give the strongest hybridisation signals may be present many times on a particular filter. This consideration effectively limits the density at which a cDNA library can be screened using the

+/- technique as the signals from the abundant sequences will mask hybridisation to other clones. Again this problem can be overcome by the use of subtracted probes. With these considerations in mind I decided to construct and screen third larval instar cDNA libraries and also screen, in conjunction with K.Kaiser, a primary genomic library to try and identify sex-specific transcripts. We felt that by screening both types of library we would maximise our chances of identifying new sex-specific genes.

3.2 RESULTS

3.2i Construction of cDNA Libraries

Drosophila male and female 3rd larval instar cDNA libraries were constructed as described in the methods section. Table 3.1 shows the yields of the cDNA products obtained. For both templates the first strand synthesis products are within the range expected (30-50%: Berger et al., 1983). The yield of globin first strand cDNA, which approaches the maximum expected, may reflect more efficient synthesis due to the small size of the template. Second strand products are again approximately as expected (90-100%), the yield of male second strand at 109% probably reflects pipetting error when dispensing aliquots for acid precipitable counting. The low yield of globin second strand was consistently observed in several experiments. No attempt was made to determine the the reason for this. Electrophoresis under alkaline conditions was used to determine the size and structure of the cDNA. Figure 3.1a shows an autoradiogram of synthesis products analysed on a 1.5% alkaline agarose gel. The size distribution (0.4 to 2.5kb) is approximately equivalent for first and second strand products indicating a low level of hairpin structure formed during first strand synthesis. The discrete bands visible at approximately 750 and 1100nt. are consistently observed when I synthesise cDNA from larval mRNA. I do not know if these represent copies of abundant mRNAs (which are not visible in neutral RNA gels) or are derived from self priming rRNA molecules. Since the mRNA was purified by three

TABLE 3.1

	FEMALE	MALE	GLOBIN
% INCORPORATION	2.7	2.4	3.0
YEILD 1st STRAND	380ng	340ng	420ng
%mRNA TRANSCRIBED	38	34	42
% INCORPORATION	2.7	2.6	1.1
YEILD 2nd STRAND	380ng	370ng	150ng
% 1st STRAND TRANSCRIBED	100	109	36

TABLE 3.2

	TITER pfu/ml L87	TITER pfu/ml NM514	L87/NM514 RATIO	ADJUSTED BACKGROUND pfu/ml	TOTAL RECOMBINANTS pfu/ml
RELIGATED VECTOR	$4x10^6$	$4x10^4$	100	——	——
VECTOR + FEMALE cDNA	$3x10^6$	$2x10^6$	1.5	$3x10^4$	$1.9x10^6$
VECTOR + MALE cDNA	$3.5x10^6$	$6x10^5$	5.8	$3.5x10^4$	$5.7x10^5$

rounds of oligo(dT)-cellulose chromatography the latter possibility is considered less likely, although an mRNA population is never completely depleted of rRNA.

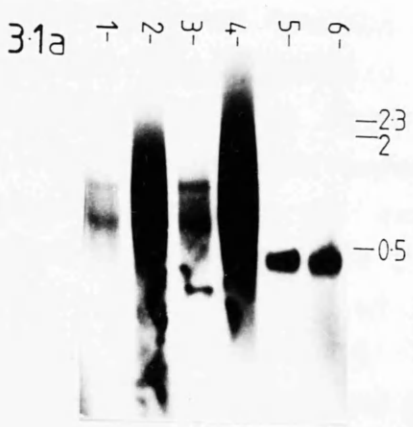
The double stranded cDNA was then methylated, EcoRI linkers added and the resulting molecules ligated into the lambda insertion vector NM1149. The recombinants were packaged into phage particles using a commercial packaging kit. A control with self-ligated vector was included to asses the effectiveness of the biological selection. The packaged phage were titered on non-selective (L87) and selective (NM514) E.coli hosts. The titers are shown in Table 3.2. The ratio of titers on L87 to NM514 should be high in the absence of insert and much lower when insert is present indicating that the biological selection is operating as expected, this is observed for both libraries. The titers also allow the number of recombinants within the library to be estimated. An adjusted background is calculated by subtracting the number of plaques expected on NM514 in the absence of inserts from the number actually obtained in the presence of the inserts (the value expected on NM514 is estimated by dividing the total number of plaques on the L87 host by the L87:NM514 ratio obtained from the self-ligated vector.). This figure however is a minimal estimate and does not take into account damage to the ends of the vector arms which may be caused by impurities present in the cDNA. Damage to the vector arms can "fool" the biological selection resulting in false positives. Both of the libraries appear to contain approximately 95% recombinants, this figure is higher than expected (70-80%) and may reflect damage to the vector arms. A more realistic figure for the level of recombinants in the library can be obtained by physical analysis. To this end 48 plaques were selected at random from each library, miniprep DNA prepared, digested with EcoRI and end labelled with ^{32}P dATP using "Klenow" polymerase. After electrophoresis in 1% agarose gels the DNA was visualised by autoradiography (not shown). In the male library 37 phage contained inserts (78%) and of these 4 (8%) contained more than one EcoRI fragment, the average size was 850bp. The female library sample contained 38 phage with inserts (79%), 9 (23%) of which contained more than one EcoRI fragment, the average size in this case was 890bp. There are two possible explanations for the presence of more than one fragment in a particular phage, one is that the cDNA contains

Figure 3.1a: Autoradiogram of cDNA synthesis products electrophoresed in a 1.5% alkaline-agarose gel. Lanes 1) Male 1st strand, 2) Male 2nd strand, 3) Female 1st strand, 4) Female 2nd strand, 5) Globin 1st strand, 6) Globin 2nd strand. Size markers indicated are in kilobases. Overnight exposure.

Figure 3.1b: Autoradiogram of sgs3 and sgs4 positive cDNA clones end labelled and electrophoresed in a 1.0% agarose gel. Lanes 1-3 female library sgs3; 4 & 5 female library sgs4; 6-8 male library sgs3; 9 & 10 male library sgs4; 11 NM1149. Marker sizes are in kilobasepairs, exposure time 2 hours.

Figure 3.1c: Autoradiogram of northern blots probed with various DNAs. Even numbered lanes contain male mRNA (1ug), odd numbered lanes contain female mRNA (1ug). Each pair of lanes were probed with the following; 1,2 aDM1523; 3,4 male sgs4 clone 9; 5,6 aDM2023; 7,8 male sgs3 clone 7; 9,10 male sgs3 clone 8. Sizes are in kilobases. The blots were washed at high stringency and exposed overnight.

Figure 3.1d: Autoradiogram of duplicate filters from male cDNA library probed with cDNA derived from male and female 3rd instar larvae as indicated. The blots were washed at high stringency and exposed overnight.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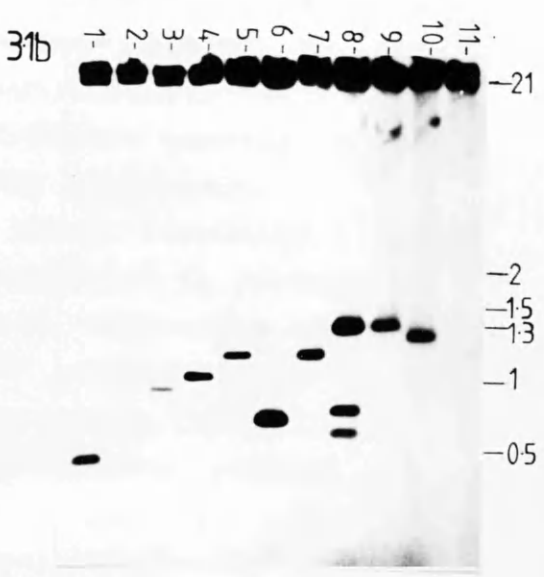
3-
4-
5-
6-
2·3
2
0·5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11-
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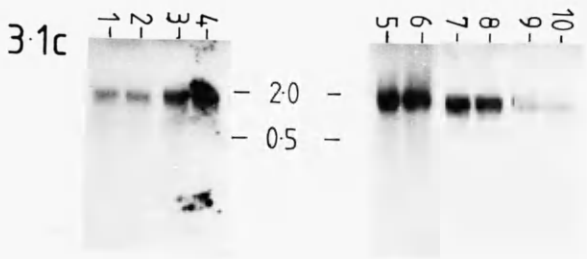
1·3
1
0·5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7-
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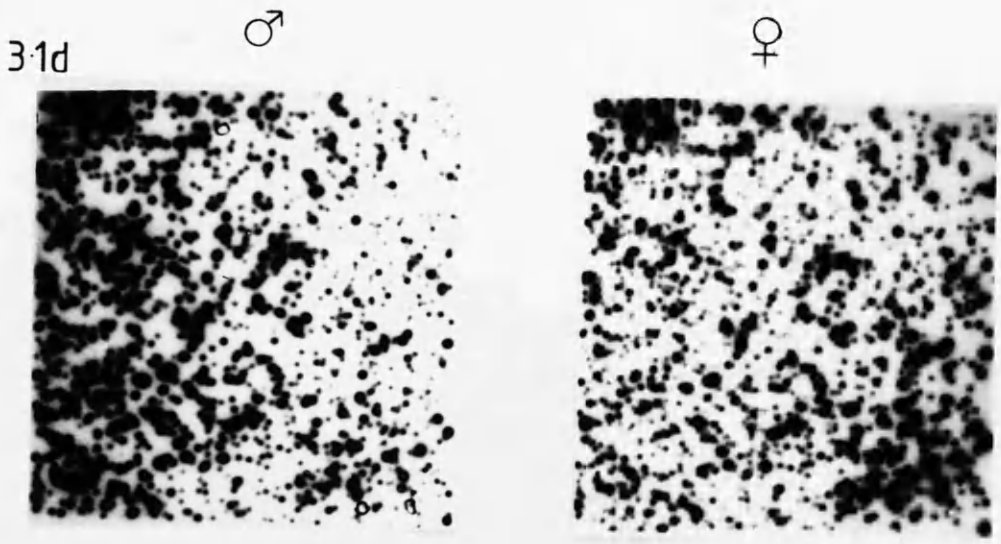
9-
10-
2·0
0·5


3·1d
♂
♀

an internal EcoRI site the other is that two different cDNA molecules have become inserted into the same phage. These possibilities may be distinguished by using the inserts as probes to northern blots. This was not carried out as it was felt that the low level of "double inserts" present in the male library would not interfere significantly with the screening. The relatively high level of double inserts in the female library may present some screening problems.

As the intention was to use these libraries for differential screening experiments I considered it prudent to examine them for the presence of known sequences at the expected abundance. To this end 7000 plaques from each library were screened with Sgs3 and Sgs4 probes (aDM2023 and aDM1523: Kaiser et al., 1986). The male library yielded 43 Sgs3 and 12 Sgs4 positives, the female 50 and 15. Although there are no direct abundance measurements of these transcripts published (the abundance of the transcripts changes through larval development), the ratio of Sgs4 to Sgs3 transcripts has been documented during studies on dosage compensation to be approximately 0.25 (Kaiser et al., 1986). This figure is in reasonable agreement with the ratios I observe with the libraries (0.28 and 0.3). Three Sgs3 and two Sgs4 positives were purified from each library. DNA was extracted by the miniprep method, digested with EcoRI and end labelled with ^{32}PdATP and "Klenow" polymerase. The insert size was determined by agarose gel electrophoresis: an autoradiogram of this gel is shown in Figure 3.1b. The sizes of the Sgs3 and Sgs4 transcripts are approximately 1100nt (Hofmann and Korg, 1987) and 1300nt (Crosby and Meyerowitz, 1986) respectively. As the autoradiogram shows the male library clones examined contain some inserts of approximately the correct size (lanes 6-10). The clone in lane 8 has more than one insert, it was not determined which one was the Sgs3 positive. The female library clones (lanes 1-5) contained smaller inserts, the clone in lane 2 has an insert smaller than 250bp which is barely visible on this exposure. DNA from three of the positives from the male library (lanes 7,8 and 9) and plasmids containing Sgs3 and Sgs4 inserts were nick translated and used as hybridisation probes to northern blots of male and female mRNA. The mRNA samples were run on the same gel, blotted together and the filter divided for hybridisation with separate probes. Figure 3.1c shows an

autoradiogram from this experiment. All the clones tested appear to hybridise to the correct transcript, the clone from lane 8 also hybridises to a weaker transcript of higher molecular weight which is consistent with the additional inserts in this phage being derived from another transcript.

To summarise, male and female third larval instar cDNA libraries were successfully constructed, the libraries contain inserts of known transcripts at approximately the expected frequency. In the case of the male library at least some of the inserts are near full length and recognise the correct transcript on Northern blots. I concluded that the libraries were suitable for differential screening experiments. Half of each library was amplified (Maniatis et al., 1982) for pilot experiments. When screened with the Sgs3 probe the amplified library gave a similar number of positives as the primary library (data not shown).

3.2ii Differential Screening Of The Male cDNA Library

Approximately 15,000 plaques from the primary male cDNA library were plated at a density of 1,500 per 10 cm^2 plate and two filter replicas taken from each plate. One set was screened with ^{32}P cDNA derived from male 3rd instar larval mRNA, the other set with ^{32}P cDNA from female 3rd instar larval mRNA. The probes were prepared as described in the methods section and were used at specific activities of $>10^8$cpm/ug of cDNA. The hybridisations were carried out in aqueous solution and washed at high stringency as described in the methods section. An example of a pair of filters from one of these experiments is shown in Figure 3.1d. A far greater number of plaques than expected show a signal after only overnight exposure. Furthermore the signals were of relatively even intensity, again an unexpected result. The number of plaques hybridising is approximately 75% of the total plated which eliminates the possibility that the probe template was contaminated with Lambda sequences. This conclusion was corroborated when screening genomic libraries with probes synthesised from the same template. The number of positives detected is in very close agreement with the number of plaques judged to contain inserts by physical analysis,

this suggests that the hybridisation was due to the presence of an insert. It is possible that the hybridisation was between the oligo(dT) tail of the cDNA probe and the dA-dT tract present in the cDNA inserts, although polyA was present in the hybridisation solution to prevent such hybridisation. To try and test this possibility I synthesised cDNA probes primed from random hexamers rather than oligo$(dT)_{12-18}$. However these experiments were hampered by high levels of background consistently observed with these probes. Similar background was also observed when the genomic library and dot blots of isolated phage DNA were screened. It is not clear why such high background was observed but it may be specific to the random hexamers and reverse transcriptase. No background was observed when plasmid probes were random primed with hexamers and Klenow polymerase, however plasmid probes were not synthesised with hexamer primers and reverse transcriptase. Alternatively the background may have been due to impurities in the cDNA which oligo-dT priming was not sensitive to. Numerous experiments were performed to try and eliminate this problem. Templates and probes were phenol extracted or filtered through nitro-cellulose membranes prior to use. Different concentrations of hexamers ranging over several orders of magnitude were tried. Unfortunately none of these measures were successful and the cause of these phenomena remain obscure. In view of the success achieved in screening the genomic library and pressure of time I decided to discontinue attempts at screening the cDNA library. The libraries however will still be useful for isolation of cDNA copies of genes identified in the +/- screens of the genomic library.

3.2iii Differential Screening Of A Drosophila Genomic Library

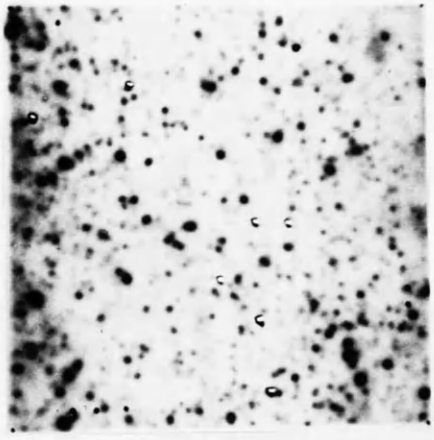
The Drosophila genomic library used was constructed in the lambda vector EMBL3 by K.Kaiser. It contains 6-18kb inserts derived from Oregon-R DNA partially digested with Sau3A. Two differential screens were carried out.

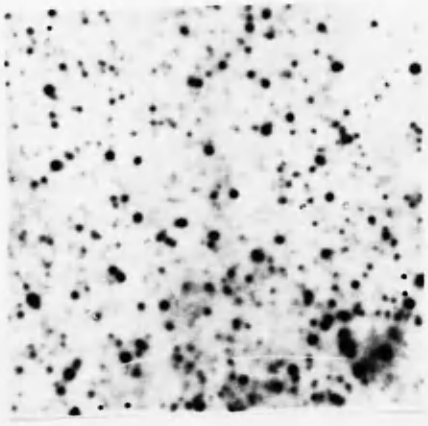
Screen 1: approximately 20,000 primary recombinants were screened at a density of 5,000 plaques per 10cm^2 plate. Duplicate filters were taken from each plate and screened

Figure 3.2a: Autoradiogram of duplicate filters from a Drosophila genomic library probed with cDNA derived from male and female L3 mRNA. See text under Screen 2 for details. Holes represent putative positives. Exposure was for two weeks.

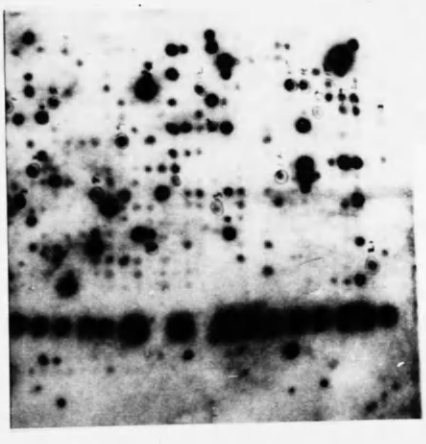
Figure 3.2b: Autoradiogram from secondary screen of positives from screen 2. See text for details. Exposure was for two weeks.

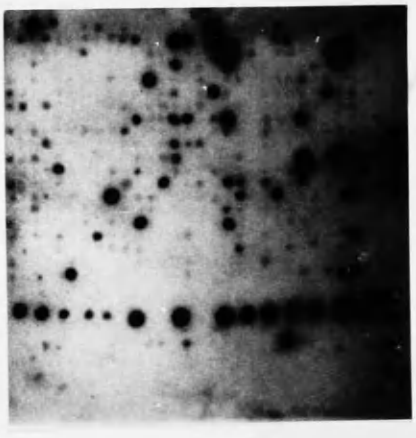
A ♂ ♀





B





3·2

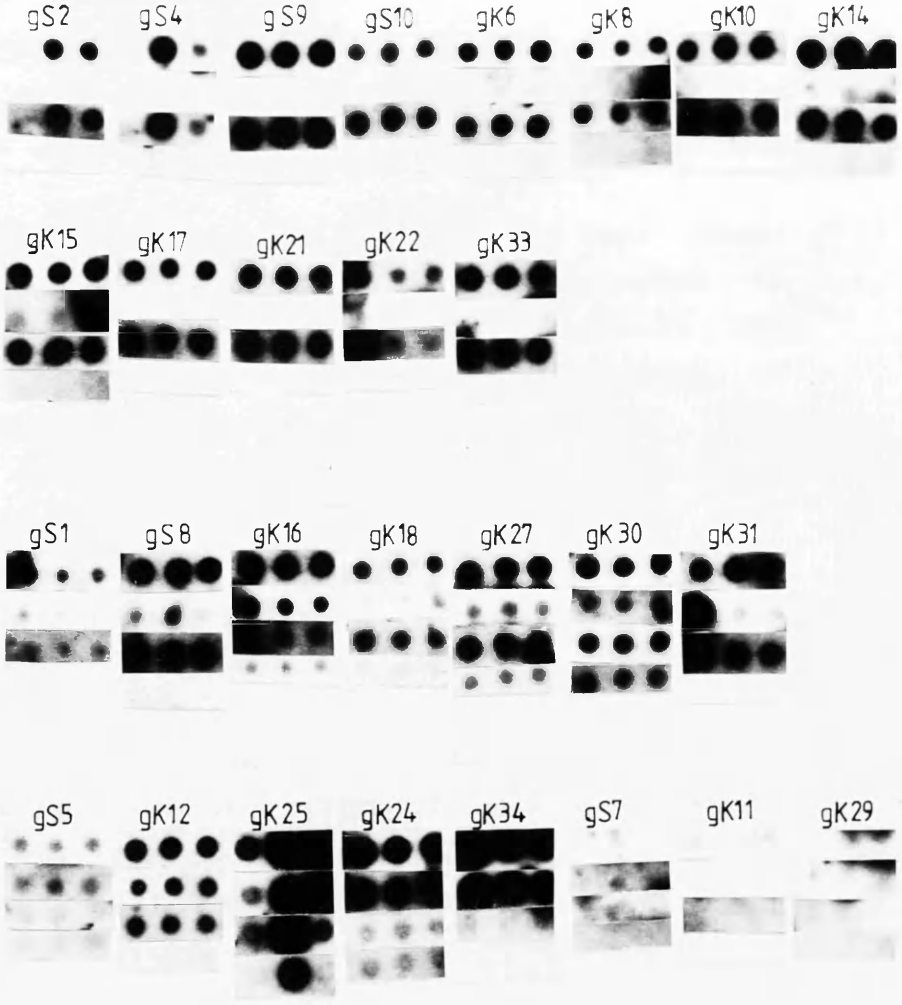
with ^{32}P cDNA from male and female third instar larvae. The probes were labelled to a specific activity of $>10^8$cpm/ug of cDNA. Hybridisations in aqueous solution and high stringency washes were carried out as described in the methods section. After exposing the filters for two weeks approximately 10% of the plaques gave signals on the autoradiograms. Of these 15 were judged to be male specific or differential. A region corresponding to the positive signal was picked and the phage streaked out to obtain single plaques. Ten plaques from each of the isolates were picked out into an array and screened again. Five of the primary positives failed to give differential signals. The remaining phage designated S1-S10 were screened again and a further 2 lost. These 8 positives were screened for a fourth time along with the positives isolated from the screen described below.

Screen 2: in this experiment 30,000 primary recombinants were screened at a density of 8,000 plaques per plate using the same conditions as above. In this case 54 plaques were identified as putative male specific/differential, the majority showing differential signals. A pair of filters from this screen is shown in Figure 3.2a. Positives were again streaked out and screened in an array. A row of Lambda clones containing an Sgs4 insert were included to asses the degree of hybridisation to each filter (Figure 3.2b). Of the 54 screened 34 positives remained these were designated K1-K34. A tertiary screen reduced this number to 20.

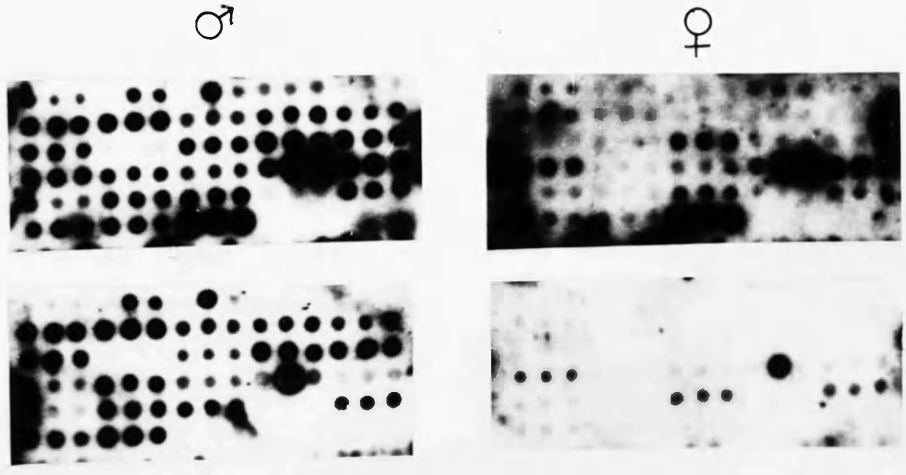
Final screening: The 28 plaques from both screens were picked out into an array (3 plaques per isolate) and 4 filters taken from the plate. These were screened with ^{32}P cDNA from new preparations of sexed larval mRNA and also with probes derived from sexed adult mRNA, the screens are shown in Figure 3.3. The upper panel details the signals for each clone individually while the lower panel shows the whole autoradiogram. The majority of the clones show similar expression with larval and adult probes which confirms that the larvae were correctly sexed. It is also apparent that some of the clones are still not single phage populations. This is probably a result of the previous screenings having been performed in close packed arrays where it is difficult to avoid some cross contamination. This is especially so when the plates are left for a two week period while the hybridisation and autoradiography are carried out. On the

Figure 3.3a and b: Autoradiograms of quaternary screening of "male-specific" positives from screens 1 and 2. The lower panels (3.3b) show the complete filters screened with cDNA derived from male and female larvae and adults as indicated. The upper panel (3.3a) has these same filters organised by individual clone for convenience. In this case the rows for each clone represent, from top to bottom: Male L3, Female L3, Male adult, female adult. The exposure time was 1 week.

3·3a



3·3b



gS1	gS2	gS4	gS5	gS7
gS8	gS9	gS10	gK6	gK8
gK10	gK11	gK12	gK14	gK15
gK16	gK17	gK18	gK25	gK24
gK22	gK21	gK27	gK29	gK30
gK31	gK33	gK34		

basis of this experiment the clones were divided into 3 groups.

Group 1: Male specific, the 13 clones in this group (gS2-gK33) show no or very little hybridisation with female probes. These clones probably contain genuine male specific genes.

Group 2: Male differential, the 7 clones in this class (gS1-gK31) show reduced hybridisation with female probes as compared to male probes. Two possibilities could account for this pattern of hybridisation; One is that the transcript is expressed at a higher level in males than in females. The other is that more than one transcribed sequence is located on the cloned fragment and only one of these is male specific. This question can be resolved by dissecting the clone into smaller fragments and analysing them individually.

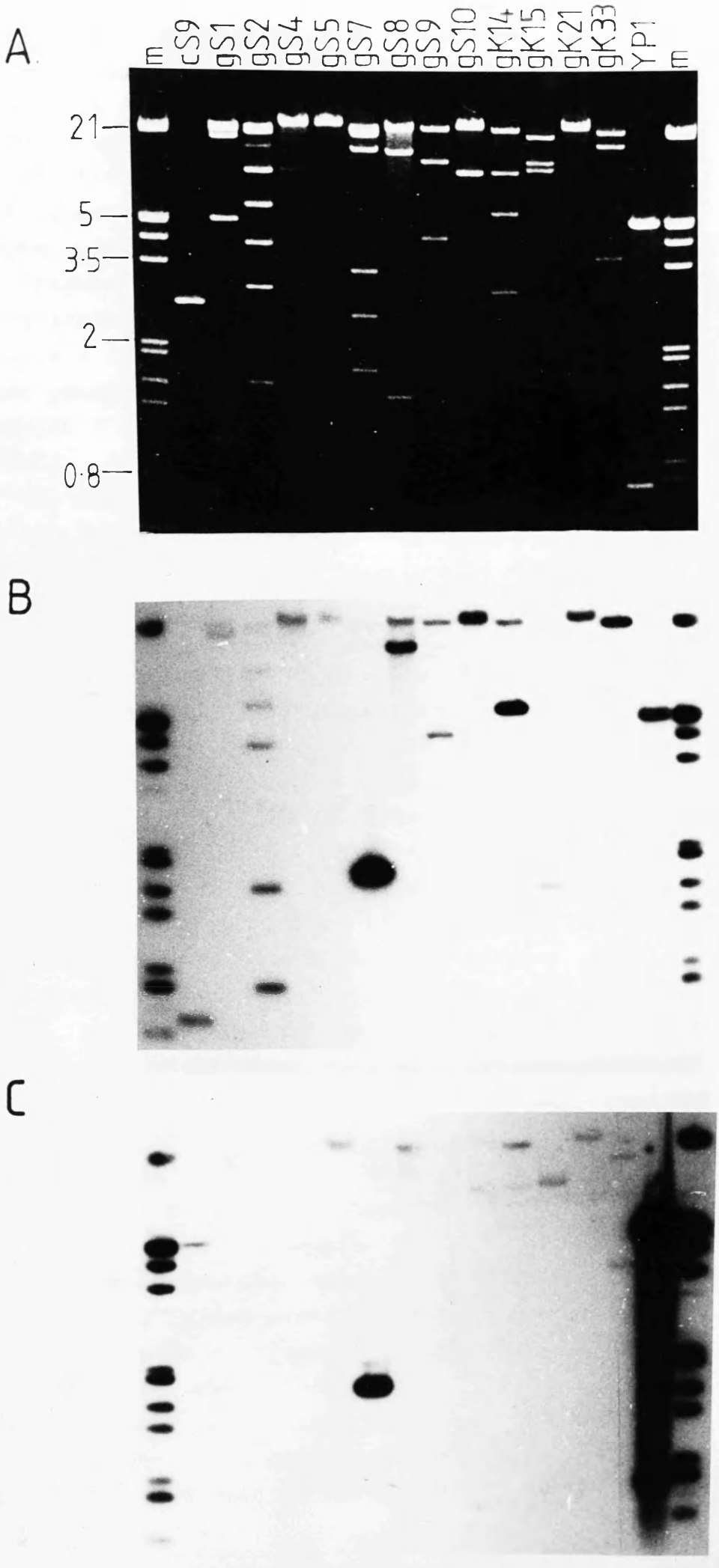
Group 3: ambiguous, the remaining 8 clones were placed in this category. Three of them showed no hybridisation, gS7, gK11 and gK29. Two appear to be larval specific, gK24 and gK34. Of the remaining three gS5 gives a weak signal and is difficult to asses, gK12 shows adult male specific expression but is not sex-specific in larvae. gK25 appears to contain 3 different phage, one is not sex-specific, one is larval specific and the third is male differential.

After screening the clones four times at least 20 remain which show male specific/differential expression. For further analysis I selected 8 clones from group 1: gS2, gS4, gS9, gS10, gK14, gK15, gK21 and gK33, two clones from group 2: gS1 and gS8, and two from group 3: gS5 as I was unsure of it's status and gS7. This clone appeared negative in this screen but not in previous screens so I went back to the isolate from the third screen and used that in subsequent experiments. The remaining clones are being analysed by another worker.

3.2iv Reverse Northern Analysis

To permit a more detailed analysis of the putative male-specific genes a "reverse northern" analysis was performed. In these experiments recombinant phage DNAs (purified on a large scale) were digested with restriction enzymes,

Figure 3.4a-c: EtBr stained 0.8% agarose gel containing BamH1 digested DNA from the set of male-specific clones selected for further analysis (0.5ug/lane). The clone numbers are given along the top. Size markers are in kilobasepairs. B is an autoradiogram of a southern blot of this gel probed with cDNA derived from male adult mRNA, C is an autoradiogram of a blot from a duplicate gel probed with cDNA from female mRNA. Both blots were washed at high stringency and exposed for three days.


A
m
cS9
gS1
gS2
gS4
gS5
gS7
gS8
gS9
gS10
gK14
gK15
gK21
gK33
YP1
m
21
5
3·5
2
0·8
B
C

electrophoresed in duplicate 0.8% agarose gels and transferred to nylon filters. The filters were probed in parallel, one with cDNA prepared from male adult mRNA, the other with cDNA prepared from female adult mRNA. Probes were prepared to specific activities of $>10^8$cpm/ug of cDNA. After aqueous hybridisation, washing at high stringency and autoradiography the filters were compared to identify restriction fragments with male-specific hybridisation. Two controls were included to asses the sex-specificity of the probes. YP1-1 is a fragment of the yolk protein 1 gene in the plasmid vector pGem1 (Kindly provided by M.Bownes). cS9 is a 650bp cDNA which I isolated during pilot experiments, it is described fully in chapter 6. At the time of these experiments an adult non sex-specific control was not available in our laboratory. Somewhat fortuitously clone gS7 appears to fulfil this function.

The reverse northern analysis was performed on phage DNA digested with SalI, BamHI and HindIII. The gel and autoradiograms for the BamHI digests are shown in Figure 3.4. The upper panel shows the ethidium bromide stained gel, the middle panel shows hybridisation with male cDNA and the lower with female cDNA. Several bands hybridise strongly with male cDNA and show little or no hybridisation with female. The male specific control cS9 only hybridises with male cDNA (band at 650bp). Weak hybridisation between the vector and female cDNA is also apparent. A band of 5kb, which represents undigested vector DNA not visible on the stained gel, is detected by female cDNA. In addition weak hybridisation between female cDNA and the arms of the Lambda vector is apparent. The reason for this spurious hybridisation is unknown. With the female-specific control, YP1, strong hybridisation with female cDNA is observed. However weak hybridisation with male cDNA is also detected. I estimate by eye that hybridisation to YP-1 with the male probe is around 100 fold lower than with the female probe. This probably reflects slight contamination of male mRNA with female, however the analysis is not unduly affected by this. The combined results of these experiments are given in table 3.3.

The majority of the clones show male specific hybridisation to a small restriction fragment. Several of the male-specific fragments were subcloned into plasmid vectors for further analysis and will be described in the following

TABLE 3.3

	SalI	BamHI	HindIII
gS1	1.6Kb (V)	(V)	V (3.7Kb)
gS2	10.5Kb (6.5Kb)	5.8,1.6,0.8Kb (4.2Kb)	(V)
gS4	V	V	V
gS5	(8.5,5.4Kb)	(V)	(8.0,5.0Kb)
gS7	(2.1,1.3Kb)	(1.8Kb)	(V)
gS8	(7.0Kb)	(12.0Kb)	(5.5Kb)
gS9	11.0Kb	4.1Kb	3.2Kb
gS10	7.5, 1.6Kb	(V)	(V), 2.6Kb
gK14	11.0Kb (3.4Kb)	6.2Kb (V)	9.0Kb (V)
gK15	1.1Kb (6.5Kb)	1.6,0.8Kb (4.2Kb)	(V) (5.0Kb)
gK21	(V)	(V)	5.5Kb (4.0Kb)
gK33	V	V	0.6Kb

Table 3.3: Restriction fragments identified by reverse northern blots. The sizes are in Kilobasepairs. Underlined fragments were sub-cloned into plasmid vectors for further analysis. Those fragments enclosed in brackets hybridised with cDNA from both sexes. V denotes hybridisation to cloned sequences which remain attatched to the Lambda vector arms.

chapters. In addition a number of restriction fragments hybridise weakly with cDNA from both sexes. This may be due to the presence of adjacent non sex-specific genes.

As is apparent clone gS7 is not sex-specific and was omitted from subsequent analyses. Clone gS5 shows very weak hybridisation which appears if anything to be female elevated. Northern blot analysis with this clone failed to detect any sex-specific transcripts (not shown). Consequently this clone will not be discussed further. Ten clones which show male-specific hybridisation remain. From the restriction digests it appears that gS2 and gK15 overlap as they share common BamHI and SalI fragments. To determine the relatedness of the other clones blots of SalI digested DNA were probed with restriction fragments from each clone (not shown). In this way the overlap between gS2 and gK15 was confirmed and cross hybridisation between gS8 and gS9 was detected although these two clones do not appear to overlap. A more detailed analysis of each clone will be presented in the following chapters.

3.3 DISCUSSION

Screening of the genomic library appears to have successfully identified cloned genomic fragments which contain genes for male specific transcripts. The isolation of 20 clones which are male differential is comparable to the screens carried out by other workers. Schafer (1986a) screened 35,000 clones and isolated 12 which defined 5 new genes, DiBenedetto et al. (1987) screened 15,000 clones and isolated 13 which defined 7 new genes and 3 previously isolated chorion and yolk protein genes. In their experiments both these groups screened amplified libraries which may be underrepresented for many sequences. By using a primary library we may have increased our chances of finding previously unidentified genes. The choice of larval templates for the preparation of the probes may have directed our screens to a different class of genes than those previously isolated. When we screen the genomic library with probes derived from sexed adults many more positives are apparent

than with the larval probes (K.Kaiser & S.Russell, unpublished data). These adult sex-specific clones probably represent transcripts derived from the highly differentiated structures which develop from the genital disc. As the gonad is the only major sexually dimorphic structure apparent during the larval stages of development, it is possible that the clones identified in our screens are transcribed specifically in this tissue. It is also noteworthy that no female-specific clones were identified in our screen. This reinforces the belief that the clones represent gonad specific transcripts as a) the female gonad is considerably smaller than the male during the larval stage and b) gametogenesis does not commence in the female until late in the pupal stage whereas primary spermatocytes can be detected in the testis as early as the 1st larval instar (Garcia-Bellido, 1964)

With regards to the screening there are steps which could be improved upon. Identification of positives on the autoradiograms of the primary screen was quite time consuming. As the plaques were distributed at random it was difficult to identify the differential signal amongst the majority which were not sex-specific. On the other hand the secondary screens where all of the plaques were ordered in an array was considerably easier. Purifying the primary positives was also problematic. I do not know how many genuine positives were lost during the purification but it would be surprising if none were. Poorly growing clones may have been missed when plaques were streaked out although every effort was made to select plaques of differing size. These problems could be overcome in a number of ways; the use of subtracted probes would make identification of positives much easier. However the purification of the positives would still be as problematic. If one were to screen the whole library in arrays identification and purification of clones would be greatly simplified. Thirty thousand plaques may seem like a prohibitively large number to plate in arrays but a device for picking out and replicating 1000 plaques at a time has recently been developed in our laboratory (MacKenzie et al., 1989). This may make such an approach feasible. The other possibility is to construct a subtracted cDNA library then relatively few clones, which are enriched for the sequences of interest, need be screened.

Disappointingly my attempts at screening cDNA libraries were unsuccessful. As discussed earlier the data suggests that the observed hybridisation was due to the presence of an insert, possibly mediated by the polyA tail. It is possible that the polyA added to the hybridisation solution was degraded and hence not effective as a blocking agent. The use of random primed cDNA should have overcome this problem but the levels of background obtained with these probes prevented this avenue from being exploited. I have little idea as to why this problem was encountered. Similar background has been observed by other workers in our laboratory, specifically with the $pD(N)_6$ and source of reverse transcriptase used in this work. Perhaps an alternative source of reverse transcriptase and/or primers or perhaps slightly longer primers may be more productive.

CHAPTER 4

IDENTIFICATION OF PREVIOUSLY ISOLATED MALE-SPECIFIC GENES

4.1 INTRODUCTION

The belief that we have isolated genuine male-specific genes would be strengthened by the identification of previously characterised genes, hence it is worth considering briefly male-specific genes isolated by other workers. Schafer (1986a; 1986b) screened 35,000 phage from the Maniatis library (Maniatis et al., 1978) with cDNA derived from size selected adult male mRNA. He identified 195 positives of which he discarded 183 as these hybridised with cDNA from embryos or adult females. The remaining 12 clones identified 5 genomic regions. Four; mst(2)Ag1 at 57D, mst(3)Ag3 at 95F, mst(2)Ag35 at 51F and a small gene family mst(3)Ag2 at 75C, all encode small (around 500nt) mRNAs expressed in the paragonia. These transcripts appear to be indirectly regulated by the somatic sex determination hierarchy. The fifth, mst(3)Gl9, at 87F encodes a 750nt transcript specific to the male germline.

DiBenedetto et al. (1987) screened 15,000 plaques from the same library with cDNA derived from male and female adult mRNA. In all they identified 13 sex-specific clones, 6 male-specific and 7 female-specific. Three of the female-specific clones were re-isolates of chorion or yolk protein genes and 2 were type 1 ribosomal spacer sequences. The remaining 2 identified a novel gene fst(3)230. Of the male-specific genes mst(3)316 at 95E encodes a 350nt transcript expressed in the paragonia. The remaining 5; mst(2)323 at 25F, mst(2)325 at 47A, mst(3)349 at 66D, mst(3)345 at 95E and mst(3)336 at 98C all encode male-specific transcripts expressed in the germline. A few other male-specific genes have been cloned and these have been discussed in chapter 1.

In an attempt to characterise our male-specific clones several experimental approaches were used. In the first instance crude restriction maps were deduced by a series of restriction enzyme double digests. These maps merely provide a working reference and it should be emphasised that they have not been confirmed by sub-cloning or partial digestion. Genomic organisation of the cloned sequences was determined by Southern blotting and in-situ hybridisation to polytene chromosome spreads. In this way genomic location and copy number could be determined.

Preliminary characterisation of the expression of the male-specific transcripts was achieved via northern blotting

experiments. Information on the developmental regulation of the transcripts was obtained from northern blots of mRNA from different developmental stages. The use of sexed material from some stages provided confirmation of the sex-specificity observed in cDNA hybridisation experiments. The SD stocks discussed in the materials section were used to generate single-sex fly populations from which mRNA was isolated.

Hybridisation to mRNA from flies carrying different mutations or defects allows preliminary separation into those sequences transcribed in the germline and those transcribed in the soma. The tra2 mutation, which transforms XX somatic tissue to phenotypic maleness, allows one to determine whether or not a particular gene is under the control of the somatic sex-determination hierarchy. If a male-specific gene is expressed in XX;tra2/tra2 pseudomales the most likely conclusion is that it is expressed in somatic tissues and regulated by dsx. It should be noted however that a few spermatocytes are detected in the rudimentary testes of pseudomales (Steinmann-Zwicky et al., 1989) so the possibility of germline expression cannot be eliminated if weak expression is detected. If a male-specific transcript is not expressed in pseudomales then it is either not expressed somatically or it is not regulated by the somatic sex determination hierarchy. One caveat to this is with regard to tissues which are regulated by the hierarchy but which fail to develop fully in pseudomales such as the somatic component of the gonad. Here a low level of expression from the reduced tissue in pseudomales may not be detected on northern blots.

Germline expression can be investigated using agametic individuals. In these experiments offspring were generated from mothers homozygous for tudor (tud)(Boswell and Mahawold, 1985) and oskar^{301} (osk)(Lehmann and Nusslein-Volhard, 1986) mutations. The offspring develop without pole-cells and consequently their gonads are underdeveloped and devoid of germ-cells. Failure to detect male-specific transcripts in these individuals is indicative of germline specific or germline dependent expression. Germline dependent expression is exemplified by chorion gene expression which occurs in the somatic follicle cells of the ovary but which requires a normally developing oocyte in order to be expressed (DiBenedetto et al., 1987). Expression of a male-specific gene in both pseudomales and agametic males argues strongly

for somatic expression. Failure to detect the transcript in either suggests germline expression (with the caveat discussed regarding the underdeveloped gonad). Expression in agametic males but not in pseudomales suggests somatic expression not regulated by the somatic sex-determination hierarchy, for example genes involved in dosage compensation. Expression of a male-specific gene in a pseudomale but not in an agametic male would indicate an early germline function but is not expected as very few XX germ cells enter spermatogenesis in pseudomales.

In the case of germline specific transcripts it would also be of some interest to investigate the effect of mutations affecting spermatogenesis. There are a large number of mutations which disrupt spermatogenesis and an investigation of them all would be somewhat time consuming. To obtain some preliminary information expression of male-specific genes was examined in males lacking a Y chromosome (XO) and in males (XY) homozygous for the tra2 mutation as both of these genotypes are associated with aberrant spermatogenesis. mRNA derived from females carrying a Y chromosome was also included on some blots (genotype XXY). For the most part hybridisation to this mRNA was identical to normal XX females. Lanes containing this mRNA are shown in some figures but will not be discussed further.

4.2 RESULTS

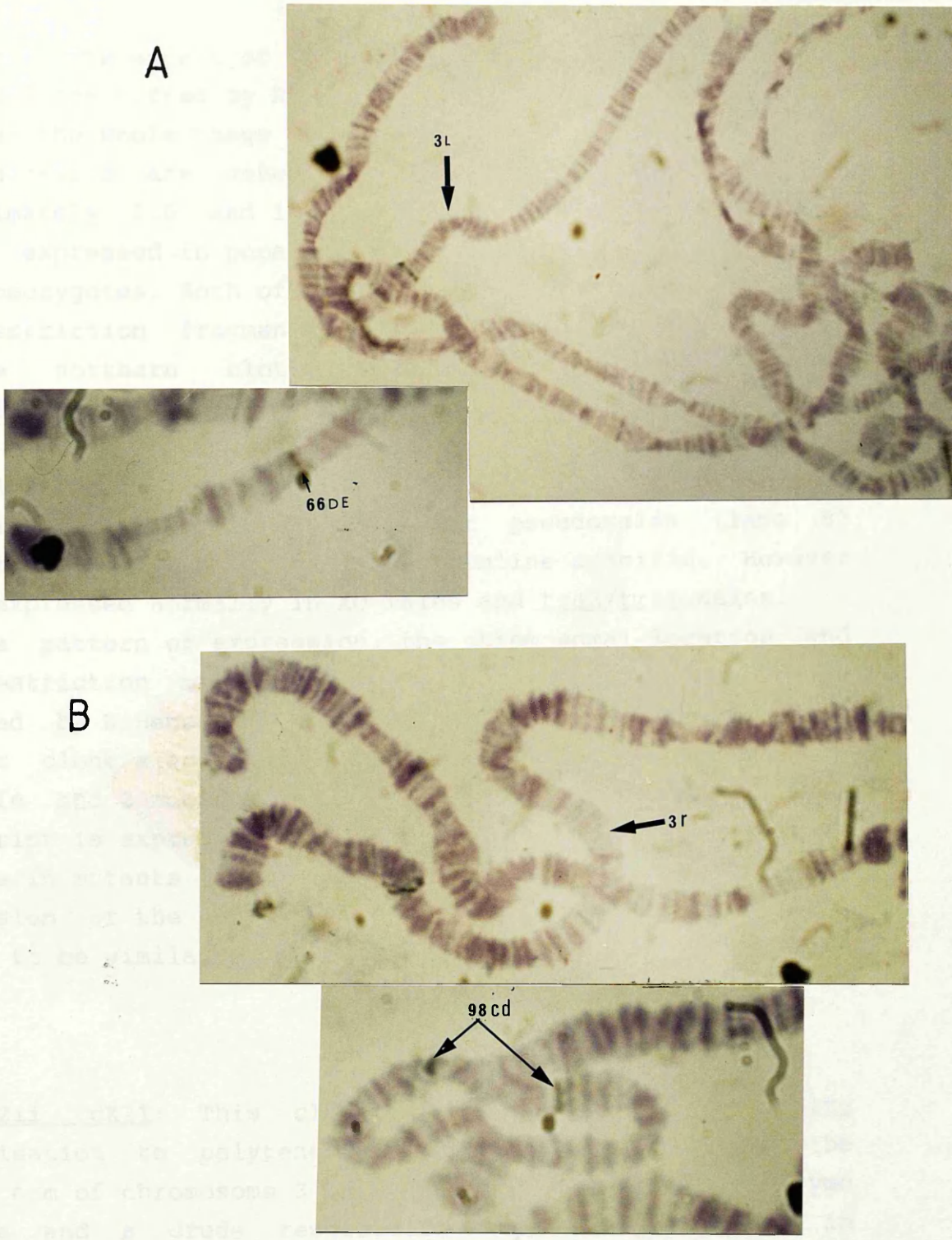
Two of the clones isolated in our screen (gK14 and gK21) appear to correspond to previously identified male-specific genes.

4.2i gK14: this clone maps to salivary gland chromosome region 66D/E as shown in Figure 4.1a. A crude restriction map of the clone is shown in Figure 4.1c. The map was derived from the double digests shown in Figure 4.2a and also by probing blots of the digests with the 3.2kb SalI restriction fragment marked on the restriction map (not shown). The restriction fragments identified on reverse northern blots are shown on the restriction map (black bar). In addition to the male-specific fragments the hatched box indicates weak hybridisation to cDNA from both sexes. The diagram also

Figure 4.1a: Photomicrographs of in-situ hybridisation to polytene chromosomes using biotinylated lambda gK14. The large panel shows hybridisation to the left arm of chromosome 3. The insert shows hybridisation at region 66DE.

Figure 4.1b: Photomicrographs of polytene chromosome spreads hybridised with biotinylated lambda gK21. The upper panel shows hybridisation to the right arm of chromosome 3. The lower panel shows hybridisation to an asynapsed chromosome at region 98CD. The signal is stronger on one of the homologues.

Figure 4.1c: Crude restriction maps of the lambda clones gK14 and gK21. Enzymes; B=BamHI, H=HindIII, E=EcoRI, S=SalI. The black bar represents hybridisation with cDNA derived from males. The hatched bar represents hybridisation with cDNA derived from either sex. The dotted line indicates the 3.2Kb SalI fragment used as a probe. The arrows depict the overlap with the mst(3)349 and mst(3)336 clones isolated by DiBenedetto et al. (1987).


A
3L
66DE
B
3r
98cd
C
S H HS HS B B H EH S
E E E E E E B
gK14
mst(3)349
S H H H H S
E E E E
gK21
mst(3)336
1Kb

indicates the extent of the apparent overlap with the clone mst(3)349 identified by DiBenedetto et al. (1987).

When the whole phage is used as a probe to Northern blots 3 transcripts are detected (Figure 4.2b). Two of these, approximately 1.6 and 1.8kb, are not sex-specific and are weakly expressed in pupae, adults, agametic individuals and tra2 homozygotes. Both of these transcripts may be encoded on the restriction fragments defined as non sex-specific on reverse northern blots. The third transcript, of approximately 2.5kb, is male-specific. It is expressed in 3rd instar larvae (L3) (the lane is underloaded and not visible in this exposure), pupae and adults. It is not detected in agametic males (lanes 9 & 11) or pseudomales (lane 8) suggesting that the transcript is germline specific. However it is expressed normally in XO males and tra2/tra2 males.

The pattern of expression, the chromosomal location and the restriction map of gK14 are very similar to those reported by DiBenedetto et al. (1987) for mst(3)349. Their genomic clone also hybridises at 66D and identifies 1 male-specific and 2 non-specific transcripts. The male-specific transcript is expressed in the same stages and shows the same profile in mutants as the male-specific transcript from gK14. Expression of the non sex-specific transcripts of gK14 also appear to be similar to those reported for mst(3)349.

4.2ii gK21: This clone was localised by in-situ hybridisation to polytene chromosome region 98C/D on the right arm of chromosome 3 (Figure 4.1b). Restriction enzyme digests and a crude restriction map are presented in Figures 4.2c and 4.1c respectively. The reverse northern analysis identified a male-specific 5kb HindIII fragment and a non-specific HindIII fragment of 4kb, these are marked on the restriction map. The apparent overlap with the genomic clone mst(3)336 identified by DiBenedetto et al. (1987) is also indicated on the diagram. Probing genomic DNA southern blots with the whole SalI insert indicates that the sequences are single copy in the Drosophila genome (not shown).

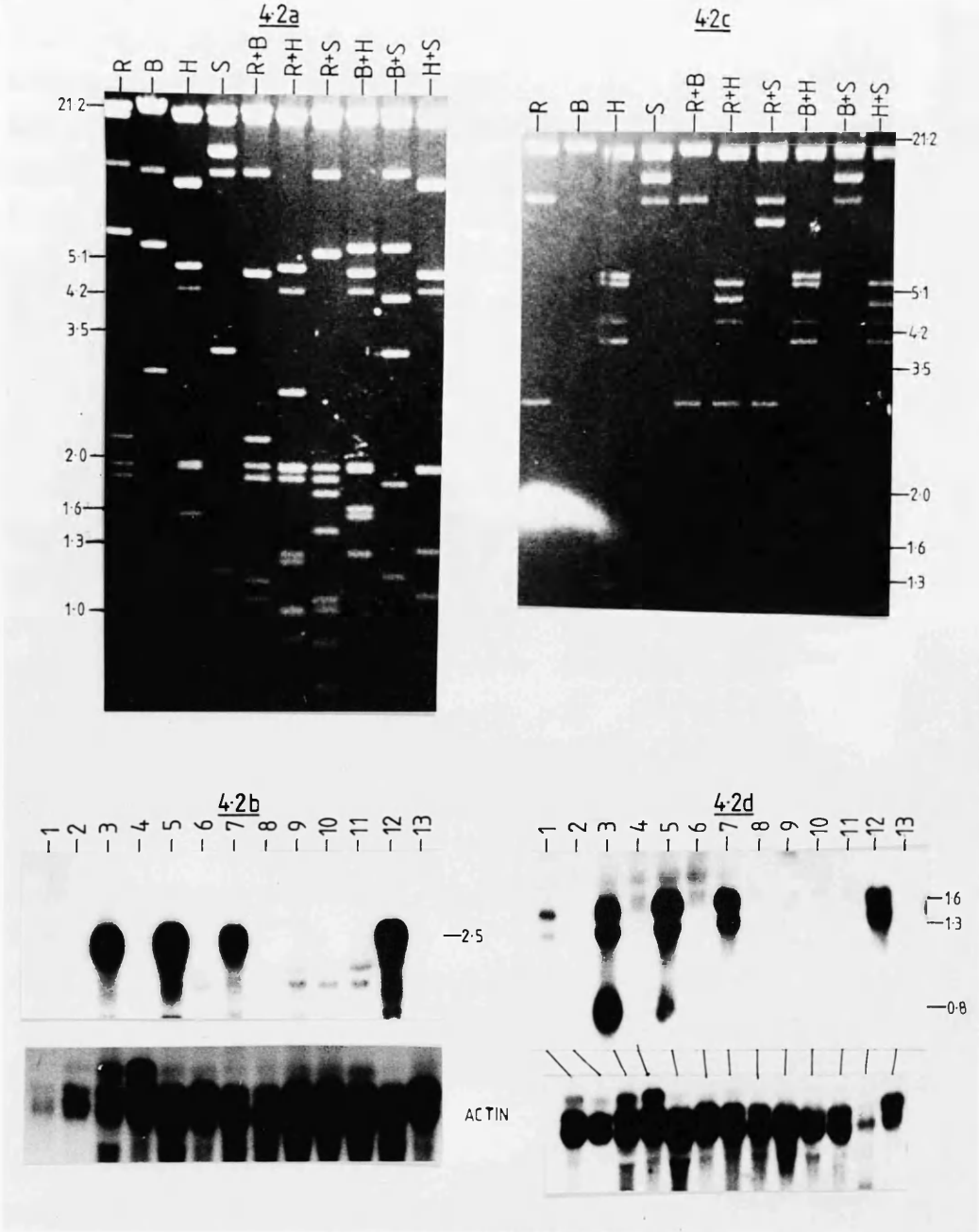
Using the whole clone as a probe to northern blots identifies 2 male-specific transcripts of approximately 1.3 and 1.6kb (Figure 4.2d). The 1.3kb transcript is detected in

Figure 4.2a: EtBr stained 0.8% agarose gel containing restriction digests of Lambda gK14 DNA (1ug/lane). Enzymes; R=EcoRI, B=BamHI, H=HindIII, s=SalI. Size markers are in kilobase pairs.

Figure 4.2b: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe used was lambda gK14, the blot was washed at high stringency and exposed for 3 days. The stripped blot was reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) Male L3, 2) Female L3, 3) Male pupal, 4) Female pupal, 5) Male adult, 6) Female adult, 7) XY;tra2/tra2, 8) XX;tra2/tra2, 9) Agametic male (tud), 10) Agametic female (tud), 11) Agametic male (osk), 12) XO male, 13) XXY female. The size is in kilobases.

Figure 4.2c: EtBr stained 0.8% agarose gel containing restriction digested lambda gK21 DNA. Enzyme designations as for figure 4.2a. Sizes in kilobasepairs.

Figure 4.2d: Northern blot analysis using Lambda gK21 DNA as a probe. Lane designations and conditions as for figure 4.2b.


4·2a
R
B
H
S
R+B
R+H
R+S
B+H
B+S
H+S
21·2
5·1
4·2
3·5
2·0
1·6
1·3
1·0
4·2c
21·2
5·1
4·2
3·5
2·0
1·6
1·3
4·2b
1
2
3
4
5
6
7
8
9
10
11
12
13
2·5
ACTIN
4·2d
1·6
1·3
0·8

L3, pupae and adults, it is not expressed in agametic males or pseudomales. An identical pattern of expression is found by DiBenedetto et al. for a similar sized transcript from mst(3)336. The 1.6kb transcript is expressed strongly in male L3, male pupae and male adults however weak expression in females agametic males and pseudomales is also detected. DiBenedetto et al. also report a 1.6kb transcript but do not report weak expression in females or mutants. The reason for this difference is at present unclear, it may be due to different sensitivity of the northern blots. Alternatively it may be a transcript encoded on the restriction fragments identified as non sex-specific on reverse northerns blots. DiBenedetto et al. do not report the probe used for their northern blots so a comparison is not possible at present. In addition to the two transcripts described above a small transcript of approximately 0.5kb is also detected. It is found in male pupae and male adults but longer exposures of the autoradiogram indicate that it is also present in females although at lower abundance. Dibenedetto et al. report a non sex-specific 0.5kb transcript but they do not indicate elevated expression in males. Given the strength of the signal for the transcript on my northern blots it is difficult to believe that this is a transfer or hybridisation artefact. Although considering the odd shape of the band this possibility should be borne in mind. Alternatively the difference between my data and that of DiBenedetto et al. may reflect strain differences. As these authors do not show an autoradiogram it is difficult to assess whether or not these differences are meaningful. Subcloning to identify transcript-specific probes will be required to confirm these observations.

A higher molecular weight transcript of approximately 2kb is also detected on this blot but not on others, this may represent a processing intermediate or a transcript from the non-specific area of the clone.

4.3 DISCUSSION

The majority of the data presented in this chapter suggests that two of the male-specific genomic clones

identified in our screen correspond to genes previously characterised. By in-situ hybridisation the clones map to the same chromosome region. The restriction maps of the clones correspond fairly well. Northern blot analysis identify very similar, although not identical, transcripts and patterns of expression. While formal proof would require cross hybridisation between our clones and those of DiBenedetto et al. the data presented are sufficient to conclude that it is highly probable that they are the same genes

DiBenedetto et al. examined the expression of their genes by in-situ hybridisation to tissue sections. In this way they determined that expression of the male specific transcripts was restricted to the lumen of the testis. These experiments complement the conclusions of my northern blot experiments which indicated that expression was germ-line specific. I extend the data of DiBenedetto et al. by finding no effect on expression of the male-specific transcripts from removal of the Y chromosome. The isolation of previously characterised genes provides powerful internal controls for the behaviour of the mutant fly lines and for the reliability of the in-situ hybridisation localisations. In this respect it should be noted that the similarity between our sequences and those of DiBenedetto et al. was not uncovered until I had completed northern blots, restriction maps and genomic localisation.

In the succeeding chapters I shall describe the characterisation of 6 novel male-specific genes.

CHAPTER 5

NOVEL GENES WITH PUTATIVE GERMLINE EXPRESSION

5.1 INTRODUCTION

Many genes appear to be involved in the process of spermatogenesis in Drosophila. This is evident from the large number of loci which can be mutated to give a male sterile phenotype. Lifschytz (1987) has suggested that these genes can be divided into two broad categories; Those with products specific to the male germline and those whose products are required in both the germline and in the soma. The latter class are believed to be male sterile because the process of spermatogenesis is likely to be more sensitive than general somatic metabolism to reductions or perturbations in the products of these genes. The rational behind this suggestion is that as the germline is the ultimate carrier of genetic information to the next generation strong selection for fitness is imposed. This hypothesis is supported by the finding that at least some male sterile mutations are allelic to organismal lethal mutations (Lifschytz, 1987).

Spermatogenesis is a cellular differentiation process which involves a complex series of morphological changes. Furthermore the majority of sperm differentiation occurs in the absence of any substantial ongoing transcription; the process relies on mRNA transcribed during the premeiotic spermatocyte stage (see chapter 1). This is not to say that there is no regulation of gene expression during sperm differentiation. On the contrary extensive translational controls appear to operate to provide functional gene products at the appropriate times in development. Thus the isolation and investigation of genes expressed in the male germline may facilitate an investigation of the regulation of tissue specific gene transcription and of the regulation of mRNA translation. The results of such studies on testis-specific B-tubulin and mst(3)gl9 have been discussed in chapter 1.

As to the function of molecules specifically involved in spermatogenesis we can envisage a requirement for at least two classes of genes.

Class 1: In the first instance structural proteins necessary for the formation of sperm-specific organelles (eg the nebenkern and axoneme). This class of molecules is exemplified by the testis specific B-Tubulin, a major component of the axoneme. In addition this tubulin subunit

has been shown, by mutational analysis, to be required for meiotic spindle formation (but not the mitotic spindle of gonial cells) and to form post-meiotic cytoplasmic microtubules (Kemphues et al., 1982). Whether or not the testis specificity of this tubulin subunit is due to structural differences with respect to other B-Tubulins or to unique sperm-specific regulatory sequences is at present unclear. Other structural molecules include the product of the testis-specific transcript mst(3)gl9 examined by Kuhn et al (1988). This transcript encodes a small 56 amino acid polypeptide composed almost exclusively of cys-gly-pro repeats. Mammalian sperm also contain cys-pro rich proteins which are believed to have elastic properties (Guraya, 1987). Nucleic acid probes from this gene detect a sperm-specific transcript in mice. The authors propose, by analogy with mammalian sperm, that the mst(3)gl9 polypeptide may be a structural component involved in sperm tail elasticity.

Hennig and co-workers (cited in Hennig, 1985) identified immunologically a 155kd sperm-specific polypeptide in D.hydei which appears to be a structural component of the nebenkern. Interestingly they find that this polypeptide is localised to a specific Y chromosome loop until it is incorporated into the nebenkern. In the absence of a Y chromosome the level of the polypeptide is considerably reduced. As the polypeptide is not believed to be Y encoded the Y chromosome may be playing a regulatory role in the translation or storage of this polypeptide. More recently Bonaccorsi et al (1988) have demonstrated similar properties for a D.melanogaster sperm-specific antigen using the same antisera.

Evidence for the presence in Drosophila of protamine like, arginine rich, sperm-specific histones such as those found in mammalian sperm is at present contradictory. Hennig (1985) has failed to detect such molecules in the sperm of D.hydei. On the other hand Das et al (1964) and Hauschteck-Jungen and Hartl (1982) have convincing evidence for these proteins being present at the time of chromosome condensation in D.melanogaster. This apparent contradiction may reflect technical difficulties in detecting these proteins in D.hydei sperm. That these differences reflect species variation appears less likely considering the similarity between these species in most other aspects of spermatogenesis.

Class 2: Another class of sperm-specific molecules that one may expect to find are those involved in the regulation of sperm-specific processes. We can imagine molecules involved in regulating tissue-specific gene expression at both the transcriptional and translational level. We may also find genes whose products direct the series of morphological changes necessary to produce a functional sperm. Although there are no firm examples of these types of molecule two interesting genes are worth mentioning.

The ref(2)P gene product is involved in sigma virus replication and is also necessary for male fertility (Contamine et al., 1989). The sterile phenotype in homozygous males, a failure to differentiate the nebenkern, results from a failure in mitochondrial aggregation. The mitochondria appear to degenerate instead of coalescing into the nebenkern (Dezelee et al., 1989). The gene has recently been cloned and shown to encode a polypeptide of 76kd which contains a mitochondrial pre-sequence and a zinc finger region (Dezelee et al., opp.cit.). The mitochondrial pre-sequence may allow the polypeptide to associate with the outer membrane of the mitochondrion and promote aggregation. The zinc finger motif could perform a regulatory role or may be involved in the storage of the polypeptide until it is required.

Leroy et al (1989) have recently isolated a murine sperm-specific transcript (PL10) which encodes a polypeptide with similarity to eIF-4A. In mammals, as in Drosophila, there is considerable translational control of stored transcripts during spermatogenesis (protamine mRNA is stored for more than a week prior to translation. Peschon et al., 1987). The finding that PL10 is related to translation initiation factors suggests that it may have a role in regulating the translation of stored transcripts. In cross-hybridisation experiments using a PL10 gene probe related sequences are detected in Drosophila. In addition there is extensive similarity with the Drosophila vasa gene product which is believed to regulate translation in the early embryo (Lasko and Ashburner, 1988). Whether or not related molecules are to be found in the male germline of Drosophila remains to be determined.

In the following chapter I will present the initial characterisation of 3 novel genes putatively expressed in the male germline. It is hoped that a structural and mutational

analysis of these genes may provide further insight into the molecular events underlying the complex process of spermatogenesis in Drosophila.

5.2 RESULTS

5.2i Clone gS1: This clone shows rather weak hybridisation on reverse northern blots. Long exposures of these blots identify a 1.6kb SalI fragment that hybridises only to male cDNA. Male-specific hybridisation to other restriction digests identifies fragments attached to the lambda vector arms. A crude restriction map of the right hand end of the clone is shown in Figure 5.2d. The region of male-specific cDNA hybridisation is indicated. The 1.6kb SalI fragment was used to isolate a 1.0kb cDNA clone from the amplified male L3 cDNA library described in chapter 3. When the cDNA library was screened only 0.03% of the plaques gave a positive signal. This low frequency is consistent with the low level of hybridisation on reverse northern blots. The cDNA was subcloned as an EcoRI fragment into the plasmid vector pEMBL18 to give plasmid pCS1. To ensure that the cDNA is related to the "male-specific" sequences within the genomic clone, gS1 DNA was digested with a variety of restriction enzymes, electrophoresed, blotted and probed with pCS1 (Figure 5.2a). The 1.6kb SalI fragment and fragments attached to the right hand arm of the lambda vector are detected. Hybridisation to undigested DNA is also apparent in EcoRI and BamHI digests. This pattern of hybridisation is identical to the hybridisation observed on reverse northern blots. These data confirm the partial restriction map for the right hand end of the genomic clone but other sites toward the left hand end of the clone have not been ordered.

The genomic clone was used as a probe to salivary gland polytene chromosome spreads and hybridises uniquely to region 82D at the base of the right arm of chromosome 3 (Figure 5.1a,b). When pCS1 is used to probe southern blots of male and female Or DNA and DNA from other Drosophila strains (Cs and m56i) single bands are detected (Figure 5.2b). The sizes of these bands are consistent with the restriction map of the genomic clone. In addition the blot confirms that the

Figure 5.1: Photomicrographs of *in-situ* hybridisation to polytene chromosome spreads with biotinylated lambda clones.

a): Probe gS1 hybridising to the base of the right arm of chromosome 3.

b): Close up view of the above spread showing hybridisation at region 82D.

c): Probe gK33, the arrow points to the weak signal on the right arm of chromosome 3.

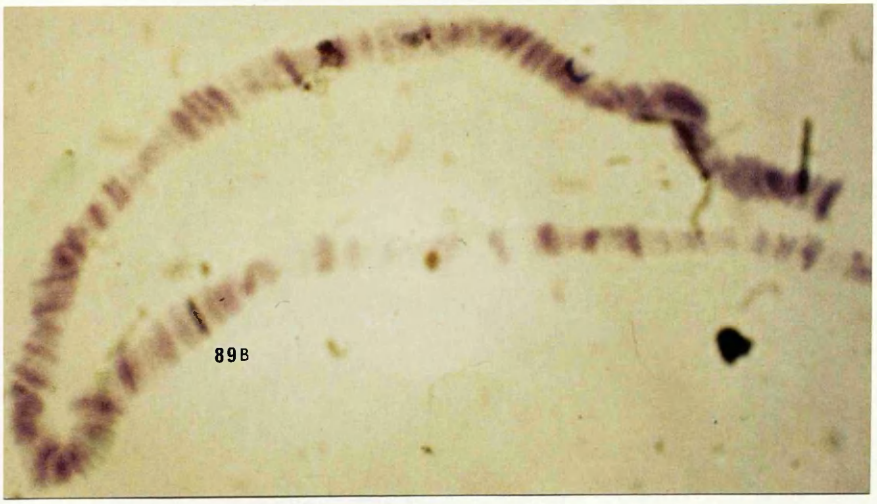
d): Close up of a different spread hybridised with gK33 showing hybridisation to region 89B.

A

B

C

D



gene is present as a single copy in the Drosophila genome and demonstrates that the sequence is conserved in other strains.

A northern blot analysis (Figure 5.2c) using the pCS1 probe detects 2 transcripts of low abundance. A major male-specific transcript of approximately 3.0kb confirms the male-specific hybridisation detected on reverse northern blots. This transcript is detected in male L3 (lane 4: barely visible on this exposure), pupae, adults, tra2/tra2 males and XO males. The transcript is not detected in agametic males, pseudomales or developmental stages prior to L3. Although this pattern of expression is consistent with expression in the male germline the low abundance of the transcript may preclude detection in cases where its level is substantially reduced. This would be the case if it were expressed in the somatic portion of the gonad. In addition low expression during the pupal stage would not be expected from a gene transcribed in spermatocytes. A more sensitive nuclease protection assay will be required to assess expression in these mutant individuals. In-situ hybridisation to tissue sections will be required to definitively localise the tissue of expression. Despite these ambiguities I can safely conclude that the presence of a germline is required for wild-type levels of expression.

A minor 0.9kb transcript of lower abundance is detected in most, if not all, developmental stages. It is not sex-specific in adults (lanes 15 and 16) but may be elevated in males. The transcript is particularly pronounced in both embryos and male pupae (lanes 1 and 6). It appears to be reduced in agametic males and females (lanes 12-14) as well as pseudomales (lane 11). It is of particular interest to note the elevated expression during embryogenesis and metamorphosis, both stages where major differentiation events are occurring. Reduced expression in individuals with rudimentary gonads is suggestive of germline associated expression. Again it will be of considerable interest to determine the site of expression by in-situ hybridisation to tissue sections.

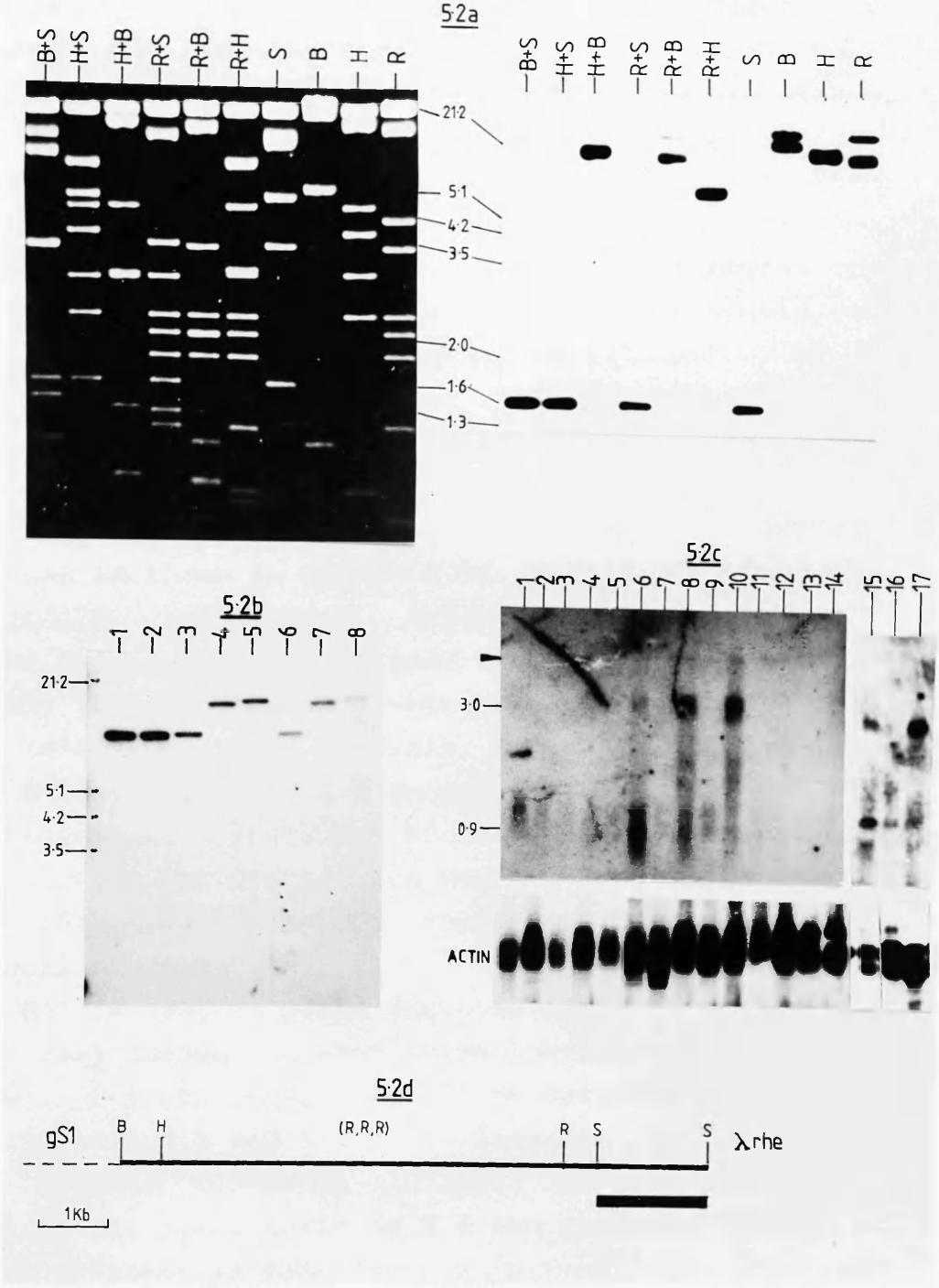
It is possible that these two transcripts are related by differential RNA processing or by the use of different transcription start-sites within the same gene. In some mRNA preparations a high molecular weight transcript of approximately 4kb is detected (arrow in Figure 5.2c lane 10),

Figure 5.2a: EtBr stained 0.8% agarose gel of restriction enzyme digested lambda gS1 DNA (1ug/lane) and southern blot probed with pCS1. The blot was washed at high stringency and exposed for 3 hours. Enzymes: R=EcoRI, H=HindIII, B=BamHI, S=SalI. Size markers in kilobasepairs.

Figure 5.2b: Southern blot of restriction enzyme digested Drosophila DNA (2ug/lane) electrophoresed on 0.8% agarose gel and probed with pCS1. The blot was washed at high stringency and exposed for 2 days. Size markers in kilobasepairs. Lanes: 1) m56i;EcoRI, 2) Cs;EcoRI, 3) Female Or;EcoRI, 4) Female Or;BamHI, 5) Female Or;HindIII, 6) Male Or;EcoRI, 7) Male Or;BamHI, 8) Male Or;HindIII.

Figure 5.2c: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe was pCS1, the blots were washed at high stringency and exposed for 2 weeks. The stripped blots were reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) Embryo, 2) L1, 3) L2, 4) Male L3, 5) Female L3, 6) Male pupal, 7) Female pupal, 8) Male adult, 9) Female adult, 10) XY;tra2/tra2, 11) XX;tra2/tra2, 12) Agametic male (tud), 13) Agametic female (tud), 14) Agametic male (osk), 15) Male adult, 16) Female adult, 17) XO male. The sizes are in kilobases.

Figure 5.2d: Restriction map of the right hand end of Lambda clone gS1. Enzymes: B=BamHI, H=HindIII, R=EcoRI, S=SalI. Sites in brackets have not been ordered. Black bar indicates male-specific hybridisation identified on reverse northern blots.


5·2a
B+S
H+S
H+B
R+S
R+B
R+H
S
B
H
R
21·2
5·1
4·2
3·5
2·0
1·6
1·3
5·2b
1
2
3
4
5
6
7
8
21·2
5·1
4·2
3·5
5·2c
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
3·0
0·9
ACTIN
5·2d
gS1
B
H
(R,R,R)
R
S
S
λrhe
1Kb

this may represent a processing intermediate.

5.2ii Clone gS10: The reverse northern analysis of this clone detects two SalI fragments (1.8 and 6.5kb) and a HindIII fragment (2.6 kb). The 2.6kb HindIII fragment was used to isolate a 0.6kb cDNA clone from the amplified male larval library. This was sub-cloned into the plasmid vector pEMBL18 to give plasmid pCS10. Only 0.05% of the plaques plated gave a positive signal, this is rather lower than would have been predicted from the strong signal on the reverse northern blots (and from the intensity of signal on northern blots, see below). This may reflect poor growth of phage containing this insert during amplification or a structural feature of the transcript which is refractory to reverse transcription. When the cDNA is used to probe blots of restriction digested gS10 DNA the fragments identified on reverse northerns are detected (Figure 5.3a). A deduced restriction map is shown in Figure 5.3d. Notice that a small (0.5kb) HindIII restriction fragment interrupts the continuity of the region showing male-specific hybridisation. This fragment is not detected by the cDNA clone (not shown) or by the reverse northern analysis. It may represent an intron or a duplication of the gene; current data do not allow differentiation between these two possibilities. Further sub-cloning and restriction mapping will be required to confirm the restriction map shown and determine the transcriptional organisation.

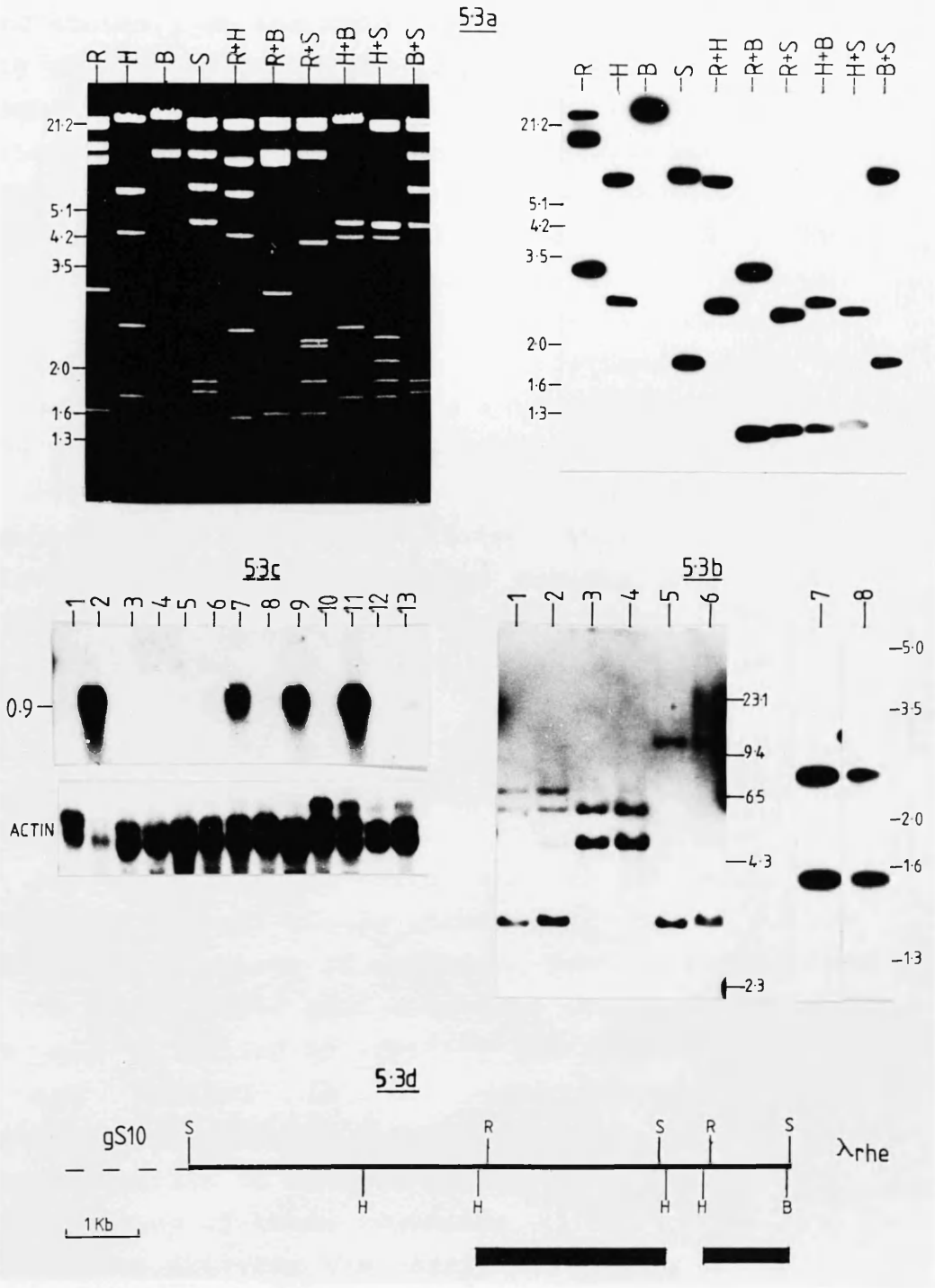
When pCS10 is used to probe Southern blots of different genomic DNAs restriction fragment polymorphisms are detected (Figure 5.3b). A 3.1kb EcoRI fragment is detected in Or and m56i DNA (lanes 1,2,5 and 6). This fragment is contained within the genomic clone. Cs DNA does not contain this fragment but in its place there is a 4.8kb fragment (lanes 3 and 4). This fragment is also visible in female Or DNA and may represent over-spill from the adjacent lane or contamination with other DNA, It is not detected in male DNA. A 6.2kb EcoRI fragment is present in Or and Cs DNA, 1.4kb of this fragment is attached to the right hand end of the vector in the genomic clone. This fragment is not present in m56i DNA, it is replaced by a fragment of approximately 10kb.

Figure 5.3a: EtBr stained 0.8% agarose gel of restriction digested gS10 DNA (1ug/lane) and Southern blot probed with pCS10. The blot was washed at high stringency and exposed overnight. Enzymes: R=EcoRI, H=HindIII, B=BamHI, S=SalI. Size markers in kilobasepairs.

Figure 5.3b: Sothern blots of restriction enzyme digested Drosophila DNA electrophoresed in 0.8% agarose gels (2ug/lane). The blots were probed with pCS10, washed at high stringency and exposed for 3 days. Lanes: 1) Male Or;EcoRI, 2) Female Or;EcoRI, 3) Male Cs;EcoRI, 4) Female Cs;EcoRI, 5) Male m56i;EcoRI, 6) Female m56i;EcoRI, 7) Female Or;HindIII, 8) Male Or;HindIII. Size markers are in kilobasepairs.

Figure 5.3c: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe used was pCS10, the blot was washed at high stringency and exposed for 3 days. The stripped blot was reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) XXY, 2) XO, 3) Agametic male (osk), 4) Agametic female (tud), 5) Agametic male (tud), 6) XX;tra2/tra2, 7) XY;tra2/tra2, 8) Female adult, 9) Male adult, 10) Female pupal, 11) Male pupal, 12) Female L3, 13) Male L3. Size in kilobases.

Figure 5.3d: Restriction map of the left hand end of the Lambda clone gS10. Enzymes: B=BamHI, H=HindIII, R=EcoRI, S=SalI. The black bars represent male-specific hybridisation identified on reverse northern blots.


5·3a
R
H
B
S
R+H
R+B
R+S
H+B
H+S
B+S
21·2
5·1
4·2
3·5
2·0
1·6
1·3
5·3c
1
2
3
4
5
6
7
8
9
10
11
12
13
0·9
ACTIN
5·3b
23·1
9·4
6·5
4·3
2·3
5·0
3·5
2·0
1·6
1·3
5·3d
gS10
S
R
S
R
S
H
H
H
H
B
1 Kb
λrhe

In addition to these strain differences a band of 7kb is reproducibly detected in both male and female Or DNA. This fragment is not accounted for in the genomic clone. When HindIII digested Or DNA is probed only the two fragments predicted from the genomic clone are detected (lanes 7 and 8). Contamination of the genomic DNA with plasmid sequences is considered unlikely as the band is not detected with other probes or by pCS10 in the HindIII digests. The presence of unrelated sequences on the cDNA clone also seems unlikely as these additional fragments are not detected in digests with other enzymes. In addition only a single transcript is detected on northern blots when pCS10 is used as a probe. Partial digestion by EcoRI is improbable as the extra bands are the same intensity in male and female DNA. A duplication of cS10 sequences to another genomic location cannot be eliminated. However this would have to alter the EcoRI sites but not the HindIII sites. Since we know that the gene is polymorphic between strains one possibility is that the Or DNA is contaminated with DNA from another strain. Alternatively the Or stock may contain a sub-population with a rearrangement at one of the EcoRI sites. Further experiments with single fly blots will be required to investigate these possibilities.

When either the genomic clone or the cDNA clone are used to probe polytene chromosome preparations no signal can be detected. Incorporation of biotin into the probe was equivalent to that achieved with control clones. Co-hybridisation with control clones showed that hybridisation and detection were operating as expected. Two possibilities may account for this; either gS10 sequences are located at a constriction and I failed to identify the signal or the sequences are located in an under-replicated or heterochromatic region. In-situ hybridisation with tritiated probes and hybridisation to mitotic larval neuroblast spreads may allow localisation of these sequences.

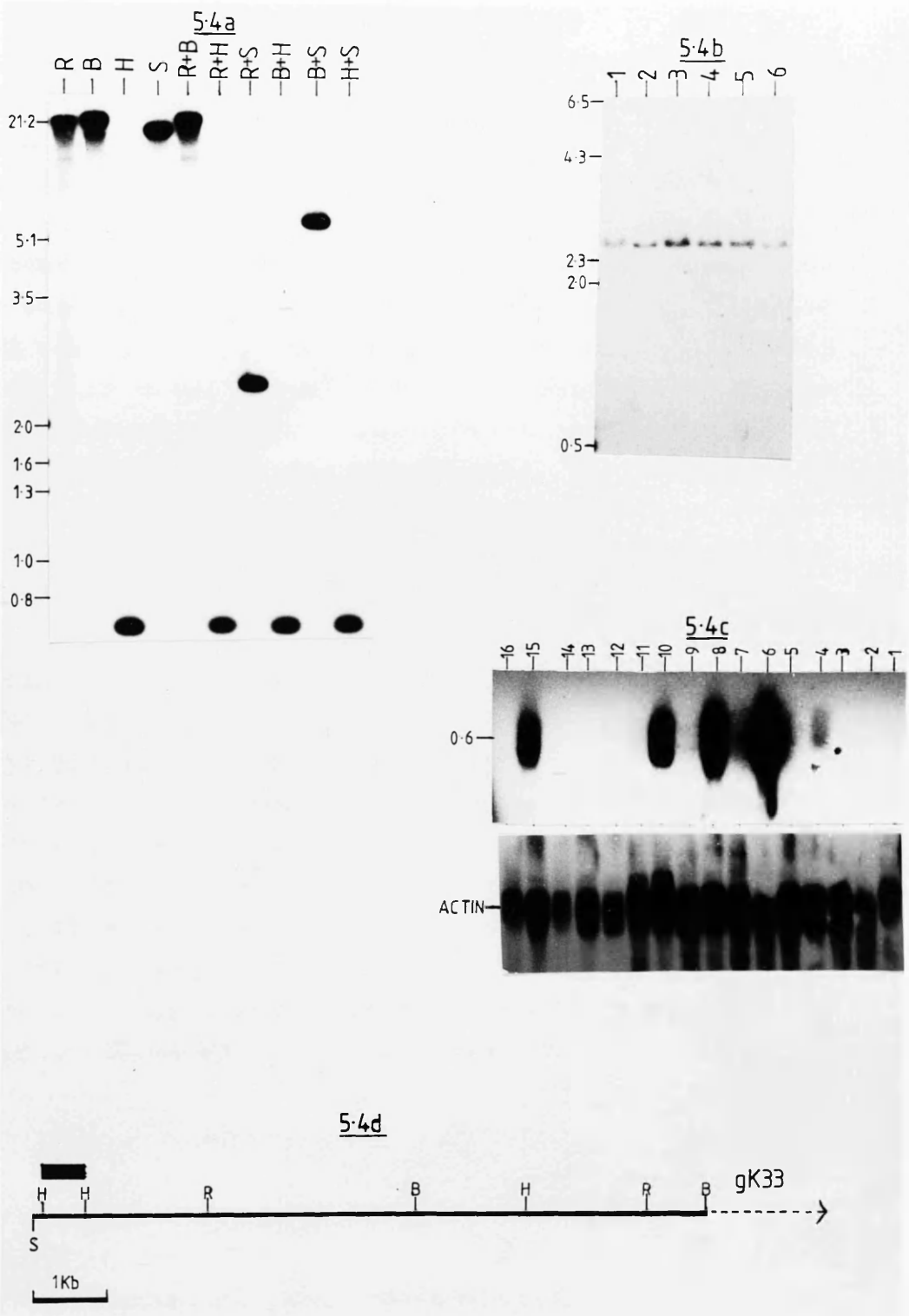
A northern blot analysis was carried out using the pCS10 probe (Figure 5.3c). A single transcript of approximately 0.9kb is present in male L3, pupae and adults. It is not detected in females, pseudomales or agametic males. This suggests germline specific expression. The level of the transcript does not appear to be reduced by the lack of a Y chromosome or by the tra2 mutation. On the contrary the level

Figure 5.4a: Autoradiogram of a southern blot of restriction enzyme digested gK33 DNA electrophoresed in a 0.8% agarose gel (1ug/lane) . The blot was probed with the subclone pGK33H, washed at high stringency and exposed overnight. Enzymes: R=EcoRI, H=HindIII, B=BamHI, S=SalI. Size markers in kilobasepairs.

Figure 5.4b: Southern blot of EcoRI digested Drosophila genomic DNA electrophoresed on 0.8% agarose gel (2ug/lane). The blot was probed with pGK33H, washed at high stringency and exposed for 2 days. Lanes: 1) Male Or, 2) Female Or, 3) Male Cs, 4) Female Cs, 5) Male m56i, 6) Female m56i. Sizes are in kilobasepairs.

Figure 5.4c: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe used was pGK33H, the blot was washed at high stringency and exposed for 3 days. The stripped blot was reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) Embryo, 2) L1, 3) L2, 4) Male L3, 5) Female L3, 6) Male pupal, 7) Female pupal, 8) Male adult, 9) Female adult, 10) XY;tra2/tra2, 11) XX;tra2/tra2, 12) Agametic male (tud), 13) Agametic female (tud), 14) Agametic male (osk), 15) XO male, 16) XXY female. The size is in kilobases.

Figure 5.4d: A restriction map of the left hand end of the Lambda clone gK33. Enzymes: B=BamHI, H=HindIII, R=EcoRI, S=SalI. The black bar symbolises the extent of male-specific hybridisation detected on reverse northern blots and also the subclone pGK33H.


5·4a
R
B
H
S
R+B
R+H
R+S
B+H
B+S
H+S
21·2
5·1
3·5
2·0
1·6
1·3
1·0
0·8
5·4b
1
2
3
4
5
6
6·5
4·3
2·3
2·0
0·5
5·4c
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0·6
ACTIN
5·4d
H
H
R
B
H
R
B
gK33
S
1Kb

of expression appears to be elevated in XO males. Confirmation of this observation will require a more quantitative analysis than the one presented.

5.2iii Clone gK33: in-situ hybridisation to polytene chromosomes with the whole genomic clone shows unique hybridisation on the right arm of chromosome 3 at 89B (Figure 5.1c,d). The reverse northern analysis identifies a small 0.6kb HindIII fragment that contains all of the sequences hybridising to male cDNA. This fragment was sub-cloned into the pEMBL vector to give plasmid pGK33h. Labelled plasmid DNA was hybridised to blots of restriction digested gK33 DNA (Figure 5.4a) and a crude restriction map of the left hand end deduced (Figure 5.4d). pGK33h detects a single 2.5kb EcoRI fragment in genomic DNA digests. The fragment is conserved in the other strains examined (Figure 5.4b).

Northern blot analysis (Figure 5.4c) with the pGK33h probe detects a smear from 0.5 to 0.7kb, this is consistent with the entire transcribed region being contained within the 0.6kb HindIII fragment when a polyA tail is taken into account. The transcript(s) are detected in male L3, pupae (where it is particularly prominent), adults and at similar levels in XO males and homozygous tra2 males. The transcript is not detected in stages prior to L3, in females or in agametic males. (Faint hybridisation to mRNA from pseudomales observed on this autoradiogram is considered to be an artefact probably due to over-spill or contamination, it is not detected on other blots.) The pattern of expression is suggestive of germline specific transcription.

5.3 DISCUSSION

Several features of the male-specific transcripts described in both this chapter and the previous chapter (gK14 and gK21) are consistent with expression in the male germline. The failure to detect the transcripts in agametic males shows that the transcripts are dependent on the presence of the male germline for expression. The lack of

expression in pseudomales strengthens this conclusion (with the caveats discussed in the introduction to chapter 4 regarding the underdeveloped gonad). The localisation of gK14 and gK21 male-specific transcripts to the germline by DiBenedetto et al (1987) confirm this conclusion, at least for these two clones. The developmental profile of expression also supports germline expression of these sequences. Low levels of the male-specific transcripts are found in the L3 stage where only a few transcriptionally active spermatocytes are found. The transcript levels increase dramatically during the pupal stage and are present at high levels during the adult stage. In addition the male-specific transcripts of gS10 and gK33 appear to be elevated during the pupal stage, paralleling the rise in spermatocyte numbers reported by Garcia-Bellido (1964). Although the evidence for germline expression is good the possibility of somatic, germline dependent, expression cannot be excluded without in-situ hybridisation data. In the case of the male-specific transcript identified by gS1 this will be particularly important as this transcript is present at low abundance. It is not likely that reduced expression of this transcript in underdeveloped gonads would have been detected by the northern blot analysis presented here. The low level of expression in the pupal stage suggests that this transcript may not be expressed in spermatocytes.

If these transcripts are involved in spermatogenesis then neither the lack of a Y chromosome nor the presence of the tra2 mutation appear substantially to affect their transcription. This observation is similar to studies on the mst(3)gl9 transcript by Schafer (1986b) and studies on sperm-specific tubulin expression (Hulsebos and Brand cited by Hennig 1985). These studies indicate that in XO males tubulin transcript levels are normal but protein levels are dramatically reduced. The apparent elevation of the gS10 male-specific transcript in XO males will require confirmation by quantitative studies.

The genomic locations of gS1 and gK33 give no clues as to their function, no male sterile genes or genes expected to have male-specific expression have been mapped in the vicinity of the clones described here. gK33 maps close to the Stubble region where there are a variety of chromosomal aberrations that could be used to generate mutations at the

gK33 locus. Alternatively a novel mutagenesis screen developed in our laboratory (Kaiser and Goodwin, in press) may be used to generate transposon insertions at or near the male-specific genes (see chapter 7).

At present the genomic location of gS10 is unknown. In the first instance the ambiguities surrounding the additional EcoRI fragments detected on genomic blots will have to be resolved. As mentioned single fly DNA blots may be the best way to investigate this question. The inability to detect the location of this clone on polytene chromosome spreads suggests that it may be located in heterochromatic DNA. As we shall see in the next chapter several heterochromatic regions have been detected which are involved in spermatogenic processes. Initially the localisation of gS10 sequences may be achieved by hybridisation to mitotic spreads, where heterochromatin is available for hybridisation. If gS10 is located in heterochromatic DNA we would expect to find moderately repetitive DNA in the genomic clone, this has yet to be determined. An alternative explanation for the failure in detecting gS10 sequences is that they are within a constriction or locally underreplicated region. Hybridisation to mitotic spreads will help to identify if this is the case.

Little can be said at present concerning the nature of the products encoded by these male-specific transcripts. The low molecular weight transcripts encoded by gK33 and gS10 are reminiscent of other transcripts expressed in the male germline. For example the transcripts of mst(3)gl9 (450nt) and the murine protamine genes (800nt: Peschon et al., 1987). The heterogeneous size of the gK33 transcript is similar to that observed for murine sperm-specific protamine genes where size heterogeneity has been shown to reflect translational regulation by transcript deadenylation (Braun et al 1989). Whether the same is true for gK33 remains to be determined. The small size of this gene makes it an ideal candidate for gene fusion experiments to identify transcriptional and translational regulatory sequences, such an approach has been successful with the mst(3)gl9 gene (Kuhn et al 1988).

Of the three genes described here gS1 looks to be the most interesting as the low transcript abundance suggests that is does not encode an abundant structural protein. It will be of some interest to elucidate the relationship between the male-specific and the non-specific transcripts.

The elevated expression of the non-specific transcript during stages undergoing major differentiation tempts me to speculate on a role in gene regulation or nucleic acid metabolism. A sequence analysis of the cDNA clone is underway in our laboratory. Perhaps this may provide a clue as to the function encoded by this transcript.

CHAPTER 6

GENES WITH UNUSUAL GENOMIC STRUCTURE

6.1 INTRODUCTION

Most heterochromatic DNA appears to be composed of highly repetitive satellite DNA and contain very few conventional genetic loci. However embedded within the simple sequence DNA mutable genetic loci are detected. Furthermore mutations at some of these loci have effects on normal spermatogenesis. It is some of these "genes" which I shall discuss below.

The Y Chromosome: The Y chromosome of D.melanogaster accounts for around 12% of the haploid genome. Cytologically it appears to be entirely heterochromatic. It exhibits intense staining with dyes specific to this class of DNA and remains underreplicated in polytene nuclei (Gatti et al., 1976). Bridges (1916) demonstrated that the Y chromosome is devoid of essential genes but that it is necessary for male fertility. Subsequent studies in D.melanogaster indicate that there are at least 6 separable loci on the Y chromosome necessary for male fertility. Two of these are on the short arm, ks-1 and ks-2, and four are on the long arm, kl-1, kl-2, kl-3 and kl-5 (Kennison, 1981). Deletions encompassing any of these fertility factors result in characteristic defects in spermatogenesis, either early or late depending on the particular factor deleted (Hardy et al., 1981). In D.hydei there appear to be 16 Y-linked complementation groups necessary for male fertility (Hennig, 1985). For many years the nature of the fertility factors remained somewhat enigmatic. However genetic, cytological and molecular studies are beginning to shed some light on these unusual genes.

Much of the early work focused on the Y chromosome of D.hydei due to the prominent structures visible in the spermatocyte nuclei of this species (Hennig, 1985, for a comprehensive review). During the spermatocyte stage the Y chromosome forms five characteristic structures reminiscent of the lampbrush loops of amphibian oocytes. The loops have each been named Nooses, Clubs, Tubular Ribbons, Pseudonucleoli and Threads on the basis of their distinctive morphology. A detailed genetic and cytological analysis has indicated that the loops correspond to at least some of the mutable fertility factors and suggests that they constitute

genetically discrete entities (Hennig, 1985). More recently Bonaccorsi et al. (1988) have demonstrated the presence of three similar loops in the spermatocyte nuclei of D.melanogaster and correlated them with 3 of the fertility factors, kl-3, kl-5 and ks-1.

A limited molecular analysis has been initiated and some Y chromosome DNA has been isolated (Hennig, 1985; Lifschytz, 1987). Broadly speaking the Y DNA so far characterised can be divided into 4 classes; 1) DNA specific to the Y chromosome but not to a specific region of the Y. This class is exemplified by the Y_LI, Y_LII and Y_LIII DNA clones from D.hydei (Trapitz et al., 1988; Wlaschek et al., 1988). These sequences are found to be highly repetitive and transcribed in several of the lampbrush loops on the Y. 2) Y-specific and loop-specific DNA such as the Y20N, Y18Cl and Y22Tr clones isolated from D.hydei by Haraven et al. (1986). These sequences appear to be restricted to a particular loop structure. Again they are repetitive and transcribed. **3 and 4)** Y associated DNA which is found on the Y chromosome as well as at other genomic locations. Class 3 DNA is highly repetitive satellite-like DNA exemplified by the D.hydei MY3 clone (Vogt and Hennig, 1986). Class 4 sequences are moderately repetitive and appear to resemble retroviral like transposable elements (Huijser et al., 1988; Brand and Hennig, 1989). These sequences are transcribed predominantly, though not exclusively, in the male germline. It is not known if they have any functional role. In addition it would appear that individual copies of these transposon-like sequences have diverged as multiple copies are only detected by a particular probe when low stringency hybridisation conditions are used.

The repetitive sequences transcribed from the lampbrush loops appear to have no protein coding potential. In addition no cytoplasmic transcripts have been detected. It is believed that these sequences are organised and transcribed in long tandem arrays hundreds of kilobases in length (Lifschytz et al., 1983). It has been proposed that the transcribed lampbrush loops perform a structural role in spermatogenesis or are required to store or compartmentalise proteins and RNA required for post-meiotic development (Hennig, 1985; Lifschytz, 1987). Several antibodies have been isolated which recognise antigens specifically associated with particular

lampbrush loops (Kloetzel et al., 1981; Tischendorf et al., 1989; Hennig, 1985). Some antigens are associated with specific structural components of the sperm at later stages in spermatogenesis, for example the 155kd antigen associated with the nebenkern discussed in chapter 5 (Hennig, 1985).

What is not clear at present is if the lampbrush loops are the only manifestation of the fertility factors. In this respect it should be noted that three of the D.melanogaster fertility factors have no known associated structures (Bonaccorsi et al., 1988). However this may reflect the destruction of such structures during the fixation process prior to cytological examination. These authors also demonstrate that some deletions of kl-3 which result in sperm dysfunction have no discernible effect on the lampbrush loop associated with this factor. At present it is still a possibility that regions of the fertility factors produce conventional protein products. In this respect we should also note that some Y-linked sterile mutations can be induced by EMS at the same frequency as X-linked male steriles (Kennison, 1983). These data suggest that some Y-linked steriles are point mutations although this has yet to be proved. Point mutations would not be expected to disrupt the function of tandemly repeated lampbrush loop structural transcripts. An argument against the occurrence of extensive point mutations is the failure of most experimenters to find complementing mutations within fertility factors. One author has reported 25 non-allelic complementation groups on the Y after EMS treatment (Williamson , 1972). Although these data are controversial and have not been confirmed recall that 16 fertility genes have been localised on the D.hydei Y chromosome. One explanation for the high level of EMS induced male steriles on the Y chromosome is that it may be more susceptible to EMS induced rearrangements than the other chromosomes. In this respect several workers have noted that X-ray induced mutations are twice as frequent on the Y than on the X (Kennison, 1983, and references therein). If indeed there are point mutations in fertility factors it will be of considerable interest to ascertain if the mutations are in coding or regulatory sequences.

X-Chromosome Heterochromatin: Approximately 50% of the mitotic length of the X chromosome is heterochromatic (Xh). In the salivary gland polytene chromosomes the Xh is not polyteneised and is associated with the chromocenter (Schalet and Lefevere, 1976). As with most other heterochromatic regions Xh appears to contain few conventional genes. However some DNA rearrangements within this region have detrimental effects on normal spermatogenesis. Unfortunately the nature of the rearrangements and how they disrupt spermatogenesis is at present poorly understood. Furthermore, if a conventional gene necessary for normal spermatogenesis were located on both the X and the Y it is unlikely that conventional genetic analysis would detect such a locus.

Cooper (1964) and Yamamoto and Miklos (1977) have identified at least two regions in Xh which are required for X-Y bivalent formation at meiotic prophase. These regions are apparently distinct from the bobbed (bb) loci containing the ribosomal RNA cistrons. Deletions of either of these regions substantially increases non-disjunction at male, but not female, meiosis (bivalent formation at female meiosis is mediated by euchromatic sequences). The precise location of the pairing sites on the Y chromosome has not been reported but seem to lie in the proximal ends of each arm (Cooper, 1964). By examining rearrangements which move these sites around the X chromosome Yamamoto and Miklos (1977) concluded that they represented specific sequences embedded within homogeneous satellite DNA. The precise nature of these pairing sequences remains to be determined.

Evidence that other Xh regions are required for proper spermatogenesis comes from the observations of several workers on sterility associated with certain Xh deficiencies. In a comprehensive study Rhaman and Lindsley (1980) examined such deletions and found that male sterility could be ascribed to some aberrations. However they found that the deletions induced sterility only when covered by duplications of Xh, carried either on the Y or on an autosome. In addition the behaviour of any particular deficiency was dependent upon the covering duplication used; a deficiency would induce sterility with one duplication but not with another. To account for their complex data they proposed the presence of three Xh sites or factors; one proximal to and two distal to bb. At present it is not clear what is the nature of these

sites nor if they are equivalent to the pairing sites previously identified (this is unlikely as the the deletions cause sterility and not non-disjunction). Equally it is also unclear whether the deficiencies uncover coding regions or sites necessary for correct chromosome behaviour during spermatogenesis. The authors favour the latter explanation and postulate that they have uncovered sites required for precocious X chromosome inactivation.

One other region which is found on both the X and Y chromosomes is the Stellate (Ste) locus (Hardy et al., 1984). The allelic state of the X-linked Ste locus determines the morphology of crystals which form in the primary spermatocytes of XO males. In Ste^{+}/O males the crystals are needle shaped whereas in Ste/O males the crystals have a star shaped morphology. The region responsible for the difference in crystal morphology was mapped to 12C-13A on the X chromosome. A specific region proximal to, but not including, kl-2 was found to be the Y chromosome site responsible for the appearance of the crystals. This was dubbed the stellate control region. The sequences on the X and Y have been analysed at the molecular level by Livak (1984). A lambda genomic DNA clone DM2L1 was isolated by virtue of its homology with a transcript elevated in the testes of XO males relative to XY males. The DM2L1 clone was found to have an unusual structure. In the first instance it was found to be devoid of recognition sites for many common hexanucleotide recognising restriction enzymes. Further examination with enzymes which have tetranucleotide recognition sequences uncovered a repeat structure; both CfoI and HincII released a tandem repeat of 1250bp. The repeats were found to be present on both the X and the Y chromosomes of D.melanogaster (related sequences were found mainly on the Y chromosome of D.mauritiana and D.simulans). In addition it was found that the Ste phenotype was correlated with the number of repeats on the X chromosome: thus Ste alleles have many copies (approximately 200) whereas Ste^{+} alleles have few copies. The homologous regions on the Y chromosome map to the stellate control region and seem to be present at a stable level of around 80 copies.

Hybrid selection experiments identify a polypeptide of approximately 17kd present in both XO and XY testes. The

nature of the polypeptide and its role, if any, in normal spermatogenesis is at present unknown. The high level of expression associated with XO testes led Livak to propose that the expression of the X-linked copies were in some way negatively regulated by the Y sequences. In this model the absence of the Y sequences results in the production of excess Ste gene product which precipitates to form the crystals. It is not known if this regulation is transcriptional, translational or at the level of product stability. The morphology of the crystals is believed to be a reflection of X-linked Ste gene dosage.

One other system affecting spermatogenesis has elements localised within heterochromatic DNA. The Segregation Distorter (SD) system is a meiotic drive phenomena which results in the enhanced recovery of one member of a pair of homologous chromosome in the progeny of "distorting" fathers. There are three major components of the system; Segregation Distorter (Sd) a dominant allele which acts in trans, Responder (Rsp) the site of Sd action and Enhancer of Sd (E(Sd)) a modifier which increases the strength of drive.

The phenotypic effect of the SD system is to cause degeneration of sperm bearing the Rsp^{S} (sensitive) allele when an Sd allele is present in the genome. It would appear that chromatin condensation is prevented in spermatids carrying a sensitive responder. It has been suggested that the transition from somatic to sperm-specific histones is disrupted (Hauschteck-Jungen and Hartl, 1982).

Sd has been mapped to region 37D on the left arm of chromosome 2 (Brittnacher and Ganetsky, 1983). Deletions appear to be without effect on otherwise wild type individuals. It is therefore unclear what role, if any, Sd plays in normal spermatogenesis. The locus has been translocated to different chromosomal locations where it is still effective in causing drive.

The Rsp locus is located within the heterochromatin of the right arm of chromosome 2, very close to the centromere. Broadly speaking Rsp has two allelic states; insensitive (Rsp^{i}) and sensitive (Rsp^{S}). All Sd bearing chromosomes isolated from the wild carry Rsp^{i} and are not subject to distortion. Rsp^{S} bearing chromosomes are recovered at a greatly reduced frequency when present in trans with an Sd

bearing chromosome (Brittnacher and Ganetsky, 1989; Lyttle et al., 1986). The Rsp locus has been cloned and appears to be composed of tandem repeats of a 240bp XbaI fragment (Wu et al., 1988). Rsp^i alleles appear to have substantially fewer copies of the repeat than Rsp^s alleles. No coding potential is detected within the repeat, nor can any transcripts be detected using the repeat as a probe to northern blots. Thus it is considered that the repeats act as a binding site for a trans-acting factor (Wu et al. opp.cit.).

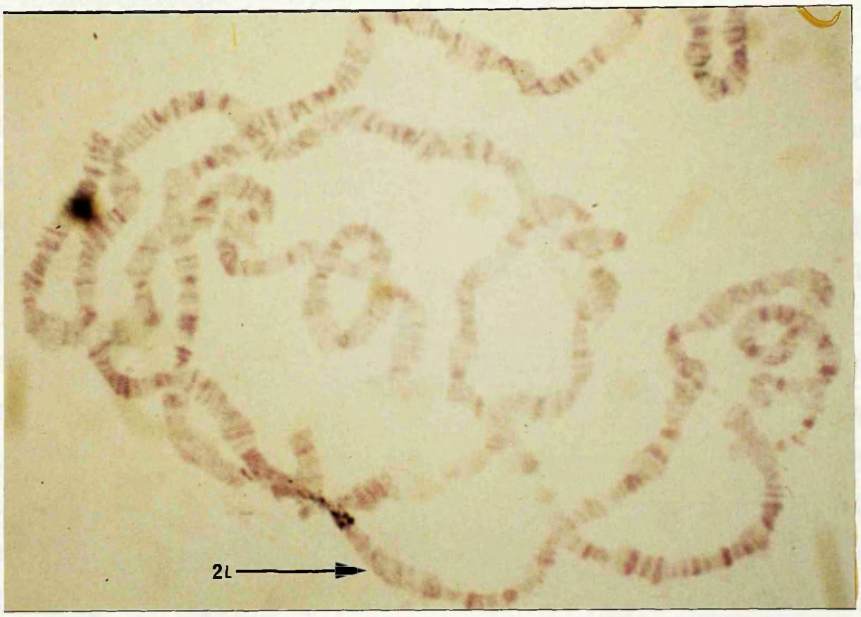
E(Sd) is also located in a heterochromatic region; in the distal end of the heterochromatin of 2L (Brittnacher and Ganetsky, 1984; Sharp et al., 1985). E(Sd) has been defined by deletion and translocation analysis. It acts in trans to increase the strength of drive and has also been reported to exhibit weak drive on its own (ie. in the absence of Sd) (Sharp et al., 1985). The heterochromatin of chromosome 2 has been extensively mutagenised by Hilliker (1976). In EMS mutagenesis screens he recovered several lethals and the previously characterised eye colour mutation light within 2L heterochromatin. It would appear that none of these are allelic to E(Sd) (T.W. Lyttle, pers.com.). The apparent failure to find EMS induced mutations suggests that the locus may have an unconventional structure. It will be of interest to discover if E(Sd) is a conventional protein coding gene, if it produces a trans-acting RNA or if it has a trans-acting structural role.

In the following results section I shall present the preliminary characterisation of three genomic clones which encode male-specific transcripts. These clones have an unusual genomic structure, one appears to be heterochromatic and the other two are located primarily on the Y chromosome.

6.2 RESULTS

6.2i Clone gS4. gS4 was localised, by in-situ hybridisation to polytene chromosomes, to the proximal end of section 40 on the left arm of chromosome 2 (Figure 6.1). As this region is associated with the chromocenter precise localisation has proved to be difficult. It appears however that this genomic clone contains DNA which only hybridises to

A



B

figure 6.1: Photomicrographs of in-situ hybridisation to polytene chromosome spreads with biotinylated lambda gS4.

a): Hybridisation to the base of 2L. There is a slight signal at the chromocenter, this is not observed in most preparations.

b): Hybridisation to a different spread showing the signal restricted to 2L at region 40.

2L. Stretched chromosome preparations show hybridisation only to 2L and not to general heterochromatin or the chromocenter (not shown).

The molecular structure of the cloned DNA has some unusual features. It was apparent that most common restriction enzymes which cut at hexanucleotide recognition sites do not cut gS4. Thus there are no sites for BamHI, EcoRI, HindIII, SalI, PstI or KpnI within the gS4 insert. When digested with SmaI or XhoI however a prominent band of 1.3kb is released, the intensity of the band suggests that several copies of a 1.3kb sequence are present in the insert of gS4 (Figure 6.2b). The simplest interpretation is that these sequences are arranged as a tandem array. In addition the two enzymes also release a fragment of 1.8kb. This may represent a diverged repeat (with the insertion of a 0.5kb fragment for example) or gS4 may contain sequences from the end of the genomic repeat. The latter suggestion is considered less likely because the 1.8kb fragment is produced by both enzymes. The size of the whole insert is estimated to be approximately 14kb which suggests there are approximately 8 copies of the 1.3kb fragment within gS4. At present the exact order of the repeats cannot be predicted as the position of the 1.8kb fragment is not known. A simple restriction map of the ordered site is shown in Figure 6.2d.

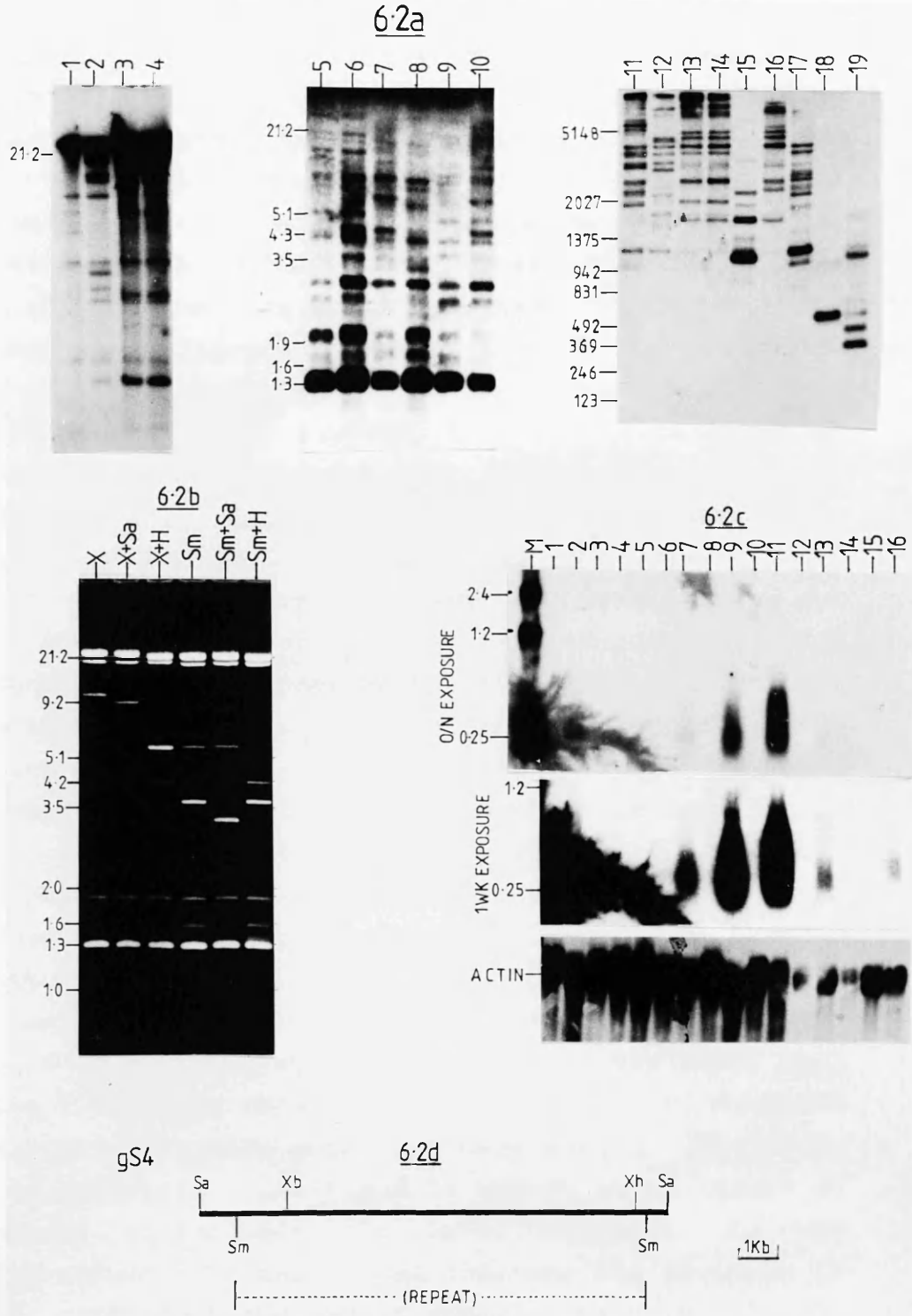
Additional repeat like structures are identified when the whole lambda clone is used as a probe to southern blots of genomic DNA. These are shown in Figure 6.2a and can be summarised as follows: With EcoRI digested DNA (lanes 1-4) most hybridisation is to DNA of greater than 20kb although lower molecular weight fragments are detected with long exposures. Some of these smaller fragments are conserved in the other strains examined. XhoI (lanes 5-10) digestion reveals the major 1.3kb band which is present in the genomic clone. In addition a ladder of bands increasing in molecular weight is also detected. Most of these bands are not multiples of 1.3kb and may represent diverged repeats. A similar, if not identical, pattern is obtained by SmaI digestion (not shown). Interestingly many of the bands are conserved in the 3 strains examined. Minor differences between male and female DNA are not believed to be significant and may reflect a degree of partial digestion (see below). Analysis of genomic DNA with enzymes which cut

Figure 6.2a: Southern blots of restriction enzyme digested Drosophila DNA (2ug/lane) probed with Lambda clone gS4. All the blots were washed at high stringency and exposed for 12 hours.
Lanes 1-4 : EcoRI digested DNA, 1) m56i, 2) Cs, 3) Male Or, 4) Female Or. 0.8% agarose gel, size is in kilobasepairs.
Lanes 5-10 : XhoI digested DNA, 5) Male Or, 6) Female Or, 7) Male Cs, 8) Female Cs, 9) Male m56i, 10) Female m56i. 0.8% agarose gel, sizes in kilobasepairs.
Lanes 11-14 : CfoI digested DNA, 11) m56i, 12) Cs, 13) Male Or, 14) Female Or,
Lanes 15-19 : Or DNA digested with; 15) HpaII, 16) HaeII, 17) HincII, 18) ThaI, 19) Sau3A. Lanes 11-19 1.2% agarose gel, size markers in basepairs.

Figure 6.2b: EtBr stained 0.8% agarose gel of restriction digested lambda clone gS4 (1ug/lane). The enzymes used were, H=HindIII, Sa=SalI, Sm=SmaI X=XhoI. The sizes are in kilobasepairs.

Figure 6.2c: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe used was lambda gS4, the blot was washed at high stringency and exposed for the indicated times. The stripped blot was reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) XXY, 2) XO, 3) Agametic male (osk), 4) Agametic female (tud), 5) Agametic male (tud), 6) XX;tra2/tra2, 7) XY;tra2/tra2, 8) Female adult, 9) Male adult, 10) Female pupal, 11) Male pupal, 12) Female L3, 13) Male L3, 14) L2, 15) L1, 16) Embryo. Sizes are in kilobases.

Figure 6.2d: A simple restriction map of the orderd sites in the Lambda clone gS4. The extent of the unmapped repeat is indicated below the diagram. Enzymes; Sa=SalI, Sm=SmaI, Xb=XbaI, Xh=XhoI.


6·2a
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2
3
4
21·2
5
6
7
8
9
10
21·2
5·1
4·3
3·5
1·9
1·6
1·3
11
12
13
14
15
16
17
18
19
5148
2027
1375
942
831
492
369
246
123
6·2b
X
X+Sa
X+H
Sm
Sm+Sa
Sm+H
21·2
9·2
5·1
4·2
3·5
2·0
1·6
1·3
1·0
6·2c
M
1
2
3
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6
7
8
9
10
11
12
13
14
15
16
O/N EXPOSURE
2·4
1·2
0·25
1WK EXPOSURE
1·2
0·25
ACTIN
gS4
6·2d
Sa
Xb
Xh
Sa
Sm
Sm
1Kb
(REPEAT)

at tetranucleotide recognition sequences reveals additional repeat-like structure (lanes 11-19). Hybridisation to ThaI digested Or DNA (lane 18) is almost exclusively to a 500bp fragment. Faint minor bands are detected at 1.0 and 1.1kb, again these may represent diverged repeats, the end of the repeats, or partial digestion. Hybridisation to HpaII and HincII digested Or DNA (lanes 15,17) reveals major fragments of 1.1kb as well as additional bands of lower intensity. Sau3A releases 3 strongly hybridising fragments of 380, 470 and 1000bp (lane 19). In contrast CfoI and HaeII do not appear to recognise repeats. The CfoI digests (lanes 11-14) suggest that there is no difference between male and female Or DNA with this enzyme. The pattern of restriction fragments with this enzyme is different between the strains examined. The Cs and m56i strains have not yet been examined with the enzymes which recognise the repeats.

It would appear then that the lambda clone gS4 is composed of SmaI, XhoI, HpaI, HincII and Sau3A repeats with a major 500bp ThaI repeat. A more detailed analysis of the structure of this clone is under-way at present in our laboratory. The pattern of restriction enzyme digestion strongly suggests that gS4 represents heterochromatic DNA.

The transcripts recognised by the gS4 sequences also show some interesting properties. Male-specific RNAs are detected when the whole clone is used to probe northern blots (Figure 6.2c). The transcripts migrate as a smear from 400-700nt. If a polyA tail is taken into account this size range is compatible with the transcripts emanating from a single ThaI repeat. Alternatively the DNA homologous to the transcripts may be located at one end of the lambda clone and be represented as a unique sequence (ie one of the faint ThaI bands or the 1.8kb SalI band). The transcripts are detected in embryos, male L3, male pupae and male adults. They are also found in tra2/tra2 males (lane 7) and XO males (lane 2) but in these individuals the level appears to be substantially reduced (in the 1 week exposure the XO lane is obscured by background however it appears reduced in the overnight exposure). No expression is detected in agametic males, pseudomales or females. This pattern of expression is consistent with male germline specific transcription.

6.2ii Clones gS8 and gS9: As mentioned in chapter 3 these genomic clones share related sequences although they do not appear to overlap. The reverse northern analysis detects restriction fragments which hybridise predominantly with male cDNA within both of these clones (see Figure 6.4b). gS8 contains a 7.0kb SalI fragment and a 5.5kb HindIII fragment. These fragments hybridise weakly, but noticeably, with female cDNA. gS9 contains 11.0kb SalI, 4.1kb BamHI and 3.2kb HindIII fragments. In this case hybridisation with female cDNA is barely detectable. Experiments to identify the sequences within these clones which hybridise with female cDNA have yet to be performed. The data presented below suggest that they are independent of male-specific sequences.

The 3.2kb HindIII fragment from gS9 and the 5.5kb HindIII fragment from gS8 were used to isolate cDNA clones from the amplified male L3 cDNA library. The gS9 cDNA fragment isolated was 650bp long and when cloned into the pUC19 vector gave plasmid pCS9. 0.4% of the plated cDNA library plaques were positive in the screen. The gS8 cDNA isolated was 500bp long and was cloned into the pEMBL vector to give pCS8. In this case only 0.2% of the plated plaques were positive. Given the homology between these clones this difference is unexpected but may be a consequence of the library screens being carried out at different times. To investigate the extent of the sequences shared by gS8 and gS9 Southern blots from restriction enzyme digests of the genomic phage clones were probed with the cDNA clones. An example is shown in Figure 6.3a. Blots of gS8 and gS9 DNA were probed with pCS9 and washed at high stringency. For both of the genomic clones the hybridising fragments are the ones identified by the reverse northern experiments. An identical pattern of hybridisation was detected with the pCS8 probe (not shown). Similar experiments allowed the construction of the crude restriction maps shown in Figure 6.4b. Thus I conclude that these non-overlapping lambda clones contain the same or very similar "male-specific" sequences.

To investigate the genomic organisation of these sequences southern blots of male and female DNA were probed with the pCS9 probe (Figure 6.4a). The majority of the hybridisation is restricted to male DNA indicating that the sequences reside on the Y chromosome. In addition it is apparent that several copies of pCS9 sequences are present on

Figure 6.3a: EtBr stained gel restriction digested DNA from Lambda clones gS8 and gS9 (1ug/lane) and a Southern blot probed with pCS9. The blot was washed at high stringency and exposed for 2 hours. Enzymes: E=EcoRI, B=BamHI, H=HindIII, S=SalI. The size markes are in kilobasepairs.

Figure 6.3b: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe used was pCS9, the blots were washed at high stringency and exposed for 3 days. The stripped blots were reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) Embryo, 2) Male L3, 3) Female L3, 4) Male pupal, 5) Female pupal, 6) Male adult, 7) Female adult, 8) XO, 9) XX;tra2/tra2, 10) XY;tra2/tra2, 12) Agametic female (tud), 13) Agametic male (tud). The size is in kilobases.

6·3a
9S+E
9S+H
9S+B
8S+E
8S+H
8S+B
21·2
5·1
4·2
3·5
2·0
1·6
1·0
0·56
9S+E
9S+H
9S+B
8S+E
8S+H
8S+B

6·3b
1
2
3
4
5
6
7
8
9
10
11
12
1·4
ACTIN

the Y. Female DNA also hybridises with the pCS9 probe though in this case the hybridisation is to a major band in each digest. In some blots weaker bands are detected in female DNA, the significance of this is at present unclear. The bands in female DNA are unlikely to result from contamination with male DNA as the intensity of the hybridisation is not consistent with this explanation. From these digests it would appear that the gS9 clone originates from the Y chromosome. The presence of a 0.5kb EcoRI fragment in this clone which is present only in male DNA is consistent with this. The predicted restriction map of gS8 correlates with the major bands detected in female DNA. In particular a 5.5kb HindIII fragment, a 4.9kb EcoRI fragment and a high molecular weight BamHI fragment are detected. Experiments to prove that gS8 is not Y-linked are underway. Blots of male and female DNA from Or, Cs and m56i (lanes 7-12) indicate that this genomic organisation is very similar in the three strains.

The 3.2kb HindIII fragment from the gS9 genomic clone was also used to probe similar genomic southern blots (lanes 13-16). Hybridisation specific to the Y chromosome is still detected however far greater similarity between male and female DNA is apparent. The number of bands detected with the genomic sub-clone is consistent with the presence of moderately repetitive DNA on this genomic fragment. When this experiment is extended using the whole gS9 genomic clone as a probe an even greater number of common sequences are detected (not shown). Again this is consistent with the presence of moderately repetitive DNA in the genomic clone.

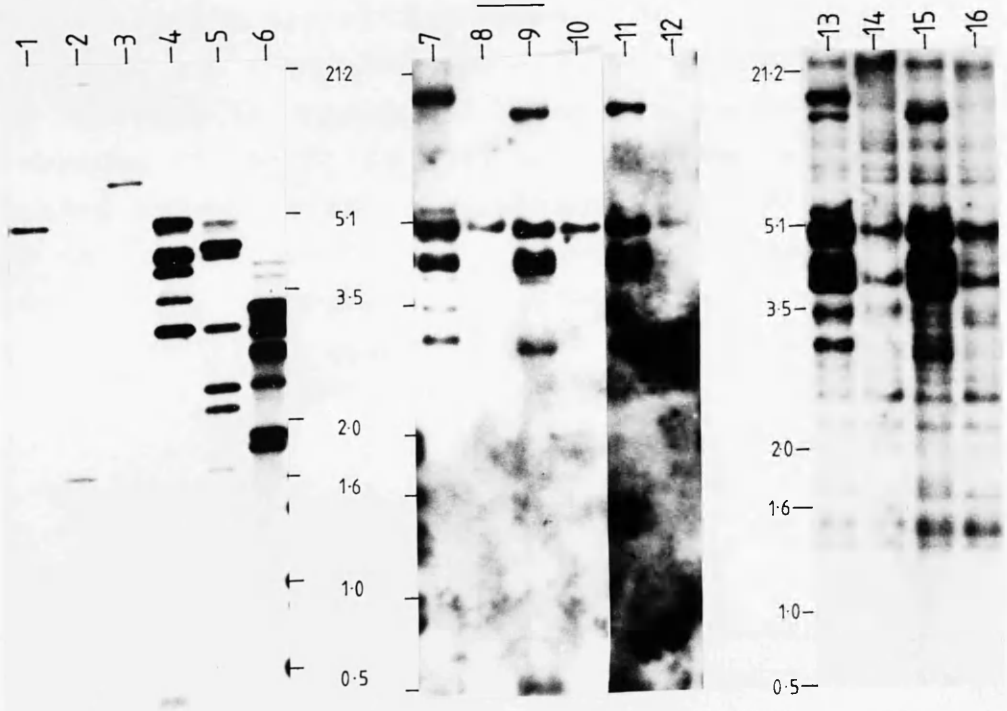
Neither the genomic clones or the cDNA clones give a detectable signal when used as probes to polytene chromosome spreads. Co-hybridisations with control clones indicate that the hybridisation and detection conditions were not unfavourable. The lack of hybridisation to Y chromosome sites is not surprising considering the Y chromosome is underreplicated in salivary glands. In the case of the non Y sequences this suggests that they may be underreplicated or heterochromatic.

Transcriptional analysis using the pCS9 clone (Figure 6.3b) detects a male-specific transcript of approximately 1.4kb. The transcript is present in male L3, while the level appears unusually high on this blot, lower levels of transcript are detected at this stage on other

Figure 6.4a: Southern blot analysis of Drosophila genomic DNA (2ug/lane). Blots were probed with pCS9 (Lanes 1-12) or the 3.2Kb HindIII fragment from gS9 marked on the restriction map below (lanes 13-16). Electrophoresis was carried out in 0.8% agarose gels. Blots were washed at high stringency and exposed for 3 days. Lanes: 1) Female Or;EcoRI, 2) Female Or;BamHI, 3) Female Or;HindIII, 4) Male Or;EcoRI, 5) Male Or;BamHI, 6) Male Or;HindIII.
Lanes 7-16 all EcoRI digests; 7) Male Or, 8) Female Or, 9) Male Cs, 10) Female Cs, 11) Male m56i, 12) Female m56i, 13) Male Or, 14) Female Or, 15) Male Cs, 16) Female Cs. Size markers are in kilobasepairs.

Figure 6.4b: Restriction maps of gS9 and the right hand end of gS8 Lambda clones. The black bar represents male-specific hybridisation identified by reverse northern experiments. The black bar under gS9 also identifies the 3.2Kb HindIII fragment used as a probe. Enzymes E=EcoRI, H=HindIII, B=BamHI, S=SalI.

6·4a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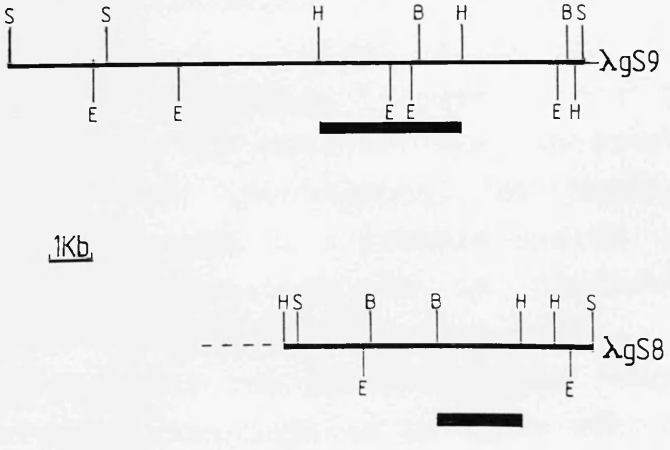
3·5
2·0
1·6
1·0
0·5

6·4b


S
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H
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λgS9
E
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1Kb
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B
H
H
S
λgS8
E
E

blots. A possible explanation for this is that in previous experiments hand-sexed wild-type larvae were employed whereas in this case the material was generated from SD stocks. This will require further characterisation. The transcript is also male-specific in pupae, adults and tra2/tra2 males. There is no expression in females, pseudomales or agametic males. In XO males the level of the transcript appears to be reduced, however it is clearly not abolished (lane 8). This indicates that the non-Y location is capable of being transcribed, at least in the absence of the Y. It does not indicate whether or not the Y linked sequences are transcribed. The transcript is not detected in females carrying a Y chromosome (genotype XXY, not shown). This pattern of expression is again consistent with transcription specific to the male germline.

6.3 DISCUSSION

The structure of the gS4 clone is reminiscent of heterochromatic DNA in that several different repeats are apparent when genomic DNA is probed with this clone. At present we are cloning and mapping the SmaI and ThaI repeats to try and develop a more detailed picture of the structure of this region. We also need to establish whether or not the repeats are the site of transcription for the male-specific transcripts. At present this seems be the most likely possibility. However transcribed sequences at the ends of the lambda clone, or within the 1.8kb SmaI fragment, cannot be excluded. It will also be of considerable interest to determine the nature of the transcripts, in particular whether they perform a structural or a protein coding role. Experiments to address this question are in progress by cloning and sequencing cDNA copies of the transcript.

As one of the major criteria for isolating this sequence was oligo-dT primed reverse transcription it seems reasonable to assume that the transcript is polyadenylated and hence most likely to be protein coding. It should be noted however that heterochromatic DNA is renowned for its high A-T content so that the presence of an A-tract within the transcript cannot be excluded. However the restriction enzymes which cut the repeats detected by gS4 all recognise G-C rich sequences,

so the presence of long stretches of A residues is considered to be less likely.

The expression pattern of the gS4 male-specific transcript argues strongly for germline specific expression. Particularly compelling is the apparent reduction of transcript levels in XO and tra2/tra2 individuals and the absence of expression in agametic males. Interestingly the phenotypes of both XO and tra2 defects on spermatogenesis can be considered as chromosomal phenomena (tra2 mutations have effects resembling defects in X chromosome inactivation). This suggests that the gS4 transcripts are sensitive to perturbations in chromosome behaviour during spermatogenesis. This may be further tested by examining transcript levels in T(X:A) individuals.

In some respects the structure of the gS4 clone is reminiscent of the Stellate locus. It too is composed of tandem repeats and produces a low molecular weight germline specific transcript. However the effect of the Y chromosome on the transcription of gS4 and Ste appears to be completely opposite. Ste expression is elevated in XO males whereas gS4 transcripts appear to be reduced. The significance of the similarity between these two loci cannot be assessed until further information on gS4 is obtained, but one is led to wonder if a repetitive structure will be found to be a more general feature of genes expressed in the male germline.

The localisation of gS4 sequences to the base of 2L is intriguing and it will be important to characterise this further using mitotic metaphase spreads. In particular we need to determine if the sequences are within a heterochromatic or a euchromatic portion of the chromosome. Somewhat fortuitously the euchromatic/heterochromatic boundary in 2L appears to be marked by a constriction in mitotic spreads. In the absence of further data it is tempting to speculate that gS4 may represent E(Sd). This locus maps at the distal end of 2L heterochromatin and appears to produce a trans-acting factor involved in chromosome behaviour during spermatogenesis. However at present E(Sd) has only been associated with aberrant spermatogenesis. It is not clear if E(Sd) has any role in normal spermatogenesis. Recall also that the Rsp locus is heterochromatic and repetitive. A repetitive nature for E(Sd) could explain the apparent lack of EMS induced mutations. I

intend to examine this possibility in collaboration with T.W. Lyttle, who is at present constructing appropriate E(Sd) deletions and rearrangements.

One further aspect of gS4 is of interest, namely the presence of transcripts in the embryo. I intend to investigate this expression in sexed embryos generated from the SD stocks discussed in chapter 2. I also wish to establish by using staged collections when during embryogenesis gS4 is transcribed. It is possible, although unprecedented, that the gene is not transcribed in the embryo but that the transcript is delivered by the sperm !).

The genomic organisation of the gS8/gS9 sequences is of considerable interest. As discussed in the introduction to this chapter other workers have identified Y associated DNA sequences. It would appear that the sequences recognised by the pCS9 cDNA clone are not related to any class of previously described Y associated sequences. The pCS9 sequences are not highly repetitive and furthermore they are transcribed as a discrete, apparently poly-adenylated, transcript. It is also unlikely that pCS9 sequences are related to the Y-associated retrotransposon elements isolated from D.hydei. The conservation of restriction pattern and Y chromosome location between the strains examined suggest that gS9 male-specific transcript sequences do not represent transposable elements, since one would expect to find transposons at a variety of chromosomal locations and at different sites in different strains. A sequence analysis of the cDNA clones is at present under-way to try and determine the nature of the gS9 gene product.

The genomic sequences flanking the gS9 transcription unit are moderately repetitive which appears to be typical of heterochromatic Y chromosome DNA. As to the location of the autosomal homologues I presume at present that they are also heterochromatic. Localisation on mitotic spreads will be required for two reasons; In the first instance to determine the autosomal location and in the second to examine the organisation on the Y chromosome. In particular I wish to determine if the sequences are clustered, perhaps at a particular fertility factor. This question may also be approached by employing the set of Y chromosome deletions generated by Kennison (1983).

The location of gS9 sequences on the Y and autosomal (or

X-linked) sites is again reminiscent of the Ste locus. Digestion of the gS9 genomic clone with restriction enzymes which release the Ste repeats fails to identify such repeats in gS9. Furthermore in contrast to the Ste transcript the gS9 transcript is not elevated in the absence of the Y chromosome.

Investigation of the transcription of gS9 sequences poses some difficult problems. We know that the autosomal locus can be transcribed in the absence of the Y, but whether or not it is expressed in the presence of the Y is rather more difficult to investigate. If the gS8 clone does indeed represent the autosomal location then we hope to find flanking sequences unique to this location. If this is the case we it may be possible to disrupt this location by transposon mutagenesis (see chapter 7). If gS8 does not represent the autosomal location this can be isolated by screening a female genomic library with the cDNA clones. This has already been carried out however the 12 positive clones isolated have not yet been analysed. Even if this is possible we may still learn little about the pattern of transcription. Another approach may be to employ duplications and deficiencies for both Y and autosomal sequences and analyse transcript levels in such individuals. Of course we must remember that it is still not clear whether or not we are dealing with a single autosomal site. Extensive southern blot analysis with calibrated blots will be required to address this question.

One final point of interest will be to examine other members of the melanogaster subgroup as well as other Drosophila species for the presence of Y linked or autosomal copies of gS9 sequences. This may allow evolutionary aspects of Y chromosome structure to be investigated.

CHAPTER 7

A COMPLEX GENE?

7.1 INTRODUCTION

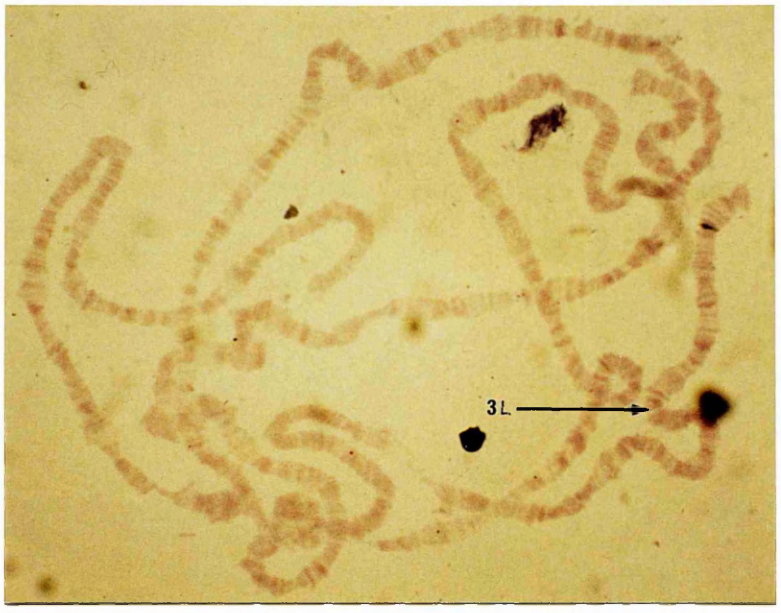
In this chapter I shall describe the analysis of the two remaining genomic clones, gS2 and gK15. These clones encode a number of male-specific transcripts which appear to be expressed in a complex way. Several Drosophila genes with complex transcriptional patterns have been characterised. The majority of these are involved in regulating developmental pathways. As was described in chapter 1 the Sxl and dsx genes produce multiple developmentally regulated transcripts. Other complex genes include Antennapedia, Ultrabithorax, Achaete-Scute and decapentaplegic (For a review of complex genes see Scott, 1987). It has been proposed that complex genes reflect informational economy in the Drosophila genome; by producing multiple products with different functions and complex regulation fewer genes may be required to "build a fly" (Scott, 1987). In this respect the number of genes in the Drosophila genome has been estimated at around 5,000 (by saturation mutagenesis of small regions, see Spradling and Rubin (1981) for a review) to 15,000 (by mRNA complexity measurements, Hough-Evans et al., 1979). Both these estimates seem rather low when one considers the complexity of the fly. Perhaps complex structures will be uncovered for an increasing number of Drosophila genes as the molecular cloning of the genome progresses.

As discussed earlier (Chapter 3) the lambda clones gS2 and gK14 are overlapping, I will present evidence that these genomic clones encode at least three male-specific transcripts and at least one non sex-specific transcript. In addition one of the male-specific transcripts is a good candidate for a gene expressed sex-specifically in the embryo.

7.2 RESULTS

Clones gS2 and gK15: The restriction fragments of gK15 that encode sex-specific transcripts, as identified by reverse northern analysis, are contained within gS2. Hence the majority of my discussion will be restricted to the latter clone.

A



B

Figure 7.1: Photomicrographs of polytene chromosome preparations hybridised with a biotinylated lambda gS2 probe.

a): A whole spread showing hybridisation toward the end of the left arm of chromosome 3.

b): Close up of a different chromosome showing hybridisation at region 61F. Identical signals are observed with the gK15 probe (not shown).

Restriction enzyme digests of both clones are presented in Figure 7.2a. Common EcoRI, BamHI and HindIII fragments are detected. The preliminary restriction map of gS2 shown in Figure 7.2d was deduced by probing Southern blots of restriction digests with various sub-clones (not shown). The reverse northern analysis identified three BamHI restriction fragments (5.8, 1.6 and 0.8kb) which hybridise to male, but not female, cDNA. Two additional BamHI fragments of 0.4 and 0.7kb separate the "male-specific" BamHI fragments. At present it is unclear whether or not the 0.4kb BamHI fragment hybridises with cDNA. This small fragment would have migrated out of the gel in the reverse northern experiments. The 0.7kb fragment from gS2 was present on the gel and does not appear to hybridise with cDNA from either sex. This fragment may represent an intron or an intergenic space. The gK15 genomic clone begins within this 0.7kb fragment. No male-specific hybridisation to the left hand end of the vector was detected in the reverse northern experiments. However in this case the reverse northern experiments were hampered by spurious hybridisation between female cDNA and vector sequences. A more extensive restriction analysis of this region is required to confirm the restriction map. In addition the 0.4 and o.7kb BamHI fragments will be used to probe northern blots to clarify their relationship to the transcripts discussed below.

A 4.2kb BamHI fragment hybridises weakly with cDNA derived from both sexes. At present this fragment has not been investigated but will be used to probe northern blots in future experiments.

The three "male-specific" BamHI restriction fragments were sub-cloned into the pEMBL18 vector and are designated pS2/5.8B, pS2/1.6B and pS2/0.8B respectively. The pS2/5.8B and pS2/1.6B plasmids have been restriction mapped and this is summarised in Figure 7.2d.

Southern blots of EcoRI digested male and female DNA from three D.melanogaster strains were probed with the pS2/5.8B plasmid (Figure 7.2b). Male and female Or and m56i DNAs show identical patterns of bands. This is consistent with the restriction map of the cloned DNA with the exception of a small 200bp EcoRI fragment, which would have migrated out of the gel. This small fragment is present in the pS2/5.8B sub-clone. Cs DNA contains a restriction fragment polymorphism.

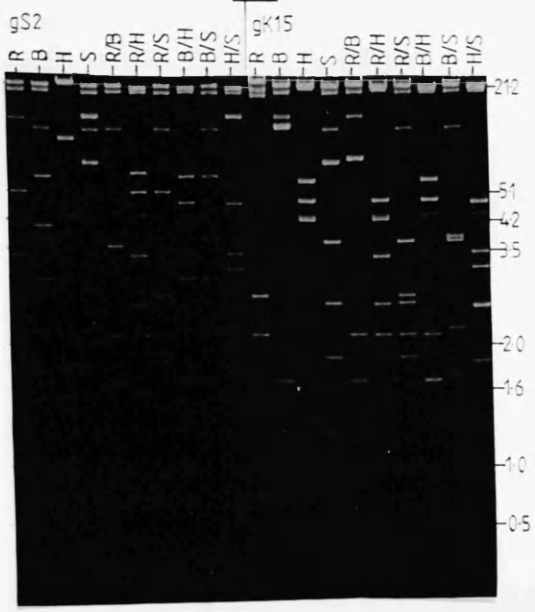
Figure 7.2a: EtBr stained 0.8% agarose gel containing restriction enzyme digests of the overlapping lambda clones gS2 and gK15 (0.5ug/lane). Enzymes R=EcoRI, B=BamHI, H=HindIII, S=SalI. The size markers are in kilobasepairs.

Figure 7.2b: Southern blot of EcoRI digested Drosophila genomic DNA electrophoresed in a 0.8% agarose gel (2ug/lane). The probe was the plasmid pS2/5.8B. The blot was washed at high stringency and exposed for 2 days. Lanes: 1) Male Or, 2) Female Or, 3) Male Cs, 4) Female Cs, 5) Male m56i, 6) Female m56i. Sizes are in kilobasepairs.

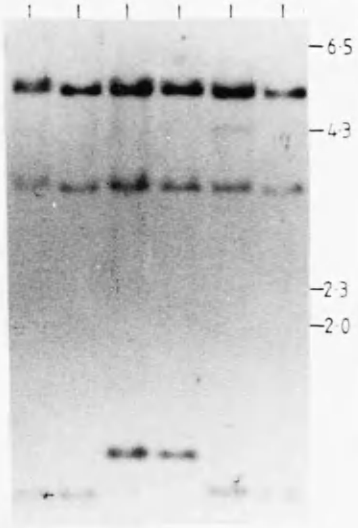
Figure 7.2c: Northern blot analysis with various poly-A+ mRNAs (5ug/lane). The probe used was pS2/5.8B, the blot was washed at high stringency and exposed for 2 weeks. The stripped blot was reprobed with actin probe Act-A2, washed at high stringency and exposed overnight. Lanes: 1) Embryo, 2) [illegible]1, 3) L2, 4) Male L3, 5) Female L3, 6) Male pupal, 7) Female pupal, 8) Male adult, 9) Female adult, 10) XY;tra2/tra2, 11) XX;tra2/tra2, 12) Agametic male (tud), 13) Agametic female (tud), 14) Agametic male (osk), 15) XO male, 16) XXY female. The sizes are in kilobases.

Figure 7.2d: Restriction maps of the lambda clone gS2 and subclones derived from it. Enzymes: B=BamHI, E=EcoRI, H=HindIII, P=PstI, S=SalI, Ss=SstI, Xh=XhoI, Xb=XbaI. The large arrow indicates the overlap with Lambda gK15. The black bars indicate male-specific, and the hatched bar indicates non sex-specific hybridisation detected on reverse northern blots.

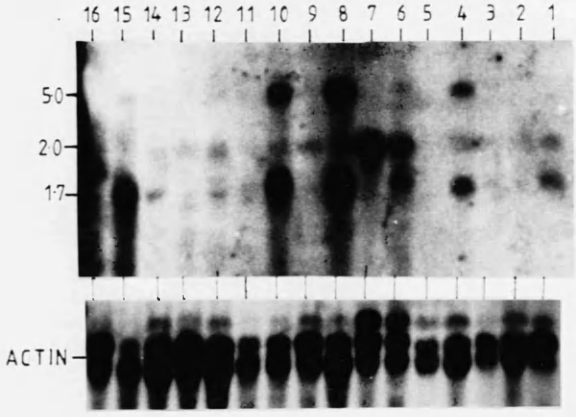
7·2a



7·2b



7·2c



7·2d

In place of the 1.0kb EcoRI fragment it contains a fragment of 1.2kb. This is consistent the absence of the EcoRI site which produces the 200bp fragment in the genomic clone, but has yet to be confirmed on high resolution gels.

When either of the two genomic clones is used to probe salivary gland polytene chromosome preparations, unique hybridisation to region 61F on 3L is detected (Figure 7.1). Interestingly a doublesex cognate has also been mapped to this region (see discussion)

A transcriptional analysis using probes from gS2 subclones has been initiated and a complex pattern of transcripts is detected. Northern blots probed with the pS2/5.8B subclone identify three transcripts of approximately 5.0, 2.0 and 1.7kb (Figure 7.2c). The 2.0kb transcript does not appear to be sex-specific. It is detected in embryos, L3 (it is not yet clear if it is sex-specific at this stage as the female L3 RNA, lane 5, is slightly underloaded), pupae of both sexes, where its level is elevated (lanes 6-7), and adults of both sexes (lanes 8-9). It also appears to be present in agametic and tra2/tra2 individuals of both sexes (lanes 10-14).

The 5.0kb transcript is expressed in male L3, reduced in male pupae and elevated again in male adults. It is not detected in females, pseudomales or agametic males and is reduced in XO males (lane 15). These data, in particular the reduced expression in XO males, suggest that the 5.0kb transcript is germline-specific.

The 1.7kb transcript is expressed in embryos, male L3, male pupae, male adults and weakly, but detectably in agametic males (lanes 12 and 14). It may also be present at low levels in pseudomales (lane 11). It does not appear to be expressed in females at any stage. This pattern is consistent with germline associated, but not germline specific, expression.

Preliminary data from the other subclones (not shown) indicate that the pS2/1.6B probe detects the 5.0kb transcript discussed above and an additional male-specific transcript of 1.2kb. This new transcript appears to be expressed in male L3, pupae and adults. It is not detected in agametic males or pseudomales. This pattern of expression suggests germline specific expression. The pS2/0.8B probe also detects the 5.0kb transcript and the 1.2kb transcript. At present I am

investigating these genomic clones further with a view to establishing the organisation of these transcribed sequences.

7.3 DISCUSSION

The region of the genome represented by these clones appears to be transcriptionally complex. Particular points of interest are: 1) The reduced expression of the 5.0kb transcript in pupae. 2) The elevated levels of the 2.0kb transcript during the pupal stage as well as its expression in embryos. 3) The expression of the 1.7kb male-specific transcript in embryos. 4) The reduced but detectable expression of this transcript in agametic males and possibly pseudomales.

The pattern of expression of the 5.0kb transcript superficially resembles a germline-specific transcript. However reduced expression during the pupal stage would not be expected from a gene transcribed in this tissue. Biological evidence indicates that the number of (transcriptionally active) spermatocytes is elevated in the pupal stage compared with the larval or adult stages (Garcia-Bellido, 1964). This is strongly supported by an examination of other germline-specific transcripts (see chapters 4 and 5). During the pupal stage the first meiotic divisions occur in the male germline. However this would not be expected to result in the reduced expression of a germline-specific transcript. Furthermore the level of the 5.0kb transcript rises again during the adult stage where there are many meiotically active cells. If this transcript is expressed in the male germline its developmental profile is highly unusual. If it is not expressed in the germline we are faced with some puzzling data. The lack of expression in agametic individuals can be explained by germline associated expression but the reduction in expression in XO males is accounted for less readily. Marsh and Wieschaus (1978) have shown, by pole cell transplantation, that the Y chromosome is not required in the male soma for normal spermatogenesis. Hence the absence of the Y chromosome may not be expected to alter the expression of a somatically expressed gene. In this respect however we should recall the observations of

Steinmann-Zwicky et al. (1989) on germline-soma interactions. Although their experiments indicate that haplo-X germ cells will initiate spermatogenesis irrespective of the constitution of the soma they will not successfully complete sperm development unless they are in male soma (XY or XO). This suggests that a germline-soma interaction is required for correct spermatogenesis. The nature of this interaction is obscure at present but probably involves the somatic cells of the gonad. It is possible that a germline autonomous disruption in spermatogenesis (eg. the lack of a Y chromosome or the absence of a germline) could affect the expression of a somatic gene involved in the germline-soma interaction.

It is tempting to speculate that the reduced expression of the 5.0kb transcript is connected with the elevated expression of the non-sex-specific 2.0kb transcript. The expression of the 2.0kb transcript is of some interest. In particular its elevated levels in the embryo as well as in pupae. A closer inspection of the expression in sexed larvae will be required to determine the status of this transcript at this stage. The function of this transcript is obscure at present, it does not appear to be associated with the germline as the levels in agametic and wild-type individuals are similar. If the expression of these two transcripts is linked it could reflect the use of an alternative transcription start site, differential polyadenylation or differential mRNA splicing. Transcript specific probes will be required to investigate this phenomena in northern blot and tissue section in-situ hybridisation experiments. Alternatively the expression of these transcripts may not be connected. Their close proximity on the 5.8kb BamHI fragment may reflect two very close but unrelated genes. This situation has been observed previously: with the tra gene for example (see chapter 1). Another possibility is that the 2.0kb transcript may be expressed from within an intron of the gene for the 5.0kb transcript. This situation has also been observed before: the sgs4 and pig1 genes are located within an intron of the dunce gene (Chen et al.,1987). However the dunce intron in question is approximately 79kb long. I have no evidence for a giant intron in the gene which encodes the 5.0kb transcript. However until the transcriptional start-site is defined this remains a possibility.

The reduced expression of the 1.7kb transcript in agametic males suggests germline associated expression. However in this case the detectable expression in germline deficient individuals indicates that if the gene is expressed in the germline then this is not its only site of expression. A leaky phenotype of the grandchildless mutations employed to generate agametic individuals is not considered likely as other, more abundant, germline transcripts described in this work are not detected in the agametic males (Chapter 4). At present the 1.7kb transcript is a candidate for a gene expressed in the somatic component of the gonad. If this is the case we may reasonably hope to find that the embryonic expression of this transcript is also male-specific. I am investigating this possibility using two approaches: By isolating mRNA from single sex embryos generated from the SD stocks described in chapter 2 and by in-situ hybridisation to tissue sections. An alternative explanation for the reduced expression in agametic males is that the gene is expressed at high levels in the male germline but at lower levels in male somatic tissue. The expression of the 1.7kb transcript in pseudomales requires clarification. The very weak signal detected on the northern blot is insufficient to firmly conclude that the transcript is expressed in these individuals. A more sensitive nuclease protection assay will be required to assess the expression in pseudomales. Expression in pseudomales argues for male somatic expression regulated by the sex determination hierarchy. Reduced expression in these individuals compared with wild-type males suggests expression in an underdeveloped tissue such as the gonad.

If the 1.7kb transcript turns out not to be expressed in pseudomales one possibility is that it represents a dosage compensation function. Dosage compensation in the embryo and in the male germline is not mediated by the msl genes (Cline,1984; Gergen and Wieschaus, 1988; Schupbach, 1985; Bachiller and Sanchez, 1986). However it should be noted there is no evidence at present for dosage compensation in the male germline. If we presume that dosage compensation is active in the germline and that the same mechanism is used to provide appropriate X-linked gene expression in the embryo then the pattern of expression of the 1.7kb transcript may reflect such a function: high levels of expression in the

embryo, low levels during the larval stage where there are only a few germline cells requiring dosage compensation and the msl genes have taken over somatic dosage compensation functions. Finally increasing expression as the germ cell population rises during the pupal and adult stages. Removal of the germline results in greatly reduced transcript levels. Residual levels of transcription may be expected by analogy with the msl genes. Male-specific dosage compensation is not the only function of at least one msl gene. The mle gene product is required in both sexes in a neurological role where it is found to be allelic to the nap mutation (B.Baker pers.comm; M. Kernan, R. Kreber, B. Ganetsky; abstract from 29th Annual Drosophila Research Conference.). Thus we might expect that genes required for embryonic and/or germline dosage compensation have other functions which are not sex-specific. This may also explain why genes involved in embryonic dosage compensation have not been isolated in genetic screens.

At present the relationship between the 1.7kb transcript and the 5.0 and 2.0kb transcripts discussed above is not known. They may be transcribed divergently from within the 5.8kb BamHI fragment or be splicing products of the same primary transcript. In order to investigate the complex transcriptional pattern of this small DNA fragment I am initiating several lines of experimentation. In the first instance the whole region will be sub-cloned and used to generate strand-specific probes to identify the transcripts on northern blots. Transcript specific probes identified in this way will be used to isolate cDNA copies of each transcript from appropriate cDNA libraries (In addition to the cDNA libraries described in this work I have also constructed libraries from embryo and adult body mRNA). Sequence analysis of the cDNAs and the genomic region will help achieve a more detailed picture of the transcriptional organisation. The sequence analysis may also provide some clues as to the function of these transcripts.

The location of a dsx cognate to the same region of the genome is of considerable interest. The dsx cognate sequences were isolated by low stringency hybridisation with a dsx gene probe (Belote et al 1985). The authors report that no sex-specific transcripts were detected when dsx cognate clones were used to probe northern blots but pupal specific

transcripts were detected. I have tried probing Southern blots of Drosophila DNA with the pS2/5.8B clone under the same conditions of stringency used to isolate the dsx cognates. No additional sequences have been detected. However the gS2 clone also contains a restriction fragment which hybridises with cDNA from both sexes and gK15 contains sequences further 3' to this. It will be of some interest to; **a)** Probe blots of gS2 and gK15 with a dsx probe and **b)** Probe Southern blots of Drosophila DNA with other restriction fragments from these two genomic clones. It is possible that the dsx cognate clone isolated by Belote et al. from this region contains only a portion of the gene. The dsx gene itself spans some 30kb of DNA, contains large introns and shows a complex pattern of transcription. At present we know that the 5.0kb transcript from gS2 spans at least 8kb of DNA and that the transcriptional pattern within this region is complex containing at least three other transcripts.

As well as a molecular approach I intend to initiate a mutational analysis of this region. This will be achieved by mobilising transposable elements and detecting insertions at or near the gS2 sequences. By using a novel method developed in our laboratory P-element insertions in cloned genes can be readily detected (Kaiser and Goodwin, in press). Briefly, insertions are detected using the polymerase chain reaction (PCR) with a primer unique to cloned sequences and another specific to the terminal repeats of the P-element. If a P-element inserts at or near the gS2 locus this can easily be detected by the appearance of a novel PCR amplification product. Such is the sensitivity of the PCR reaction that one individual carrying the insertion can be detected in a population of several thousand flies which do not. Furthermore if the crosses are arranged such that a female is carrying the insertion in a heterozygous form a sib-selection scheme can be employed. With this method DNA is prepared from the embryos produced by a large population of mutagenised females (one of which hopefully carries an insertion in the gene of interest). The PCR reaction will detect the presence of desired insertions in this population. The females may then be subdivided into smaller populations and the process repeated. After a few rounds of selection the population will be reduced to one or a few females carrying the insertion. Progeny from these females may then be thoroughly examined

for phenotypic and molecular disruptions. Such a method of generating mutations circumvents many of the uncertainties of conventional genetic analyses. In particular the sib-selection procedure is carried out using heterozygous females. Thus insertions may be obtained without biasing for phenotype. In addition this scheme also allows the recovery of silent insertions and insertions with very weak phenotypic effects which may go undetected in conventional mutagenesis strategies. Undoubtedly this method will have wide applications in the mutagenesis of cloned genes and hopefully will provide phenotypic information on the function of some of the genes described in this work.

CONCLUDING REMARKS

I initiated this project in order to isolate genes expressed only in one sex. In particular I am interested in sexually dimorphic gene expression in the embryo. This is primarily because the Drosophila embryo is a good source of reliable nuclear extracts for transcription, DNA binding and splicing studies (Biggen and Tjian, 1988; Rio, 1988). The availability of nuclear extracts may permit the identification and characterisation of regulatory molecules which act at specific promoter elements. In addition regulatory proteins, once identified in this way, can be isolated from cDNA expression libraries by virtue of their ability to recognise specific DNA sequences (Vinson et al., 1988). A second reason for wishing to identify genes expressed sex-specifically in the embryo is to investigate the mechanisms which regulate germline sex. As I described in chapter 1 very little is known about germline sex determination. The isolation and characterisation of genes expressed early in the development of the germline may allow an investigation of this problem using the methods outlined above.

Whether or not the aim of the project has been successfully realised remains to be determined. Twenty lambda clones were isolated which appear to encode male-specific or male-elevated transcripts. Ten of these have been investigated in some detail. Two genomic clones, gS2 and gS4, encode transcripts expressed in embryos. These transcripts are male-specific in the larval, pupal and adult stages. At present the expression in sexed embryos has not been determined. If they are sex-specific in the embryo then I am in a position to initiate the lines of investigation outlined above. The clone gS2 discussed in chapter 7 is particularly interesting in view of the complex transcriptional pattern observed on northern blots. In addition one of the transcripts is detected in agametic males, indicating somatic expression. The data suggests that the expression of this transcript is germline associated. It is this clone which I intend to concentrate my efforts on at present. The lines of experimentation I intend to follow were discussed in chapter 7.

Some of the other genes which I characterised have interesting features which merit further investigation. The Y associated sequences detected by gS8 and gS9 appear to be unusual. No other Y chromosome DNA with this particular organisation has been reported: Multiple copies on the Y chromosome and probably a single copy elsewhere in the genome. It should be noted that low stringency hybridisations with the pCS9 cDNA probe does not detect additional sequences in female DNA (not shown). This suggests that these sequences do not represent diverged remnants of mobile genetic elements. The expression of the male-specific transcript appears to be restricted to the male germline. No expression is detected in agametic males or females (even when the females have a Y chromosome). In addition the expression in males is not dependent on the Y chromosome, although the levels of the transcript appear reduced in XO males. Experiments are at present underway in our laboratory to map the structure of these two genomic clones more carefully. It will also be important to try and identify the genomic location of these sequences by hybridisation to mitotic chromosomes.

The repetitive structure of the gS4 clone is also unusual. As discussed in chapter 6 it would appear that this genomic clone consists of heterochromatic-like DNA. This is suggested by a number of different repeat-like structures recognised by different enzymes and by the localisation of these sequences near the chromocenter. At present it is not clear how the repeats are organised. No simple structure which accommodates the 1.3kb SmaI and 0.5kb ThaI repeats springs to mind. This clone is being dissected and mapped in our laboratory at present.

The isolation of the gS4 and gS9 clones, which appear to contain repetitive DNA, justifies the decision to use a primary genomic library for these screens. An amplified library may well have been depleted of repetitive DNA due to recombination during propagation in E.coli. In this respect a recD E.coli strain (nm621) was used throughout this work to propagate recombinant phage. The recD mutants appear to be more tolerant of repetitive DNA that standard recA strains (Whittaker at al., 1988).

Of the remaining genes gK14, gK21, gS10 and gK33 appear to be genes transcribed in the male germline. There is good,

although not conclusive, evidence that gK14 and gK21 represent previously characterised male germline-specific genes. All four of the above clones identify transcripts with very similar patterns of expression: weak in male L3, very strong in male pupae and lower in male adults. This is a pattern of expression which fits well with the counts of spermatocyte numbers during development (Garcia-Bellido, 1965). In addition these transcripts are not detected in agametic males or chromosomal females transformed to somatic maleness by the tra2 mutation. These data strongly support the belief that the genes are only expressed in the male germline.

The expression of the male-specific transcript detected by the gS1 clone is unusual. Although I included this gene in the "putative male germline" section (chapter 5) there are suggestions that it may not be expressed in this tissue. In particular the weak expression during the pupal stage as compared to the adult stage. The increasing expression from L3 to adults and the apparent lack of expression in agametic males leads me to suspect that this gene may be expressed in the gonadal soma. If this is the case then this gene may be the best candidate for a gene expressed sex-specifically in the embryo. Indeed if we consider that the male gonad in the embryo contains fewer than a hundred cells (Sonnenblick, 1950) we may not expect to detect a gene expressed in this tissue by northern blotting. This suggests to me that the embryonic expression observed for the gS2 and gs4 transcripts may not be sex-specific, as they are germline associated during later stages in development.

In general the +/- screening technique used in this work has been successful in isolating genes expressed sex-specifically. Most, although not all, of the genes described in this work appear to be expressed at fairly low levels during the larval stage (the stage used to prepare the cDNA probes). In order to isolate genes expressed at a much lower abundance, as may be expected of the transcripts for regulatory proteins, I am considering initiating a new screen. I intend to use the SD strains (chapter 2) to produce sexed embryo populations and generate subtracted probes to screen genomic libraries. In this way I hope to identify regulatory molecules expressed male-specifically in the early embryo. The reason for screening for male-specific genes is

that it is far easier to transform a female into a male, using somatic sex determination mutations, than perform the converse male to female transformation. The hope is that by characterising sex-specific molecules, in particular the way in which they interact with the known sex determination genes, a more thorough understanding of the control of sex-specific gene expression may be achieved.

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