IDENTIFICATION AND CHARACTERIZATION OF A HIGHLY CONSERVED GENE, WHICH IS PREDOMINANTLY EXPRESSED IN THE HUMAN TESTIS, IN SILKWORM *Bombyx mori*

THESIS

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Dedicated to
my Parents

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STATEMENT

I hereby state that the work presented in this thesis entitled **"Identification and characterization of a highly conserved gene, which is predominantly expressed in human testis, in Silkworm *Bombyx mori',*** has been carried out by me under the supervision of Dr. **Lalji Singh** and that this has not been submitted for any degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that Ms. Anju Singh has carried out the research embodied in the present thesis under my Supervision and guidance for the full period prescribed under the Ph.D. ordinance of the University. I recommend her thesis entitled "Identification and characterization of a highly conserved gene, which is predominantly expressed in human testis, in silkworm *Bombyx mori*' for submission for the degree of Doctor of Philosophy of this University. This work has not been submitted for any degree or diploma of any other University or Institution.

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ABBREVIATIONS

aa	Amino acid
A	Adenine
AMV	Avian myeloblastosis virus
AMH	Anti mullerian hormone
ATP	Adenosine 5'-triphosphate
Bkm	Banded krait minor satellite
bp	Base-pairs
Bis-acrylamide	N, N'-Methylene-bis-acrylamide
BLAST	Basic local alignment search tool
BSA	Bovine Serum Albumin
C	Cytosine
CIP	Calf intestinal phosphatase
cDNA	Complementary DNA to RNA
cm	Centimetre
CO_2	Carbon dioxide
Ci	Curie
CSD	Chromosomal sex determination
cpm	Counts per minute
cps	Counts per second
°C	Degree centigrade
da	*daughterless*
DAPI	4'6-diamidino-2-phenyl indole
DDW	Double distilled water
d.p.c.	days post coitum
DEPC	Diethyl pyrocarbonate
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic acid
dl	decilitre
dATP	2'-deoxyadenosine 5'triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
ddNTP	2^1, 3'-dideoxynucleotide 5'-triphosphate
dNTP	2'-deoxynucleotide 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DTT	Dithiothreitol
dsx	*doublesex*

dsRNA	-	double stranded RNA
dsRBM	-	double stranded RNA binding motif
EDTA	-	Ethylene di amine tetra acetic acid
eng	-	*engrailed*
ES cells	-	embryonic stem cells
EST	-	expressed sequence tag
FACS	-	Fluorescence activated cell sorter
fern	-	*feminization*
Fig	-	figure
FISH	-	Fluorescence *in situ* hybridization
fog	-	feminization of germline
fru	-	*fruitless*
G	-	Guanine
g	-	gram
GR	-	Genital Ridge
^3H	-	Tritium
h	-	hour
HEPES	-	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid
her	-	*hermaphrodite*
Hg	-	Mercury
HMG	-	high mobility group
hox	-	*homeobox*
IPTG	-	β-D-isopropyl-thio galacto pyranoside
IU	-	International units
ix	-	*intersex*
Kb	-	kilobase pairs
lb	-	pound
l	-	litre
LB medium	-	Luria-Bertani medium
M	-	mole
MEM		Minimum essential medium
ml	-	millilitre
µl	-	microlitre
mg	-	milligram
µg	-	microgram
µCi	-	microcurie
min	-	minutes

mm	-	millimetre
mM	-	millimole
μM	-	micromole
mRNA	-	messenger RNA
MOPS	-	3-[N-Morpholino] propanesulfonic acid
MW	-	molecular weight
NaOH	-	Sodium hydroxide
NCBI	-	National Centre for Biotechnology Information
nm	-	nanometre
ng	-	nanogram
NGF	-	nerve growth factor
nM	-	nanomole
OAT	-	Ornithine delta amino transferase
OD	-	Optical density
ORF	-	Open Reading Frame
^{32}P	-	Phosphorus-32
PCR	-	Polymerase Chain Reaction
PGC	-	Primordial germ cells
PGK		Phosphoglycerate kinase
PI	-	Propidium Iodide
PPO	-	2, 5-Diphenyl oxazole
POPOP	-	1, 4-Di-2-(5-phenyl oxazolyl)- benzene
PHA	-	Phytohaemagglutinin
PEG	-	Polyethylene glycol
Pfu	-	Plaque forming unit
pg	-	picogram
rDNA	-	ribosomal DNA
RNase	-	Ribonuclease
RNA	-	Ribonucleic acid
rpm	-	rotations per minute
RT	-	Reverse transcription
SCF	-	Stem cell factor
sdc		sex and dosage compensation
sec	-	seconds
SDS	-	Sodium dodecyl sulphate
SET		Saline-EDTA-Tris
SF-1	-	*Steroidogenic factor-1*
sis	-	*sisterless*

Sry	-	Gene in sex determining region of the Y-chromosome
SSC	-	Sodium-saline-citrate
STS	-	Steroid sulfatase
sq. cm.	-	Square centimetre
Sxr	-	Sex reversed
Sxr^b	-	Smallest sex determining region of the mouse-Y chromosome
Sxl	-	*Sex-lethal*
T	-	Thymine
Tas	-	T-complex locus
TAE	-	Tris-acetate-EDTA
TCA	-	Trichloroacetic acid
Tda		Testis determining gene on autosome
TDF	-	Testis Determining Factor
Tdy		Testis determining factor on the Y-chromosome
Tfm	-	Testicular feminization
TE	-	Tris-EDTA
TEMED	-	N, N, N', N'-Tetramethyl ethylenediamine
TLC	-	Thin layer chromatography
tra	-	*transformer*
Tris	-	Tris (hydroxy methyl) aminomethane
TPE	-	Tris-phosphate-EDTA
TSPY		spermatogenesis gene on the Y-chromosome
UTR	-	Untranslated region
UV	-	Ultraviolet
V/cm	-	volts/centimeter
v/v	-	volume/volume
W		dominant white spotting locus
W^e	-	allele of W locus
WT-1	-	*Wilm's tumor - 1*
w/v	-	weight/volume
X-gal	-	5-Bromo-4-Chloro-3-Indolyl-P-D-Galactoside
xol	-	XO lethal
Zfy	-	Zinc finger gene on Y-chromosome

ABSTRACT

Nature has evolved an astonishing variety of genetic and epigenetic sex-determining systems which all achieve the same result, the generation of two sexes. The mystery of two sexes, how they arise and how the sexual dimorphism is produced, has puzzled scientists and laymen alike for centuries. The fascinating insights that we have today are mainly derived from four groups of organisms in which the analysis has preceded down to the molecular level. These are the fruitfly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans,* the mammals like man, mouse and the yeast *Saccharomyces cerevisiae.*

The banded krait minor satellite *(Bkm)* sequences originally isolated from female-banded krait are 'W specific repeats and a conserved component of eukaryotic genome. *Bkm* sequences show a tendency to be concentrated on the sex chromosomes and for that they are thought to be involved in the sex determining process or in the allocyclic behaviour and evolution of sex chromosomes. For example, in mouse, *Bkm* is associated with the sex determining 'Sxr" region of the Y-chromosome whose translocation to the X-chromosome during meiosis leads to the male sex reversal and in snakes and birds they are clustered on W-chromosome. Sequencing of *Bkm* positive clones from snake, mouse, human and Drosophila revealed that the tetranucleotide repeats 'GATA' are the major conserved component of *Bkm.* Expecting a similar association between *Bkm* and sex chromosomes in silkworm, a silkworm genomic library was constructed in *X*- DASH vector and screened with *Bkm.* Twenty five positive clones were obtained of which three clones designated as $A_{1.2}$, C1.2 and $C_{2.1}$ were selected at random for further characterization.

After detailed restriction analysis of all the three genomic clones, the smallest size *Bkm* positive fragment of the genomic clone was subcloned into Bluescript plasmid vector and sequenced. All the three genomic clones were found to contain GATA repeats. However it was imperfect repeat having the interspersion of GAA in between GATA stretches i.e. GATA $[GAA(GATA)_3]_3$

GATA loci were flanked by AT-rich SAR (Scaffold Attachment Regions) sequences.

Homology search with the 1769bp *Bm(GATA)$_n$* nucleotide sequence against various genomic databases revealed a high degree of homology between *Bombyx mori* GATA repeats and GATA sequences reported from Humans, Mouse, *Drosophila,* etc. Homology search in silkworm database showed only 3-4 hits and all of them show homology only with a small stretch of sequence flanking the GATA repeats suggesting that GATA repeats have not been reported in silkworm earlier.

Southern hybridization of *Bm(GATA)$_n$* with silkworm male and female genomic DNA digested with different restriction enzymes showed an identical pattern of hybridization in both the sexes with no apparent polymorphism between the sexes or individuals. Evidently, GATA sequences seem to be distributed throughout the silkworm genome without any preferential concentration on W-sex chromosome.

In an alternative approach *Pϕ2*, a *Bkm* positive highly conserved gene located on human X-chromosome region Xp11.23 was used as a probe to identify and characterize its homologue from silkworm. *Pϕ2* is expressed predominantly in the testis of mouse, rat, chicken and snake irrespective of their male or female heterogamety. The protein coded by *Pϕ2* belongs to WD-family of proteins. Presence of *Pϕ2* gene on 'P' arm of X-chromosome and its abundant transcriptional activity in embryonic testis of mouse suggests that it may be involved in complex pathway of sex determination and differentiation.

Silkworm genomic library was screened with human *Pϕ2* cDNA clone and the three positive clones were designated as 4.3.1, 7.2.1 and 19.1.1. The strongest *Pϕ2* positive silkworm genomic clone 4.3.1 selected for further characterization was found to have insert of 16kb. The 11.2kb and 3.2kb stretches of the 16kb genomic fragment have been completely sequenced. *BmPϕ2* appears to be transcribed maximally in the embryonic stages and 1st instar larva. The expression of *BmPϕ2* starts around 5-6th day of embryonic

development when the gonadal ridge is formed and it starts declining with age and reaches a zero level in the mature fifth instar larva. Fluorescence *in situ* hybridization revealed that the expression was restricted to gonadal ridge and nerve ganglions in embryos and in 1st instar larva. The transcripts were predominantly localized in the testis along with low levels of transcripts in nerve ganglions and ovary. The expression of *BmPϕ2* was found more than two-fold higher in testis compared to that of in ovary. Fluorescence *in situ* hybridization on silkworm metaphase chromosomes and interphase nuclei using *BmPϕ2* as probe, showed that *BmPϕ2* is located on the Z-chromosome.

Detailed *in silico* analysis of the genomic clone revealed that *BmPϕ2* gene contains 6 putative exons of the size of 87bp, 248bp, 149bp, 252bp, 129bp and 145bp respectively. However 3' end of the gene is not complete and there are exons present further downstream of the 6th exon. Homology search in the human genome database for X and Y chromosome with putative *BmPϕ2* cDNA revealed that *BmPϕ2* shows maximum homology to the region Xp11.23 where human *Pϕ2* is located. Homology search in the EST database of D. *melanogaster* identified two ESTs, GM05201 and LD11521 belonging to the same gene CG1800. A similar gene has been identified in *C. elegans.*

The putative *BmPϕ2* cDNA contains an open reading frame of 146 amino acids, which starts in the third exon and continues till the end suggesting that 3' end of cDNA is not complete. The putative protein coded by *BmPϕ2* shows 65-70% homology with the protein predicted from CG1800 gene of *Drosophila.* CG1800 is located on chromosome 3R of *Drosophila* and is a RNA binding protein.

In other words, localization of *BmPϕ2* on homomorphic sex chromosome 'Z', gonad specific and sexually dimorphic pattern of expression during embryogenesis and later on in the development, and homology to RNA binding protein from *D. melanogaster* and C. *elegans* suggests that *BmPϕ2* probably plays an important role in sex determination, differentiation and spermatogenesis.

Chapter I

Introduction

Silkworm has been the source of silk industry since the dawn of human civilization. The earliest silk textile is nearly five thousand years old (Kuhn, 1988). In modern times, *Bombyx* has been used as a model for genetic studies since the birth of genetics as a formal Science in the early 1900's. As early as in 1905, Toyama, one of the founders of silkworm genetics, was breeding genetic hybrids between Thai and Japanese silkworms for improved vigor and silk production (Yokoyama, 1968). He first reported discovery of a chorion mutation that affects the shape and transparency of the eggshell in 1910 (Tazima, 1964), the same year as the publication of Morgan's famous white-eyed mutant of *Drosophila melanogaster*. Although studies with many lepidoptera have made important contributions to genetics, today with more than two hundred mutations mapped, the silkworm stands as the only member of this taxonomic group whose genetic system is established well enough to consider adopting it as a molecular genetic model for solving a broad range of fundamental biological problems.

Investigations to understand embryonic development of the organs like testis, ovary, liver, heart, kidney have been active areas of research using model organisms such as *Drosophila melanogaster, Caenorhabditis elegans* and mouse. One such area has been molecular basis of sex-determination. There are diverse sex-determining mechanisms present in different animal taxa. This diversity demands evolutionary reason for its occurrence and it is in this respect that the study of sex determination in silkworm, *Bombyx mori*, assumes significance as it has not been investigated from evolutionary and developmental point of view in as much detail as it has been studied in *Drosophila* (Slee and Bownes, 1990), the nematode (Hodgkin, 1990; Villeneune and Meyer, 1990), and the mouse (Singh and Jones, 1984; Eicher and Washburn, 1986; Gubbay *et al.*, 1990). A comparison of sex-determining mechanisms in these model systems raises important questions about convergent evolution and, the extent to which the developmental processes are evolutionarily conserved.

It is now possible to isolate specific genes controlling organ development and differentiation, study their structure in detail, identify and compare the time and site of their expression in the embryo and predict the final product. This was the approach that led to the discovery of the existence of homeotic genes (or *Horn* genes) in *Drosophila* (Ruddel, 1994; Carvoll, 1995). The *Horn* genes are known to act as master switches for organ specificity during embryogenesis in *Drosophila.* When studies were extended to other animals like frog, mouse and human, they were also found to have homeotic genes of *Drosophila* type that operated in the similar way. These studies reveal basic similarity in genetic planning of embryogenesis in diverse group of animals. This also suggests that homeotic genes are evolutionarily conserved and function in taxonomically diverse groups of animal systems (Akam, 1989; Quiring *et al.*, 1994). Genetic mechanisms of sex determination and differentiation, therefore needs an in depth study to understand whether they too have evolved from a common genetic program.

Sex-Determination

Molecular tools in form of recombinant DNA techniques are being used to discover the similarity and differences in genes causing heteromorphic sex differentiation in various animals. There are predominantly, three kinds of chromosomal sex determining mechanisms. In one, males are heterogametic, like mammals, in the other females are heterogametic like the lepidopteran insects, snakes and birds (Tazima, 1964; Traut and Mosbacher, 1968; Robinson, 1971; Singh, 1972; Bull, 1983; Strunnikor, 1983; Jones and Singh, 1984) and in the third kind, males are heterogametic sex but sex is determined based on the ratio of X-chromosomes and autosomes like *Drosophila* and nematodes. There is yet another mechanism of sex-determination found in turtles, alligators and muggers, where temperature controls the development of a particular sex. For example, turtles develop into females at warm temperature and into males at cool temperature. Alligators and muggers on the other hand develop into females at cool temperatures and males at warm temperatures (Bull, 1980; Deeming and Ferguson, 1988). In the insect, *Apis mellifera,* the sex is chromosomally determined but in a slightly different way: diploid individuals

become female and haploid individuals develop into males (Bull, 1983; Hodgkin, 1992). These examples, thus, indicate operation of different sex-determining mechanisms in the development of heterogametic male and female phenotypes.

Function and Consequences of Sex

Sex is a morphological expression of being a male or a female and sexual structures like male and female gametes of complementary nature participate in production of a diploid embryo leading to the development and organization of a diploid male or female individual. These individuals in their subsequent sexual reproduction generate haploid gametes by meiotic cell division. During meiosis, homologous chromosome pairs recombine leading to the production of gametes with parental and recombinant genetic constitution. Thus, the function of sex and sexual reproduction constitutes a genetic mechanism for producing genetically variable gametes and, in turn, resulting in genetically variable offspring required for successful survival under varying natural conditions.

Studies on the mating behavior in birds, mammals and other animals suggests the involvement of competition over mates for sexual reproduction. The concept of sexual selection based on competition over mates suggests that both males and females enter into competition in choosing appropriate partners for the act of mating and sexual reproduction (Andersson, 1994; Petrie, 1994; Hasselquist *et al.*, 1996). Since, it is known that the 'Y' is the male determining chromosome in mammals and W the female determining chromosome in lepidoptera, birds and snakes, selection pressure will favour localization of genes on the respective sex determining chromosomes for successful mating and reproduction (Hastings, 1994). Recent studies on genetic information located on Y-chromosome clearly shows that it contains genes for sperm production, body size and tooth development, the traits assumed to be important in male-male contest for female selection (Roldan and Gomendio, 1999). Accordingly, sexual selection might be the main driving force for sex chromosome heteromorphism in heterogametic systems.

Another most pertinent question in this context would be the revelation and understanding of the evolution of molecular mechanisms favoured by sexual selection pressure that function in the development of heteromorphic sex chromosomes.

Sex-Determination and Dosage Compensation in Heterogametic Systems

Bombyx mori exhibits chromosomal sex-determination with heterogametic females and homogametic males. The heteromorphic chromosomes are designated as W and Z. In contrast to *B. mori,* other species like *C. elegans, D. melanogaster*, *M. domestica* and *Homo sapiens,* which also exhibit chromosomal sex determination, have male heterogamety with heteromorphic sex chromosomes designated as the X and Y.

W-chromosome exhibits developmentally regulated partial or complete heterochromatinization depending upon the nature in Lepidopteran species. They share this property with snakes (Ray-Chaudhuri *et al*., 1971; Singh, 1972), birds (Stefos and Arrighi, 1971) and with the Y-chromosome of different animal groups (John, 1988). It is important to mention here that Z-linked genes in ZZ/ZW are not dosage compensated (Cock, 1964; Johnson and Turner, 1979; Stevens 1997; Lucchesi, 1998). The reason for such fundamental difference in the dosage compensation system of XX/XY and ZZ/ZW system is not clear.

The standard strains of *M.domestica* are heterogametic males with dominant male determiner 'M' located on the Y-chromosome which functions to produce maleness by repressing the activity of female determining gene 'F'. Accordingly, the male determiner is epistatic to female determiner in this system (Schmidt *et al*, 1997a, 1997b).

Studies on this aspect in the three model organisms i.e., *Drosophila, C. elegans,* and humans have shown simultaneous operation of genetic systems controlling both, sex heteromorphism and dosage compensation. In *Drosophila,*

XX individuals are female and XY are male. Y-chromosome of the male does not function in determination of maleness but is mainly required for functional sperm production as it carries fertility genes (Sternwann-Zwicy, 1992). In *C. elegans,* XX individuals are female in their soma and both male and female in their germ line. 'XO' individuals remain male both in somatic cells and germ cells. In these two systems the ratio of number of the X-chromosomes to the number of sets of autosomes generates the primary signal for development of maleness or femaleness (Hodgkin, 1990). In mammalian system, the genes for maleness are located on Y. The 'XX', 'XO' and 'XY' systems of sex-determination result in inequality of dosage of genes present on the X, as there are two copies of such genes in XX individuals (female) and one copy in XY individuals (male). In spite of such differences in the dosage, the level of product of almost all X-linked genes is found equal in the two sexes. This is achieved by dosage compensation due to silencing of one of the two X-chromosomes.

There are three basic dosage compensation regulatory strategies. In mammals, inactivation of one X-chromosome in females is the mechanism for equalizing the products of X-linked genes in female individuals (Lyon, 1974). In *D. melanogaster* and *C. elegans,* X:A ratio is responsible for dosage compensation. The *Sxl* is the master control gene, involved in determination of sexual phenotype and dosage compensation in *Drosophila* while *Xol1* gene in association with *Sdc1* and *Sdc2* genes determine sexual phenotype and dosage compensation in *C. elegans* (Villeneuve and Meyer, 1987; Miller *et al*., 1988; Nusbaum and Meyer, 1989).

Mechanisms of Heteromorphic Sex Chromosome Evolution

First step in chromosomal sex determination and evolution of heteromorphic sex chromosome probably involved acquisition of male (M) and female (F) determining gene(s) at the sex specific locus of the otherwise homomorphic sex chromosome pair. In other words, heteromorphic chromosomes evolved from a pair of homomorphic chromosomes with allelic difference of a single gene at the sex-determining locus (Ohno, 1967; Bull, 1983; Jablonka and

Lamb, 1990). This contrasts with the current status of the mammalian sex-determinaton genetics in which genes causing maleness on *Y* and femaleness on 'X' are not the alleles of a single gene (Jimnez and Burgos, 1998). Heteromorphic changes in the organization of homologous chromosomes around the sex-determining locus in heterogametic sex is proposed to have been the primary cause of their non-homology in the sex-determining region. The development of such sex-locus-specific non-homology in otherwise homologous sex-chromosome pair prevents genetic recombination between their non-homologous regions. Thus, acquisition of sex determining function and suppression of recombination in the sex determining non-homologous region are the two significant biological reasons for the sex chromosomes to undergo differentiation during evolution (Bull, 1983; Charlesworth *et al.,* 1986; Jablonka and Lamb, 1988; Steinemann 1993), either by Mullers ratchet, sex specific acquisition of *Bkm* sequences (Singh *et al.,* 1976) or by genetic hitchhiking (Rice, 1987).

Two basic evolutionary pathways termed conformational and structural have been proposed to be the cause of origin and development of sex chromosome heteromorphism. According to conformational pathway, a change in chromatin conformation in the region of heterogametic loci is assumed to be the initial evolutionary reason for sex chromosome heteromorphism. The structural pathway proposes that structural change such as an inversion or a translocation involving originally homomorphic sex chromosomes was the initial event in sex chromosome heteromorphism (Haaf and Schmid, 1989). Singh ef *al.,* have proposed an alternative model for rapid evolution of heteromorphic sex chromosomes in snakes, which entails the involvement of a transposon like element to be the primary cause of conformational heteromorphism leading to isolation and degeneration of heteromorphic sex chromosomes (Singh *et al.,* 1976).

Developments in molecular methodologies have provided impeccable molecular tools to study the physical organization of genes and a class of repetitive sequences called satellite DNA in order to understand the role of these sequences in regulating the structural and functional integrity of the

genes. During the past 25 years, Singh and his group have carried out extensive study in a variety of animal system using *Bkm* satellite DNA as a probe to find out the role of such elements in sex determination in heterogametic animals. Cumulative evidence resulting from these studies clearly indicates a definite and decisive role for this satellite DNA in assigning heteromorphic status to W and the Y- chromosomes.

Clues from the Silkworm

Silkworm shows female heterogamety *i.e.,* ZZ/ZW system. The main aim of present study was to find out whether *Bkm* like sequences have played any role in the origin and evolution of W chromosome in the silkworm. For this purpose, *Bkm 2(8),* a DNA marker developed and characterized as a reliable genetic probe by Singh and his group (1995), was used to screen the silkworm genome to identify, isolate and characterize the related sequences on its W-chromosome. This was decided in the light of following findings:

1. *Bkm* sequences are highly conserved (Singh *et al.,* 1981; Singh and Jones, 1982).

2. *Bkm* is preferentially associated with the sex chromosomes of *Drosophila* (Singh *et al.,* 1981) and Snakes (Singh *et al.,* 1979, 1980, 1981; Singh and Jones, 1982; Jones and Singh, 1984).

3. *Bkm* is predominantly associated with the *Sxr* region of the mouse Y-chromosome, which is necessary and sufficient to convert a female into a male mouse (Epplen *et al.,* 1982; Singh and Jones, 1982; Singh *et al.,* 1984, 1994).

4. *Bkm* sequences $(GATA)_n$ are predominantly located along the length of the snake W-chromosome which remains highly condensed in all somatic cells but undergoes extensive decondensation in the germ cells in response to sex and tissue specific Bkm-binding proteins (BBP).

Though the highly conserved component of *Bkm* sequences is a tetra nucleotide repeat GATA, no other simple repeat is as consistently associated with the sex chromosome as are the *Bkm* repeats. Therefore, it was considered appropriate to screen silkworm genomic library with *Bkm* for isolating sex-specific (or W-chromosome specific) *Bkm* sequences in this system. This molecular approach demonstrated genomic distribution of $(GATA)_n$ with no preferential localisation on autosomes or on sex-chromosomes in *B. mori*. In an alternative approach, we also performed experiments to identify novel sex chromosome specific genes whose expression was restricted to the gonads.

There has been no study to identify and localize sex specific genes on sex chromosomes of *B. mori* or any other lepidopteran insect. The reasons could be many, but one of them appears to be the failure of mutational approach through classical genetic methods. Modern DNA techniques, however, allow alternative approaches to this problem, and one of these would be to use sex specific heterologous gene probes from other organisms like *Drosophila,* mouse and human and identify their counterparts in the silkworm genome.

Singh and his group have also developed a testis specific probe, *P<p2,* from human testis cDNA library. This gene is highly conserved and is predominantly expressed in the testis. It is localized on the X-chromosome region Xp11.23. In this study, *Pφ2* has also been utilized to isolate its homologue in the silkworm and characterize the gene. Present study describes identification, isolation, and molecular characterization of a gene, which is Z-chromosome specific in silkworm and may be involved in the complex pathway of sex-determination, differentiation and spermatogenesis.

chapter II

Ontogeny of the Silkworm

The mulberry silkworm, *B. mori* has been the source of silk since time immemorial. Thus, its cultivation and domestication too dates back to that period. Developmental stages of silkworm include fertilization of the egg, embryogenesis, hatching of the larva and its passage through larval instars, pupal stage and the adult moth. Duration of developmental stages varies and it can be controlled throughout the life cycle by regulating environmental conditions mainly the temperature and the nutrition. The developmental character of the egg, either diapausing or non-diapausing can also be controlled by conditioning the female parent with the temperature and photoperiod cycles during the incubation period. Furthermore, even the date of hatching can be rescheduled by applying artificial hatching treatment in combination with cold storage.

The diploid chromosome number in *B. mori* is 56. The haploid genome contains nearly 0.5pg DNA and its size is $5.3X10^8$ bp (Gage, 1974a; Rasch, 1974). Thus, *B. mori* genome is approximately three and half times the size of the fruitfly genome (140 million base pairs, Rasch *et al.*, 1971). Like most of the lepidopterans, the chromosomes of silkworm are holocentric i.e., they possess centromeres throughout the chromosome (Murakami and Imai, 1974). *B. mori* chromosomes are usually highly condensed (1-4μm long) and appear dot shaped at most of the mitotic and meiotic stages. Apart from the centromere, lepidopteran chromosomes display features similar to those of other eukaryotes. Diffused centromeric nature of the chromosomes renders them difficult to characterize cytogenetically.

B. mori was the first insect shown to have an interspersed pattern of repetitive and non-repetitive sequences typical of the mammalian genomes (Gage, 1974; Gage *et al.*, 1975). Silkworm genome harbors abundant retroposons (Short Interspersed Nucleotide Element Repeats, SINEs) like *Bm1* and *Bm2* elements, which represent 5% to 10% of the total genome similar to the *Alu* and *Alu*-like elements found in mammals (Eickbush, 1995; Ogura *et al.,* 1995). *Bombyx* also contains several diverse elements some of which having long terminal repeats like *Pao, Mag, R1Bm*, *R2Bm* and *BMC1* but lack long terminal

repeats similar to the LTRs *(Copia, Gypsy)* and non-LTR elements (*R1Dm, R2Dm, Jockey,* etc.) described in *Drosophila* (Robertson and Asplund, 1996).

2.1. Development of the Embryo

The embryo starts developing once the eggs are fertilized. The embryo grows, forms various organs and hatches as the larva. Development up to hatching stage is known as embryonic development, and the embryos hatch in about 10 days after fertilization at 25°C (Fig. 2.1). The various physiological and morphological changes found during the course of embryonic development can be divided into different stages (Table 2.1).

2.2. Post Embryonic Development

The silkworm is a holometabolous insect. In about 50 days they complete their life cycle of four different metamorphosing phases: egg, larva, pupa and adult. Of this life cycle, about half is the larval stage, the only stage at which they take food. The range of food selection of this insect is very narrow almost limited to mulberry leaves hence the silkworm is classified as a monophagous insect.

The larval body grows rapidly and functions actively while the imaginal analgens grow little and remain quiescent until metamorphosis. When the larva transforms to a pupa, the imaginal analgens grow rapidly and differentiate into the adult organs. The larval stage is a very important period for silk protein synthesis and egg formation. The pupal stage is a closed system, but the metabolic activity is very high due to histolysis and histogenesis.

The larva just after hatching is about 3.0 mm in length, has many setae on the body surface and the body color is black. At about 3 days after hatching, larva stops eating for 24 hours (molting). During this time, larva produces a new cuticle and sheds the old one (ecdysis). Since molting is repeated 4 times during the larval period, there are 5 feeding periods or instars (table. 2.2)

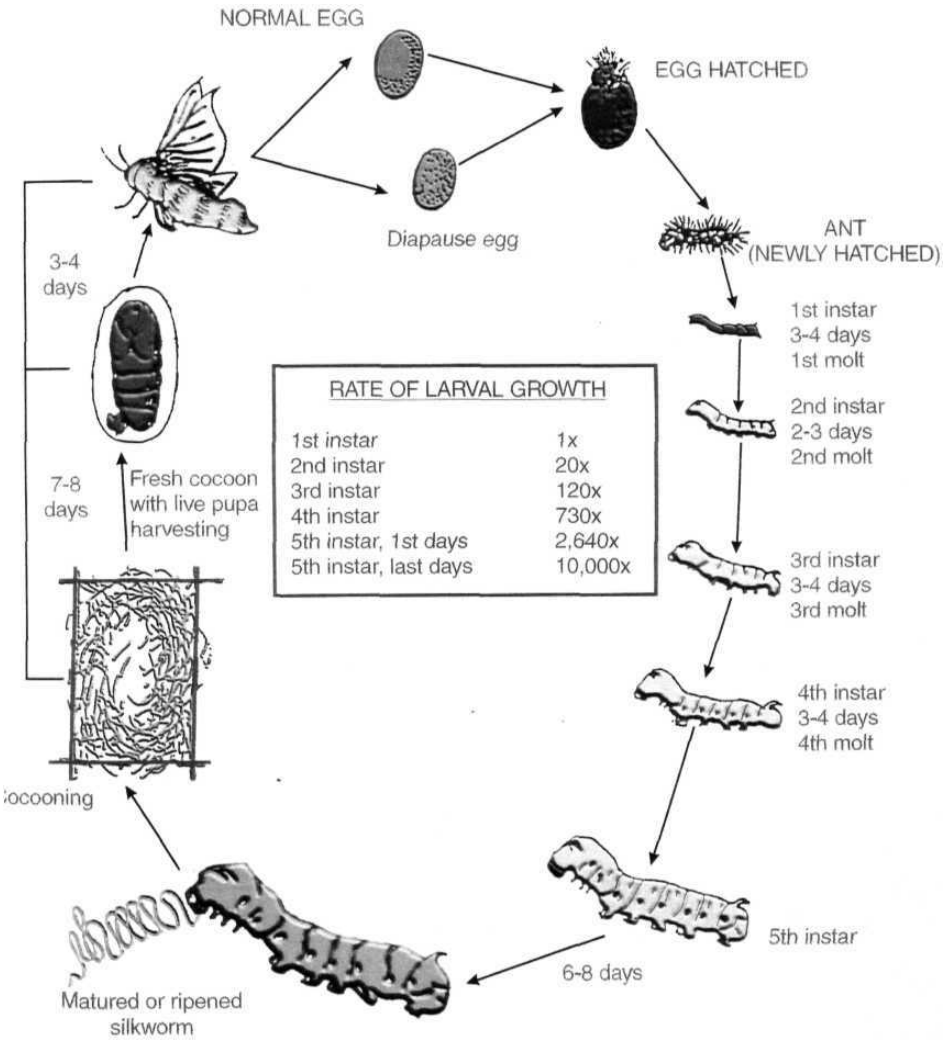


Fig. 2.1 Life cycle of the silkworm

Table. 2.1.

Course of Development from Fertilization to Embryo

Duration	**Development** stages
0-2 hours	Fertilization stage
2-10 hours	Cleavage stage
10-20 hours	Blastulation stage
20-24 hours	Yolk cleavage stage or Germ band formation stage
24-30 hours	Daruma stage
2 days	Appearance of abdominal appendages
3 days	Constriction stage
4 days	Pre involution
4 days and 12 hours	Involution
5 days	Involution completed
6 days	Appearance of tubercles
7 days	Tracheal colouration stage
8-9 days	Blue spot stage
9-10 days	Hatching

Table. 2.2

Time course of development of silkworm, from larval stage to adult moth

Stages of Growth	Feeding (time in days)	Molting (time in days)	Total (time in days)
I instar larva	**3.0**	**1.0**	4.0
II instar larva	**2.5**	**1.0**	3.5
III instar larva	**3.0**	**1.0**	4.0
IV instar larva	**3.5**	**1.5**	5.0
V instar larva	**6.0**	-	6.0
From spinning to pupation			4.0
From pupation to emergence	-	-	10
Total	-	-	**36.5**

In the 5th instar, when the larvae develop fully and stop eating, they attain a maximum length of about 70mm. The larval skin becomes transparent at this stage. These mature larvae are usually placed in a spinning nest, this process being called mounting. After about two days, larvae cease spinning the silk thread. The state from this time to the larval pupal ecdysis is called the pharate pupa and the duration is about 24 hours. Through the steps of pupal stage for 3 days and the pharate adult stage for 5 days, the adult moth emerges by pupal adult ecdysis. It is difficult to discriminate between ovary and testis in the larva just after hatching. However, by third instar the ovary becomes triangular in shape and is distinguishable from the kidney shaped testis.

2.3. Gametogenesis

2.3.1. Spermatogenesis

In males, testis becomes differentiated from the embryonic mesoderm. The primordial germ cells give rise to spermatogonia, primary spermatocytes, secondary spermatocytes and finally spermatozoa.

In the embryo, just before hatching, the testis is divided into four spermatic chambers. The primordial germ cells undergo repeated cell divisions in the peripheral regions particularly at the apical end. Later, one of the spermatogonia undergoes repeated divisions to form saccular cells. The primary spermatocytes further undergo maturation divisions twice inside the sac producing 256 spermatids. Each spermatid forms spermatozoa and they are grouped into bundles, each enclosed by a common membrane. Among the spermatozoa formed, some are nucleated (Eupyrene) and the others are non-nucleated (Apyrene). The nucleated spermatozoa are formed from the cells, which have completed the reduction division by the time the larva attains maturation stage, and the non-nucleated spermatozoa are formed from the cells, which complete the reduction division late after the larval maturation stage. It is the nucleated spermatozoa that enters the ovum and fertilizes it.

During the late pupal stage, the spermatozoa are released by the rupturing of the membrane and some of them are found in the lower part of the vas-deferens. These spermatozoa enter the seminal vesicle and during mating of the male and the female moths, they become covered by a thin membrane secreted from the ejaculatory duct thus forming spermatophores. Along with spermatic fluid, the spermatophores are ejected into the bursa copulatrix of the female where they are stored for some time. Due to the peristaltic movement of the wall of the duct leading from the accessory glands of the seminal receptacle and the secretion of the gland, the spermatophores rupture, liberating the spermatozoa that now reach the seminal receptacle of the female moth. The spermatozoa enter the swollen part of ovipositor from the seminal receptacle and finally penetrate the egg through the micropile as the egg descends from the ovarioles. The spermatozoa remain dormant at the anterior part of the egg until the egg nucleus completes the maturation division (Tazima, 1964).

2.3.2 Oogenesis

During oogenesis, eggs are formed from the primordial germ cells of the ovary that is differentiated from the embryonic mesoderm. When the development is complete and embryo is ready to hatch, the ovary divides into four small ovarioles. These contain a mixture of oogonial cells formed from the primordial germ cells and also the cells that later become the covering cells of egg. By the time a larva attains the early third instar, a single oogonial cell undergoes three repeated divisions resulting in a group of eight cells. Of these, one becomes the oocyte (no.1) and the rest i.e., seven cells become nutritive or nurse cells. By fourth larval stage, the most well developed part of the ovariole has several groups, each with one oocyte and seven nutritive cells arranged alternatively and regularly. These ovarioles are enveloped by follicular cells and can be divided into two parts namely a nutritive part or nurse chamber and a reproductive part or the egg chambers. The two chambers are inter-connected and the cytoplasm of the nutritive cells flows into the egg chamber and the oocytes grow by utilizing the nutritive contents. As the oocytes grow in size, the follicular cells become oval and yolk granules fill the oocytes. At a later stage, yolk-membrane and egg shell forming material are secreted from the follicular cells and eggs with complete egg shell can be seen in the oviduct of six or seven day old pupa. In the eggs with complete shell, the nucleus of the cell has progressed upto the middle stage of the first maturation division and the eggs descend the ovarioles filling up the ovarian tubule. The maturation division is resumed only after mating with male moth followed by the entrance of spermatozoa into the ovum and laying of eggs (Tazima, 1964).

chapter llI

Sex Determination: An Overview

Reproduction, variation and inheritance of variations are the three basic features of any living system. Genetics is the science of genes and their inheritance, and DNA the genetic material, is inherently organized to undergo reproduction, variation and inheritance of variation in cellular organisms. The basic sources of such variations are mutation and recombination that cause biological evolution. Sex implies being male and female and sexual reproduction involves mating of male and female for production of genetically variable progenies that result from gametic recombination of parental genomes during sexual reproduction. Genetics of sexuality therefore comprises genes of maleness and femaleness as well as genes that cause and promote gametic recombination. There are variety of sexual systems functioning in microbes, plants and animals with the sole common purpose of promoting genetic recombination and producing genetically different recombinant progenies. Genetic knowledge of sex and sexual reproduction is known to have played indispensable role in the past in generating breeds of animals and plants with improved products for our ever-growing need of survival and civilization. Sexual reproduction is also a natural means to repair and maintain the structural and functional integrity of genome. It is therefore, not surprising to know that genetics of sex-determination and evolution of sex chromosomes have attracted best minds of the world to understand their evolutionary causes and consequences.

Studies on genetics of sex-determination in various model animal systems have shown an astonishing variety of genetic and epigenetic sex-determining systems that operate to achieve the same purpose of producing two sexual types, the male and female. Now it is clear that a common principle operates behind the observed variations in genetic mechanism of sex-determination. This common principle consists of a primary sexual signal that can be genetic or environmental and is different in males and females, and a key sex-specific regulatory gene, whose state of activity is transmitted through a cascade of subordinate regulatory genes to the sex-differentiation genes, determining maleness or femaleness. Does such a common principle imply that the genes and the molecular mechanisms of sex determination in various animals have been conserved in evolution? The available information suggests that the

nature of the genes and the associated molecular mechanisms have not been conserved during the course of evolution (Schütt and Nothiger, 2000). This fact would become self evident during the brief description of genes involved in determining and controlling sexual phenotypes, in *D.melanogaster*, *M. domestica*, *C. elegans* and humans.

3.1 Sex Determination in Lepidoptera

Diptera (flies) and lepidoptera (moths and butterflies) are the insects of monophyletic group but the former has XY system of male heterogamety and the later has a ZW system of female heterogamety. However, variations in sex chromosome system are known to occur with XX female and XO male in diptera and ZZ male and ZO female in lepidoptera. Partial or total heterochromatinization is another feature of lepidoptera sex chromosomes (Traut and Marec, 1997). In the somatic cells of lepidoptera, W-chromosome forms a conspicuous heterochromatic mass. Due to its derivation from 'W'and its female specific presence the heterochromatin is designated as "W chromatin" or sex-chromatin (Traut, 1999).

B. mori females are designated as ZW and males as ZZ. Females of other lepidopteran species may be ZO, ZW, ZZW or ZZWW, the last being the case of presumed fragmentation of W (Traut and Mosbacher, 1968; Soumalainen, 1969; Robinson, 1971). Hashimoto (1933, cited in Tazima, 1964)) was the first to suggest that the W-chromosome has a female determining role in the silkworm on the basis of the fact that silkworms with ZW, ZZW and ZZWW were all females (cited in Tazima, 1964). Tazima confirmed this model of sex determination in *Bombyx* and showed that sex determinants are localized at one end of the W chromosome (Tazima, 1964; Strunnikov, 1983). Changing in the ratio of sex chromosome to autosome had no effect on sex-determination and gynandromorphs (i.e., sexual mosaics), instead of intersexes, were produced. This suggested that there is a balance of active factor on both W and Z-chromosomes (Tazima, 1964; Robinson, 1971).

Unlike males, lepidopteran females have no crossing over. W and Z-chromosomes are paired in the pachytene stage. Chromosomes with all the signs of non-homology do synapse along their entire length. However, complete alignment of homologous chromosomes by synaptonemal complex in pachytene does not lead to crossing over and chaismata formation. The absence of crossing over has led to the evolution of a substitute for chaisma at diplotene. The substitute consists of modified synaptonemal complexes that are not eliminated at the end of pachytene as in the case in chiasmatic meiosis (Traut and Marec, 1996).

Deficiencies of the Z-chromosome are lethal in females where as deficiencies of one of the Z-chromosomes do not have any effect on the sex expression in males (Tazima, 1964). The presence of a piece of W-chromosome, not including the female determinant (transferred to chromosome-3 by reciprocal translocation) has a lethal effect in males while the loss of this portion of W chromosome has no effect on sex expression in females. These findings indicate that the Z and W chromosomes in *B.mori* have at least some non-homologous segment containing sexually antagonistic genes (Tazima, 1964; Rasmussen, 1978; Traut and Marec, 1996).

The lepidopteran sex heterochromatin (W-chromatin) is transcriptionally inactive. This has been demonstrated by its failure to label in the presence of tritiated uridine in the silkglands of *Ephestia* (Traut and Scholz, 1978). Nevertheless, uridine incorporation is associated with heterochromatin in nurse cells indicating that at least part of the W-chromosome remains active in tissues involved in sex- determination (Guelin, cited in Traut and Scholz, 1978). These kinds of observations have led to the suggestion that heterchromatinization serves to reduce or eliminate the function of the W-chromosome in selected tissues, analogous to the role of chromosome diminution in Ascaris, Cyclops and Sciara (Traut and Scholz, 1978; Crouse, 1990).

3.2 Sex Determination in *Drosophila melanogaster*

Expression of *Sxl* in somatic cells is regulated by a cell autonomous signal, the X:A ratio but its expression in XX germ line requires both the cell autonomous signal of X:A ratio and an inductive signal generated by somatic cells (Bakerc and Beloti, 1983; Steinmann-Zwicky *et al.*,1989). On the contrary, in *C. elegans,* the same set of genes that control the somatic sex also control germline sex but with some basic difference in their mode of action in the two cell lines (Schedl and Kimble, 1987, 1988; Villeneure and Meyer, 1990).

SXL protein controls *Sxl* and *tra* transcript splicing directly by binding to poly U-rich stretches in pre-mRNA (Sosnoski *et al* 1989; Inove *et al* 1990; Valcarcel *et al* 1993). The gene *snf, vir* (*virilizer*), and *fl(2)d [female-lethal (2)d*] generate products that facilitate SXL's positive autoregulatory splicing (Granadino *et al,* 1990; Flickinger and Salz, 1994; Hilfiker *et al,* 1995).The switch gene *tra,* the direct regulatory target of *Sxl* requires a partner *tra-2* (*transformer-2*) (Fujihara *et al,* 1978). Together these two genes control the sex-specific alternative RNA splicing of *dsx (double sex),* an unusual bifunctional gene (Nagoshi *et al,* 1988; Mattow *et al,* 1990). A likely target for *tra* in the sex determination pathway is *fru (iruitless)* (Gailey and Hall, 1989; Gailey ef *al* 1999).

Dsx (doublesex), is the gene controlling terminal differentiation of somatic or germ cells into male/female phenotype. It acts downstream of *tra* and *tra-2* (Baker and Ridge, 1980). DSX protein works both by repressing gene expression that is appropriate for differentiation of opposite sex, and by inducing gene expression appropriate to the same sex (Coschigano and Wensink, 1993; Jursnich and Burtis, 1993). It is assisted by partners (genes) which are not sex specifically regulated: *ix* (intersex) and *her (hermaphrodite)* (Cline and Meyer, 1996).

The *Sxl* gene responds to the dosage of cis elements under the influence of X:A ratio in early embryogenesis and becomes set in one of the two modes,

either active (females) or inactive (males). The early phase of control also involves the activity of other genes such as *da, liz, fl(2)d.* The active state of SXL protein inturn acts on the *tra* gene transcript to promote its correct splicing for functional TRA protein. The combination of active TRA protein and TRA-2 protein, a distinct product of *tra-2* gene together function in correct splicing of *dsx* gene transcript to produce DSX^F protein which causes development of female phenotype by activating female pathway genes and suppressing the transcription of male specific genes. The whole sequence of events thus described is characteristic of female sexual phenotype development in XX individuals. In males, in the absence of either TRA or TRA-2, male specific default splicing results in DSX^M proteins (Cline and Meyer, 1996).

In contrast, the product of *Sxl* gene in XY individuals, where X:A ratio is 0.5 remains inactive. Since active state of *Sxl* is required for production of active TRA protein, the *tra* gene product naturally remains inactive. Under such situation *dsx* gene transcript undergoes default mode of splicing leading to the production of DSX^M protein that inhibits expression of female specific genes and promotes expression of male specific genes thus causing the XY individuals to become males (Cline and Meyer, 1996) (Fig. 3.1).

3.3 Sex Determination in *Musca domestica*

M. domestica is a dipteran member like Drosophila and exhibits male heterogametic type of sex determination system. However, it differs from Drosophila in the following basic respects: It has a transposon like male determiner gene 'M', epistatic to female determiner gene 'F,' and known to function as determiner of male phenotype by inactivating the female determining gene 'P. In this system, absence of 'M' in homogametic XX system (F+/F+) results in induction of female pathway (Schmidt *et al.*, 1997a; Schmidt *et al.*, 1997b). This is in sharp contrast to Drosophila system in which the master sex specific switch *Sxl* controls not only sex-specific pathways but also dosage compensation pathways. Such functional involvement of *Sxl* in Drosophila suggests that its sex-determining mechanism has become refractive

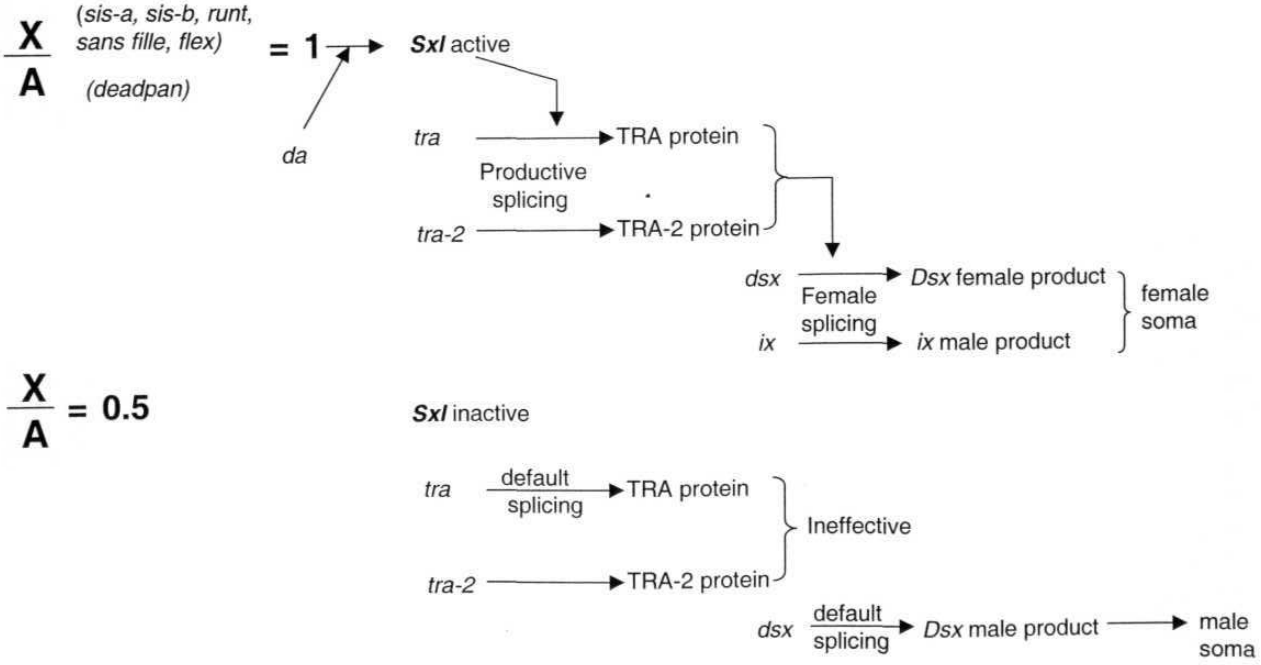


Fig.3.1. The somatic cascade for sex determination in *Drosophila melanogaster*
(Adapted from Hodgkin, 1990)

to further evolutionary changes. This is not the case in *M. domestica* as it lacks dosage compensation and its key sex-specific genes seem to function exclusively in sex- determination (Dübendorfer *et al.,* 1992; Schutt and Nothiger, 2000). It is worthwhile to mention here that *M. domestica* contains homologue of Drosophila *Sxl,* whose expression is not controlled by the primary sex-determining signal of X:A ratio therefore not equivalent to 'F' gene, whose expression is regulated by primary sex determining signal 'M'. Similar results have been obtained in other non-Drosophilid species. In conclusion, it thus seems that original function of *Sxl* gene was not specific to sex-determination in non-Drosophilids and that its role as a coordinator of sex-determination and dosage compensation was acquired during evolution of sex in Drosophila (Meise *et al.,* 1998; Schutt and Nothiger, 2000).

3.4 Sex Determination in *Caenorhabditis elegans*

The level of HER-1, the product of the key regulatory gene *her-1,* determines the activity of other sex-determining genes lying downstream of *her-1.* The sequential interaction of sex-determining genes in *C. elegans* unlike that in *D. melanogaster* is negative (Hodgkin, 1987). In other words, *her-1* gene product is inhibitor of *tra-2, tra-3* gene products, which in turn inhibit the activity of *fem 1,2,3* genes. Such sequential interactions result in high level of *tra-1* gene product in XX individuals thus causing them to develop into female. In XO individuals, the similar negative interaction results in low level of *tra-1* gene product leading to male soma. Infact, the state of *dsx* gene product regulates maleness and femaleness in Drosophila and the level of *tra-1* gene product regulates maleness/ femaleness in *C. elegans* (Hodgkin, 1986; Cline and Meyer, 1996) (Fig.3.2).

3.5 Sex Determination in Mammals

The observation of heteromorphic sex chromosomes in humans, an XX pair in females and an XY pair in males, suggested a chromosomal basis of sex determination but was not informative as to the mechanism. The description of

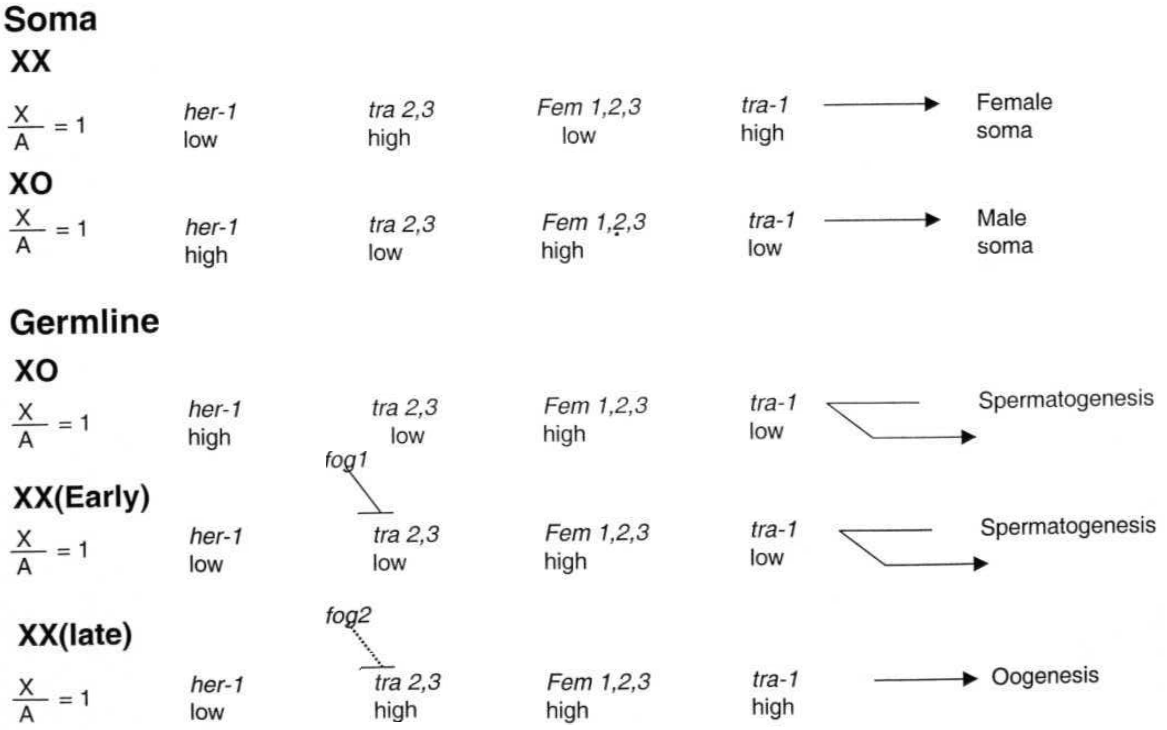


Fig. 3.2. The Somatic and Germline cascade inferred for *C. elegans*.
(Adapted from Hodgkin,1990)

human phenotypic female with a single X-chromosome (Ford *et al.,* 1959) and a phenotypic male carrying two X-chromosomes and a Y-chromosome (Jacobs and Strong, 1959) demonstrated that, rather than X:A ratio acting as a signal for sex determination, the presence of Y-chromosome directs male phenotypic development and in its absence, a female sexual phenotype results (Schafer and Good fellow, 1996).

Discovery of a mutant mouse with chromosomal complement of a female (XX), but developing as a male, designated as Sxr (sex reversed) by Cattanach *et al.,* (1971), was an important step in understanding the mechanism of sex determination. Singh and Jones (1982), using the highly conserved *Bkm* probe showed for the first time that sex reversal in these mice was due to translocation of a very small part of the Y-chromosome to the distal end of the long arm of the X-chromosome. *Bkm* studies revealed that the Sxr region of the mouse Y-chromosome is strongly male determining and is necessary and sufficient to convert a chromosomally female mouse into a male (Singh and Jones, 1982). A systematic analysis of XX males and XY females has led to the detailed mapping of Y-chromosome (Guellaen *et al.,* 1984; Disteche *et al.,* 1986; Page *et al.,* 1987) and isolation of *ZFY* (Zinc finger on the Y-chromosome), earlier thought to be testis-determining factor (TDF) on the Y (Page *et al.,* 1987) and *SRY* (Sinclair *et al.,* 1990), the gene in the sex reversal region of the Y which has now been shown to be the regulatory switch for sex-determination. *SRY* is required for triggering the differentiation of bipotent gonad primordium into 'sertoli' cells leading to formation of testis. Once the testis is formed, the male hormones they produce give rise to the male phenotype. In addition to *SRY,* several other genes like *DAX1, SF1, WT1, DMRT1* are suggested to be involved in determination of male and female sexual phenotypes in mammals (Jimenez and Burgos, 1998; Raymond *et al.,* 1999). The activities of *SF1* and *WT1* are essentially required for the development of bipotential gonads from genital ridge (Kreidberg *et al.,* 1993; Luo *et al.*, 1994; Graves 1998).

In addition, *SF1* gene is also expressed in sertoli cells during gonad formation, which implies an important role of this gene in testis development (Ikeda *et al.,* 1994). Further studies on the role of this gene in testis development have shown its essentiality for the activation of anti-mullerian gene (AMH gene) and steroidogenic gene (Shen *et al.,* 1994). *DAX1* has been shown to act as the inhibitor of *SF1* gene function leading to inhibition of testis development (Swain *et al.*, 1998).

SRY gene product is proposed to induce testis development indirectly by antagonizing the suppressive effect of *DAX1* gene on *SF1* gene product (Jimnez and Burgos, 1998). Consequently, such interaction between *SRY* and *DAX1* gene product results in normal activity of *SF1* gene product, which in turn activates the male pathway of testis development. The testis thus developed produce male hormone for determining male phenotype as well as anti-mullerian hormone that inhibits the pathway of ovarian development (Jimenez and Burgos, 1998).

Recently, a gene with DM-domain similar to *mab-3* and *dsx* has been identified in humans and designated as *DMRT1* (Raymond *et al.,* 1998). *DMRT1* maps to the small region on 9p24.3 implicated in sex reversal and is a strong candidate gene for this trait (Raymond *et al.,* 1999). *DMRT1* is expressed exclusively in the genital ridge before sex-differentiation (the only other gene which shows this pattern is *SRY,)* and soon after is expressed only in the testis (Raymond *et al.,* 1999), thus *DMRT1* seems to be involved in mammalian sexual development (Ellegren, 2000).

According to the current status of sex determination in mammals, a model has been proposed to account for the genetic basis of maleness in XY individuals and femaleness in XX individuals (Fig.3.3). However, the fact that a large number of individuals having normal *SRY* and other genes implicated in sex-determination, with no mutation at all, develop as females and a large number of individuals without the presence of *SRY* develop as males and none of the hermaphrodites contain any 'Y' DNA at all suggests the involvement of other genes, which have not yet been identified (Thangraj *et al.,* 1998).

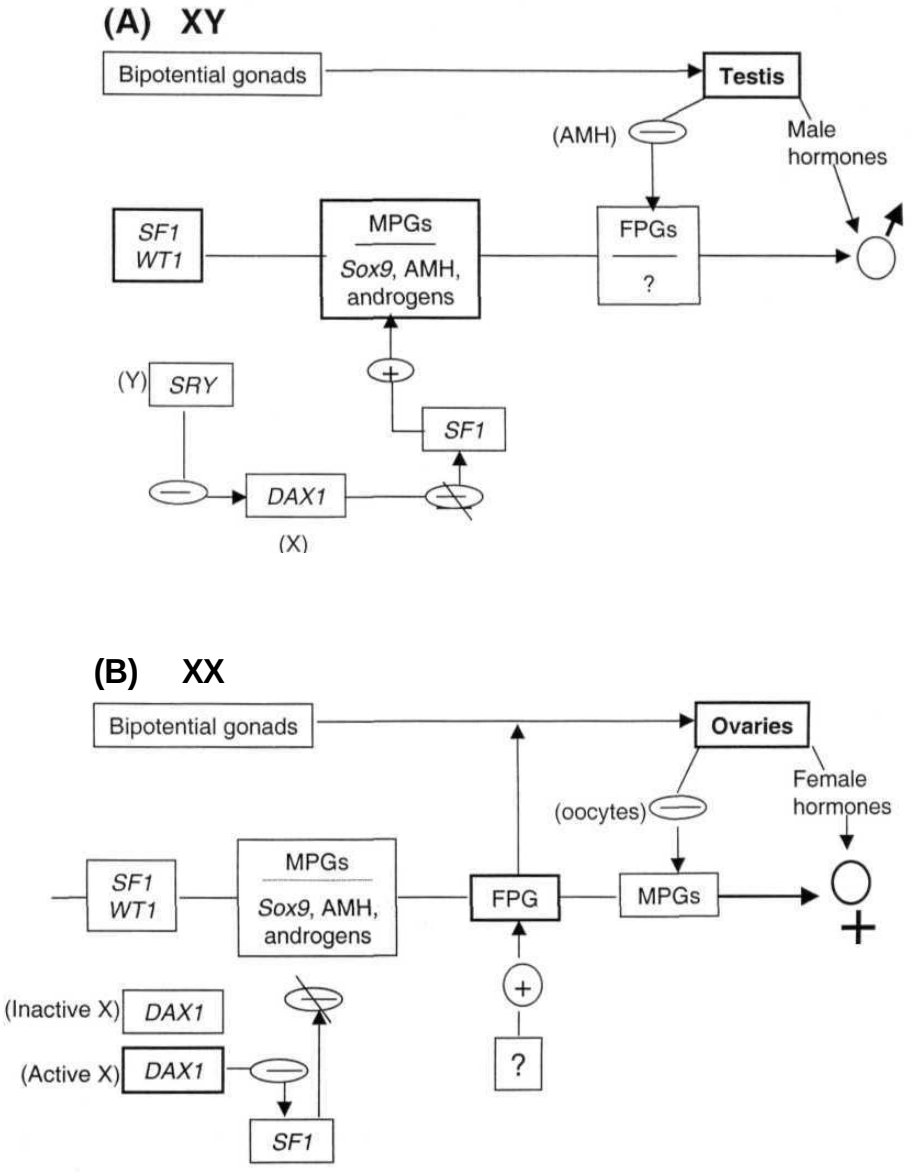


Fig. 3.3. DSS gene based model for sex determination in mammals. (Adapted from Jimnez and Burgos, 1998).

3.6 Dosage Compensation

The evolution of heteromorphic chromosomes will in most cases lead to a significant imbalance in the dosage of the genes located on the sex chromosomes which may have nothing to do with sex determination but are nevertheless present in two doses in one sex and single dose in the other. Some form of dosage compensation is likely to occur in order to equalize the activity of all these genes between the two sexes (Hodgkin, 1992). The experimental analysis shows that haploidy or triploidy for more than a few percentage of the total genome is severely deleterious or lethal so evidently compensation will be an essential process. This experimental finding has led to the concept that dosage compensation may not occur for those sex linked genes which are very few in number or are required to express only in one sex or the genes which are part of gene families distributed throughout the genome. Accordingly, lack of dosage compensation for sex-linked genes in birds and butterflies has been explained on the basis of very small size of sex chromosomes carrying very few genes (Johnson and Turner, 1979; Baverstock era/., 1992; Hodgkin 1992).

However, the lack of dosage compensation in various systems reported so far still requires scientific explanation as to why dosage compensation is not required for the viability of ZZ/ZW sexual system but required for the viability of the XX/XY system.

3.6.1 Dosage Compensation in Lepidoptera

In female heterogametic organisms like birds, butterflies it has been confirmed that the sex linked genes are not dosage compensated (Cook, 1964; Johnson and Turner, 1979; Barestock *et al.,* 1982; Suzuki *et al.*, 1997). In *Heliconius melpomere* and *H. elato*, the activity of 6-phosophogluconate dehydrogenase (6-PGD) located on the Z-chromosome is not compensated between the two sexes (Johnson and Turner, 1978). As in the case with other lepidoptera, *B. mori* has female heterogamety and does not show dosage compensation. The

level of transcription of Z-linked genes shows a two-fold difference between males and females. As for example *Bm kettin* gene, homologue of the Drosophila *Kettin* gene, is located on the Z-chromosome and is not dosage compensated, as its transcript level is two fold higher in males than in females. Unlike the dosage compensated species such as mammals in which one copy of the homogametic chromosome pair (XX) is inactivated in the somatic tissues by heterochromatinization (Lyon, 1974), in many lepidopteran species as well as other taxa with heterogametic females like birds and snakes there is ample evidence that it is W-chromosome that becomes inactivated (Cock, 1964).

Lack of dosage compensation supports the idea that female limited characters will be predominantly autosomal whereas male limited sexual characters can be both sex linked and autosomal (Johnson and Turner, 1979). Among butterflies, a large proportion of genes controlling female mate selection are located on the Z-chromosome (Grula and Tayler, 1980) and the genetic data on these phenotypes suggest that most, if not all, of the Z-chromosome lacks dosage compensation (Johnson and Turner, 1979; Grula and Taylor, 1980). Thus, it appears that there is a close relationship between an absence of dosage compensation of Z-linked genes and sexual dimorphism in phenotype.

3.6.2. Dosage compensation in *Drosophila melanogaster*

Drosophila has impressive list of molecules that associate with dosage compensated X-chromosome of XY individuals thus causing two-fold increase in the transcription rate of X-linked genes. They include male X-chromosome proteins like MLE, MLS-1, MLS-2, MLS-3, MOF and non-coding RNAs like *roX-1* and *roX-2* (Gorman *et al.,* 1995). The male X-chromosome protein and *roX-1* and *roX-2* are required together for binding to the target sites located on the male X-chromosome where they catalyze acetylation of histone H4 at its 16-lysine residue. This interaction opens the chromatin for two-fold rise in transcription of X-linked genes (Baker *et al,* 1994). In males the dosage compensation system works mainly because SXL protein in male cells is rendered inactive thereby not interfering with the activity of *msl-2* gene product,

MSL-2 protein (Bashaw and Baker, 1995; Kelley *et al.,* 1995). In comparison, in females SXL protein remains active leading to inhibition of the activity of MSL-2 protein. Since MSL-2 protein in association with other protein components and *roX-1* and *roX-2* RNA, is a basic requirement for two-fold rise in transcription from X-linked genes (Birchler, 1996), its inactivity in female cells naturally results in no influence on basic rate of transcription of X-linked female genes. This is how equalization of the level of X-linked gene product occurs in XX and XY cells of fruit fly (Cline and Meyer, 1996; Stuckenholz *et al.,* 1999).

3.6.3. Dosage compensation in *Caenorhabditis elegans*

In nematodes, the dosage compensation mechanism acts to down regulate transcription of X-linked genes to 50% in XX individuals and not in XO individuals (Hodgkin, 1983). The reason is that SDC-2 and SDC-3 proteins are XX individual specific and not XO individual specific. Since, the complex down regulating the transcription of X-linked genes is made of protein subunits like SDC-2, SDC-3, DPY-26, DPY-27, DPY-28, MIX-1 and since SDC-2 and SDC-3 are made only in XX individuals and not in XO individuals, naturally the down regulation of X-linked genes occurs specifically in XX individuals and not in XO individuals (Lucchesi, 1998).

In XX individuals, (high X:A ratio) expression of *Xol-1* gene is reduced. Since the product of *Xol-1* gene inhibits the formation of *Sdc-1* and *Sdc-2* genes product, the level of SDC-1 and SDC-2 protein naturally remains high in XX individuals. High level of these two gene products in turn reduces the product of *her-1* gene and causes the formation of active protein complex called DPY complex down regulating these expression of X-linked genes by 50%. The sequence of events found occurring in XX individuals is reversed in XO individuals where *her-1* gene product level is raised and as a result the production of active DPY complex fails to occur. Thus, DPY complex system is the mechanism of dosage compensation in *C.elegans* (Cline and Meyer, 1996; Lucchesi, 1998).

3.6.4. Dosage compensation in mammals

In mammals, dosage compensation involves inactivation of all X-chromosomes except one regardless of sex chromosome constitution (XO, XY, XX, XXY, XXX etc). Obviously, dosage compensation mechanism in mammals must be capable of sensing number of X-chromosome present for inactivation. The mechanism of X-inactivation is known to result from the activity of *Xist* gene whose product is a 15kb long non coding and cis acting signal originating from the Xic centre (Kelly 1995; Penny *et al.*, 1996; Backdroff *et al.,* 1998). The *Xist* RNA produced from the inactive X-chromosome coats its source. The Xic locus is a multifunctional locus involved in cis propagation of X-chromosome inactive state through *Xist* RNA production, as well as in the decision of how many and which chromosome to be inactivated. The molecular details of such inactivation process are far from clear (Brown *et al.,* 1991; Backdroff ef *al.,* 1992; Rastan, 1994).

3.7. Evolution of Sex Chromosome

The existence of specialized sex determining chromosomes poses a range of fascinating and fundamental questions, few examples of which have received any satisfactory answers. Why, for example, has the evolutionary process led to such developments? Why do some, but not all species exhibit them? How it is that an entire chromosome, originally containing many other genes unconnected with sex, can become subverted to a singular developmental programme? Why does the functional monosomy, involved in the specialization of the Y or W sex-determining chromosome, not lead to chromosomal imbalance and consequent lethality? Related to this, why is there compensation for X-linked gene dosage in mammals, but dosage effect in respect of Z-linked genes in reptiles? Could it be that dosage compensation is not the primary function of X-inactivation? We do not know the answers to many of these questions. However, attempts have been made to answer some of these questions.

Chromosomal sex determination and sex chromosome evolution are the two dissimilar distinct biological processes linked evolutionarily. It is an observed fact that Y or W chromosome (or part of them) forms a genomic compartment that does not recombine with the respective X or Z-chromosome. Suppression of recombination between X /Y and Z/W results in differentiation of the two chromosomes Y and W by seperate evolutionary mechanisms of degenerative nature. Taxonomic distribution of heteromorphic sex chromosomes suggests that sex chromosome heteromorphism must have evolved independently on many occasions. This indicates that conditions predisposing the specialization of sex chromosomes are wide spread amongst eukaryotes and the probability of their evolving is relatively high. Most often it has been assumed that the heterochomatin, which is a common feature of sex-determining chromosomes throughout vertebrates, is the end point of a long and complex process of mutational drift to functionlessness associated with gradual 'compensation' for hemizygosity (Ohno, 1967; Charlesworth, 1978).

The Z and W-chromosomes of birds share many features with mammalian X and Y-chromosomes respectively. Both avian chromosomes are metacentric, pair during meiosis and a synaptonemal complex is formed at the ends of the short arm of the two chromosomes, therefore a small pseudoautosomal region exists (Stefos and Arrighi, 1971; Ellegren, 2000). W-chromosome forms a sex-chromatin (W-chromatin) in female snake somatic tissues. It remains in the condensed state during meiosis and conformationally it is out of synchrony with the Z-chromosome during pachytene (Ray-Chaudhari *et at.,* 1971; Ray-Chaudhari and Singh, 1972). Similar behavior of W-chromosome has been shown in moth *Ephestia kuchniella* (Traut and Scholz, 1978). Such a relatively discrete condensation cycle obviously would be consistent with the activity of one, or few, genes and its timing is appropriate for early embryonic development including, presumably, sex determination. Inactivated mammalian X-chromosome becomes reactivated in oocytes (Gartler *et al.*, 1972; Lyon, 1974).

It has generally been accepted that sex was determined initially by an allelic difference at a locus born on a homologous pair of autosomes. The two primordial sexes consisted of individuals heterozygous or homozygous at the sex-determining locus. The transformation of the autosomes bearing the sex-determining gene in question into heteromorphic sex chromosomes is thought to have resulted from preferential accumulation of loss of function mutations in the neighborhood of the sex-limited allele of the gene. The retention of such mutations was facilitated by a general reduction in the rate of recombination in the genome of individuals bearing the sex-limited allele or atleast in the chromosomal region adjacent to the allele (Lucchesi, 1999). A number of authors such as Brian, Charlesworth, James Bull and others have proposed models that would account for a reduction in recombination between the homologues bearing the sex-determining genes. These models are all derived from R.A. Fisher's idea that, if mutations with opposite effect on the sexes occur on the chromosomes bearing the sex-determining locus, a tighter linkage of these mutations to the sex-determining gene will be selected in order to maintain the appropriate favorable allele in the appropriate sex (Bull, 1983). Sex-limited mutations benefiting one sex may accumulate if they harm the other sex due to counter selection (Lucchesi, 1999).

Heterochromatin preceded gross structural alteration of the W in evolution. Structural alterations of the W-chromosome have played a minor causal role, if any, in its evolution and it also implies that the molecular alterations involved in becoming heterochromatic can occur relatively rapidly in evolutionary time. This further implies that functional monosomy, which accompanies this process of chromosomal inactivation, can also be accommodated without a prolonged evolutionary adjustment or 'compensation' for altered gene dosage. The fact that there is no evidence of dosage compensation in reptiles, birds or lepidoptera (Ohno, 1967; Johnson and Turner, 1979; Baverstock et al., 1982) further suggests that there was little or no barrier to prevent a rapid evolution of the W-chromosome (Jones, 1983).

In other words, acquisition of sex-determining function and suppression of recombination are prerequisites for chromosome pair to undergo differentiation by Muller's Ratchet (Muller, 1964, Charlesworth, 1978), by sex specific acquisition of *Bkm* sequences (Singh, 1976) or by genetic hitchhiking (Rice, 1987). Why does such suppression of recombination between hetreromorphic sex chromosome pair occur at all? Bull (1983) suggested that most likely reason is that if there are sex linked alleles with opposite effect on fitness in the two sexes, there is a selective advantage in reducing recombination between the sex determining locus and these genes. According to Rice (1987), suppression of recombination is selectively favored by the accumulation of sexually antagonistic genes on the sex chromosome.

Meiotic cell division is a known mechanism for chromosomal recombination to occur in eukaryotic cells. The function of meiotic recombination has been found to be the means for producing genetically variable progenies. In addition, it is also known to play indispensable role in the repair of both genetic and epigenetic defects in DNA and in the elimination of selfish DNA like transposons from the germline (Bernstein, 1977, Martein, 1977, Holliday, 1984, Ettinger, 1986]. Accordingly, recombination deficiency can result in the accumulation of mutation and other genetic changes in the heteromorphic chromosomes. The significance of suppression of crossing over between the sex chromosomes in the heterogametic cells was first pointed out by Muller (1914, 1918). He suggested that lack of crossing over in XY heterogametic males would cause accumulation of deleterious recessive mutations on Y-chromosome leading gradually to its progressive heteromorphism, a phenomenon termed as Muller's Ratchet. There is an alternative view that finds experimental reasons to conclude a specific role of *Bkm* sequences in heteromorphic degenerative evolution of Y or W sex- chromosome and where the entire chromosome seems to function like a super gene (Singh, 1995) According to Jones (1983) W-evolution is triggered by mutations, which cause the control of the process of mitotic W-condensation to be modulated by the sex-determinants. According to this model, the cycle of somatic inactivation and germ cell activation of the W-chromosome signifies the cycle of sex gene

expression. In species where W has continued to specialize, the sex determiner assumed control over the center, involved in chromosome condensation. A spreading effect or read through from the sex gene was such that when it turned off, the chromosomes also condensed; the sex determiner thus fortuitously but effectively had hijacked the entire chromosome. In primitive species, the model postulates that the condensation center was not located sufficiently close to the sex determiner for there to be a similar interaction between them. In evolutionary biology, sexual selection based on sexual heteromorphism seems to have played a very important role in the evolution of various systems for regulating the genetic interest of the two parents. Few examples of such systems include dosage compensation, genomic imprinting and haplodiploidy system (Moore and Haig, 1991).

Meiotic pairing is normally dependent on structural and conformational homology between the two chromosomes involved. In respect of sex chromosome this can be illustrated in terms of behaviour of sex chromosomes in somatic and reproductive cells. In heterogametic XX/XY system, the XX females exhibit heterochromatinization of one of its X-chromosome in somatic cells and its euchromatinization in oocytes. In contrast, XY males exhibit euchromatinization of its X in somatic cells and heterochromatinization in spermatocytes. The problem of inadequate pairing in non-homologous region is avoided when these regions either become heterochromatinized or euchromatinized. These ideas have led Jablonka and Lamb (1990) to suggest that the need to avoid effects of pairing failure may have played an important role in the evolution of sex-chromosome heteromorphism. According to them, reduced crossing over between the sex-chromosomes, functional and structural degeneration of the Y-chromosome, sex-chromosome imprinting and dosage compensation may all be evolutionary consequences of the requirement for pairing at meiosis. There are two pathways, conformational and structural, known to be associated with initiation of reduced recombination in the non-homologous regions of the heteromorphic sex chromosomes. Conformational pathway assumes that the event initiating evolutionary changes leading to sex-chromosome heteromorphism was a change in chromatin conformation in the

region in which the sex determining genes were located. The structural pathway assumes that the initiating event was a structural change resulting from an inversion or a translocation in the originally homomorphic sex chromosomes (Jablonka and Lamb, 1990).

3.7.1 Conformational Pathway

There are many examples in both vertebrates and invertebrates where the only known difference between the sex chromosomes is a conformational difference. The genus *Triturus has* XY system of sex determination and the two sex chromosomes in somatic cell can be recognized only by specific staining for constitutive heterochromatin, which is found greater in amount on Y chromosome (Schimdt *et al.*, 1979). Similarly, in the Cyprinodont fish, *Poecilia sphenops* v. *melanistica* with WZ system, the W-chromosome of the female differs from Z in having a large terminal heterochromatic segment (Haaf and Schmid, 1984). Singh *et al.*, (1976) showed that W-chromosome to differs from Z-chromosome in having a specific satellite DNA in evolutionarily intermediate group of primitive snakes containing otherwise seemingly homomorphic sex chromosomes.

The conformational difference between chromosomal regions reflects differences in the level of condensation of chromatin (Schweizer *et al.,* 1987), the timing of replication in 'S' phase (Holmquist, 1987) and level of sensitivity to Dnase I (Kerem *et al.*, 1983, 1984). Such chromatin organization confers on it a conformationally active or inactive state. Reduced recombination in conformationally heteromorphic region of XY chromosomes in species of *Triturus* has been experimentally demonstrated (Schmid, 1983), thus confirming the fact that conformational heteromorphism between the regions of homologous sex chromosomes is the cause of suppression of crossing-over. Ray-Chaudhari *et al.,* (1971) suggested another mode of chromatin conformational heteromorphism resulting from late replication of DNA in the W-chromosome of WZ snake system. According to them, late replication of DNA in 'W' is the first step in the differentiation of 'W' from the 'Z' chromosome in snakes (Ray-Chaudhari and Singh, 1972). Schempp and Schmid (1981), have

made similar observation with European frog, *Rana esculenta.* Singh and his group have shown involvement of sex specific *Bkm* sequences (GATA sequences) in controlling Y or W-chromosome heteromorphism associated with their male or female specific gene activity. According to this model, *Bkm* sequences would also result in suppression of recombination between non-homologous regions of the sex chromosome (Jones, 1983; Jones and Singh, 1984).

3.7.2 Structural Pathway

There is considerable evidence suggesting that in many different groups the evolution of sex chromosome heteromorphism has involved a structural change such as pericentric or paracentric inversion or a translocation (Ohno, 1967). The structural change, which usually involves only one of the two homologues, can have two rather different effects, which can contribute to the functional degeneration of 'Y' or 'W chromosome. First, a small inversion or a translocation can result in incomplete pairing either within the rearrangements or in adjacent region (White, 1973). To counteract the adverse effects of pairing failure during meiotic prophase, any mechanism that inactivates the unpaired region will be selected. In this way both X and Y-chromosomes could come to have genetically inactive regions.

Structural change may also contribute to the functional degeneration of Y chromosome through its effect on recombination. Crossing over between structurally heterozygous chromosomes frequently results in production of inviable gametes carrying chromosomal duplications or deletions. Evidently, many structural changes act as apparent crossover suppressors thus causing progressive degeneration of Y-chromosome.

Lepidoptera contains WZO/ZZO system of sex-determination in which W is suggested to have arisen from free homologue of the fused autosome to Z chromosome (Traut and Marec, 1997). Another example is *Drosophila miranda.* This species has a neo Y-chromosome formed by a fusion between the ancestral Y and an autosome. Steinemenn (1993) has shown that the

originally autosomal part of the neo Y has some regions, which are heterochromatic and have accumulated repetitive DNA sequences which are not present on the original homologue thereby implying a role of repetitive DNA in degeneration of Y-chromosome.

3.8 Sex Chromosome Evolution In Lepidoptera

Marked differences are observed between 'advanced' families that form the clade ditrysia comprising 98% of all lepidopteran species and the primitive non-ditrysian families in respect of sex chromatin. All ditrysian families include species with sex chromatin (WZ/ZZ system) and sporadically species without sex chromatin (Z/ZZ system). In contrast none of the species investigated from non-ditrysian families had sex chromatin (Z/ZZ system). The inference is that the W-chromosome had not evolved when these non-ditrysian lineages diverged from the common lepidopteran stem (Traut amd Marec, 1997; Traut, 1996).

A possible scenario for the acquisition of the W is fusion of an autosome with the Z-chromosome. The free homologue of the fused autosome is then transmitted as a W-chromosome in female lineage. All molecular and morphology differentiation of the WZ pair must have taken place since then including the loss of the W-chromosome in some ditrysian species. Lepidoptera thus, displays the full evolutionary life cycle of a univalent sex chromosome, through genetical and structural differentiation, rearrangement with autosomes and eventual loss (Traut and Marec, 1997; Traut, 1999)

3.9. Junk DNA and Sex-Determination

Early work on organization of eukaryotic genome by using renaturation kinetics (Britten and Kohn, 1968) revealed that a large fraction of the genome consists of non-coding repetitive DNA sequences, also called as Junk DNA. It has been estimated that 97% of the human DNA is junk or non-genic and the remaining 3% is genic (Nowak, 1994). However, recent studies on this aspect are clearly providing evidence for indispensable role of Junk DNA in the regulation and

organization of centromere, telomere, chromosomal architecture and gene activity. The following gives a brief account of the known families of the junk DNA (repeated DNA sequences) characteristic of eukaryotic systems with their possible known functions.

1. **Introns:** Most eukarotic genes contain introns that do not code for proteins. Some of the introns are now known to code for small nucleolar RNA (sn RNA) believed to be essential for the assembly of ribosomes (Elder and Turner, 1995).

2. **Satellites:** They are short DNA sequences 140-180bp long, repeated 100-1000 times at a stretch. They mainly occur at the ends and centers of the chromosome. They are essential for the structural and functional integrity of chromosomes as their absence leads to chromosomal disintegration.

3. **Minisatellites:** Similar to satellites but are shorter up to 40bp long, occur throughout the genome. Defective minisatellites are found to be associated with cancer disease.

4. **Microsatellites:** They are shorter than minisatellites, usually 2-5bp long. GATA sequences of *Bkm* are examples of microsatellite and are known to be essential for the organization and activity of heteromorphic sex chromosome (Singh, 1995). In addition, GATA motif and GATA family of transcription factors are known to be essential for the activity of globin and other erythroid specific genes (Evans *et al.,* 1988; Mignotte *et al.*, 1989).

5. **SINES** (non-LTR) and **LINES** (LTR): They occur in numerous copies. One example of SINE is 300bp Alu sequences that occur 500,000 times in human genome. LINES are similar to SINES but longer up to 700bp apiece. Both LINES and SINES hop about the genome and cause mutation if they land in a gene (Nowak, 1994; Elder and Turner, 1995).

Haploid genome of silkworm, *B. mori contains* 560 million bp/silkgland cell which is approximately 3.5 times the size of *D. melanogaster* and 1/6th to the size of human genome (Nagaraju, 2000). Molecular characterization of repeatitive DNA elements have shown the occurance of microsatellites of dinucleotide repeats $(CA)_n$ and $(CT)_n$, transposon like elements characteristic of Drosophila genome and retroposons typical of mammalian genome. In addition, it also contains SINES like *Bm1* and *Bm2* representing 5-10% of its total genome. The additional DNA sequences present in silkworm genome are diverse types and include *Pao, Mag, R_1Bm, R_2Bm2, BmC1, RDM, R_2DM, Jockey, Fgi,* and *mariner* (Nagaraju, 2000).

With the advent of recombinant DNA technology, it became quite feasible to probe the occurrence and activity of non-coding regulatory DNA sequences like GATA and coding genic sequences like globin gene, *DAX1, SRY,* etc. *Bkm* sequences have been used as a probe to locate its presence on autosomes and sex chromosomes in various animal systems belonging to snakes, birds, mouse, and humans.

The female Banded Krait (*Bungarus fasciatus)* is the source of *Bkm* DNA sequences (Singh *et al.,* 1980). Further studies showed the presence of cross-hybridizing *Bkm* sequences in various eukaryotes from slime molds to humans (Singh *et al.,* 1984; Arnemann *et al.*, 1986). Cloned *Bkm* positive genomic fragments of Drosophila and mouse contain long clusters of tetranucleotide GATA repeats, as a component (Singh *et al.,* 1984). Further studies showed long stretches of GATA repeat alone giving similar hybridization pattern as did the original *Bkm* probe (Schaffer *et al.*, 1986; Traut 1987).

Bkm sequences exhibit a tendency to be concentrated on sex chromosomes thereby implicating their definite role in the sex determining process or in the allocyclic behavior and evolution of sex chromosomes (Jones 1984; Singh *et* al., 1984). They are predominantly located on the W-chromosomes of snakes (Singh *et al.,* 1980) and on the X-chromosome of *D. melanogaster* (Singh *et al.,* 1981). Similar studies in mice showed the presence of a cluster of *Bkm*

sequences in the proximal regions of the Y that is essential for the sex determination. Close linkage of *Bkm* to sex determination can be seen in Sxr mice in which sex reversal is regularly associated with an exchange of *Bkm* cluster to the X-chromosome (Singh and Jones, 1982). However, *Bkm* sequences are known to occur on autosomes as well in *Ephestia kuehniella* (Traut, 1987) and mouse (Kiel-Netzger *et al.,* 1984).

chapter IV

Materials and Methods

Materials

4.1 Bacterial Strains

1. **DH5α:** F X, *recA1 endA1 hsdR17* (r_k^-, m_k^+) D (*lacZYA-argF*) *U169* (*ϕ80 lacZ* d*M15*)*supE44 thi-1 gyrA96 relA1* (BRL, 1986).

2. **JM103** : *endA1, hsdR, SupE, SbcB15, thi-1, StrA, D(lac-pro), (F', tra D36, proAB, laclqZDM15),*(Hanhan,1983).

3. ***XL-Blue MR*** *A* ***(P2)*** *: (mcrA) 183 (mcrCB- hsdSMR- mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac (P2* lysogen).

DH5a was used for all the routine transformations, plasmid isolation and selection of recombinant plasmids etc.

4.2 Plasmid and Phages

1. **PBS KS(+)**: A high copy number plasmid derived from pBR322 (Messing, 1983; Yanish-Perron *et al*, 1985) was used in all the cloning experiments.

2. *X* **DasHII** : λ*sbh*λ1°*b*189 <T3 promoter-polycloning site srIλ3° *nin*L44 *boi* polycloning site-T7 promoter> KH54 *chiC srλ4° nin5 shn*dIIIλ6° srIλ5, *red*⁻*gam*⁻.

4.3 Clones/Probes

1. **BKm 2(8)** : A *Bkm* positive subclone of the Drosophila clone CS314 containing 545bp insert having 66 copies of tetranucleotide repeat GATA. The insert was cloned into the PstI/BamHI sites of M13mp9 (Singh *et al.,* 1981; 1984).

2. **Pϕ2 cDNA clone** : A Bkm positive human testis cDNA clone of 2.4kb. The insert was cloned into the EcoRI site of Bluescript Plasmid vector (Rajyashri, 1995).

4.4 Bacterial Media, Antibiotic and Solutions

1. Ampicillin : 100μg/ml solution in sterile double distilled water.
2. LB (Luria-Bertani medium) : 1.0% bactotryptone, 1.0% NaCl, 0.5% bacto-yeast extract ; pH adjusted to 7.0 with 0.1 N NaOH.
3. LB agar (bottom): LB containing 1.5% (w/v) bacto agar.
4. SOB : 2% bactotryptone, 0.5% bacto-yeast extract, 200μl of 5M NaCl, 150μl 1.0M of KCl, 1.0ml of 100mM $MgSO_4$ in a total volume of 100ml.
5. SOC : 200μl of 1.0 M glucose in 100ml SOB.
6. Top agar : LB containing 0.7% (w/v) bacto agar.
7. IPTG : 1.0M stock of isopropyl thio-β-D-galactoside in sterile double distilled water.
8. X-gal : 5.0mg/ml of 5-bromo-4-chloro-3-indolyl-p-D- galactoside in di-methyl formamide.
9. RF-I : 1.2% $RbCl_2$, 1.0% $MgCl_2$, 1.5% w/v glycerol, 0.15% $CaCl_2$, 3.0ml of 1.0M CH_3COOK (pH 7.5) ; pH was adjusted to 5.8 with 0.2M CH_3COOH and volume was made up to 100ml.
10. RF-II : 2.0ml of 0.5M MOPS [3-(N-morpholino) propane sulfonic acid] pH 6.8, 1.1% $CaCl_2$, 0.2% $RbCl_2$, 15% w/v glycerol; pH was made upto 6.8 with NaOH and volume was made upto 100ml.
11. TE : 10mM Tris-HCl (pH 7.5-8.0), 1.0mM EDTA.
12. DNA Extraction Buffer : 10mM Tris-HCl (pH 8.0), 0.1 M EDTA, 0.5% SDS and 20μg/ml RNase.

13. Denaturing solution : 0.5M NaOH, 1.5M NaCl.

14. Neutralising solution : 1 .0M Tris-HCl (pH 8.0); 1.5M NaCl.

15. 1.0 X SSC : 8.765gm of NaCl, 4.41 gm sodium citrate in double distilled water. pH was adjusted to 8.0 with NaOH and the volume was made up to one litre.

16. SM : 0.58% NaCl, 0.2% $MgSO_4$, 50mM Tris-HCl (pH 7.5) and 0.01mg gelatin in 100ml double distilled water.

17. Phosphate Buffered Saline : 8.0gm of NaCl, 0.2gm of KCl, 1.44gm of Na_2HPO_4 in 800ml distilled water, pH was adjusted to 7.4 with HCl and volume was made up to 1.0 litre with distilled water.

18. DNA Loading Buffer : 0.25% bromophenol blue, 0.25% xylene cyanol FF and 50% glycerol in water. Stored frozen at -20°C.

19. 10X TAE : 0.9M Tris base, 10mM EDTA, pH adjusted to 8.0 with glacial CH3COOH.

20. 10X TBE : 0.9M Tris base, 10mM EDTA, pH adjusted to 8.0 with boric acid.

21. TE : 10mM Tris-HCl (pH 8.0), 1 mM EDTA.

22. 25X TPE : 2.25M Tris base, 50mM EDTA, pH adjusted to 8.0 with 85% phosphoric acid (1.679g/ml).

23. Proteinase-K : 20mg/ml in H_2O.

24. Denhardt's Reagent : 5.0gm of Ficoll, 5.0gm of polyvinylpyrrolidone, 5.0gm of bovine serum albumin, and H_2O to 500ml.

4.5 Laboratory Animals

Different Inbred strains of silkworm were obtained from Seri-Biotech Research Laboratory, Bangalore. Inbred strains of Balbc mice was obtained from the animal house, CCMB, Hyderabad.

4.6 Chemicals

All the chemicals used in this study were obtained from commercial sources as listed below :

Agarose NA, β-Mercaptoethanol, BSA (Fraction V), Cesium chloride, Calcium chloride, DTT, Ethidium bromide, Formamide, Glycerol, Guanidinium thiocyanate, Heparin, HEPES, Lysozyme, Magnesium chloride, MOPS, N-Lauryl sarcosine, PEG 8000, Proteinase-K, SDS, RNase, DNase, Tween-20, Salmon sperm DNA, Rubidium Chloride and Tris base were purchased from Sigma chemical company. Acrylamide, Bis-acrylamide, Sodium acetate and TEMED were bought from BDH. Ficoll and Sephadex G-25 were obtained from Pharmacia. Nitrocellulose filter discs of 0.45μ and 0.22μ pore sizes were purchased from Millipore. *X* DASH-II vector Arms and the packaging extracts were purchased from Stratagene, Hybond-N, Hybond-N^+ membranes and filters and T4 DNA'Ligase were obtained from Amersham. Nick Translation Kits and Random Priming kits were obtained from Amersham and BARC. Restriction Enzymes were bought from New England Biolabs, Pharmacia, Bethesda Research Laboratories and Bangalore Genei. Sequencing kits were purchased from Promega. PHA was obtained from Welcome laboratories. Calf Intestinal Phosphatase, IPTG, X-gal and λHind III digested DNA markers; DIG-High prime DNA Labeling and Detection kit, Propidium Iodide, DAPI were all bought from Boehringer Mannheim. PCR kit, BiGDye cycle sequencing kit, were purchased from Perkin-Elmer Cetus Corporation. The Kination/ Dephosphorylation kit was obtained from BARC. Whatman filter papers, 3.0mm and 1.0mm were from Whatman International Ltd. X-ray films were obtained from Konica Corporation. Intensifying screens were purchased from DuPont. α-^{32}P-dATP was obtained from Amersham and from BARC. Bacto-agar, Bacto-tryptone and bacto-yeast extract were purchased from Hi-Media and Difco laboratories.

All other chemicals were purchased from local manufacturers and were of analytical grade.

Methods

4.7 Silkworm rearing

Rearing was done according to the standard procedure described by Krishnaswami (1978). Silkworms were reared on fresh mulberry leaves and maintained at Seribiotech Research Laboratory, Central Silk Board, West of Chord Road, Mahalaxmipuram, Bangalore.

4.8 Sterilization

All glassware's were sterilized by baking overnight at 250°C. Plastic wares were autoclaved at 15-lb/square inch pressure at 120°C for 20 minutes. Solutions were prepared in double distilled water or Milli-Q water, filtered through 0.45μ nitrocellulose filters and sterilized by autoclaving. Bacterial growth medias were sterilized by autoclaving.

4.9 Siliconization

For all manipulations involving minute quantities of nucleic acids the glassware's, eppendorf and gilson tips to be used were siliconized with 5.0% solution of dichloro-dimethyl-silane in chloroform. The siliconized labwares were heated at 80°C for 2 hours, rinsed thoroughly in double distilled water and autoclaved.

4.10 Construction of Silkworm Genomic Llibrary

4.10.1 Isolation of High Molecular Weight DNA from Posterior Silkgland

The tissue was homogenized in liquid nitrogen and 10 volumes of extraction buffer was added. RNase was added to a final concentration of 100μg/ml and incubated at 37°C for 1 hour. Then Proteinase-K was added to a final concentration of 100μg/ml and gently mixed. The suspension of lysed cells was

placed in a water bath for 3 hours at 50°C. The solution was cooled to room temperature and an equal volume of phenol equilibrated with 0.5M Tris-HCl (pH 8.0) was added and gently mixed by slowly turning over the tube for 10 minutes. The two phases were separated by centrifugation at 5,000g for 15 minutes at room temperature. The viscous aqueous phase was transferred to a clean centrifuge tube and the extraction with phenol was repeated twice. After the third extraction with phenol, the pooled aqueous phases were dialyzed at 4°C for four times against four litres of a solution of 50mM Tris-HCl (pH 8.0) and 10mM EDTA (pH 8.0) until the $O.D_{270}$ of the dialysate was less than 0.05. The absorbance of the DNA was measured at 260nm and 280nm. The ratio of A_{260} to A_{280} should be greater than 1.75.

4.10.2 Partial Restriction Enzyme Digestion of High Molecular Weight DNA

Partial digestion with restriction enzymes that recognize frequently occurring tetranucleotide sequences within eukaryotic DNA yielded a population that was close to random and yet could be cloned directly.

Pilot experiments were carried out to establish conditions for partial digestion of eukaryotic DNA. The aim was to maximize the yield of fragments whose size would be appropriate for insertion into a bacteriophage λ vector. After conditions had been established in pilot experiments, 100 μg of high molecular weight DNA was digested with the appropriate amount of Sau3A for the appropriate time. The digested DNA was gently extracted with phenol: chloroform twice. The DNA was precipitated by adding two volumes of ethanol at 0°C and redissolved in 200μl of TE (pH 8.0). 10%-40% continuous sucrose density gradient in a Beckman SW-40 Polyallomer tube (or its equivalent) was prepared. Sucrose solutions were made in a buffer containing 10mM Tris-HCl (pH 8.0), 10mM NaCl, 1.0mM EDTA (pH 8.0). Samples were heated for 10 minutes at 68°C, cooled to 20°C and loaded on to the gradient. The gradient was centrifuged at 22,000 rpm for 22 hours at 20°C in a Beckman SW-40 rotor (or its equivalent).

10µl of every other fraction was analyzed for the size of the DNA in each fraction by using 0.5% agarose gel electrophoresis and appropriate DNA markers. Following electrophoresis, the gradient fractions containing DNA fragments of the desired size (9.0-22 kb) were pooled. The pooled fractions were dialyzed against 2.0 litres of TE (pH 8.0) at 4°C for 12-16 hours, with a change of buffer every 4-6 hours. Dialyzed DNA was extracted several times with equal volume of 2-butanol until the volume was reduced to about 1.0ml. DNA was precipitated by adding ammonium acetate to a final concentration of 2.0M and 2.5 volumes of ethanol at room temperature. DNA was dissolved in TE (pH 8.0) at a concentration of 300-500µg/ml. An aliquot of the DNA (0.5µg) was analyzed by electrophoresis through a 0.5% agarose gel to check that the size distribution of the digestion products was correct.

4.10.3 Ligation

Partially digested genomic DNA (1.0µg) was mixed with 1.0µg of digested Lambda Dash II/BamH1 arms. To this, Ligase buffer (1X), 10mM rATP and one unit of T4 DNA ligase was added and incubated at 4°C overnight.

4.10.4 Packaging

Packaging extracts were used to package recombinant lambda phage with high efficiency, which increases the size of genomic libraries. Gigapack-11®-XL packaging extract is an in vitro packaging extract which preferentially size selects for extra large inserts while maintaining the highest packaging efficiencies commercially available. Gigapack-11 packaging extracts have been developed to increase the efficiency and representation of libraries constructed from highly methylated DNA. The packaging extracts were *hsd, mcrA⁻, mcrB⁻ and mrr⁻*.

Sonic extract (yellow tube) was removed from -80°C freezer (from the kit) and allowed to thaw on ice. Simultaneously the 2nd extract (red) tube (from the kit) was held between fingers until it just began to thaw. The DNA (1.0µl of ligation

mix) was immediately added to the freeze-thawed extract (red) tube and placed on ice. Quickly 15µl of the sonic extract was added to the freeze-thawed extract containing the DNA. The contents were gently mixed without introducing air bubbles and incubated at room temperature (22°C) for 2 hours. 500µl of SM buffer and 20µl of chloroform were added. It was mixed gently and spun briefly to sediment the debris. The supernatant became ready to be titred. It was stored at 4°C.

4.10.5 Preparation of Host Bacteria

The glycerol stock of XL1-Blue MRA (P2) was streaked on to the LB plate and incubated overnight at 37°C. 10ml of LB media supplemented with 10mM $MgSO_4$ and maltose (0.2% v/v) was inoculated with a single colony of *XL1-Blue MRA(P_2).* It was grown at 37°C, shaking for 4.0-6.0 hours (it was not grown past OD_{600} = 10). The bacteria were pelleted at 2,000 rpm for 10 minutes. The cells were gently resuspended in half the original volume of sterile 10mM $MgSO_4$ and diluted to OD_{600} = 0.5 with sterile 10mM $MgSO_4$

4.10.6 Titering Procedure

Dilutions of the final packaged reaction were made in SM Buffer. 1.0µl of the packaged material to 100µl of host cells diluted in 10mM $MgSO_4$ to OD_{600} = 0.5. 1.0µl of 1:10 dilution of packaged material in SM buffer was also added to 200 µl of host cells. The phage and bacteria were incubated for 15 minutes at 37°C to allow the phage to attach to the cells. 2.5-3.0 ml of top agar (48°C) was added and plated on LB plate, the phages were counted and the plaque-forming unit per millilitre (Pfu/ml) concentration of the library was determined as

$$\frac{\text{Number of plaques x dilution factor X total packaging volume (1000µl)}}{\text{Number of micrograms packaged X number of microlitres mix plated}}$$

Only the recombinant phage grew on XL1-Blue MRA (P_2). Plaques were visible after 8-12 hours of incubation at 37°C.

4.10.7 Amplification of the Library

It is usually desirable to amplify libraries prepared in lambda vectors to make a large stable quantity of a high titer stock of the library. However, more than one round of amplification is not recommended since slower growing clones may be significantly under represented.

The host bacterial cells were prepared and diluted to OD_{600} = 0.5 in 10mM $MgSO_4$. 600µl of OD_{600} = 0.5 cells were added per 150mm plate. Aliquots of the packaged library suspension containing ~50,000 plaque forming bacteriophage were mixed with 600µl of OD_{600} = 0.5 host cells in falcon tubes. For screening 1.0 x 10^5 plaques, 10 aliquots of 10,000 plaques each were plated. The tubes containing phage and host cells were incubated for 15 minutes at 37°C. 6.5 ml of melted top agar was mixed with each aliquot of infected bacteria and spread evenly onto a freshly poured 150mM plate of bottom agar. The plates were incubated at 37°C for 6-8 hours. The plaques were not allowed to get larger than 1.0-2.0mm.

The plates were overlayed with ~ 8.0-10ml of SM Buffer and stored at 4°C overnight with gentle shaking. The bacteriophage suspension was recovered from each plate and pooled into a sterile polypropylene container. Chloroform was added to a 5.0% final concentration. It was mixed well and incubated for 15 minutes at room temperature. The cell debris was removed by centrifugation for 10 minutes, at 2,000xg. The supernatant was recovered and transferred to a sterile polypropylene or glass bottle. Chloroform was added to a final concentration of 0.3% and stored at 4°C. The aliquots were stored in 7.0% dimethyl sulfoxide (DMSO) at -80°C. 1.0µl of the amplified library was checked using appropriate amount of host cells and serial dilutions of the library. The final titre of the library was found to be 10^9 pfu/ml

4.11. Preparation of Frozen Competent Cells

The competent cells were prepared according to Hanahan (1985) with slight modification. A culture of *E.coli* DH5α grown overnight in 1.0ml of SOB was inoculated into 100ml of SOB (1:100 dilution) medium and incubated at 37°C with vigorous shaking till the O.D. at 550 nm reached 0.6-0.7. The culture was chilled on ice for 15 minutes and the cells were pelleted by centrifugation at 4,000 rpm for 10 minutes at 4°C. The supernatant was drained thoroughly, the cells were resuspended in 0.33 volumes of RF-I. After keeping on ice for 2 hours the cells were pelleted down as before and resuspended in 0.5 volumes of RF-II and left at 4°C for further 15 minutes. Aliquots of cells (100μl) were distributed into eppendorf tubes, flash frozen in liquid nitrogen and stored at -70°C. Cells kept frozen for 2.0-3.0 years could also be efficiently transformed.

4.12 Transformation

The frozen competent cells were thawed slowly on ice just before use. The DNA (10-100ng) used for transformation in a maximum volume of 10μl was mixed with the competent cells and was incubated on ice for 40 minutes. 900μl of LB media was added to the tubes and incubated at 37°C for 1 hour with gentle shaking. Transformed cells (50-100μl) were plated on LB agar plates (LB media containing 1.5% bactoagar) containing 100μg/ml of ampicillin, 10mM IPTG and 25μg/ml X-gal. The plates were incubated at 37°C overnight and recombinant colonies picked up the following day. Bacteria carrying the recombinant plasmids of PBS (KS+) formed white colonies.

4.13 Precipitation of DNA

DNA samples were precipitated by addition of 0.1 volume of 3.0M Sodium acetate (pH 5.2) and 0.6 volumes of isopropanol or 2.5 volumes of distilled ethanol. Genomic samples were precipitated at room temperature whereas DNA samples of low molecular weight were precipitated by incubation at -20°C for 2 –12 hours. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes. The excess salt was removed by washing three times in

70% ethanol. The DNA samples were dried under vacuum and dissolved in appropriate volume of TE (10mM Tris-HCl, pH 7.5, 1 .0mM EDTA).

4.14. Large Scale Isolation of Supercoiled Plasmid DNA

Plasmid DNAs were prepared by the alkaline lysis method (Birnboin and Doly, 1979) described by Sambrook ef *al.* (1989) with minor modifications. Bacterial pellet from a culture grown overnight in 500ml LB containing appropriate antibiotic was washed in 100 ml of ice-cold STE (0.1 M NaCl, 10mM Tris-HCl pH 8.0, 1.0mM EDTA). The cells were resuspended in 20ml of solution I (50mM glucose, 25mM Tris-HCI pH 8.0, 10mM EDTA) containing 20mg of lysozyme. The contents of the tubes were mixed gently and incubated at room temperature for 10 minutes. 40 ml of freshly prepared solution-II (0.2 NaOH, 1.0% SDS) was added and the tubes were left on ice for 10 minutes. 20 ml of ice-cold 5.0M Potassium acetate (pH 4.8) was added to the above. The contents were mixed and chilled on ice for 15 minutes. The genomic DNA and bacterial debris were pelleted at 8,000 rpm in a Sorval SS-34 rotor for 30 minutes at 4°C. The plasmid DNA in the supernatant was precipitated with 0.6 volumes of iso-propanol at room temperature for 30 minutes. The DNA was pelleted at 10,000 rpm for 30 minutes at room temperature. The pellet was washed in 70% ethanol, dried under vacuum and dissolved in 8.0ml of TE (10mM Tris-HCI, pH 8.0, 1.0mM EDTA)

4.15. Small Scale Isolation of Plasmid DNA

The same protocol, as above, was scaled down according to the volume of bacterial culture used for isolation of the plasmid DNA. The DNA was dissolved in TE, treated with RNase and the proteins were extracted with phenol, phenol: chloroform and chloroform: isoamyl alcohol. The purified plasmid DNA was reprecipitated, washed in 70% ethanol, dried and dissolved in TE.

4.16 Purification of Plasmid DNA For Sequencing

Plasmids were purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation. 4.2gm of cesium chloride was added to 4.2ml of the plasmid in TE and ethidium bromide was added to a final concentration of 400μg/ml. The solution was centrifuged at 72,000 rpm in a Beckman VTi80 rotor for 5 hours at 18°C. The lower band containing supercoiled plasmid DNA was collected in a syringe fitted with a 21-gauge needle. The ethidium bromide was extracted with water saturated butanol and the plasmid DNA was dialyzed against TE (pH 8.0) for 12-16 hours at 4°C. The DNA was precipitated, washed twice with 70% ethanol, dried and dissolved in TE (pH 7.5). All purified plasmids were stored either at -20°C (long term) or at 4°C (short term).

4.17 Small Scale Isolation of Plasmid DNA for Sequencing

Overnight grown culture (3.0ml) was spun at 12,000rpm for 1 minute. The pellet was resuspended in 100μl of double distilled water. 100μl lysis buffer (1% SDS, 10mM EDTA, 0.1 N NaOH) was added. The sample was boiled in a boiling water-bath for 2 minutes (puncture tops with a fine needle to avoid popping). 50μl of 1.0M $MgCl_2$ was added and mixed by tapping. It was kept on ice for 2 minutes and spun at 12,000rpm for 2 minutes. In the same tube, 50μl of 5.0M potassium acetate was added mixed by tapping, kept on ice for 2 minutes and spun at 12,000rpm for 2 minutes (pellet was not disturbed). The supernatant was transferred in a fresh tube. 600μl of isopropanol was added, kept on ice for 5 minutes and finally spun at 12,000rpm for 2 minutes. Finally the pellet was washed with 70% alcohol, dried and dissolved in TE or water.

4.18 Preparation of Cells for Electroporation

Single colony of DH5a was inoculated in 10ml of LB media and grown at 37°C with shaking. 5.0ml of overnight grown culture of DH5a was added to 500ml of LB and grown till $O.D_{600}$ = 0.6 - 0.8 (do not exceed 0.8). The cells were spun down and washed with equal volume of water in 500ml (1 .0X). It was spun at

10,000 rpm for 5 minutes at 4°C. The supernatant was discarded, washed and resuspended the pellet in 250 ml (0.5X) of Milli Q H_2O. It was spun and the pellet was resuspended in 125 ml (0.25X) of water. The cells were pelleted by spinning at 10,000 rpm for 15 minutes at 4°C. 1.0ml of 10% glycerol was added. The pellet was resuspended in glycerol and was collected by spinning at 10,000rpm for 10 minutes. Finally the cells were dissolved in 0.002 volume (1.0ml) of 1% glycerol. Aliquots of 40µl each were made. It was flash frozen in liquid nitrogen and stored at -70°C.

4.19 Electroporation

Ligation mix (1µg/µl in H_2O or low salt TE) was added to 40µl aliquot of DH5a cells into the cuvette and the cuvette was flicked to mix and settle the cell mixture. The cuvette was chilled on ice for 1 minute. The Electro Cell Manipulator 600 was set at 129 ohms resistance (R5), and 2.45 KV charging voltage. The desired field strength was set at 12.25 kV / cm and finally an electric pulse of 5-6 milli seconds was given. Immediately after giving the electric pulse, 960µl of LB was added into the cuvette. The mixture was transferred into an eppendorf tube and incubated at 37°C for 1 hour.

4.20 Elution of DNA from Agarose Gel using Freeze Thaw Method

The agarose gel piece was cut out and put in an eppendorf tube. Sufficient volume of Tris-saturated phenol was added so that the gel piece was completely submerged. It was frozen in -70°C for 2 hours (immediately). From -70°C, it was transferred to -20°C for 30 minutes. After 30 minutes, it was spun immediately at 12,000rpm for 10 minutes. The aqueous phase was taken out in a separate eppendorf tube and phenol: chloroform and chloroform extraction was carried out. The aqueous phase was precipitated with 1/10th volume of 3.0M Sodium acetate and 2.5 volumes of absolute alcohol. The pellet was washed with 70% alcohol twice and dissolved in TE.

4.21 Nick Translation

Plasmid DNAs were radiolabeled by Nick translation as described by Rigby *et* a/., (1977). The reaction was carried out in 50µl volume containing 200-300 ng of DNA, 0.5mM of each unlabeled dNTP, 30-40µl of α^{32}PdATP, 50mM Tris-HCl pH 7.2, 10mM MgCl2, 0.1 mM DTT, 2.5µg nuclease free BSA, 50pg DNasel and 5.0 units fo E.coli DNA Polymerase-I. After 90 minutes of incubation at 15°C, the reaction was stopped by adding EDTA to a final concentration of 10mM. 10µg of sheared and denaturated *E.coli* DNA was added as carrier and the DNA precipitated at 0°C overnight. Subsequently, the probe was pelleted by centrifugation at 10,000rpm for 15 minutes. The unincorporated radioisotope was removed by washing the pellet three times in ethanol. The probes were vacuum dried and dissolved in TE (pH 8.0).

4.22 Multiprime Labelling

50-200 ng of double stranded DNA was denatured in a boiling water bath for 10 minutes followed by chilling on ice. 75ng of random hexanucleotides, 5.0µl of 10x reaction buffer (900mM HEPES adjusted to pH 6.6 with NaOH, 10mM $MgCl_2$ and 40mM DTT), 4mM each of unlabeled dNTPs, and 50-60µCi of a- ^{32}P-dATP and 2.0 units of Klenow (E.coli DNA polymerase - large fragment) were added to the DNA in a total volume of 50µl. The samples were incubated at 37°C for 2 hours. The reaction was stopped and the probe was prepared as above.

4.23 Measurement of Radioactivity in Nucleic Acids

The radiolabeled probe (1.0µl) was spotted on a piece of nylon membrane, dried and washed in ice cold 10% trichloroacetic acid. After 10 minutes, the filter was rinsed in ethanol at room temperature for 10 minutes. The filter was air dried, immersed in scintillation fluid (0.5% PPO, 0.03% POPOP in toluene) and the percentage of incorporation of radioactivity determined by counting in a Packard scintillation counter. Usually nick translation results in probes with a

specific activity of 2.5x10 cpm/μg while the probes prepared by random priming or single strand synthesis are of ten-fold higher specific activity.

4.24 DNA Sequencing

Nucleotide sequencing was performed using the di-deoxy termination method of Sanger (Sanger *et* a/., 1977) modified by Chen and Seeburg (1985). Cycle sequencing was carried out with the help of M13 universal primers and custom made oligos. Sequencing was carried out with the ultrapurified supercoiled plasmid DNA. Big Dye terminator cycle sequencing kit (Perkin Elmer) provided the reaction mix containing all the dNTP's and the differentially labeled four deoxynucleotides along with Taq DNA Polymerase in the sequencing buffer. Four deoxynucleotides were labeled with different fluorescent dyes.

The reaction mix made by mixing 8.0μl of terminator ready reaction mix (Perkin Elmer sequencing manual), 5.0pmoles of primer and 500ng of plasmid DNA in a final reaction volume of 20 μl. Cycle sequencing was carried out in thermal cycler, Gene Amp PCR System 9600, by first giving a rapid ramp to 96°C and holding at this temperature for 30 seconds. This step was followed by rapid thermal ramp to 50°C and holding for 15 seconds. Last step was rapid thermal ramp to 60°C and holding for 4 minutes. The cycle consisting of the above three steps was repeated 30 times. After 30 cycles, the reaction was rapidly brought to 4°C. The unincorporated dye terminators were removed by ethanol precipitation.

The pellet was resuspended in 4.5μl of loading buffer (deionized formamide and 25mm EDTA pH 8.0, in a ratio of 5:1). The sample was vortexed, spun, heated at 95°C for 2 minutes to denature and placed immediately on ice. The samples were loaded on a pre-electrophoresed 4% polyacrylamide gel. Electrophoresis was carried out for 10 hours at a voltage of 2 KV and at the current of 50mA. The temperature of the gel was maintained at 15°C.

4.25 Sequence Analysis

Raw data collected after electrophoresis was analyzed using Sequence analysis and Autoassembler software (Perkin Elmer).

4.26 Restriction Enzyme Digestion of DNA

Restriction digestion of plasmid DNA samples was carried out with 2.5 units of restriction enzyme per μg of DNA. For digesting genomic DNA up to 5.0 units of restriction enzyme per μg of DNA was used. The buffers and incubation conditions for digestion were followed as given by the manufacturer. While digesting genomic DNA, spermidine was also added to a final concentration of 2.0mM. Most of the digestions were stopped by incubating at 65°C for 15 minutes and only if the need arose, phenol: chloroform purification was performed.

4.27 Dephosphorylation of Vector DNA

The vector DNA digested with appropriate restriction enzyme was purified by phenol: chloroform method. The supernatant was ethanol precipitated and dissolved in TE. This linearized vector was dephosphorylated using calf intestinal phosphatase (CIP) as per the protocol of Tabor (1989). To 1.2μg of linearized vector, CIP dephosphorylation buffer (composition of 1X **CIP** dephosphorylation buffer: 1.0mM ZnCl2, 1.0mM $MgCl_2$ and 10mM Tris pH 8.3), and 0.1 unit of CIP were added and incubated at 37°C for 30 minutes.

CIP was inactivated by heating at 75°C for 10 minutes in the presence of 5.0mM EDTA. Further purification was done by phenol: chloroform extraction. Since, the acidic pH could lead to the precipitation of EDTA, sodium acetate of neutral pH (pH 7.0) was used for ethanol precipitation. The precipitate was washed with 70% alcohol and redissolved in TE (pH 8.0).

4.28 Agarose Gel Electrophoresis and Southern Transfer

Agarose gels were used for restriction digest analysis and for checking the quality of DNA samples. 10μg of genomic DNA was digested to completion with 4.0 units/μg of appropriate restriction enzyme at the recommended temperature for 16-20 hours. The digested DNA samples were mixed with an appropriate volume of 6X dye (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) and size fractionated by electrophoresis in 0.8-1.2% agarose gel in TPE (15mM Tris-HCl pH 7.2, 18mM sodium dihydrogen phosphate, 2.0mM EDTA) at 2.0-5.0 volts/ cm. After ethidium bromide staining (0.5μg ethidium bromide/ml of TPE), the DNA was visualized under UV, photographed and depurinated with 0.25N HCl for 15-20 minutes. Southern transfer (Southern, 1975) was done by capillary transfer on the vacuum blotting procedure of Olszewska and Jones (1988).

4.29 Vacuum Blotting

The DNA was transferred to Hybond- N^+ membrane at a constant vacuum of 70mm Hg using a vacuum blotting assembly. Transfer was carried out first in denaturing solution (0.5N NaOH, 1.5M NaCl) for 40 minutes, followed by neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 1.0mM EDTA) for 2 hours. The Hybond-N^+ membrane was used directly for hybridization after air-drying without baking.

4.30 Southern Hybridization

Blots were prehybridised in a solution of 0.5M sodium phosphate buffer (pH 7.5) and 7.0% SDS at 60-65°C for 2 hours. 200μl of prehybridization mix was used per sq.cm. of filter area. Hybridization was carried out in the fresh mix of the same composition containing ^{32}P labeled probe (specific activity 10 - 10^9 cpm/μg) with a probe concentration of 1-5x10 ^{8} cpm/ml at 60-65°C (depending on the sequence composition) 16-24 hours.

4.31 Post-Hybridization Washing and Autoradiography

Low stringency wash: The blots were washed twice in 2.0X SSC+0.1% SDS at 60°C for 30 minutes each.

High stringency wash: The blots were washed thrice in 0.1XSSC+0.1% SDS at 65°C for 30 minutes each.

After washing, the blots were rinsed thrice in 2.0XSSC at room temperature for 5 minutes each to remove SDS. The blots were exposed to X-ray films at -70°C for varying lengths of time in X-ray cassettes with intensifying screen.

4.32 Isolation of Total RNA

Total RNA was prepared from various tissues using the guanidinium isothiocyanate method of Chemozynski and Sacchi (1987).

4.32.1 RNA Isolation from large amounts of Tissue

1.0 gm of tissue was homogenized in 10 ml of ice cold GITC (4.0M guanidinium isothiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarkosyl, 0.1 M p-mercaptoethanol) in a glass homogenizer. 0.1 volume of 2.0M sodium acetate (pH 4.0) 1.0 volume of water saturated distilled phenol and 0.2 volumes of chloroform: isoamyl alcohol (49:1v/v) mixture was added initially on ice with thorough mixing after the addition of each reagent. The final suspension was shaken vigorously for 1-2 minutes, incubated on ice for 15 minutes and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was dissolved in 3.0ml GITC and extracted again with an equal volume of phenol and 0.2 volumes of chloroform: isoamyl alcohol (48:1 v/v). The RNA was re-precipitated with an equal volume of isopropanol at -20°C for 4-6 hours. The RNA was pelleted as before, washed three times with 70% ethanol, vacuum dried and dissolved in sterile double distilled water /formamide. The quality of the RNA preparation was checked by electrophoresis using a 1% agarose gel in TBE (0.089M Tris, 0.089M boric acid and 2.0mM EDTA) containing 0.5μg/ml

of ethidium bromide. The RNA was quantitated by estimating its absorbance at 260nm using a spectrophotometer.

4.32.2 Preparation of mRNA

Poly (A)+ RNA was separated from the total RNA using oligo (dT) cellulose column chromatography as described by Sambrook *et al*. (1989) with minor modifications. Columns plugged with autoclaved glass wool were packed under gravity with oligo (dT) cellulose (presoaked for 10-12 hours in sterile double distilled water). The oligo (dT) cellulose column was washed in 0.1N NaOH till the pH of the flow through was greater than 10, following which; it was equilibrated with 1.0X binding buffer (10mM Tris-HCl pH 7.5, 0.5M lithium chloride, 1.0mM EDTA) till the pH of the flow through reached 7.5. Total RNA in 1.0X binding buffer was denatured by heating at 65°C for 10 minutes before loading. It was applied to the column at a concentration of 1 .0mg/ml. The flow through was collected, denatured and loaded again on the column. After 4.0-5.0 cycles of loading, the column was washed with 5.0-10 column volumes of 1.0X binding buffer till O.D. of the eluate at 260nm reached zero. The column was then washed in 1.0X wash buffer (10mM Tris-HCl pH 7.5, 150mM lithium chloride, 1mM EDTA) till the O.D. of the eluate at 260nm reached zero. The poly (A)+ RNA bound to the column was finally eluted with elution buffer (2.0mM EDTA pH 7.5). Elution was continued till the O.D. of the eluate at 260nm reached zero.

The poly (A)+ RNA was precipitated with 0.1 volume of 3M sodium acetate (pH 4.0) and 2.5 volumes of ethanol at -20°C for 8-12 hours. The RNA was collected by centrifugation at 12,000 rpm for 20 minutes at 4°C. The precipitate was washed once in 70% ethanol, vacuum dried and dissolved in sterile double distilled water. Poly (A)+ RNA used for Northern blots were purified twice by oligo (dT) cellulose column chromatography.

4.32.3 Preparation of DNA free RNA

All RNA samples used for reverse transcription were treated with RNase free DNase before use. Total RNA (100-500μg) in a buffer containing 0.1M sodium acetate (pH 5.0) and 1mM magnesium sulphate was treated with 20-100 units of RNase free DNase at 25°C for 2 hours. The DNase was removed by digestion with proteinase-K (100mg/ml) at 37°C for 2 hours followed by phenol, phenol: chloroform (1.0:1.0) and chloroform: isoamylalcohol (24:1.0) extractions. The RNA was then precipitated with 0.1 volume of 3.0M sodium acetate (pH 4.0) and two volumes of isopropanol at -20°C overnight. When the amount of RNA was less than 10μg, 20-50μg of *E.coli* tRNA was added as carrier. The RNA was centrifuged at 10,000 rpm at 4°C for 1 hour and the precipitate was washed in 70% alcohol, vacuum dried and dissolved in sterile double distilled water.

4.32.4 Northern Blotting and Hybridization

40μg of total RNA in a buffer containing 1.0X MOPS (40mM morpholino propane sulfonic acid pH 7.0, 10mM sodium acetate, 10mM EDTA), 50% formamide and 6.5% formaldehyde were denatured at 65°C for 15 minutes. 0.2 volumes of the loading dye (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol 1mM EDTA) was added and the samples were loaded on a 1.0% agarose gel containing 2.2M formaldehyde. After electrophoresis the gel was washed thoroughly in sterile double distilled water and soaked in 10X SSC for 15 minutes. RNA was transferred to Hybond N^+ membrane in 10X SSC by capillary transfer or using the vacuum blotting apparatus. The excess salt was removed by washing the membrane in 2XSSC. The membrane was air dried and baked under vacuum at 80°C for 2 hours.

Blots were prehybridized in 0.5M sodium phosphate buffer (pH 7.5), 7.0% SDS at 65°C for 4 hours. The hybridization mix was changed, 100 μg/ml of sheared and denatured *E.coli* DNA was added and incubation was continued at 65°C for a further 3-4 hours. $3\text{-}5 \times 10^6$ cpm/ml of denatured radiolabeled probe was

added to the hybridization mix and incubation was further continued for 16-20 hours at 65°C.

Blots were washed twice in 2.0X SSC, 0.1% SDS at 65°C for 20 minutes, followed by washing in 0.5X SSC, 0.1% SDS for 15 minutes. The blots were exposed for autoradiography.

4.32.5 RNA Dot/ Slot Blot

Requisite amount of RNA was made up to 100µl in sterile double distilled water. An equal volume of denaturing mix [20X SSC: formamide (6:4)] was added and the samples were denatured at 65°C for 10 minutes. The denatured samples were chilled on ice and blotted on to Hybond-N^+ membrane (presoaked in 10XSSC for 20 minutes) under vacuum. The slots/dots were washed with 200µl of 10XSSC. The membrane was air dried and baked under vacuum at 80°C for 2 hours. The blot was then prehybridized and hybridized under conditions identical to those used for hybridization of Northern Wots.

4.33 Construction ofTC-T cDNA Library

4.33.1 Isolation of RNA

Total RNA was isolated from 1st instar larva using the guanidium isothiocyanate method. Poly (A)+ RNA was separated from the total RNA using oligo (dT) cellulose column chromatography as described by Sambrook *et ai,* (1989).

4.33.2 First Strand cDNA Synthesis

Poly (A)+ RNA (1 .0µg) was heat denatured at 70°C for 5 minutes. First strand of cDNA was synthesized using TX primer (2.0-3.0 p moles) The mixture of RNA and primer was heated at 70°C for 10 minutes and cooled to 25°C for 5 minutes. To this mixture, 4.0µl of First strand synthesis buffer and 200 units of AMV Reverse Transcriptase were added. Water was added to make up the

volume to 20µl and incubated at 42°C for 50 minutes. The enzyme was inactivated by heating at 75°C for 5.0-10 minutes.

---------- AAAAAA poly (A)+ RNA
---------- TTTTTT XXXX cDNA TX primer

4.33.3 Spin Column Purification of cDNA

The reaction mix was purified by amicon column (80k-100k). The aliquot containing cDNA was diluted to 2.0ml with distilled water and loaded on 100k-amicon column. Column was centrifuged twice with 2.0ml-distilled water to wash the cDNA product. Finally the purified cDNA aliquot was collected (20µl).

4.33.4 Oligo dG Tailing

Oligo dG tailing of the cDNA was carried out using terminal deoxynucleotidyl transferase (TdT). To 10µl of cDNA template 3.0µl of 5X TdT buffer (50mM sodium cacadylate pH 7.2, β-mercaptoethanol, 1.0mM CaCl2) 200µM GTP and 2.0 units of TdT were added. The final volume was made to 15µl with water and incubated at 37°C for 30 minutes. After 5 minutes, an aliquot of 5.0µl was taken out from the reaction mix and chilled on ice. The same process was repeated after 15 and 25 minutes.

— GGGG———————— I I I I I IXXXXXX cDNA with poly dG tail

4.33.5 PCR Amplification With TC and T Primers (Hot Start PCR)

To 3.0-4.0µl of oligo dG tailed cDNA, 10pmoles of TC primer 20-30 pmoles of T primer, 2.5 units of Taq polymerase and PCR buffer (4.0mM tricine KOH, pH 9.2 at 20°C, 10mM potassium acetate, 3.0mM $MgCl_2$, 50µg/ml BSA) and 200µmoles of each of dNTPs were added. Final volume was made up to 50µl with distilled water.

1st cycle of PCR:.(94°C - 30 seconds, 53°C - 1 minute, 72° - 2 minutes, 53°C - 10 seconds, 72°C-2 minutes).

18 cycles of: 94°C - 10 seconds, 56°C - 20 seconds, 71 °C - 2 minutes.

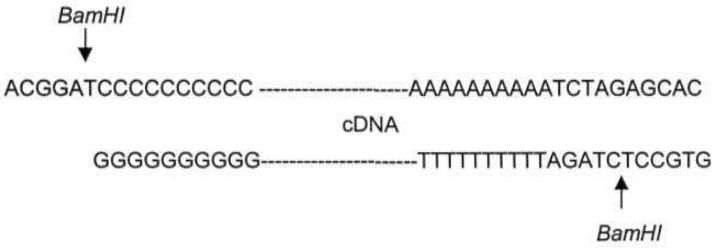
Final cycle: 94°C - 10 seconds, 56°C - 20 seconds, 71 °C - 2 minutes, 56°C - 20 seconds and 71 °C - 2 minutes.

Finally, 5.0 units of pfu Taq was added and elongation at 72°C was carried out for 5 minutes.

Primers used for construction of TC-T library

T primer (21 bp): GTGCCTCTAGATIIIIIIIII
TC primer (35bp): GTGCCTCTAGATIIIIIIIIGGATCCCCCCCCCC
C primer (16bp): ACGGATCCCCCCCCCC
X primer (19bp): GGCCAACGGTATGGTGTGC
TX primer (36bp): GGCCAACGGTATGGTGTGCCTCTAGATIIIIIIIII

4.33.6 Restriction Digestion of the cDNA Product and Vector



Restriction enzyme digestion of cDNA was carried out using 15-20 units of BamHI enzyme. 1.0μg of cDNA in a 100μl reaction was digested using the appropriate buffer and incubated at 37°C for 2 hours. The plasmid vector PBS KS(+) was also digested in similar fashion. Reactions were stopped by incubation at 65°C for 10 minutes. An equal volume of distilled phenol at room temperature was added to this mixture, vortexed for 3-5 minutes, centrifuged at

5,000 rpm for 15 minutes at room temperature and the upper aqueous phase collected. Phenol: chloroform (1:1 v/v) and chloroform: isoamylalcohol (24:1 v/v) extractions were carried out. The DNA was precipitated, washed twice in 70% ethanol, vacuum dried, dissolved in 10µl of TE (pH 7.5).

4.33.7 Ligation

Ligation was carried out at 16°C for 20-24 hours in 10µl reaction volume containing ligation buffer (50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 10mM DTT and 1.0mM ATP).

4.34 Detection of RNA by Fluorescence *In Situ* Hybridization

4.34.1 Subbed Slide Preparation

Slides were soaked in 0.1 N HCl for 0.5 to 1 hour, rinsed in distilled water three times and finally soaked in 95% ethanol for overnight. Subbing solution was prepared by adding 5.0gm of gelatin to 1.0 litre of distilled water (40-50°C). To this, 450mg of chromium potassium sulfate was added and filtered through Whatman no.1 filter paper. Completely dried slides were placed in subbing solution for 2 minutes and then dried in a refrigerator for 30 minutes. After 3-4 rounds of subbing, slides were completely dried and stored at room temperature.

4.34.2 Tissue Fixation

Tissues were fixed in 4.0% paraformaldehyde dissolved in 0.1 M phosphate buffered saline (PBS), pH 7.4. Three microns thick tissue sections were made from paraffin embedded tissues on gelatin-coated slides. The slides were baked at 42°C for 72 hours and the modified protocol of Lawrence *et* a/, 1988 was followed. Tissue sections (3.0µm) were dewaxed with xylene for 30 minutes and cleaned with 100% ethanol (2 x 5 minutes). Slides were washed in PBS twice for 5 minutes each. Then the slides were extracted with 0.01%

TritonX-100 in PBS for 3 minutes. The sections were over-layed with 10μg/ml Protinase-K (freshly diluted in PBS from a 20mg/ml stock) and left for 5 minutes. The excess liquid was removed and washed in 2.0mg/ml glycine solution (in PBS) for 5 minutes. The slides were post-fixed in 4% paraformaldehyde for 5 minutes and washed thrice in 1.0xPBS for 5 minutes each. The slides were then passed through graded alcohol series i.e., 70%, 80%, 90% and finally in absolute alcohol for 10 minutes each and air dried.

4.34.3 *In Situ* Hybridization

Tissues were prehybridized with 25μl of hybridization solution (50% deionized formamide, 10% dextran sulphate, 0.5% SDS, 2.0xSSC, 50mm phosphate buffer, 5.0X Denhardt's solution, 100μg/ml yeast tRNA) in a humid chamber for 3hours at 45°C. After draining, the slides were overlayed separately with 25μl of fresh hybridization solution containing 1ng/ml Digoxigenin labeled cDNA probe (Probes were labeled by PCR using Boerienger and Mannhem kit), sealed under a 22 x 50mm coverslip and incubated in a humid chamber at 45°C overnight (Sammer and Tantz, 1991).

4.34.4 Detection

After careful removal of the coverslip slides were washed twice in 2.0X SSC at room temperature. These slides were further washed in 0.5X SSC and 0.1X SSC, three times in each solution at 45°C (15 minutes for each wash). After thorough washing, the slides were rinsed in 1.0XPBS and then kept for blocking in 1.0X blocking solution at 37°C for 2 hours. Blocking solution was removed and the slides were overlayed with anti-Digoxigenin antibody and incubated at 37°C for 1 hour. Finally the slides were washed three times in 1.0XPBS+0.2%Tween-20 at 37°C and finally mounted in propidium iodide and antifade and scanned using a confocal microscope.

4.35 Chromosome preparation from Silkworm Embryos

Fertilized eggs (48 hours old) were collected and washed in 0.05% sodium hypochlorite for 15 minutes. The eggs were washed thoroughly with distilled water and then finally with insect ringer solution. The eggs were crushed in ringer solution. Colchicine was added to the final concentration of 0.001% and incubated at 37°C for 2 hours. The cells were spun at 1,000 rpm for 10 minutes, supernatant was removed and the pellet was resuspended in 0.075 M KCl. After incubation at 37°C for 45 minutes, immediately two drops of fixative (3:1, methanol: acetic acid) was added to stop the reaction. It was spun at 1,000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in chilled 3:1 (methanol: acetic acid) fixative. The cells were washed atleast 7-8 times with the fixative. Finally the cell pellet was resuspended in a small volume of fixative. 1-2 drops of cell suspension were placed on clean glass slides. Slides were air dried and stored at 4°C.

4.36 Chromosomal Fluorescence *In Situ* Hybridization (FISH)

The slides were baked at 65°C for 1 hour. RNase (100μg/ml in 1.0XPBS) was added on to the slide, covered with a coverslip and incubated at 37°C for 1 hour in a humid chamber. The slides were rinsed twice in 2.0XSSC, 10 minutes each at room temperature. Excess water was drained off and proteinase-K was added at a concentration of 2.0mg/ml for 2 minutes. The slides were immediately rinsed in the 1.0XPBS (3 times - 5 minutes each). The chromosomes were fixed on to the slide by putting the slides in 50mM $MgCl_2$ (in 1.0XPBS) for 10 minutes followed by 10 minutes incubation in PBS containing 1.0% formaldehyde and 50 mM $MgCl_2$.

The slides were washed in 1.0X PBS thrice, 10 minutes for each wash and then passed through graded alcohol series of 70%, 80%, 90% and absolute alcohol. The slides were air dried and baked at 65°C for 1 hour. The slides were denatured in 70% formamide and dehydrated by passing through alcohol series i.e. 70%, 80%, 90% and finally absolute alcohol.

The probe was denatured at 85°C for 10 minutes. The denatured probe along with the hybridization mix (hybridization mix contained 100ng of DIG-labeled DNA probe, 10% dextran sulfate, sheared *E.coli* DNA as competitor, 50mM phosphate buffer, 2.0X SSC, 5.0% Denhardts, etc) was added on to the slide and hybridization was carried out at 42°C for 16 hours.

After the hybridization, slides were washed at appropriate stringency and incubated for 1 hour at room temperature with 50 ml 1.0% blocking solution in a coplin jar to block non-specific antibody binding. After blocking, the slides were drained on a Whatman paper. Working concentration of first antibody (anti-DIG) was added on the slide and covered with a coverslip. It was incubated for 1 hour at 37°C in a humid chamber. The slides were washed three times shortly in washing buffer (1XPBS with 0.01% Tween-20). The slide was then incubated with working solution of antibody-2 (anti DIG mouse-lg-DIG) for 1 hour at 37°C in a humid chamber. The slides were washed three times in washing buffer and then working concentration of antibody-3 (anti-DIG fluorescein) was added on to the slide, covered with a coverslip and incubated for 60 minutes at 37°C in a humid chamber. Finally the slides were washed thoroughly (3-4 times for 10 minutes each) at 37°C in washing buffer. The slides were then mounted in glycerol containing propidium iodide (5.0-10 μg/ml), DAPI (0.2-0.5μg/ml) and antifade.

4.37 Databases Scanned For Sequence Analysis

http://www.ncbi.nlm.nih.gov.
http://bdgp.org-flybase
http://www.ebi.ac.uk
http://www.ab.a.u-tokyo.ac.jp-Silkbase

chapter v

isolation and characterization of Bkm 2(8) Positive silkworm Genomic clones

(Results and Discussion)

RESULTS

5.1 Strategy

Bkm sequences are found in almost all eukaryotes studied till date and in most cases they are predominantly localized on the sex chromosome. In Snakes, which have female heterogamety (ZZ/ZW), *Bkm* sequences are predominantly concentrated along the length of the W-sex chromosome. Silkworm also displays female heterogamety. Therefore, it was anticipated that *Bkm* sequences might similarly be concentrated on the W-chromosome of silkworm and screening of silkworm female genomic library with *Bkm 2(8)* might lead to isolation of gene(s) or sex-specific sequences closely associated with the sex-determining region of sex chromosome. Presence of *Bkm* $(GATA)_n$ sequences in silkworm genome was already proven by using Bkm2(8) as a probe for characterization of silkworm races(Nagaraju *et a*/., 1995; Nagaraju and Singh, 1997). It was therefore proposed to screen the genomic library with *Bkm* ***2(8).***

5.2 Screening of Silkworm Genomic Library with *Bkm 2(8)*

Silkworm genomic library was constructed in λ DasHII/ BamHI vector and screened with P labeled *Bkm 2(8)* probe. *Bkm 2(8)* was radiolabeled by primer extension using M_{13} primers. About one-lakh plaques were plated on five 120mm plates at a density of 20,000 PFU/plate. The plaques were transferred to Hybond- N membrane, denatured, neutralized and immobilized. These membranes were hybridized with *Bkm 2(8)* for 16 hours and washed at a stringency of 0.5 x SSC + 0.1% SDS at 60°C. After primary screening, twenty-five positive clones were obtained which were further purified and confirmed by secondary and tertiary screening. After tertiary screening, three clones out of 25 positive clones were selected at random for further characterization. The clones were designated as $A_{1.2}$, $C_{1.2}$ and $C_{2.1}$.

5.3 Analysis of *Bkm 2(8)* Positive Silkworm Genomic Clones

The three genomic clones selected were first restriction analyzed to find out whether they were all the same or different. These clones were digested with different restriction enzymes like SalI, SacI, PstI, EcoRI, run on 1% agarose gel, transferred to Nylon membrane and hybridized with *Bkm 2(8)* probe. All the three clones were found to be different, as they did not show identical restriction pattern and size of the positive fragment after hybridization was also different (Fig. 5.1 & 5.2).

5.4 Subcloning and Sequencing of *Bkm 2(8)* Positive Silkworm Genomic Clones

The smallest size positive fragments obtained using these restriction enzymes were subcloned into Bluescript plasmid vector digested with appropriate restriction enzymes (table. 5.1).

Table- 5.1

Bkm 2(8) positive genomic clones (digested with different restriction enzymes)

S.No.	Clone name	Size of insert	Fragment cloned/Enzyme
1.	$A_{1.2}$	15Kb	4.5Kb, 3Kb & 3.5Kb - SacI
2.		15Kb	800bp - SacI
3.	$C_{2.1}$	16Kb	3.5Kb and 2.2Kb - SalI

These genomic subclones were sequenced using M13 forward and reverse primers and Big Dye terminator cycle sequencing kit. All the subclones except two $C_{2.1}$-2.2Kb/SalI, and $C_{1.2}$-800bp/SacI were found to contain $(GATA)_n$ repeats, GATA [GAA $(GATA)_3]_3$ $(GATA)_{11}$. However, it is an imperfect repeat. The $(GATA)_n$ loci were flanked by AT rich SAR (Scaffold Attachment Regions) sequences. In the subclone $C_{2.1}$–3.5Kb/SalI, another microsatellite locus $(GAGT)_n$ was found to be present along with $(GATA)_n$. $(GAGT)_n$ is also an

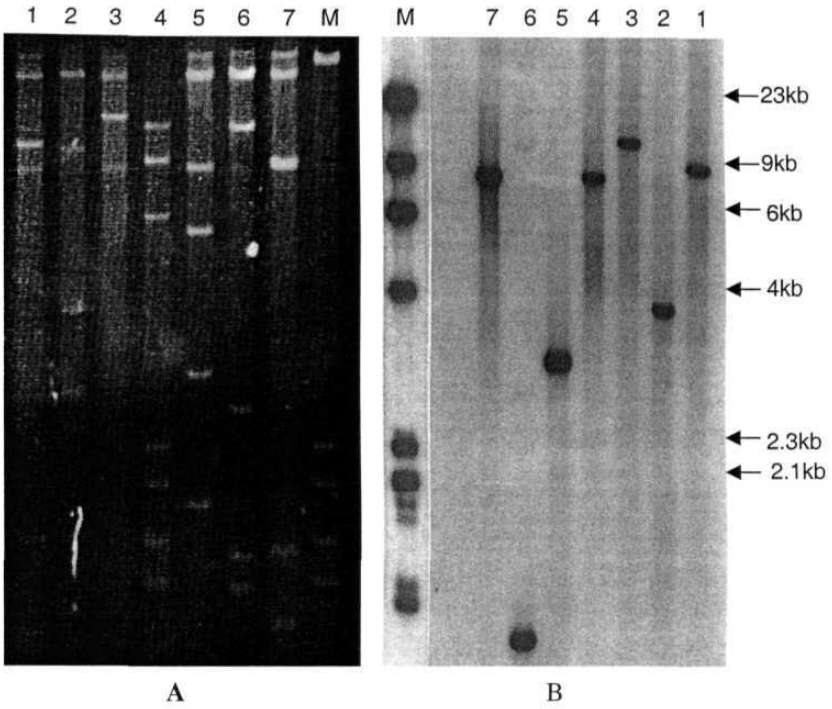


Fig:5.1. Southern hybridization of GATA positive silkworm genomic clones with ^{32}P labeled *Bkm2(8)*

3μg of Phage DNA was digested with different restriction enzymes, electrophoresed on 0.4% Agarose Gel (A) and hybridized with radio labeled *Bkm2(8)* at 60°C **(B).**

Lanes- 1: A, $_2$ digested with SalI, 2: A, $_2$ digested with SacI, 3: A, $_2$ digested with ECoRI, 4: A1.2 digested with PstI, 5: $C_{1\,2}$ digested with SalI, 6: $C_{1\,2}$ digested with SacI, 7: $C_{1\,2}$ digested with ECoRI, M: Marker.

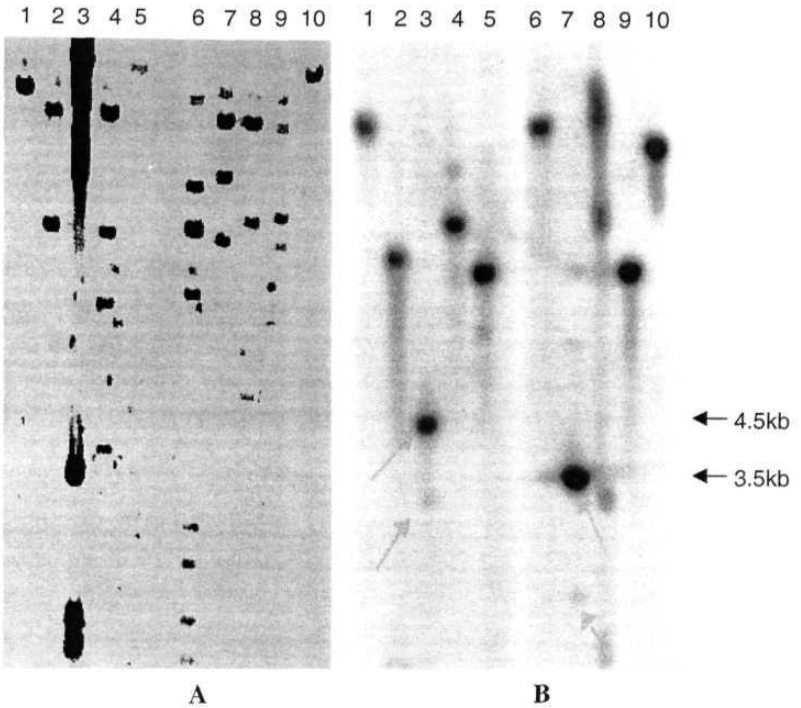


Fig. 5.2. Southern hybridization of GATA positive silkworm genomic clones $A_{1\,2}$ and $C_{2.1}$ with ^{32}P labeled *Bkm2(8)*

3µg of Phage DNA was digested with different restriction enzymes, electrophoresed on 0.4%Agarose Gel (A) and hybridized with radio labeled *Bkm2(8)* at 60°C **(B).**

Lanes- 1: $A_{1.2}$ uncut DNA, 2: $A_{1\,2}$ digested with SalI, 3: $A_{,\,2}$ digested with SacI, 4: $A_{1\,2}$ digested with ECoRI, 5: $A_{1\,2}$ digested with PstI, **6:** $C_{2\,1}$ uncut DNA, 7: $C_{2\,1}$ digested with SalI, 8: $C_{2\,1}$ digested with SacI, 9: $C_{2\,1}$ digested with ECoRI, 10: $C_{2.1}$ digested with PstI.

example of imperfect repeat i.e., $[GATT(GAGT)_{41}]_2GAAT(GAGT)_4$. All these clones were further subcloned using frequent cutters like DraI and ClaI.

5.5 *In Silico* Analysis of 1769bp *Bm(GATA)$_n$*

Nucleic acid database search using 1759 bp query sequence showed many hits because GATA sequences have been reported from most of the eukaryotes. The top three hits in silkworm show homology with the query sequences in the region flanking the GATA sequences (Fig. 5.3). There was no hit showing homology with the query sequence in GATA rich or GAGT region from silkworm suggesting that existence of GATA microsatellite or GAGT microsatellite loci have not been reported in silkworm (table. 5.2).

Table- 5.2

Various hits obtained from database using 1759bp query sequence

Database	Clone name	Score	P/N
Genbank-Silkbase	*Bombyx mori* gene for silk protein	927	2.8e-36
Genbank-Silkbase	*Bombyx mori* gene for Nd-sD mutant	770	3.2e-25
Genbank-Silkbase	*E. Kuchniella* repeat region	506	2.2e-15
Genbank-Silkbase	*Xenopus leavis* transcription factor	507	8.4e-13
Genbank-Silkbase	*D. melanogaster* DNA, GATA-4 tandem	484	2.3e-14
Genbank-Silkbase	MouseSxr (Bkm-like) DNA, sex determining	415	4.4e-11

All the hits obtained from silkworm EST database show 85-95% homology with the query sequence in the region 1026-1365 bp present between the two-microsatellite loci. Most of these ESTs are tissue/stage specific (table. 5.3).

ATCGATTTAAGAATACAAGAACAAATTATATCCAAACTATAAAACACCGT
ATGTTTTCAATAAAGAAACCGAGCGACCATCGAACGTCGGATCACGAGAT
GGAAAGACAATCAAGACATAAATTCGATGCCAATAGTTTCTATGTTATTA
CCTTCCAATACGAACTGATCGTTCATATCACGCAACCCAAAAAAAACGAA
ATAGTGATCGTGTACCAATCATACCGTACAGGAATGCCTTTTTAAGTTGT
TCGGAATCGTGATTCTTATTGTGTGCCCTTACTATACTCGTGAGTGAGTG
AGTGAATGATTGATTGAGTGAGTGAGTGAGTGATTGAGTGAGTGAGTGAG
TGAATGAGTGAGTGATACCACCATTGAACGTCTAGTTTCTTTGACAGAAA
CCTTGGGTATGGATCGAAATGTGAAATGTTTGAGTTAATAAAATCACGGA
GCAAAGACCGGTATATTGATAGTTAAGACTAACTATTCAACTATAAAACA
TACTGTTTTAACTAAAAACTACTTTTACGATTTAATCTCCCATTCATTGT
CATTGGTTGCGACCTTTAGAATACTTCAGAGTTTTCATGTATACCTAAGG
ACAATTAAGATGGGCCTGCTATTCGTGGACTATTGAATATTGTGATAACT
GCTGTTGAATTTTCTAACTTAACAGTAGAGGTAGTTTAATTGTATCGAGT
CACGATTCCATTTGCTAGCGTATTAAAATTATGTTATTACTATTATTTAT
TAGCTACATGTGCTGTCAGTTGGTGCCGAGTAGTCAGTGAGAGTCATAGA
TAAGACTACGCGAAATCTCAGTTCATTGCATTTGGAATAACGACTGTGTT
TAGCTTCAAAGCAGAACGCATATTTTTTGGACGAGCTCACAGGCCACCTC
GTGTTAAGTGGTTACTGGAGCCCATAGACATACAACCGAAATCACCACCC
ATTCATCCGCCACCCATCTTAAGATATAAGTTCTAAGGTGTCAGTATAGT
TACAACGGCTGCCCCACCCTTCAAACCTACTCGTGCGGACTCACAAGAGG
TCCTACCACCAATAAATTTATATTTATTATTAGCTGACCCGGCAGACTTC
GTAGTGCCTCAATCGATAAATAAAAAGATCTAAACTTTTGTATAAAATAAA
CTAAAACAAACAAAAGGTATCCGTCCGACGGGGGACACATCAAAGGTAAA
ACAAAATTGTTATTTTTATTTAATTCCGAGCATTTTCATATTTATCTACC
TTTTAAACCTTCTCTGGACTTCCACAAATAATTCAAGACCAAAATTAGCC
AAATCGGTCCAGCCGTTCTCAAGTTTTAGCGAGACTAACGAACAGCAATT
CATTTATATATACTATATATAGATAGAAGATAGATAGATAGAAGATAGAT
AGATAGAAGATAGATAGATAGAAGATAGATAGATAGATAGATAGATAGAT
AGATAGATAGATAGATAGATGTATTTACTTATGCAGACGCACGATAGGCC
ACGCTATCAGTAAAAGAAGAAAAAAAAACATTAAACATTCTCATATAACA
AATATAAATAAAACTTACCCAAGTCCATAGTGTACCCGCAACCAGTGCCC
ACGTCATTGGATTCGTCCACTACATTCTGTAGGTAGTCAAATACGTTCCA
CTGCTGTTCTCTGGCAGCGTAGTCAAAGGCAGTCAGCCCGTCGAGGTCCC
GTACATAGGGATCTCCTCCGCAGCCGAGCAAGAGTTTCAGGTTGTCGACG
GTATCGAT

Fig. 5.3. Bkm(8) positive Silkworm **genomic** clone showing **$(GATA)_{22}$** repeats , **$(GAGT)_{12}$** repeats (bold) and SAR motifs (underlined).

Table-5.3

Various hits obtained from EST database of silkworm using 1759bp sequence

Database	Clone name	Score	P/N
Silkbase-EST	Wd V30052	1379	1.3e-63
Silkbase-EST	br—1036	1374	5.5e-58
Silkbase-EST	mg—0721	1344	1.3e-56
Silkbase-EST	fbVm 0413f	1317	2.3e-55
Silkbase-EST	wdV 30968	1187	1.7e-49

To find out the coding potential of 1759bp sequence, we carried out ORF search in the NCBI database. However, all the ORFs obtained were very small in size coding for a very small protein of 40-60 amino acids. These proteins did not show significant homology to any of the known proteins in the database.

5.6 *In Silico* Analysis of 1228bp *Bm(GATA)$_n$* Flanking Sequence

Nucleic acid database search, using 1228 bp query sequence showed only 4 to 5 hits (table. 5.4).

Table-5.4

Various hits obtained from Silkworm database using 1228bp query sequence

Database	Clone name	Score	P/N
Genbank-Silkbase	*B. mori* gene for silk protein	837	2.2e-37
Genbank-Silkbase	8. *mori* gene for Nd-sD mutant	740	1.8e-26
Genbank-Silkbase	*B. mori* gene for cuticle protein	678	5.5e-35
Genbank-Silkbase	*B. mori* gene for *Xanthine dehydrogenase*	442	4.9e-15

Most of the EST hits obtained using 1228bp query are tissue/stage specific. However, only a small stretch of 310bp i.e., 430 to 740bp shows 85-90% homology to these sequences. Rests of the flanking sequences do not show homology to any sequence in the database suggesting that it is unique (Fig. 5.4).

To find out whether this 1228bp sequence contains any potential Open Reading Frames (ORF), ORF search was carried out using ORF Finder Program of NCBI. The biggest ORF was found in region 541-834 in +1 reading frame, coding for 97 amino acid protein. However, the protein coded by the ORF does not show significant homology to any known protein in database. All the other ORFs found in different reading frames are very small in size coding for 30-40 amino acids.

5.7 Characterization of *Bm(GATA)$_n$* Subclone

To find out the distribution of $(GATA)_n$ sequences in silkworm genome, southern hybridization of male and female genomic DNA digested with different restriction enzymes was carried out using ^{32}P labeled 500bp *Bm(GATA)$_n$* subclone as a probe.

Digestion of genomic DNA with HinfI, AluI and PstI and hybridization with radiolabeled *Bm(GATA)$_n$* at low stringency (60°C -2XSSC + 0.1%SDS), yielded a single major band of equal intensity in both the sexes along with few minor common bands (Fig.5.5 & 5.6). The size of the major band was smaller with the 4 base cutters, 2.5kb with HinfI and 2.8kb with AluI, while it was 10kb with PstI, a 6 base cutter. No polymorphism was seen between different individuals/sexes when probed with ^{32}P labeled $(GATA)_n$. The *Bm(GATA)$_n$* probe is 500bp long with 100bp long GATA stretch flanked on each side with 200bp long AT-rich sequences. Use of such a probe for localization of GATA sequences in silkworm genome may result in false signal because of flanking sequences, which are locus specific. Cross hybridization of this $(GATA)_n$ clone with human genomic DNA was carried out as a control.

(A)

GTCGACGCGGCCGCGTAATACGACTCACTATAGGGCGAAGAATTCGGATC
TCACAAAACGCCTAACCACCAGTAAAACTGACTTGACAACTTAAAAATAA
CTAAACATAAAAACCGCATATTTTTATTGTCCTTTACGGCCATCTGGTGG
TGACTGGTTACTGTAGCTCATGAATGCTCAGCAATGCCAGGGGTAGAGAC
GTCACGAGAGCTACTTTTAAGTTTTTTATTTTTATTTTGTGTTCAAATAC
AGTAATTCTAAGTTACCCAACGTATTTTAGGGTTCGATTACGAACGATAA
TCTAATAAATGGAACTGATGACCGTTAAGTTCACGGAATATTATTGATTT
GATTCACATTCCAATGGCGTAATTTGTACAAAGCTATTGTCTTTGAAAAT
GAATATACATTTTACAGTAAACATATAGTGTTTTGAATTGAATTTTATTT
ATTACTAGCTGACCCGGCAGACTTCGTAGTGCCTCAATCGATAAATAAAA
GGCTTAAACTTTTGTATAAAATAAACTTAAAACAAACAAAATGAATCCGT
CCGATGGACGGACGGACACATCAAAGGGAAAACAAAAGTGTTATTTTTAT
TTAATTCCGAGCATTTTCATATTTATCTACCTTTTAAACTTTTTCTGGAC
TTCCACAAATAATTCAAAACCAAAATTAGGCAAATCCGTCCAGCCGTTCT
CGAGTATTAGCGAGACTAACGAACAGCAATTCATTTTTATATGTATAGAT
TTATACGCGAGTGACGAATACGCATTGTGGACATTTTATAGAATGCTTTT
GAAGTTACTCAATGTTCAAAGAAGGAATAATTGACAAACAATGCATTCTT
CATTTGTCATATAGTTCACTCGGCCTGTACAATGAACATTGTCAGCTGCC
CGTATCGAAGATCAGAGGTTAACGCGAGCCACTGCGAGATAATAAAAGTT
CAATTCAATTATGTTTTTCTTGGTAACTGCATTGAAAAATGTTTGATAGT
TTGAGATTAGTTAAGGAAGGGTGAATAGGTCATTTAAGTCATATTCAACG
TTCGTCCAAAAGGAAAAGATAAACATAGTAGTAAAAAACAACTCAGCTGT
GTTTATGTTGTTTATTTATTGCGCATTTTTTCCCCTACCAATGATCACAA
TCCCCCTACCAAATTCTCAAGAGCTGCTAAGATTGATTATTTATTCTGAA
TCGAATTTAAAATGACAAAGTAATCGAT

(B)

CTACAAACGTTAGGGTTACGCTGAAATTGCCCCTAAGGCTATCAGCTT
AGGTAGGAAAAAAAAAAAAACAAAAACAAAAAAAAAATTGCTTTTTGG
GATGAGACACATTTATCATGTACGTACCTATGTCTATGACGGACGTGT
TCAATTAAAACGTTGTTGGTTTGTCACTTTAATGCAACAGTCAATTTC
CAAGTAGGATATGAAAATTTAACCGGATGTTTTTGTTAAACGGGATTA
ATGAAAAAGTTCATATACACAATTATTAAAAAGTCATTTTGTGTTATT
GCTTTAAAAGCACAACATTTGGGCCTGCTGTTAACTCTGGAAATTTAA
TATAGCAAGGGTAAGAAATATTAAAAGATGAAA

Fig. 5.4. Both A and B are the sequences from the GATA positive silkworm genomic clones.They are present adjacent to the GATA rich region but do not contain GATA repeats.

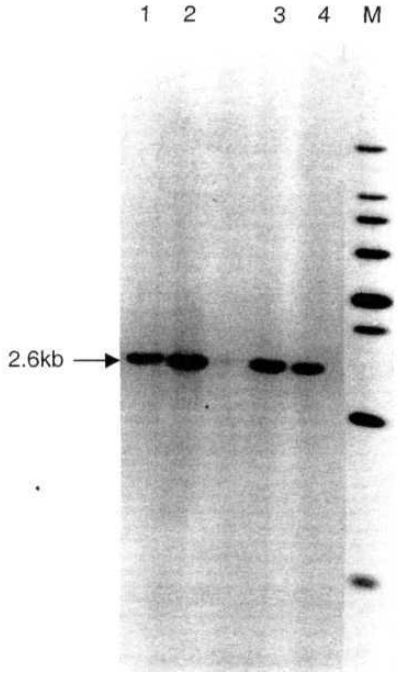


Fig.5.5. Southern hybridization of silkworm genomic DNA with *Bm(GATA)$_n$*

10μg of genomic DNA was digested with HinfI, electrophoresed on 0.8% agarose gel and hybridized with ^{32}P labeled *Bm(GATA)$_n$* subclone.

Lanes- 1&2: silkworm male genomic DNA/HinfI, 3&4: silkworm female genomic DNA/HinfI, M: 1kb ladder

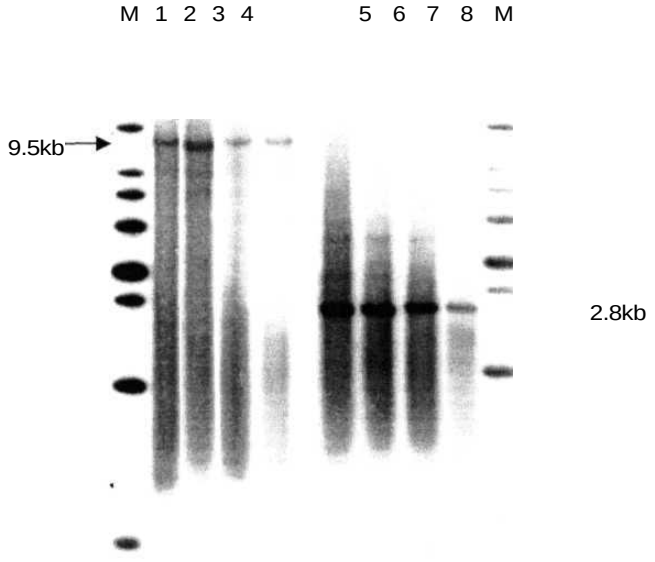


Fig.5.6. Southern hybridization of silkworm genomic DNA with ***Bm(GATA)$_n$***

10μg of genomic DNA was digested with PstI and AluI electrophoresed on 0.8% agarose gel and hybridized with ^{32}P labeled *BmGATA* subclone.

Lanes- 1&2: silkworm male genomic DNA/PstI, 3&4: silkworm female genomic DNA/PstI, 5&6: silkworm male genomic DNA/AluI, 7&8: silkworm female genomic DNA/AluI, **M:** 1 kb ladder

When *Bm(GATA)$_n$* subclone was radiolabeled and hybridized with human Genomic DNA digested with frequent cutters like HaeIII, BstN1, the hybridization pattern was indicative of GATA being uniformly distributed both on autosomes as well as sex chromosomes in the genome and the polymorphism between the individuals was also clear (Fig. 5.7).

5.8 Characterization of (GATA)$_n$ Microsatellite Loci

After sequencing the (GATA)$_n$ containing subclones primers were designed from the sequences flanking GATA microsatellite loci

Forward primer - 5' TAGCGAGACTAACGAACAG 3' - 19 mer
Reverse primer - 5' TACTGATAGCGTGGCCTAT 3' - 19 mer

After PCR amplification, two bands of 100bp and 170bp were seen which were further cloned and sequenced. However, only 170bp product was found to contain (GATA)$_n$ stretch (Fig. 5.8).

5.9 Characterization of (GAGT)$_n$ Microsatellite Loci

Primers were digested from the sequences flanking (GAGT)$_n$ microsatellite loci

Forward primer - 5' TCGTGATTCTTATTGTGTGC 3'
Reverse primer - 5' TTCGATCCATACCCAAGT 3'

After PCR amplification, two products of size 400bp and 192bp were seen. No polymorphism was seen between different diapausing and non-diapausing strains of silkworm (Fig. 5.9).

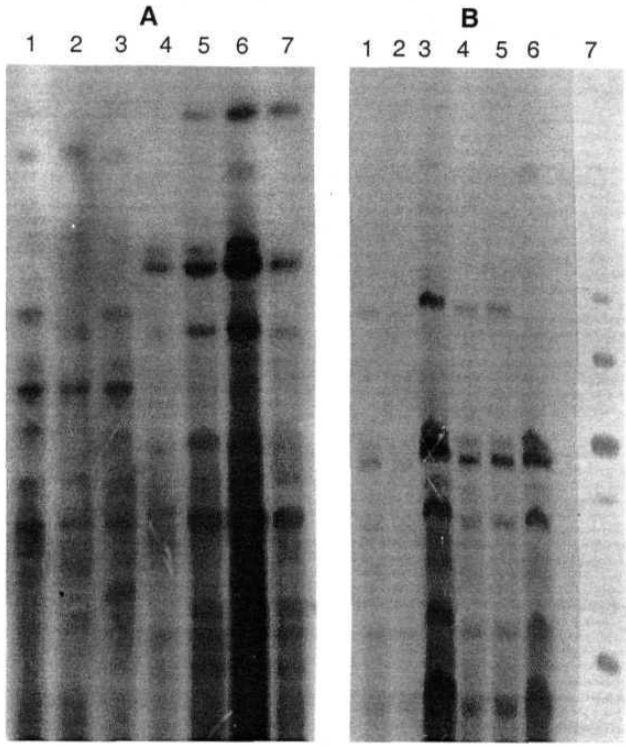


Fig.5.7. Southern hybridization of ^{32}P labeled 500 bp $(GATA)_n$ subclone of silkworm to human genomic DNA.

15 μg of genomic DNA digested with HaeIII and BstN1 was electrophoresed on 0.8% agarose gel and hybridized with $(GATA)_n$ at 60°C and washed at a stringency of 2XSSC + 0.1% SDS. Note the pattern of hybridization is indicative of $(GATA)_n$ being a repetitive sequence uniformly distributed in the genome.

A: Lanes 1-4 : random human samples/Hae III
Lanes 5-7 : random human samples/BstN1

B: Lanes 1-2 : twins/HaeIII
Lanes 3-6 : family with twin sons (4&5)/HaeIII, (3&6)/HaeIII are father and mother respectively
Lane 7 : marker

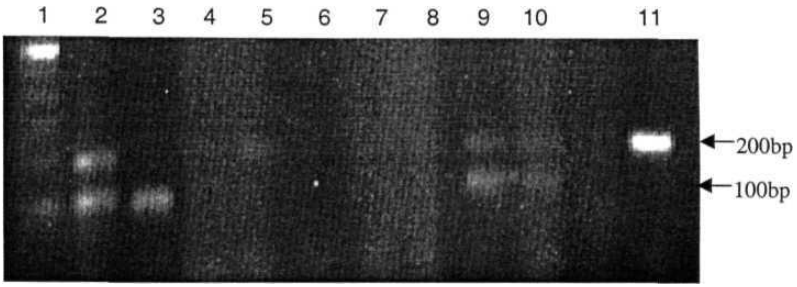


Fig. 5.8. PCR amplification of GATA repeats from genomic DNA of various diapausing and non-diapausing strains.

50 ng of genomic DNA of different silkworm species was amplified with primers flanking the GATA repeats for 30 cycles.

Lanes- 1: 100bp ladder, 2: *B.mori*, 3: *B.mandarina*, 4: *P.cynthia ricni*, 5: *A. assamensis*, 6: *A.proylei,* 7: *A.roylei,* 8: *A.mylitta*, 9: *A.pernyi*, 10: *A. yamamai,* 11: *B.mori.*

Note: there is very faint amplification in *P. cynthia ricni* and no amplification in *A.* roy/e/and *A. mylitta*

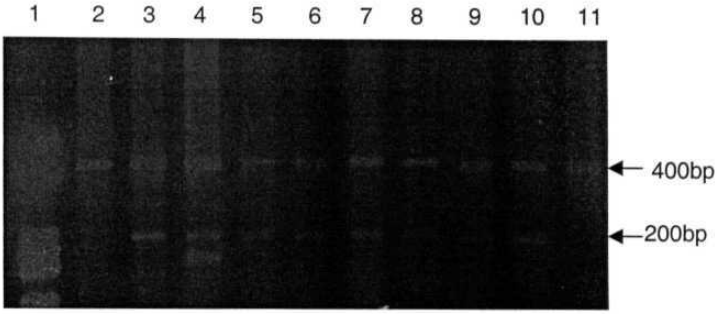


Fig.5.9. PCR amplification of GAGT repeats from genomic DNA of various diapausing (D) and non-diapausing (ND) strains.

50 ng of genomic DNA of different silkworm species was amplified with primers flanking the $(GAGT)_n$ repeats, for 30 cycles.

Lanes- 1: Marker V, 2: Nistari (ND strain), 3: KA (D strain), 4: NB_7 (D strain), 5: Sarupat (ND strain), 6: NB_1 (D strain), 7: *C. Nichi* (ND strain), 8: NB_{18} (D strain), 9: Hu_2O_4 (D strain), 10: NB_4D_2 (D strain), 11: Nistari (ND strain)

Discussion

In the present study, *Bkm* was used as a probe to study the occurrence and distribution of *Bkm* sequences in male and female genome of silkworm by southern hybridization technique. The present approach was based on the findings of Nagaraju *et al.* (1997), which showed the presence of *Bkm* sequences in silkworm and suggested its use as RFLP probe for estimating the genetic diversity among diapausing and non-diapausing strains of silkworm.

The basic premises of the given experiments with *Bkm* sequences were as follows:

1. If *Bkm* sequences were located on the autosomes alone then the southern hybridization pattern would remain identical in the genomes of both male and female silkworm.

2. If the *Bkm* sequences were located on the sex chromosomes, WZ/ZZ alone, then the southern hybridization pattern of male with ZZ constitution would be different from the hybridization pattern of female with WZ constitution.

3. If the *Bkm* sequences were distributed on both autosomes and sex chromosomes, then the southern hybridization pattern may or may not be different between male and female, depending on the fact whether these sequences are preferentially and predominantly concentrated on a specific region of the W-chromosome or not.

The present study also revealed that GATA sequences were flanked by AT-rich regions. AT-rich regions are known to represent SAR (Singh *et al.,* 1994). Association of SAR with GATA is proposed to play a definite role in decondensation of genetic loci inactive in somatic cells and active in germ cells. The proposed role of GATA binding protein (BBP) in this process is to cause decondensation of the GATA associated regions of chromosomes for

transcriptional activation (Singh *et al.*, 1984) in germ cells. Since GATA repeats present in silkworm are also flanked by AT rich regions, it is suggested that similar mechanisms of BBP dependent GATA mediated activation of GATA associated genes may occur in silkworm. Sequences flanking the GATA repeats are transcribed as they show homology to several ESTs from the silkworm database. However, the GATA repeats as such are not transcribed.

The results of occurrence and distribution of GATA repeats in the male and female genome of *B. mori* shows an identical pattern of hybridization in both the sexes with no apparent polymorphism. Evidently, distribution of GATA sequences does not seem to be predominantly concentrated on the W-sex chromosome in the silkworm. This is contrary to snakes, in which *Bkm* sequences are predominantly concentrated along the length of the W-chromosome. The obvious inference is that, perhaps *Bkm* sequences are distributed throughout the genome without any preferential concentration on a specific chromosome or chromosomal region. The silkworm shows ZZ/ZW system of chromosomal sex-determination where female sex promoting genes are located on the autosomes and male promoting genes are on the Z-chromosome (Traut, 1987; Traut, 1999). The present result showing absence of preferential concentration of *Bkm* sequences on the sex chromosomes suggests probable localization of GATA sequences on autosomes proposed to contain female sex-determining genes. Such localization of GATA sequences as a regulatory sequence for sex determining genes is quite in agreement with its role in sex-determination of heterogametic females and homogametic males. Such an interpretation is also supported by similar observations and interpretation made by Traut, *et al.* (1987) in *Ephestia kuehniella,* a moth with silkworm type of chromosomal sex determination. The present finding also confirms the observation of Sharma *et al.,* (1999) that *Bkm* sequences are not distributed in a sex-specific manner. A similar relationship of an autosomal location of *Bkm* (GATA repeat) and sex-determining factors has been proposed in mice where *Bkm* sequences in addition to being preferentially concentrated in the sex-determining region of the short arm of the mouse Y-chromosome are concentrated near T-locus /MHC gene cluster on mouse chromosome-17b,

approximately in a region where deletion produces hermaphrodites (Kiel-Metz-Ger *et al*., 1984).

Further, support to the present observation comes from reports on identical content and distribution of *Bkm* sequences in males and females of primitive snake (Singh *et a*/., 1999). The apparent absence of preferential concentration of *Bkm* sequences (GATA) in W-chromosome of silkworm indicates that perhaps such sequences have not played an important role in heterochromatinization and heteromorphic evolution of W-chromosome in silkworm. This is contrary to the observation in snakes as reported by Singh and his group. However, present observation only excludes preferential concentration of GATA repeats on the W-chromosome in the silkworm. It does not exclude its presence on the W-chromosome in limited copy number. This can be confirmed only after making W-chromosome specific library and sequencing the entire W-chromosome DNA. It is very pertinent to mention that in humans no sex-specific pattern is seen on southern blot using *Bkm* probe although many STRs on the human Y-chromosome are actually Bkm (GATA) repeats.

Heterochromatinization and heteromorphism of W-chromosome in silkworm is as pronounced as that found in W-chromosome of snakes and birds (Traut, 1999). Since heterochromatic regions of chromosomes are known to contain many copies of repeated sequences, the highly heterochromatic W chromosome of silkworm must result due to the presence of repetitive sequences other than the GATA repeats. The present findings therefore, suggest molecular dissection of silkworm W-chromosome in order to identify and characterize the supposed repetitive sequences present in it.

The chromosomal mechanism of sex determination (CSD) in XYO/XXO system and WZO/ZZO system of insects has been the subject of intensive studies. *M.Scalaris,* a dipteran member is known to contain a mobile male determining gene 'M' of epistatic nature. It moves like a transposon from one homologous pair to another pair with a characteristic transposition frequency thereby

creating new XY pair with every transposition. This system has provided a model for studying the primary nature of sex determining genes and also to examine the degeneracy of newly created *V* as a function of age (Traut, 1994). Evidences have been presented that newly created male determining Y-chromosome always remains associated with GATA sequences (Traut, 1994). This observation again implicates a definite role of GATA sequences in maintaining functionally male determining character of 'Y'. The next chapter dealing with identification and characterization of *Pø2* gene in silkworm will provide a summary view of the known and hypothetical mechanism of sex-determination in various animal systems.

The present and previous findings on the role of GATA sequences in sex determination leads to the following conclusions:

1. *Bkm* or GATA sequences are preferentially associated with the sex-determining region of the sex chromosomes or autosomes or on both and perhaps play an important regulatory role by bringing about chromatin conformational change.

2. Role of W-chromosome in female sex determination does not seem to suggest requirement of GATA sequences in silkworm and *Ephestia* (lepidoptera). However, until W-chromosome is sequenced, complete absence of GATA from W-chromosome and its role in W-chromosome evolution cannot be completely ruled out. This observation raises the question regarding the mechanism by which W-chromosome of silkworm, *Ephestia* or any other female heterogametic system exercises its epistatic influence on female development. This aspect of W-chromosome activity would be discussed in some detail in the next chapter dealing with characterization of silkworm homologue of human *P02* gene.

Chapter VI

Isolation and characterization of Genomic and cDNA clones of BmPΦ2

(Results ani Discussion)

RESULTS

6.1 Objective

Pø2 is a novel gene present on human X-chromosome region Xp11.23. It is highly conserved in both the sexes in all the vertebrates and invertebrates studied. Protein coded by *Pø2* gene belongs to WD family of proteins. The presence of *P02* gene on 'P' arm of X-chromosome and its abundant transcriptional activity in testis of mouse suggests that it may be involved in complex pathway of sex determination and differentiation. Since silkworm is a holometabolous insect with a life span of 50 days and shows female heterogamety, it was decided to use silkworm as a model system to study the structure and function of *Pø2* gene in silkworm.

6.2 Screening of the Library

Female silkworm genomic library was constructed in *X* DasH II/BamHI vector with an average insert size of 9-22Kb. The library had a titer of $10^{9\text{-}10}$ PFU/ml. The library contained only recombinant clones, as *X* DasH II is a replacement vector and the *X* arms without insert DNA cannot be packaged. The *E. coli* host strain used for infecting the phage was *MRA* P_2.

Fifteen filters containing 3×10^6 plaques were screened with ^{32}P labeled human *P02* cDNA clone. The filters were hybridized with radiolabeled human *Pø2* cDNA clone at 60°C for 16 hours and washed at stringency of 0.5X SSC + 0.1% SDS for 30 minutes. Twenty plaques, which hybridized to *Pø2* with varying intensities, were picked up after primary screening. These plaques were subjected to secondary and tertiary screening. Three plaques showed hybridization even after tertiary screening. These three genomic clones were designated as 4.3.1, 7.1.1 and 19.1.2 and digested with NotI to release the insert (table 6.1).

TABLE. 6.1

Pϕ2 Positive genomic clones digested with NotI

Clone name	Size of Insert
4.3.1	16 Kb
7.1.1	16 Kb (approx.)
19.1.2	14 Kb (approx.)

The clone 4.3.1 showed maximum intensity of hybridization to *Pø2* cDNA clone and clone 7.1.1 and 19.1.2 showed very weak signal when hybridized with *Pø2* suggesting that they contain a very short stretch of DNA which is homologous to *P02.*

6.3 Characterization of 4.3.1

Genomic clone 4.3.1 was digested with rare cutters like NotI to release the insert. The size of the insert was found to be 16Kb (Fig. 6.1). About 2-3μg of phage DNA was digested with different restriction enzymes, like EagI, EcoRI, HaeIII, HindIII, SacI, SalI, etc., run on 0.4% agarose gel and transferred to Hybond-N membrane by capillary transfer. The membrane was hybridized with ^{32}P labeled *Pø2* cDNA clone. The positive fragments were subcloned into Bluescript plasmid vector digested with appropriate enzymes (Fig. 6.2). Silkworm genomic DNA digested with HindIII was hybridized with 4.3.1 genomic clone to confirm that 4.3.1 represents a fragment of Silkworm genome (Fig 6.3).

6.4 Characterization of 7.1.1 and 19.1.1

Genomic clone 7.1.1 was also analyzed by digesting the clone with different restriction enzymes like EagI, EcoRI, HaeIII, HindIII, MnlI, SacI, SalI, SmaI and XhoI, etc. About 2.0-3.0μg of digested phage DNA was run on 0.4% agarose

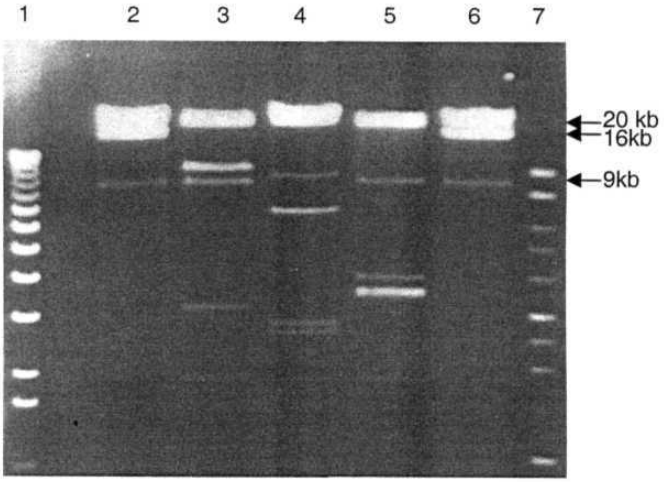


Fig.6.1. Genomic clone 4.3.1 digested with different restriction enzymes

Lanes- 1. Marker-X, 2. NotI, 3. SalI, 4. SacI, 5. PstI, 6. NotI, 7. Marker

Note: A clone 4.3.1 has an insert of 16 Kb which is released as a single band by NotI

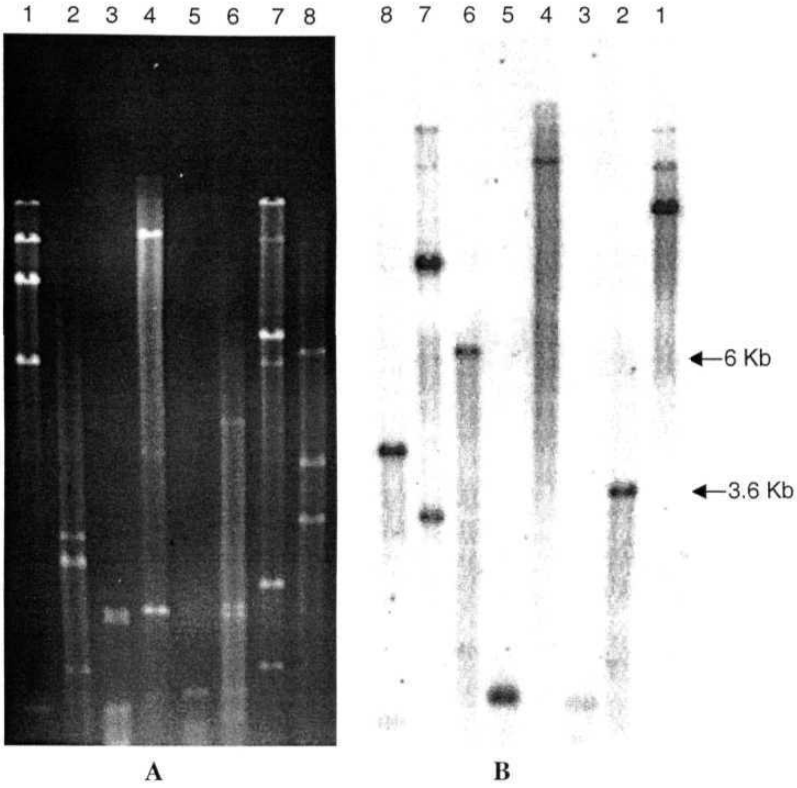


Fig.6.2. Southern hybridization of *Pϕ2* positive silkworm genomic clone with ^{32}P labeled human *Pϕ2* cDNA

A. Size fractionated 0.4% agarose gel stained with ethidium bromide, showing restriction digestion of 3µg of *Pϕ2* positive A clone 4.3.1 with different restriction enzymes: EagI (lane1), EcoRI (lane2), HaeIII (lane 3), HindIII (lane4), MnlI (lane 5), SacI (lane 6), SalI (lane 7), SmaI (lane 8), and XhoI (lane 9).

B. Size fractionated gel (A) transferred on nylon membrane and hybridized with ^{32}P labeled human *Pϕ2* cDNA.

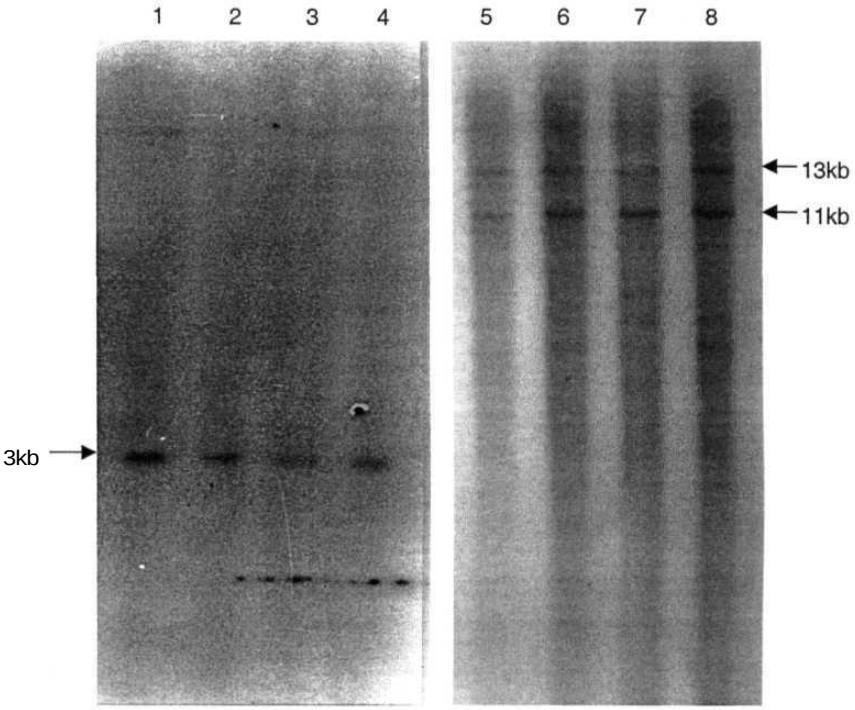


Fig.6.3. Southern hybridization of silkworm male and female genomic DNA with ***BmPø2*** genomic clone

10µg of silkworm male and female genomic DNA was digested with SacI and HindIII, electrophoresed on 1% agarose gel and hybridized with ^{32}P labeled *BmPø2* at 65°C and washed at a stringency of 0.1 XSSC+0.1 %SDS.

Lanes- 1&2: SacI digest of male DNA, 3&4: SacI digest of female DNA, 5&6: HindIII digest of male DNA, 7&8: HindIII digest of female DNA.

gel and transferred on to Nylon membrane by capillary transfer. The membrane was hybridized with radiolabeled *Pø2* clone. However, after washing very few enzymes showed positive fragments, which hybridized with *Pø2*. The intensity of the signal was also low (Fig. 6.4). A 700bp fragment, which was positive for *P02,* was subcloned in Bluescript plasmid vector and sequenced using M13 universal primer and Big Dye cycle sequencing kit.

Genomic clone 19.1.2 was also analyzed similarly but was not selected for further characterization because it showed very weak hybridization signal when hybridized with ^{32}P labeled *P02* cDNA clone.

6.5 Sequencing of clone 4.3.1

The *P02* positive silkworm genomic clone having 16kb insert was designated as *BmP02* sequenced first by subcloning them in Bluescript plasmid vector and then by walking through lambda clone with internal primers (table 6.2).

TABLE- 6.2

Different *Pø2* positive subclones of genomic clone 4.3.1

Size of the fragment cloned in Bluescript	Enzyme
2.5 kb & 5 kb	PstI
3.4 kb	EcoRI
2.8 kb	SmaI
6 kb	SacI

All these subclones were further subcloned using frequent cutters like HaeIII, Sau3A, DraI, Ava II, etc., so that overlapping clones were obtained. Subclones were sequenced using M13 forward and reverse primers and Big Dye cycle

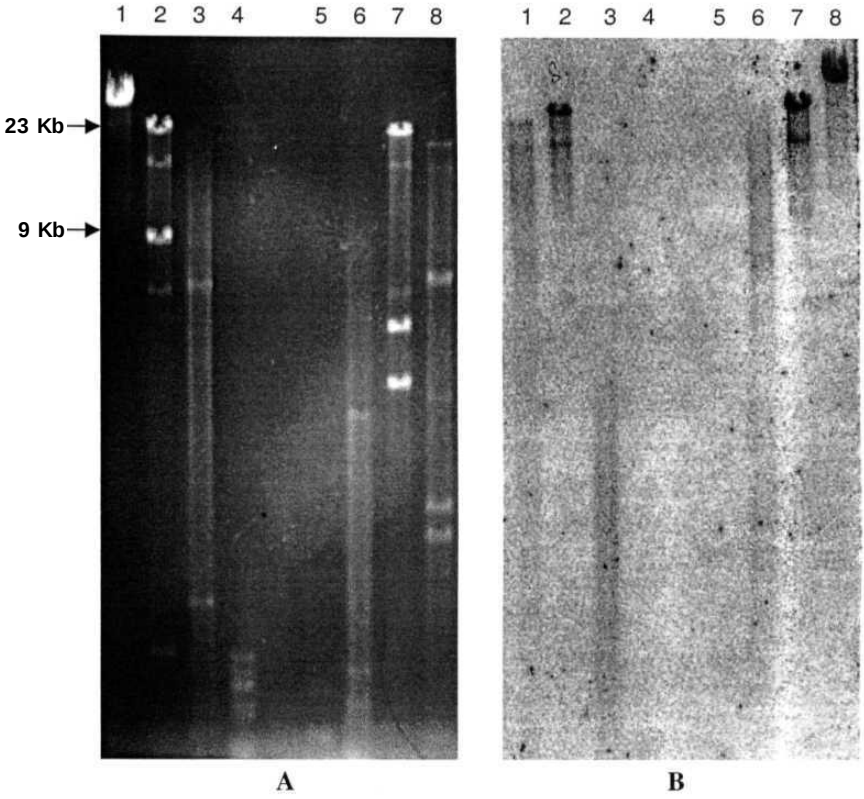


Fig.6.4. Southern hybridization of *Pϕ2* positive silkworm genomic clone with ^{32}P labeled human *Pϕ2* cDNA

A: Ethidium bromide stained gel photograph showing restriction digestion of 3µg of *Pϕ2* positive A clone 7.2.1 using restriction enzymes: undigested A DNA (lane 1), EagI (lane 2), EcoR1 (lane 3), HaeIII (lane 4), MnlI (lane 5), SacI (lane 6), SalI (lane 7), SmaI (lane 8), XhoI (lane 9), size fractionated on 0.4% agarose gel by gel electrophoresis.

B: Size fractionated gel (A), transferred on nylon membrane and hybridized with ^{32}P labeled human *P<p2* cDNA.

sequencing kit. The 11.2kb and 3.2kb fragments of 16kb genomic clone were completely sequenced (Figs, 6.5a, 6.5b, 6.5c).

6.6 Expression of *BmPø2* in different Silkworm Tissues

To study the expression profile of *BmPø2* gene (silkworm homologue of human *P02)* in different silkworm tissues, like 5th instar larval testis, 5th instar larval ovary, silk gland, pupal testis, pupal ovary, haemolymph etc., were dissected out. RNA was isolated from these tissues using GITC method. About 40ug of total RNA from each tissue was loaded on to 1% agarose gel. RNA was transferred to Hybond-N membrane by capillary transfer in 10X SSC. The RNA blot was hybridized with ^{32}P labeled human *Pø2* cDNA clone at 42°C in 50% formamide + 5X Denhardts and washed in 0.5 X SSC and 0.1% SDS. After hybridization, expression was seen in both early 5th instar larval testis and early 5th instar larval ovary. However, no expression was seen in pupal testis and pupal ovary. Since the intensity of the expression was found to be weak with human *Pø2* cDNA clone, it was decided to use silkworm genomic clone as a probe (Fig. 6.6).

The expression level of *BmPø2* gene in early 5th instar larval testis and early 5th instar larval ovary was very low. Therefore, RNA was isolated from several other tissues at a much earlier stage of development like fertilized eggs, 1st instar larva (immediately after hatching) and adult moth testis and ovary. About of total RNA from each of these tissues was run on 1% agarose gel, transferred on to nylon membrane and probed with P labeled *BmPø2* positive genomic clone. After high stringency washing, the expression was seen only in Ist instar larva. (Fig. 6.7)

To find out when the expression of *BmPø2* gene starts during early embryonic development, RNA was isolated from the fertilized eggs, every 3rd day after the fertilization till the 1st instar larva hatches out. For e.g., 3rd, 6th, 8th, 9th, 10th day etc. Here again 40μg of total RNA from these stages was loaded on to 1% agarose gel along with total RNA from silkgland and haemolymph serving as

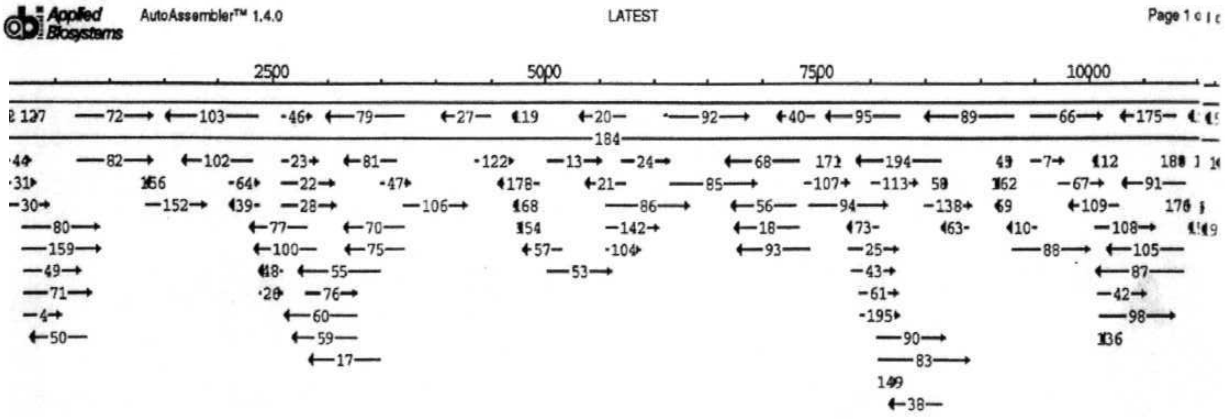


Fig. 6.5(a) Sequencing strategy of *BmPϕ2* gene

ll,163bp Sequence

5'

```
   1  TTCCTGTCCTTCTTGAGGGTCAGCATCTGCCTGGCTTCCTGGAAACTTGA   50
  51  AAAGTAGCTATTGATGCCTCTCTCTATGGCTGAAACAGCAGTGAAGATGG   100
 101  TAAAAGAAAAAAAAAAATAGAAGGGCATTCACATACTCCACCTTTCCCTGG   150
 151  TCCTTTTCCTGAGAGCGGAAGGCCCTTCAAGTTCTCCTGATCCAACCTAA   200
 201  CACTAAGCCCTGGAATCACAGAGGGGTCAGGAAGCTAGAGAAGGAGGGGG   250
 251  CCAAGGAAAGACAAACATCAGGAATAAGAATACAACCAGCAGCTCACCAA   300
 301  CAAGTAACCAGTCCGTACTGTAGCACACTACACACCAGAGCAGCCTCCGC   350
 351  ACCCGAGTTAGGGTCTACACAGTCACATTTTAAACTCATTGAAAGGAATA   400
 401  TTAAATATATGTGGTTCTCTAGTTATTTATAAATCATTAGAATTGAAATA   450
 451  TACAAATTTTAAAAAATTGTCTCTAAAAAAGAAAATAACGGTTTGAAGGC   500
 501  CAATAACATAGTTCGGCATGAATGTTGACACGTTCATTAACTCCGGTACC   550
 551  TATTGTTACTTACACTTTAAAACAAAATATTTTATGAACATGACACTTAT   600
 601  TTACAGCTGTTTCAACATAAATATTCTACTGCAAACGCTACGAATTACTT   650
 651  ACAACTTTGTATAAATTAACAGTTCCGCATTCGACTCCAGTGGACACTCG   700
 701  CACAACTCTAGTACGTTTCCATGTGATATCGTAAATGAATTTTCAGATTA   750
 751  TCGGTTCTCCAAAACGAGTTGACGGAAGAGAGGGGCTTGGCGGCGGCCAT   800
 801  GGCCAGCAGCCAGTCGGAGTGGCAGGCCAGGGCTAAAGAAAAGGAGGAGA   850
 851  ATTTCACCAAAGAGGTTTGCTCCACGTTTAGCGTCGATTAGTAGATGTGC   900
 901  CCGTGGTCCTCGGTGAGGTCGCCGCGGTGACACTCGAGACAACTGGTCCG   950
 951  GTAGGCTAAGTTTGGATGATTTGATTAAATAGAAAAAGGGGGGGGTATAC  1000
1001  CCGCACCATCGTGCGGCGCGGGGGGCAGGGGGGGCGGGGGATGGGGGTCC  1050
1051  CACGAGTCGCTGTCAATTAATTTATCTTCGCACGTTATAAAAATGGAGAC  1100
1101  AATATTGAACAAGCTTCAGGACGGGACGATGTAACACCAGCACAGCGCCG  1150
1151  CGTCAGCGAGCCCACACTATAACGGCATTTGTCTTTTGGTGGGAACAGGG  1200
1201  CATAGGAGTAATGGGACTTTTTTATTTTGATACAAAACGTTAGAAGAGAA  1250
1251  ACGTAAGAACGTAAGAATTTTATAAAACGTTCGATAGTTATTTAACTAAT  1300
1301  ATTGGTTTAATCTTTCGCTAAGCGTTTTATTATAAGAAACAATATTTACC  1350
1351  TATGTAAAGACCTAATAAATGGAAATAGATTTCCCCTAAACTCCATTCTG  1400
1401  TAGTTCCTGCTTTTTTTAGAGGACTATCTCGATAAATGTACTTTTTGTTA  1450
1451  GATCTAATACGCACTAGTAGTCAGTTTAGTTCACCGTACTATATGCCACT  1500
1501  CTATCACTACTTTGGATTGCTTTTCCTTCGTAGCGAATTGTCTCGCTTCA  1550
1551  TGCTTTTCAAGTATTTTGACCTTCGACGTAGCCACGCTCGTGTCCGAGTC  1600
1601  AAATTGCTATTTGGCCAAAAGACAGCTTTCAGTGCCTCTGGGTGCACTTG  1650
1651  CAATATGAACACAAACTTAGAAAAACGTGATTGAGGTCATAATTTAAGGC  1700
1701  GCGAGCGCAGCTGGAGCGGTCCGACGCCGTGTCGCGCGACGAGCTGGCCG  1750
1751  CCGCCACGCTGACCGTGCCGCCCCGCCACGCCGCGCCGCGCCGCCGCGC  1800
1801  TCGGTAGGGCCACGCAGCCGTCGTCGCGCCCCGCCGCGTCGCGCCCCGCC  1850
1851  CCGCCCCGCCGCGAGCTCCAACACCTTCCTTTGTAATCTTGAACATTAGT  1900
1901  ATGTAAATATGAAAAATTCTGTGATAAATGTTTAGAAGACTATGTACTTG  1950
1951  ATTTATTACATATGTGTGTTTTGCTGAGATAATTAAATTTATTTGTTCCA  2000
2001  ATGCTGTTTCTAAACTAGCTCTGAAATGAAGAACATAATAAATAAATAAA  2050
2051  AAAGAGTGTTGTATGTTTTTTGGCCCTAATCCTTGCATTCGCTTAAGTTT  2100
2101  TATCTCATGTAGTAAAAGCTATTGACTATTGAACACACATGTACCTATAT  2150
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2151 GATGAATTCGAAGACGCAATTGTATTGCCAGGACTGGGGTTCTCAACGCA 2200
2201 AACTATTGAACAGATTACACCAAACTGACGATACCTAGTCACATTGACTC 2250
2251 GATTTCACCAGGATCTAATACGCACTAGTAGTCAGTTTAGTTCACCGTAC 2300
2301 TATATGCCACTCTATCACTACTTTGGATTGCTTTTCCTTCGTAGCGAATT 2350
2351 GTCTCGCTTCATGCTTTTCAAGTATTTTGACCTTCGACGTAGCCACGCTC 2400
2401 GTGTCCGAGTCAAATCTTGCTATTTGGCCAAAAGACAGCTTTCAGTGCCT 2450
2451 CTGGGTGCACTTGCAATATGAACACAAACTTAGAAAAACGTGATTGAGGT 2500
2501 CATAATTTAATGATCAAACAAGTTAATTTAATTGTTTTTTAGGTTTATTC 2550
2551 GTGTAATGTCTCAGATTAATGGCGAATAGTTCGTGCGCACCACGACCACA 2600
2601 TATAGAACAGCTTCTATTTCTCTTTAATTAGTGAACATAGAATTTCAAAT 2650
2651 CCGTACGTCGTTTGTTTCGGGTGCCACGTAGAGGTTCTGTCCTATTATCC 2700
2701 TGTTAAGTGTGTTATATATGATTCTATCTGTGATTTGAATATACCCATAC 2750
2751 TATTAAGGAGAGTAGAAGTAACGAGCACTATCTCAGTGTAAAAAGTGTCC 2800
2801 GCTGAAAGAGAGAAAACAAGGTGGCGCCATAGTAGCAAGTAGAACGATTA 2850
2851 AAAACAGCGCCTTAACCACACTTCAATTTAATTCAAGCACACTGCACTCT 2900
2901 GTTTAAAACTGCTATACTCATATTATTTCCACTTCCTCCACTAGCGCTAT 2950
2951 TTGGGTGAGTCTTACAACAATAATTTGTTGTTTACAGGTAGACACTTTTT 3000
3001 TGCTTGTCCTCTATACAAGATGGTACATTTCTTTTTTTCCTACCTAAGCT 3050
3051 G**AG***TGCCTTGAGAGGCTATATCAGCGTAGCCTTA*ACTAGTAGGTGAGCTC 3100
3101 *ACGGGGCTCAAACCTGATGACGTTGCTAACACGTA***GT**AACACGTTACTGG 3150
3151 TGGTAGGACCTCTTGTGAGTCCGCGCAGTTAAGTACCACCGCCCTGCCTA 3200
3201 TTTCTGCCGTGAAGCAGTAATGCGTTTCGATTTGAAAGGTGGAGCAGCCG 3250
3251 TTGTAACTATACTGAGATCTGAGAACTTATATCTCAAGGTGAGTGGCGCA 3300
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7051 CCCCAAATCTCAGTCGGTAACCAACTATCTAAAAAGTATATCAGATACAG 7100
7101 GGTGTCCCTCAAGAGGGTGCGTCAAGCCGACAACTGTAGGTAGTTCTTTA 7150
7151 TTAGAATAATCGAAGTAAATAGTTTTGGAATAATGAGTACTTGTACTTAG 7200
7201 CGAACAACCTCTTCTTCCGCCAACCCTTGAACTGTGTTTTACAGAAGGTA 7250
7251 TCATAATATGATTCGTAGTTAACATTAAGGCTGGCCACATATATTAAAAG 7300
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8851 TTATTGCTTCACGATAACGCGAGACCTCATACAGCACGAGAAACCGTTTT 8900
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8951 TGCTCCCTCGAACTACAATTCCTTTCGTGATTTGGACAATTTTCTATGTG 9000
9001 ATAAAAAGTTTTCTTCCCAGGAGGCAGTACAAAATGCTTTCACACAGTAA 9050
9051 GTAGAATCTAGATCATCAGAGTTCTATCGCAAAGGCATAAATGACCATCC 9100
9101 TATTAGATGGCAGCAATGTATAGATAATAATGGTAGATATTTTGATTAGA 9150
9151 TAAATATGTTAAAACGAAATTAATAAAACGAATAAAAAATTTTCAGTACA 9200

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 9251  TGAGTGAACCATTTATTTTGACCCTATGTGTACGATGCGCAGATGGCACC  9300
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 9351  ATTGTGTTAATTGTCTTGGTACAAAATTAAATTAATTATTTGTACGTAAC  9400
 9401  ACGTTACATATATGGGGTCATTCAAGTGCGTTATGCGAGAATACTGCTAC  9450
 9451  GCGTGTTTTCTATAACGCGGAAGCAATCTACAGTGTTATAGGGGGATTAC  9500
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 9701  TATACTACCGTTAATAAACACTAAAAAAATAAATGTTATTCTATTAAACG  9750
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 9901  GCGACGAAGGACTCGACGAGCGAATTAACCCACGGACACAGCCCACTGAG  9950
 9951  TTTTCTGGCCGGATCTTCTCAGTGGCTCGCGTTTCCGATCCGGTGGTAGA  10000
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10151  TCCAACGTTATTAAAATGCTTGTTGACTC**AG***ACAAGGGCGCCAACCACTT*  10200
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10301  *TTCCTCGGGAAAGGGAACATCCGG***GT**AACTATACTCCGCACCCAGCCTGC  10350
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11001  CATGGTCACCGTTCGGTGTGTGTGTGTGATGTGTACTGCGAGGAATTGTA  11050
11051  GACAATGGTGGAGAAGCGAATCTCTGGCCAGCTTTACTTCTGCAACGATC  11100
11101  GAGCGAAGAGTACCGTTTTTTGCCACCATGGGGATCATGACCTCCCTTCT  11150
11151  GTCAGCATCGGAC                                       11163
                                                           3′
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Fig. 6.5(b). **11,163bp** nucleotide sequence of ***BmPϕ2*** gene
Bold letters AG and GT indicate intron-exon junctions and sequences in italics represent exons.

3,261bp sequence

```
   1  AACCCTTCCAATTAATTAATTTGTTACATAGGGGTTTAAGACCATTAGAC  50
  51  TTAAATCTCACAATGTCGTTACGACGCTTAAAACCCAGTAATGGACCCTT  100
 101  CCCATGTCATGATTGTTTGCGCAGCAAAAGATCGAACGGCTGTAAGTAGG  150
 151  CAGGGTGAGCGAGTCACTTAGAGTGAATACCTCGCCTAGTTGGACGCTTT  200
 201  ATGGCTTTCGAACCGGATTTGCCTCGGTGTTTGCCTTTGAGGTAGGTTTG  250
 251  GGGGTTCGGTTTTTGGTTTGGGAAGGCTTGTAAACAGGAGCGTATTGTGC  300
 301  TGCAGGATATCAAGAAGCCAGAGAAAGACGAATGGGGCAACAGTCTAGAT  350
 351  ACCTTAGAACATGCCCTGCAGCTGTAGAAAGAGGTTAACCAGGCTTTGCT  400
 401  GGATCTGCACAAGCTGGCAACTGAGAAAGCAGACCCTCATCTCTCCGACT  450
 451  TCCTGGAGAACGAATTTTTGGAAGAGCAAGTGAAGGCCATCAAACTGCTG  500
 501  GGAGACCACTGTACCAACTTGAAGCGCCTGGGGCTGCCCCAGAACGGCAT  550
 551  GGGCGAATACCTGTTTGACAAGCTCACCTTAAGCGAGAGCAGCTGAACCT  600
 601  TGTCCCGTTGATTTATGCAGTTTGTTTTGGGGGAAATGCAAATAAACGAA  650
 651  AAAATTGTTAAAAAAATCTAAGTCATAATTATGTAGTAGTTTCGTGCAAA  700
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 751  CAAGTTACAGGTGCCGTGTTACTCCGATGGAAGTTAAGGACGTCGCAAGG  800
 801  CTCCCGGTTCCGGCGGTACATCTAACCGCGTCGTCAAACTTCTACCCGCC  850
 851  CAACTCATCGTGATGTTGGCATCTATTTTTAAAGCCGCTATGTCAACTGC  900
 901  ATCTTTCTGACACCTGACATCTGCCTCGTGCCTGAAGGCTCCGCGCGTGG  950
 951  CCGAGTGAGCGGGCGTCGACTCGCCATGCTCCGGAAACGGATTTATATTC  1000
1001  CATGATGATTTGTAGCTGTTGCTTGAAGCTACTGAAGTTACCGTTAAAAT  1050
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1101  CCCGTACCTCCACATCCACGTGTCACAGATGAGGCATATCCAGAGCGTGC  1150
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1301  CAGGATGGCGGATCCCACCCGCGCGTCCTCACAACAACATTCTACGACCG  1350
1351  GTCAAGAAATCTACTTCAATTTTCAAAGGAGAATGCCCATTGCAGTATGG  1400
1401  AATTTGGTTTCAAGTTGTGCTCTCATGTAACTTACCTCATTTTGTAGGGT  1450
1451  TTAATATGTTAATTCATTTATAAAAAAATATATATCTCTAGTTCGCCGGA  1500
1501  GTTTCTAGTAAAAGACCAATGAAGTTTATAGAATTAGGTTATTTTTTGTA  1550
1551  CAAGACAGCAGTGGATGTTTTTTGATTATGCTGAGTTCTTACATACTTAA  1600
1601  AAACATACGTATAAAGTATTTTATTAATGAAAACATGTTTATAGCATAAA  1650
1651  CTTATTTACCTAGCATAGTATTTGTGTGGCTAGGGCCACTACGTCCATTG  1700
1701  GTTAAGTAAAGGTACTATTACGGCAATTCAAAATAAAACAAAAATAGACA  1750
1751  TAAAATAATTTATTTCTCATATTATGCCGATGTATCATTATACCTAGCTC  1800
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1851  AGCTGTAGTAATAACTAGGGATTGTATTAATAGTATTAGCAACGTTTTCA  1900
1901  TCGCGATTAATTAATGTGTACCGTATAATACATAAATTAATTCTGGCTCC  1950
1951  AATATGCCTGGACTTCTGTCTAACAAAATAACCGAATATGACCAACGAAA  2000
2001  CACATGATTTAAAACTCAATCGGAAACATGAGAACGCGCGGCGCCAGTAA  2050
2051  ATCAAAATATAGCTAGATCGTTGAGTCTCCCGTACAGATGTGATGGAGTA  2100
2101  TGTATGCGTGGTCTGAACGCGTAGTGTAATATGTAAGACCTGAGTAGGCG  2150
2151  CCGTCGATGTTCGGATAATAATTAAATACGACACCTTGCACACTAATGAA  2200
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2951  GGGGTTCAGAATGTCTTCGATTCTGTGCCTGTTAAGCAACGCAAGGAAAA  3000
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3101  AGTTTTAATGCTAACGTTTAAAGCTGCTGCTAAGTCAGCGATAGACAGAT  3150
3151  TATCGTCAGCTTTCACTATCGCCTTTAATTCGTCGATGATGACTTTAGTT  3200
3201  TACCGGCGACCACGGGATTCATTTTTTAGATCATAATGTCCATGTCGCAT  3250
3251  CATTCGGACC                                          3261
```

Fig. 6.5(c). 3,261 bp nucleotide sequence of *BmPϕ2* gene

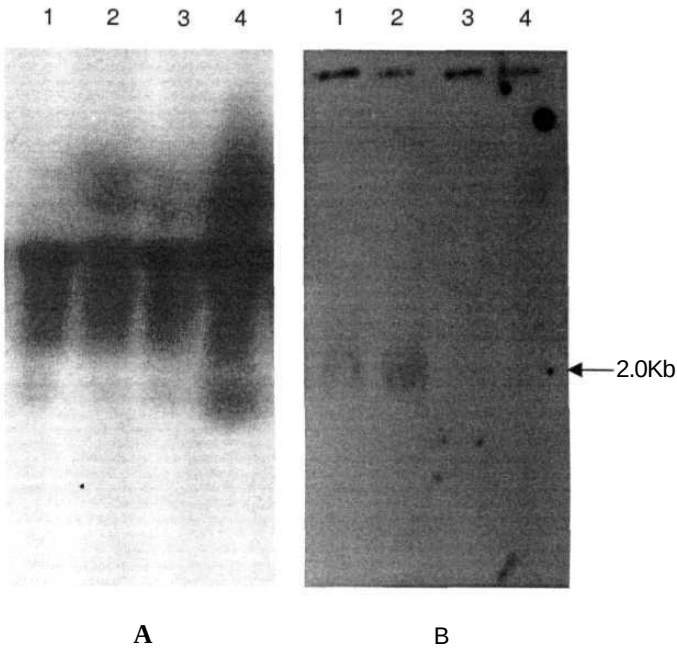

Fig.6.6. Northern hybridization of total RNA from larval and pupal gonads with ^{32}P labeled *BmP<p2.*

40µg of total RNA isolated from testis and ovary was electrophoresed on a denaturing gel (A) and hybridized at high stringency to ^{32}P labeled *BmPϕ2* **(B).**

Lanes- **1:** Early 5th Instar larval testis
2 : Early 5th Instar larval ovary
3 : Pupal testis 4. Pupal ovary

Note: Stage specific hybridization to both larval testis and ovary.

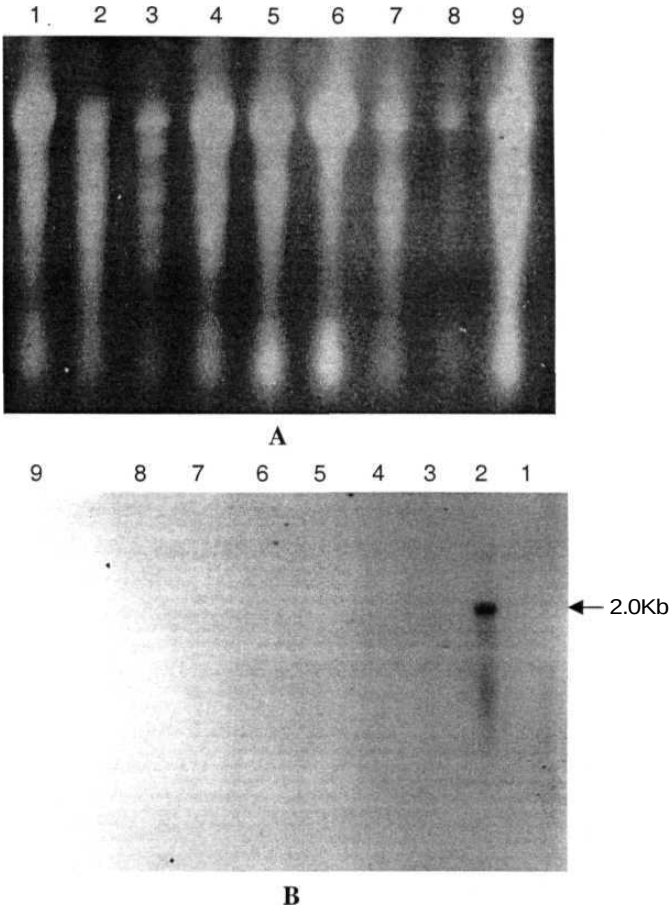

Fig.6.7. Northern Hybridization of total RNA from different tissues/ developmental stages of silkworm with ^{32}P labeled ***BmPφ2***.

30μg of total RNA from different tissues/developmental stages was electrophoresed on a denaturing gel (A) and hybridized at high stringency to ^{32}P labeled *BmPφ2* (B). Note stage specific hybridization to a 2.0Kb mRNA in Ist instar larva.

Lanes- 1: Mouse liver, 2: Ist Instar larva, 3: 5th Instar larval testis, 4: 5th instar larval ovary, 5: Pupal testis, 6: Pupal ovary, 7: Moth testis, 8: Moth ovary, 9: Silkgland.

negative control. After hybridization with P labeled *BmPø2*, the autoradiograh showed that the expression of *BmP02* gene started around 5-6th day after fertilization. The level of expression was found to be very high during the late embryonic period. Northern blot hybridization revealed the presence of 2-3 transcripts suggesting the possibility of alternative splicing (Fig. 6.8).

6.7 RNA FISH

Since first instar larvae were too small (5mm long and 1mm wide) to dissect out various tissues, whole larvae were used for isolation of total RNA. In order to identify the different tissues in embryonic stages and 1st instar larva, which show *BmPø2* expression, young developing embryos were dissected out from the fertilized eggs using dissecting microscope, fixed in 4% paraformaldehyde and 3μ thick paraffin embedded sections were obtained. These sections were processed and RNA *in situ* hybridization was carried out using DIG-labeled *BmP02* genomic clone. After stringent washing and incubation with anti Dig antibodies, the sections were scanned in confocal microscope.

Sections from 1st instar larva showed localization of *BmPø2* transcripts in developing testis as well as in nervous ganglions. The testis could easily be identified in the larva because of its morphology and high level of *Bmpø2* expression (Fig. 6.9). Unfortunately, ovaries could not be identified in the 1st instar larval sections. Sections of the developing embryos (larvae developing within the eggs) did show expression in the posterior end of the larva where genital ridge is localized. The expression was also seen in nerve ganglions. Some background signals were also obtained in the outer layers of the body surface because of the chitinous outer covering enclosing the larval body. The chitinous membranes have been reported to trap the fluorescence (Fig. 6.10).

The *BmP02* transcript level was found to be very low in early 5th instar larval testis and ovary. Therefore, testis and ovary were dissected out from the 4th instar larval stage and paraffin embedded sections were made. RNA *In situ* on these sections using DIG-labeled *BmPø2* genomic clone as a probe showed

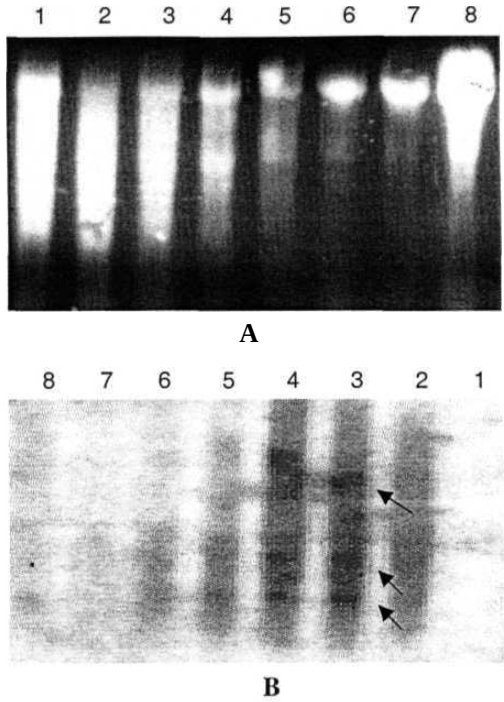

Fig.6.8. Northern Hybridization of total RNA from different tissues/developmental stages with ^{32}P labeled silkworm *BmP<p2* homologue.

30μg of total RNA from different tissues/developmental stages was electrophoresed on a denaturing gel (A) and hybridized at high stringency with ^{32}P labeled *BmPϕ2* **(B).**

Lanes- 1: fertilized eggs, 2: 4 days old embryo, 3: 6 days old embryo, 4: 9 days old embryo, **5**. 1st Instar larva, 6: 5th instar larval testis, 7: 5th instar larval ovary, 8: Silkgland

Note: the expression starts around **5-6**th day of embryonic development and 2-3 transcripts of different sizes are seen.

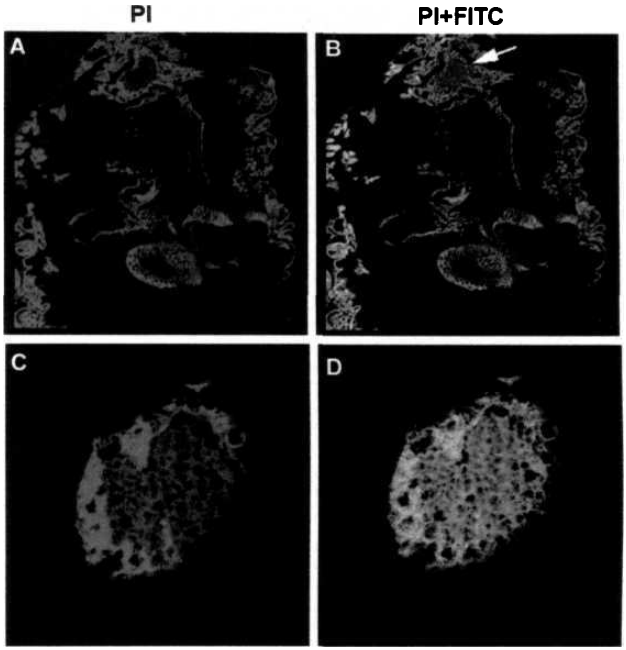

Fig.6.9 RNA *in situ* hybridization on Ist instar larva using DIG labeled *Bmpϕ2* DNA probe.

A: PI stained section of 1st instar larva (10X),

B: FITC+PI stained section of 1st instar larva (10X),

C: PI stained section of testis of 1st instar larva (60X),

D: FITC+PI stained section of testis of 1st instar larva (60X).

Note: arrow indicates testis showing high level of expression in 1st instar larva.

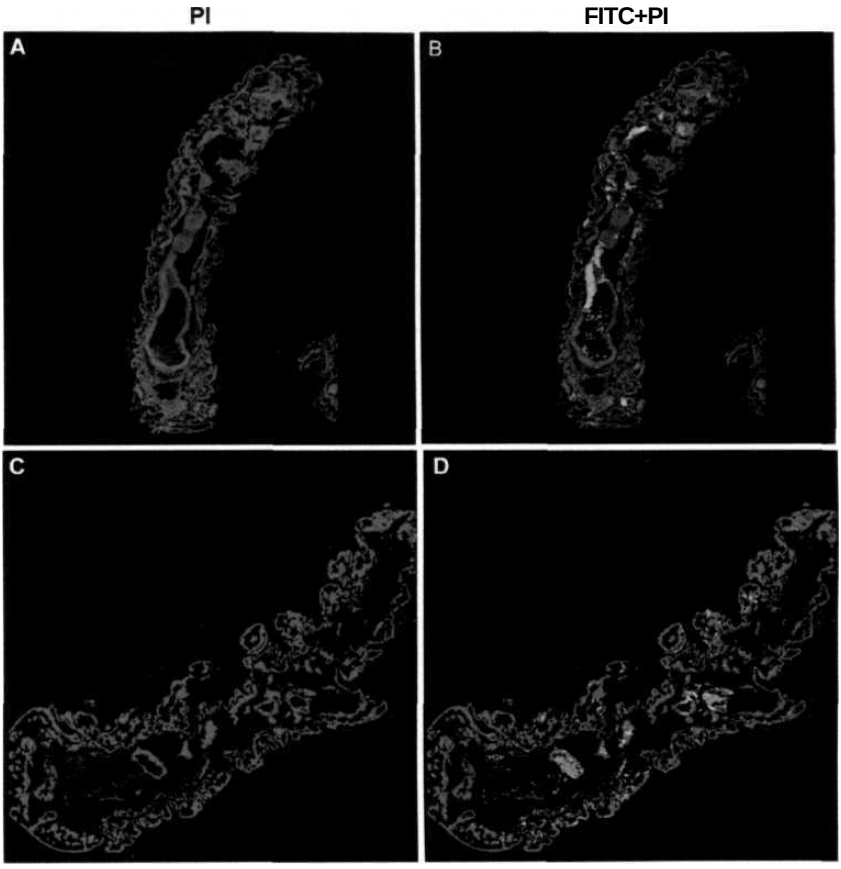

Fig.6.10 RNA *In situ* hybridization on 5-6 days old silkworm embryos with DIG-labeled *BmPϕ2* DNA probe.

A & C: PI stained section of 5-6 day old embryo of silkworm (10X).
B & D: FITC+PI stained section of 5-6 day old embryo of silkworm (10X).

good expression in testis but in ovary the level of expression was almost two fold lower as compared to testis. Within the testis, the expression was seen in all the cells. However in the ovary, the transcripts were preferentially localized in the developing oocytes (Fig. 6.11).

Human *P02* gene is predominantly expressed in mouse testis. The expression is also seen in mouse ovary, brain and heart but the level of expression is very less as compared to testis. So paraffin embedded sections of mouse testis and mouse ovary were made and hybridized with DIG-labeled *BmPø2* gene to find out the localization of the *BmPø2* transcripts in mouse testis and ovary. The pattern of expression of *Pø2* in mouse testis was found to be the same whether human *Pø2* cDNA or silkworm *P02* homologue was used as a probe, thus confirming that the two probes are really homologous to each other (Fig. 6.12).

6.8 Construction of cDNA Library from 1st Instar Larval Stage

The expression of *Pø2* gene was found to be very high in Ist instar larva, so mRNA was isolated from 1st instar larval stage and a cDNA library was constructed in Bluescript /BamHI vector using TC-T primers. 1µl of the ligation mix was electroporated that yielded about 100,000 colonies of which 90% were recombinant white colonies.

6.8.1 Screening of cDNA Library

The cDNA library in Bluescript plasmid vector was screened using P labeled *BmP02* as a probe. After primary screening, positive colonies were obtained of which two were selected for further studies. These two cDNA clones were designated as A_2 and A_{25} and were characterized further.

6.8.2 Characterization of cDNA clone A_2

A_2 plasmid was double digested with EcoRI and SacI to release the insert. It contained a 450bp insert, which was completely sequenced using M13 forward

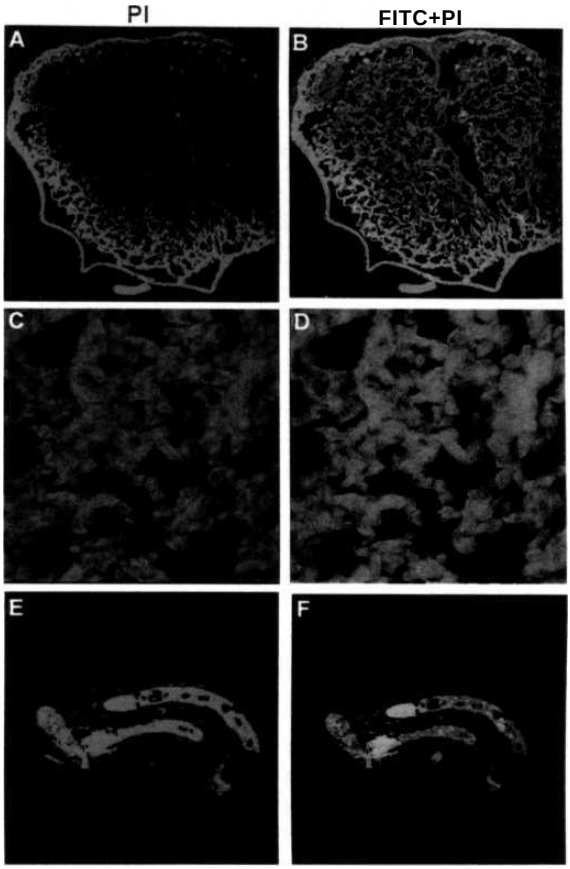

Fig.6.11 RNA *in situ* hybridization on 4th instar larval gonads using DIG-labeled *BmPϕ2* DNA probe.

A: PI stained section of 4th instar larval testis (10X),
B: FITC+PI stained section of 4th instar larval testis (10X),
C: PI stained section of 4th instar larval testis(60x),
D: FITC+PI stained section of 4th instar larval testis (60X),
E: PI stained section of 4th instar larval ovary (60X),
F: FITC+PI stained section of 4th instar larval ovary (60X).

Note: expression of ***Bmpϕ2*** in testis is much higher when compared with ovary

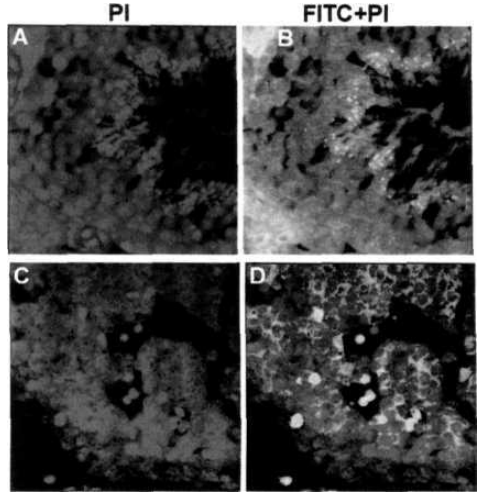

Fig. 6.12 RNA *in situ* hybridization on adult mouse testis and ovary using DIG labeled *BmPi>2* gene as a probe.

A: PI stained adult mouse testis section (60X),
B: PI+FITC stained mouse testis (60X),
C: PI stained adult mouse ovary (60X),
D: PI+FITC stained adult mouse ovary (60X).

Note: expression of *BmPϕ2* is much higher in testis than in the ovary.

and reverse primers and Big Dye cycle sequencing kit. However, it was a false positive and did not show homology with *BmPø2* genomic clone at nucleotide sequence level. Silkworm EST database search using this 450bp sequence as query identified 72 ESTs from different tissues/stages. The ESTs showing 99% homology were e40h0883, wdV41020, prgV0556, NV120108 and br - - 1930 etc.

The cDNA clone A_2 has no open reading frame (ORF) suggesting that it is not a full-length cDNA clone. Two 700bp ESTs, e40h0883 and wdV41020, showing 99% homology to A_2 had potential ORFs. The putative protein coded by this ORF does not show any significant homology with any of the known proteins in the database.

6.8.3 Characterization of $A_{25.1}$

The second cDNA clone $A_{25.1}$ was also double digested with EcoRI and SacI to release the 5kb insert which was further subcloned in Bluescript plasmid vector. The 5kb insert was sequenced using M13 forward and reverse primers and Big Dye cycle sequencing kit. When the cDNA sequence was aligned with *BmPϕ2* genomic sequence, cDNA was found to be completely co-linear with the genomic sequence having both introns and exons, suggesting that it was an unprocessed pre-mRNA. The cDNA clone $A_{25.1}$ had an insertion of 500bp sequence which was not present in the genomic clone. Since it was a unprocessed pre-mRNA, it was not characterized further (Fig. 6.13).

6.9 In Silico Analysis of *Bmpø2* Genomic Clone

Two major fragments of 11.2kb and 3.2kb from *Bmpϕ2* genomic clone were sequenced completely. All the genomic databases of NCBI, GenBank, EMBL, Silkbase and Flybase were searched using 11.2kb sequence and 3.2kb sequence as query. The query sequence did not show homology to any of the known genes in various databases at nucleotide level. However Nucleic Acid database search identified the positions of *BmC1* repeats in the genomic clone.

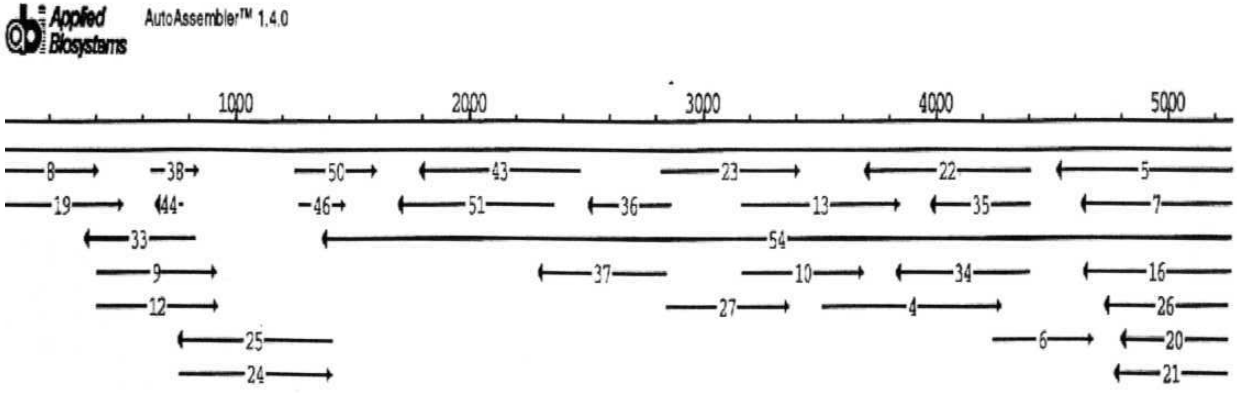

Fig. 6.13. Sequencing strategy of *Bmpϕ2* cDNA. Allignment of different subclones of *Bmpϕ2* cDNA to generate 5.2kb sequence

BmC1 elements were present at three sites in the genomic clone i.e., 1038bp-1400bp, 2471-2550bp, 7607-8020bp. Similarly, all the EST databases were scanned using 11.2kb and 3.2kb sequence as query. Many silkworm ESTs showing homology to 11.2kb sequence were obtained but none of them showed any homology to 3.2kb sequence (table 6.3).

TABLE- 6.3

Various ESTs from Silkworm EST database showing homology to *Bmpϕ2* genomic clone

Database	Clone name	Region of genomic clone showing homology	Orientation of the sequence	% homology
Silkbase-EST	WdV40619	3048bp - 3135bp	(+/+)	99%
Silkbase-EST	e96h0003	3676bp - 3924bp	(+/+)	99%
Silkbase-EST	wdS00697	4184bp – 4333bp	(+/+)	99%
Silkbase-EST	wdS00697	5428bp - 5680bp	(+/+)	99%
Silkbase-EST	WdS00697	6612bp – 6741bp	(+/+)	91%
Silkbase-EST	WdS00697	10179bp – 10324bp	(+/+)	96%

In silico analysis of 11.2kb genomic sequence and the three ESTs, wdS00697, e96h0003 and wdV40619 identified six putative exons in the 11.2kb genomic sequence. Since all these ESTs are sequenced in 5'-3' direction, the orientation of the *BmPϕ2* gene could be identified.

The 11.2kb genomic sequence contains six putative exons with first exon of 87bp identified from wdV40619, second exon of 248bp from e96h0003 and the last four exons of 149bp, 252bp, 129bp and 145bp respectively, identified from wdS00697. The 100bp sequence downstream to sixth exon (145bp) towards the 3' end of the EST wdS00697 did not show any homology with the genomic clone and they did not have polyadenylation signature sequence 'AAATAA' and poly-A tail suggesting that there are exons present downstream of the last exon of 145 bp. The 5' end of the EST wdS00679 was also not complete as 9bp

sequence present upstream to the third exon of 133bp had no transcription initiation codon ATG or Kozak sequence. Upstream to the first exon of 87bp, transcription initiation codon or the Kozak sequences were present. However, a detailed promoter analysis will have to be carried out to find out the exact position of promoter and the transcription start site (Fig. 6.14).

Homology search with Silkworm EST wdS00697 and putative *BmPϕ2* cDNA in the *Drosophila* EST database identified two EST's GM05201 and LD11521, both of which are from the same gene CG1800. TBLASTX identified EST GM05201, from *Drosophila* with a score of 299 and e-value of 2.1e-26 and 69% similarity in +1 frame. The 5' end of the EST GM05201 is similar to the 5' end of cDNA clone yk192c4 from hermaphrodite male *C.elegans* which has not been characterized in detail. The Drosophila EST GM05201 is from Germanium stage-6 and belongs to the predicted gene *CG1800* that has a transcription unit length of 3135bp.

Homology search in Human genome database for X and Y-chromosome was performed to find out whether *BmPϕ2* cDNA maps to the same region Xp11.23 where *pϕ2* cDNA is located. The best Blast hit obtained using *BmPϕ2* cDNA maps to Xp11.23 thus confirming that *BmPϕ2* cDNA and human *Pϕ2* cDNA are indeed homologous.

6.10 Analysis of Potential ORFs in the Putative Bmpϕ2 cDNA

The putative cDNA having all the six exons was analyzed for Open Reading Frames. The cDNA was found to contain an ORF of 146 amino acids, starting in the middle of 3rd exon and continuing till the end suggesting that 3' end of the cDNA is not complete. The EST wdS00697 having the exons 3-6 shows very small open reading frame (ORF) of 65-amino acids in the +1 reading frame and 64-amino acids in +3 reading frame. The other two ESTs e96h0003 and wdV40619 also have very small ORFs.

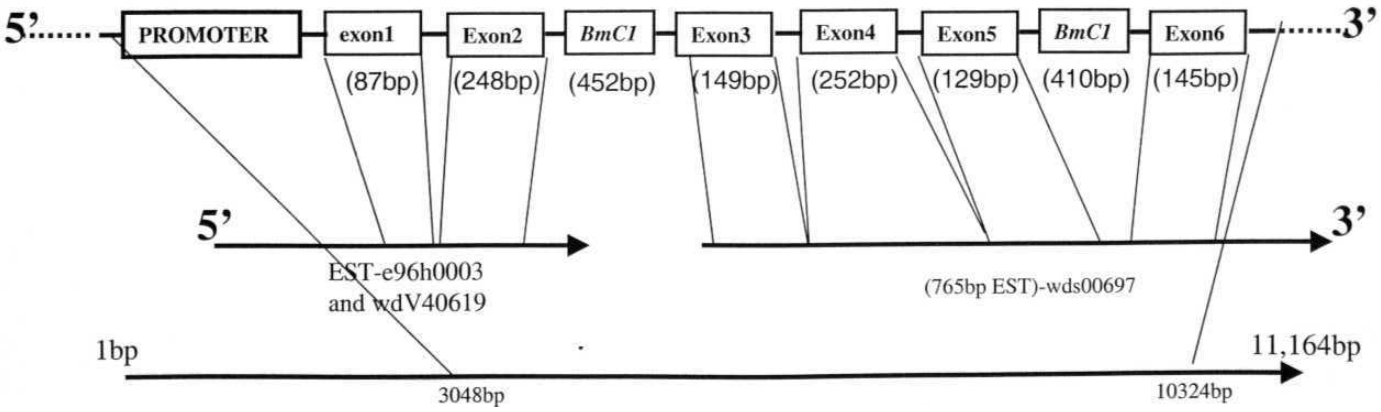

Fig. 6.14. Putative structure of *BmPϕ2* gene

The proteins coded by these ORFs do not show significant homology to any of the proteins in the Database. Homology search for the 145-aminoacid putative protein of *BmPϕ2* against different protein databases also did not show any significant homology to any protein in the database. To find out whether similar kind of protein exists in *Drosophila,* Blast search was carried out in *Drosophila* protein database using amino acid sequence of *BmPϕ2* as a query. The search identified a predicted protein *CG1800* showing 65-70% homology to the putative protein coded by *BmPϕ2* in +1 reading frame with a score of 400 and e-value 1.7e-30 (Fig. 6.15). The *Drosophila* gene *CG1800* is located on chromosome 3R and has a transcription unit length of 3135bp. It codes for a protein of 642 amino acids. The function of this gene has been categorized as RNA-binding and has DSRBD (double stranded RNA binding domain) domains. *Drosophila* protein database search with EST wdS00697 also identified the same gene *CG1800* with a score of 264 and e-value of 8.4e-29. Significant homology between the proteins coded by *BmPϕ2* and CG1800 suggests that *BmPϕ2* probably performs a similar function of RNA-binding in silkworm genome. However, all these interpretations have to be further confirmed by isolation of full- length cDNA clone and a detailed computational analysis of the cDNA as well as the protein coded by it.

6.11 Chromosomal Localization By Fluorescence *In situ* Hybridization (FISH)

To study the localization of *BmP02* gene on silkworm chromosomes, mitotic chromosomes were prepared from 48-hour old fertilized eggs. The chromosomes after processing were hybridized with DIG-labeled *BmPø2* genomic clone. After hybridization, the chromosomes were incubated with primary and secondary antibodies and finally anti-DIG FITC. The chromosomes were counter stained with propidium iodide. After staining the chromosomes were observed in fluorescence microscope using UV lamp and FITC filter.

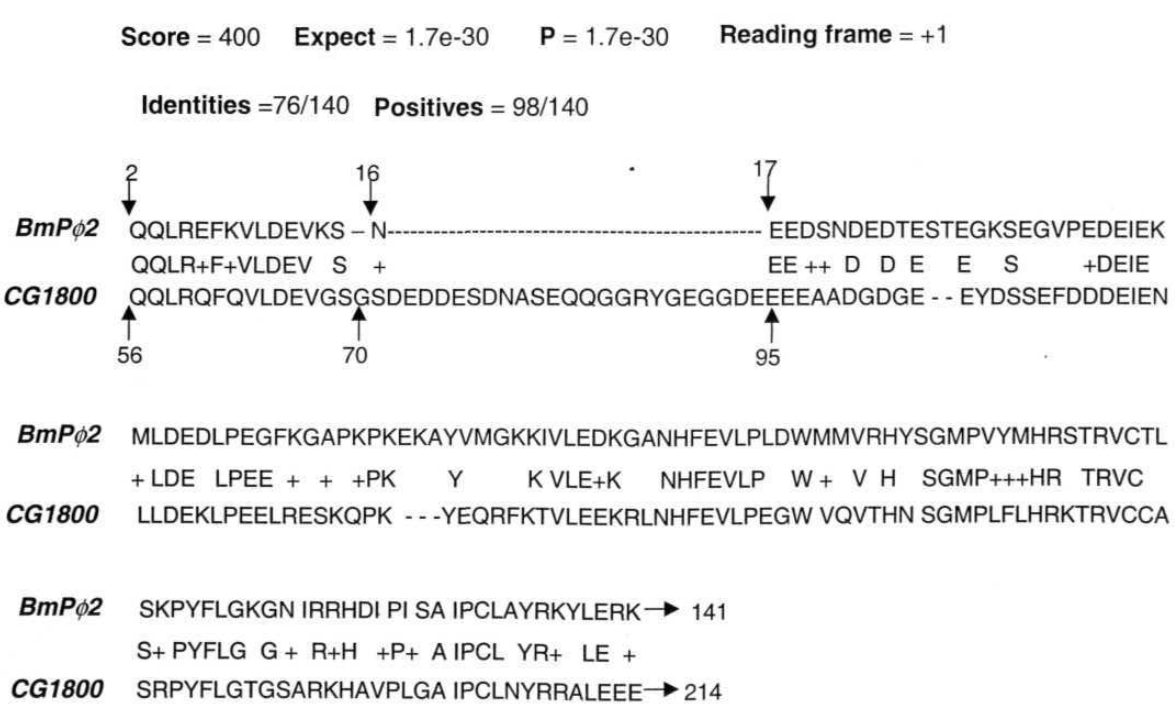

Fig. 6.15. Alignment of amino acid sequence of *CG1800* gene of *Drosophila melanogaster* and *BmPϕ2*.

All the interphase nuclei and metaphase spread were showing a uniform distribution of either single or two FITC signal. The ratio of single signal to two signals was almost one. Since the chromosomes were prepared from the eggs where there is equal population of male and female cells the above results clearly indicate that *BmP02* gene is located on the Z-chromosome in silkworm.

To further substantiate the above results, chromosomes were prepared from testis and hybridized with the same DIG-labeled probe. However, in this case all the metaphase plates and interphase nuclei showed two FITC signals, which is proportional to the presence of two chromosomes in testis. This confirmed the above result that *BmP02* gene is located on the Z-chromosome in silkworm.

Many polyploid cells were also photographed. In case of polyploid cells the number of FITC signal was directly proportional to ploidy level in the cell. For e.g. six signals were seen in a hexaploid cell, three in a triploid cell. Sperms having a haploid genetic content showed only one FITC signal (Fig. 6.16)

In situ hybridization was also carried out on chromosomes prepared from Uzifly, *Exorista bombysis,* a Dipteran fly. It is an endoparasite of *B.mori* and has XY/XX system of chromosomal sex-determination. It has two pairs of autosomes and one pair of sex chromosomes. All of them are morphologically similar. Chromosomes were prepared from testis and hybridized with DIG-labeled *BmP02* genomic clone. All the metaphase plates and interphase nuclei showed only one FITC signal testifying that *BmPø2* gene is located on one of the sex chromosomes (Fig. 6.17).

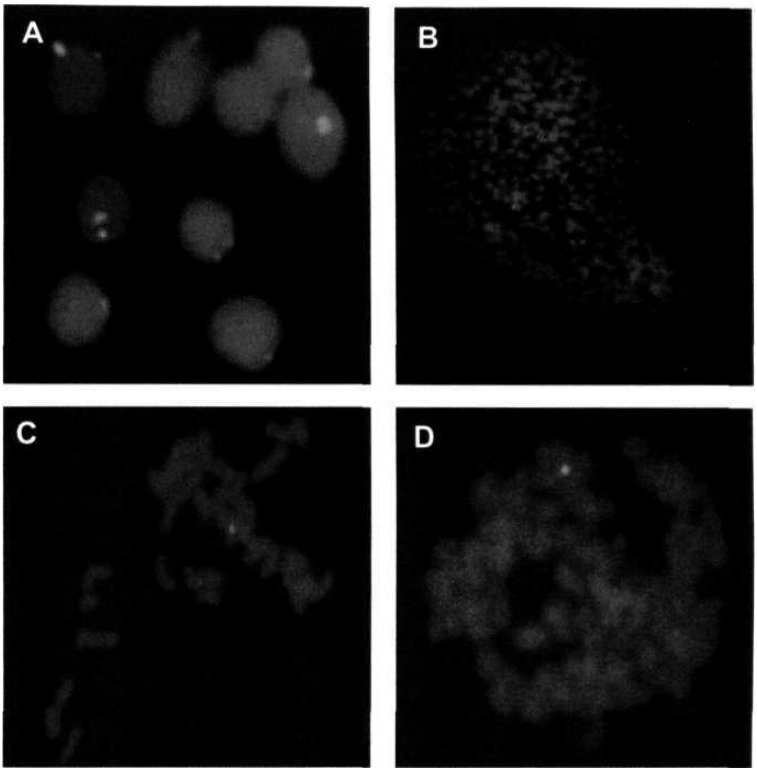

Fig. 6.16. Fluorescence *in situ* hybridization of *B.mori* chromosomes using DIG-labeled *Bmpϕ 2* probe.

A: Interphase nuclei from silkworm eggs,
B: Polyploid cell from silkworm eggs,
C: Testis metaphase chromosomes,
D: Egg metaphase chromosomes.

Note interphase nuclei from eggs showing a uniform distribution of single and two FITC signal. Metaphase chromosomes from testis show two distinct FITC signal suggesting that *Bmpϕ* 2 is located on Z-chromosome

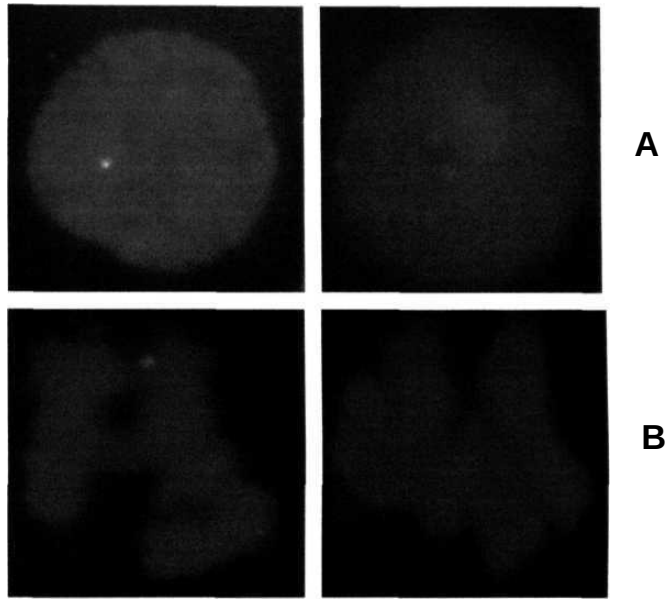

Fig. 6.17. Fluorescence *in situ* hybridization on the male **Uzi fly chromosomes using DIG-labeled *Bmpϕ2* gene**

Panels: A: Interphase nuclei, B: Metaphase chromosomes

Note both interphase nuclei and metaphase chromosomes of male Uzi fly show single FITC signal suggesting its localization on one of the sex-chromosomes.

DISCUSSION

Until today there has been no concrete information about the nature of sex determining genes localized on Z/ W sex chromosomes or on autosomes that are essential for the development of bipotential gonads and their differentiation into ovary and testis.

One of the basic reasons responsible for lack of information on sex determining genes in silkworm or any other Lepidoptera has been the very small size of chromosomes not suitable for recognizing structural changes resulting from mutational causes cytologically. The other reason seems to have been the success of crossbreeding experiments with naturally occurring genetic stocks in generating silkworm strains economically useful in silk industry.

The advent of modem genetic engineering tools has now armed the scientists to identify, characterize and sequence any gene in any biological system with the help of a heterologous probe. This was the approach adapted in the present study to identify the silkworm homologue of human ' *Pø2*'. This study led to the successful isolation and cloning of *Pø2* homologue from silkworm. Presence of *Pø2* homologue in silkworm genome clearly suggests that it is a highly conserved gene. Success of such a study encourages the silkworm geneticists to examine the genetic structure of sex chromosomes in silkworm with the help of heterologous probes of X and Y-chromosome specific human genes. Similar approaches can be utilized to identify, isolate and characterize silkworm autosomal genes as well. The results of the given studies would then help us to know the number and nature of tissue specific, organ specific homeotic genes as well as regulatory and house keeping genes. Table 6.4 provides a list of mammalian genes that find immediate application in the study of silkworm genes.

TABLE- 6.4.

Gene	Functional role in *Drosophila*	Localization in Humans	Functional role in Humans
DIAPHONOUS	*dia* affects spermatogenesis and oogenesis, mutation leads to sterility (Banfi *et al.*, 1997)	**Xp22**	Interrupted by a break point in patients with familial premature ovarian failure (Sala *et al.*, 1997)
Odd Oz	*odz* is a late acting pair rule gene that encodes a large signaling protein and is involved in development of CNS, adult eye, limb, wing, etc. (Levine *et al.*, 1994)	Xp25	These genes are expressed at early developmental stages as well as in adult in CNS, sensory epithelia, etc. They are expressed in an intricate overlapping pattern suggesting that each has its own critical role (Tali Ben-Zur & Wides, 1999)
Msl	*msl* genes are known to regulate transcription from the male X-chromosome in a dosage compensation pathway that equalizes X-linked gene expression in males and females (Gormman *et al.*, 1995)	Xp22.3	*MSL3L1* performs a similar function in chromatin remodeling and transcription regulation. Its a candidate gene for several developmental disorders that have been mapped to this region (Prakash *et al.*, 1999)
SCM *a)SCML1* b) *SCML2*	*scm* gene is a member of polycomb group genes. These genes are involved in regulation of homeotic genes and they act as transcriptional repressors (Bornemann *et al.*, 1996) -do-	Xp22 -do-	*SCML1* is involved in transcriptional repression of *Hox* genes and mutation in this gene may cause developmental malformation (Vosse *et al.*, 1998) It maps very close to *SCML1* and has been identified as a candidate gene for Xp22 linked developmental disorders including Oral-facial-digital (OFDI) syndrome (Montini *et al.*, 1999)
DACHSHUND	It encodes a nuclear protein required for normal eye and leg development (Mardon *et al.*, 1994)	13q21	*DACH* is a candidate gene for Bardet-Biedl syndrome. It is expressed in limbs and retina (Kozmik and Cvek., 1999)
Doublesex	*dsx* is a bif unctional gene functioning actively in alternative modes in the two sexes to induce either male or female differentiation (Baker and Ridge, 1980; Burtis and Baker, 1989).	9p24.3	*DMRT1* a protein with DM-domain, expressed only in testis, maps to distal short arm of chromosome 9, a location implicated in human XY sex reversal (Raymond *et al.*, 1998; Smith *et al.*, 1999)

Tissue and Developmental Stage Specific Expression of *BmPø2*

The presence of *Pø2* homologue in silkworm genome does not tell whether it is a house-keeping gene or developmentally regulated autosomal gene or sex-chromosome specific gene. To study the expression profile of *BmPø2* in silkworm, total RNA isolated from different tissues, and different developmental stages, was hybridized with *BmPø2* genomic clone. Among the different larval, pupal and adult stages studied for expression, very high expression was seen in the newly hatched 1st instar larva and a low level of expression was also detected in early 5th instar larval testis and ovary. In order to correctly identify the time at which this gene is switched on during embryonic development, total RNA was isolated from different embryonic stages, and hybridized with *BmPø2*. The results showed that its expression started around 5th-6th day of embryonic development when the gonadal ridge is formed. The expression of *BmPø2* was maximum at the late embryonic stages.

Since in all these experiments, total RNA isolated from whole embryo or whole 1st instar larva was used the results did not give any idea about tissues expressing the *BmP02.* However, it did suggest that the expression was not limited to one sex as it was observed both in early 5th instar larval testis and ovary.

To identify the different tissues where *BmPø2* was expressed during the embryonic developmental stages, RNA in *situ* hybridization was carried out on sections made from different embryonic stages using DIG-labeled *BmPø2* genomic clone as a probe. In the sections made from 5-6 day old embryonic stage, the expression was localized to gonadal ridge. In addition to gonadal ridge, the expression was also seen in nerve ganglion. In newly hatched larva, fully formed testis could be visualized and *BmP02* transcripts were predominantly localized in the testis with a significant level of expression in nervous tissue. All the methodological approaches on the organ/tissue/stage specific pattern of expression revealed that (i) Its expression started at 5th -6th day of embryonic development when gonadal ridge was formed with dual

potential of differentiating into testis or ovary, (ii) Maximum expression of *BmPø2* was seen in the embryonic stages, (iii) *BmPø2* expression was significantly higher in testis than in ovary, (iv) *BmPø2* was also expressed in nerve ganglions, (v) The temporal pattern of expression with maximum expression in the later embryonic stages of development was observed, which started declining with age and reached zero level in the fully matured 5th instar silk spinning larva. These findings clearly indicate that the *BmPø2* is a developmentally regulated gene predominantly expressed in gonads.

Chromosomal Localization of *BmPø2*

Fluorescence *in situ* hybridization results revealed localization of *BmPø2* gene on the Z-chromosome. The Z-localized genes in silkworm and other lepidopteran species (Traut and Marec, 1996) are not dosage compensated and are therefore expressed both in testis as well as ovary with higher level of expression in the former than in the latter. The observed expression pattern of *BmPø2* follows the well-documented expression pattern of Z-linked genes like *Bm kettin* gene, *T15* gene, *6-phosphogluconate dehydrogenase*, etc. In chicken, the DMRT1 homologue is located on Z-chromosome. It is expressed in the genital ridge from the time it starts to form and the expression is higher in male embryos than in female. DMRT1 is implicated in avian sex-determination because of its expression profile and Z-chromosome localization (Raymond *et a*/., 1999; Ellegren, 2000).

Sequence Analysis of *BmPø2* Gene

To examine whether *Bmpø2* encodes for a WD-repeat kind of protein or any other protein of regulatory nature, we have sequenced the 11.63kb and 3.2kb stretch of 16kb genomic clone of *Bmp02.* Detailed *in silico* analysis of *Bmpø2* using ESTs from Silkbase revealed that it contains six putative exons of 87bp, 248bp, 149bp, 252bp, 129bp and 145bp respectively in the 11.2kb genomic fragment. In addition to these 6 exons, there are exons present further downstream of the 6th exon, as the sequences present at the 3' end of EST

wdS00697 are not present in the genomic clone and the poly adenylation signal, poly-A tail also is not present at the 3' end of the EST. However, the 5' end of the gene is complete because the transcription start site or the Kozak sequences are present upstream to the 1st exon.

Human *Pø2* cDNA is a 2.49kb sequence with a GC content of 60%. Both human and mouse *Pø2* genes are made up of 9 exons, and show 50-55% homology with *BmPø2* exons at nucleotide level. However only exon 2, 3, 4, 5 and 6 of human *Pø2* show homology with *BmPø2* genomic clone or homologous silkworm EST.

Homology search in the Human genome database of X-chromosome using putative *BmPø2* cDNA sequence as query revealed that it shows maximum homology to the region Xp11.23 where human *Pø2* gene is located thus confirming that *BmPø2* and human *Pø2* are indeed homologous.

The putative *BmP02* cDNA shows 65-70% homology to Drosophila EST-GM05201 from Germanium stage-6, EST-LD11521 from the embryonic stage (0-24hours) both belonging to the same gene CG1800 and 5' end of *C.elegans* cDNA clone yk192c4 from the gene *T22A3.5.* Silkworm EST, wdS00697 also identified the same EST form *Drosophila* and *C.elegans.* The EST-LD11521 from embryonic stage suggests that expression of *CG1800* starts very early in embryonic development. Identification of similar EST's from *Drosophila,* and C. *elegans* further consolidates the fact that *Pø2* is a highly conserved gene.

Putative Protein Coded by *BmPø2*

ORF analysis of putative cDNA having 6 exons was carried out. The longest open reading frame was found in +1 frame. The ORF starts in the 3rd exon and continues till the end suggesting that 3' end of cDNA is not complete. The 145 amino acid protein coded by the putative *BmPø2* shows 65-70% homology to the CG1800 protein coded by the Drosophila gene.

CG1800 gene in *Drosophila* is located on chromosome 3R and has been cytologically mapped to the region 100D2. It has a transcription unit length of 3135 bases. The function of this gene has been categorized as RNA binding based on the sequence similarity with the other RNA binding proteins. It has domains like DSRDM (double stranded RNA binding domain), dsrm, and encodes for a 642-amino acid protein. There are no recorded mutant alleles for this gene. Similar gene *T22A3.5* has been identified in *C. elegans. T22A3.5* is located on 1^{st} chromosome. cDNA clone yk192c4 belongs to this gene . It encodes for a 790-amino acid protein and has a double stranded RNA binding domain.

RNA-binding proteins play central role in the post-transcriptional regulation of gene expression. These proteins contain regions, which function as RNA-binding domains, and auxiliary domains that mediate protein-protein interaction and subcellular targeting. The double-stranded RNA-binding domain is a 65-amino acid motif that is found in a variety of proteins that interact with double-stranded RNA (Burd and Dreyfuss, 1994).

Drosophila *lark* gene encodes an essential RNA binding protein of the RNA recognition motif (RRM) that is required for the embryonic development. The *lark* homologous mouse and human gene expresses a single 1.8kb size class of mRNA in most or all tissues including brain. Both mammalian proteins contain two copies of RRM type consensus RNA binding motif. It has been postulated that the LARK protein executes an RNA binding function related to translation control or RNA processing (Jackson et al., 1997). Another murine gene, *Zfr* (zinc finger RNA binding) contains a domain that has been conserved in a collection of frog, mouse, humans, *C. elegans* and *Drosophila. Zfr* probably functions in germ cell development, and perhaps in other developmental pathways in these organisms (Jackson *et al.,* 1997).

The human gene *Pø2,* investigated by Singh and his group codes for a protein having WD-motifs (unpublished data). The best-characterized WD-protein is the G-p subunit of heterotrimeric G-protein, which forms a tight dimer (G-β-γ)

with G-γ subunits. G-proteins like transducin are very well characterized components of the transmembrane signaling machinery in eukaryotes (Smith *et al.,* 1999). In addition to G-proteins, several other proteins that have WD-repeat are known to function in signal transduction. They include a subunit of a phosphatase (Neer *et al.,* 1993; Ron D *et al.,* 1994; Chen R *et al.,* 1995) However, the other known functions of WD-repeat protein include RNA processing, by being a member of RNA processing complex and transcription regulation by being a subunit of the TATA box binding protein complexes (Hoey ef *al.,* 1993). WD-proteins are also known to play a role in cytoskeleton assembly and mitotic spindle formation, regulation of vesicle formation and vesicular trafficking, controlling various aspects of cell division and regulation of sulphur metabolism in fungi (Smith *et al.,* 1999). The two completely sequenced eukaryotic genomes, S. *cerevisiae* and C. *elegans,* have 55 to 70 WD-repeat proteins respectively. Of these, at least 12 proteins form pairs that are predicted to have similar surfaces and would therefore be expected to be functional homologues in the two organisms. Among these, 12 are proteins that play roles in RNA processing, signal transduction, cell division as well as proteins whose functions are not known.

The 146-amino acid putative protein coded by *BmPø2* cDNA does not have any WD-motif. However, only partial amino acid sequence of *BmP02* protein is available. It is possible that WD-motifs might be present after the initial stretch of these 145-amino acids in the full-length *BmPø2* protein. Characterization of full-length *BmP02* cDNA and protein will give us clear-cut idea about the presence of WD-motifs in *BmPø2* protein and will also tell us whether this gene is functionally homologous to human *Pø2.*

On the face of reported enormous diversity in the function of WD-repeat protein, it is difficult to assign any specific function to WD-protein of human *Pø2* gene with any authority. Nonetheless, in view of the location of *Pø2* on homomorphic sex chromosome 'X[1] in humans, its homology to *BmPø2* of silkworm, *CG1800* gene of Drosophila and *T22A3.5* gene of C. *elegans,* all having dsRNA binding domain, the reported range of function of *Pø2* can be

narrowed down to sex-specific transcription and RNA processing. But such inferences will require experimental verifications to become definitive conclusions.

Mechanism of Sex-Determination in Lepidoptera?

Working hypothesis for genetics of sex determination in *Drosophila* (Hodgkin, 1990), worms (Cline and Meyer, 1996), Musca (Schmidt *et* a/., 1997) and mammals (Jimnez and Burgos, 1998) has always preceded the planning of relevant genetic experiments in order to arrive at the correct genetic model. Unfortunately, there has been no such attempt of constructing a genetic model of sex determination in silkworm or any other lepidopteran member (Traut and Marec, 1997). It is therefore very tempting to propose a working hypothesis for genetic determination of sex in 6. *mori* and other lepidopteran insects based on already available relevant genetic information, which is as follows (Traut 1987; Traut and Marec, 1996):

1. Members of lepidoptera exhibit sexual dimorphism with heterogametic females having WZ or ZO constitution and homogametic males having ZZ constitution. Some heterogametic females with ZO constitution (members of family micropterigidae) are evolutionarily most primitive and some others like *Antheraea assamensis*, *Ipimorpha pleonectusa,* etc., with identical constitution are evolutionarily more advanced and are considered to have arisen by the secondary loss of W-chromosome from WZ ancestor (Traut and Marec, 1996).

2. *B.mori* embryos with ZZ chromosomal constitution develop as males and with WZ chromosomal constitution grow as females.

3. Ratio of sex chromosome to autosomes in ZZ/Z0 system determines the nature of primary sexual signal. When the ratio of Z:A is 1.0, the primary sex signal is male-specific and when Z:A ratio is 0.5, the sex signal is female- specific.

4. There is virtually no definite information on the location of primary sex determining genes controlling maleness and femaleness.

5. It is accepted that all ZO/ZZ chromosome systems are balanced mechanisms with male promoting factors on the Z-chromosome and counteracting female promoting factors on the autosomes (Traut and Marec, 1996). In WZ or WZZ system of heterogametic sex, the Z: A ratio generated sex signal is hypostatic to dominant W-linked female determiner as is the case in 6. *mori.*

6. In the present study, use of GATA containing *Bkm* sequences for identification of sex-specific genes showed the presence of such sequences throughout the genome including the autosomes. Similar results were obtained by Traut (1987) in *Ephestia kuehniella,* and he concluded from the results the possible presence of GATA associated sex genes on autosomes as well. The present results of *B. mori* can also be taken to infer that GATA repeats may similarly be associated with genes with sex-specific functions, which may be located on autosomes.

Drosophila is demonstrated to have balanced mechanism of sex determination with female determining genes on the X-chromosome and male determining genes on autosomes (Bridges, 1925). Accordingly, ratio of X:A determines the nature of primary sexual signal. In *M. domestica,* dominant male determining factor 'M' is known to occur on Y-chromosome, autosomes as well as on X-chromosome and the postulated 'F', the female determiner hypostatic to male determiner may be autosomally or X-chromosome located. In this system, ratio of X:A has no role in determining the nature of primary sexual signal. The male factor 'M' determines the nature of primary male specific signal by repressing the expression of female determining gene 'F (Schütt and Nothiger, 2000).

The female determinants of lepidopteran system (ZZ/ZW) like male determinants of *M. domestica* function by repressing the activity of male determiner. However, the other lepidopteran systems (ZZ/Z0), appear to use ratio of Z-chromosome to autosomes, like Drosophila uses X : A ratio in

producing primary sex-specific signal. But this system differs from Drosophila where the primary sexual signal when present functions like activator of female determining key master sex switch gene from its early promoter (Sxl_{pe}) and in lepidoptera primary sexual signal when present functions as repressor of female pathway. Thus it seems that lepidopteran system of chromosomal sex determination shares the sex determining mechanism of Drosophila and *M. domestica*.

Sexual differentiation in sexually dimorphic animal system comprises sex-specific morphology, physiology and behaviour, dosage compensation or lack of dosage compensation and sex-specific development of the germ cells. A variety of different genetic or environmental mechanisms have evolved to achieve the sexually dimorphic program of development and differentiation. A careful analysis of various systems reveals the operation of a common principle, which consists of a hierarchly built control system consisting of:

(a) Genes determining the nature of primary sexual signal.
(b) Key master switch gene capable of reading the signal and responding by being in a state of activity (on) or inactivity (off).
(c) A genetic double switch (dsx) responding to the on/off state of master switch gene and then accordingly activating the specific pathway.

According to the given principle, it is proposed that members of lepidoptera (ZZ/ZO) contain a Z-located male determining gene 'M' leading to the production of repressor signal for 'F' (female promoting gene), whose state of activity is then transferred to the genetic double switch dsx_m activating the male pathway of development. This mode of sex determination operates in individuals with a Z: A ratio of 1.0. When the Z:A ratio is 0.5, the Z-generated male specific signal is not enough to repress 'F'gene. This results in activation of 'F' gene producing a product which modulates the transcription of *dsx* gene in a way that results in production of female specific *dsx* product designated dsx_f inhibiting male pathway and activating female pathway genes. In Drosophila, X-located numerator constitutes the X-dose counting mechanism,

which in XX individuals generates transcription activator signal of female pathway. In lepidoptera, an analogous Z-dose counting mechanism represented by male determining factor is proposed to occur which in ZZ individuals generates male specific signal by inactivating F^+ and in ZO individuals generates an inactive male signal thus rendering the female gene in active state. WZ system of lepidoptera like 8. *mori* appears to have evolved as a variant of ZO system carrying a mutant dominant form of female determiner F abbreviated here as F . The epistatic female determining behavior of 'W is a strong evidence for occurrence of wild type female determining F^+ gene located on autosomes.

Does this mean that such regulatory genes of sex-determination have been conserved in evolution? Available knowledge and information on this aspect in *Drosophila*, *Caenorhabditis,* mammals and *Musca* suggests that it is not so. For example, although *Drosophila, Caenorhabditis* and lepidopteran members make use of the sex chromosome to autosome ratio as the primary signal, the molecular nature of the regulatory genes and the mechanism by which these genes control sexual development are very different (Hodgkin, 1990; Cline and Meyer, 1996). In contrast with this, apparently different modes of sex determination found in various strains of *M. domestica* is the result of variation from a common genetic and molecular theory. It has been demonstrated that various allelic forms of female determining gene 'F and transposon like male determining factor 'M' together can account for all the observed apparent variation in sex determination mechanism (Schutt and Nothiger, 2000). It therefore requires to analyze the nature of 'M', 'F and *dsx* proposed to function in regulation of sexual dimorphism. If 'M' is male determining, it has to be testis specific. In the present study in *B. mori,* the *Pø2* homologue of silkworm (*BmPø2*) is gonad specific in expression, shows maximum expression in the testis and is developmentally regulated. But whether the silkworm homologue is representation of 'M' can be resolved only when its transcription and translational product is analyzed. However, the verification of the proposed working hypothesis for sex determination in silkworm and other members of lepidoptera requires investigation of the occurrence and organ / tissue / stage

specific expression pattern of proposed sex determining gene by using their homologues found in *Drosophila, Musca, Megaselia,* etc.

Wilkins (1995) proposed a model for sex determination cascade to be evolving from bottom up and not from top down. According to this model, different upstream global sexual regulators appear to be unrelated atleast between nematode and arthropods where they have been most extensively characterized. The *dsx* gene is the end regulator of sex determination cascade. Recent studies have shown that this gene is highly conserved not only in its structure but also in its expression and also possibly in its function (Raymond *et al.,* 1998). As in Drosophila, the *dsx* homologue of *Megaselia* (Sievert *et al.,* 1997) *Bactrocera* (Shearman and Fromner, 1998), *Musca* (Schutt and Nöthiger, 2000) and *Ceratitis capitata* (Saccone *et al.,* 1998; Schutt and Nöthiger, 2000) express male and female specific transcript, the intron-exon structure is also highly conserved. A gene with a DM domain similar to *mab-3* and dsx has been identified in humans, chicken, and alligator and has been designated as *Dmrt1*. *Dmrt1* has a gonad-specific and sexually dimorphic expression profile during embryogenesis in mammals, birds and reptiles *(Alligator mississippiensis).* The sex specific expression pattern in all the organisms studied so far is suggestive of its sex-determining role (Raymond *et al.,* 1999; Ellegren, 2000; Schutt and Nbthiger, 2000). Other downstream sexual regulators, for example, *fruitless, intersex,* and *hermaphrodite* genes of Drosophila may also be conserved across the phyla. It has been proposed that the downstream regulatory genes in sex-determining pathways are the most ancient and upstream regulatory genes are recruited subsequently (Dubendorfer *et al.,* 1992; Raymond *et al.,* 1998). For example, upstream regulators like *tra, and Sxl,* were recruited by different taxonomic groups during evolution (O'Neil and Belote, 1992; Raymond *et al.,* 1998). Downstream regulators such as *dsx* and *mab-3* may be less likely to evolve because they control multiple genes and modifying such downstream regulators might also require modification of all of their targets (Marin and Baker, 1998; Raymond *et al.,* 1998; Schutt and Nbthiger, 2000). The available data suggest that *BmPø2*

is probably one such downstream regulatory gene in sex determination, which has been conserved across a wide variety of eukaryotes.

It is very fascinating to point out that the gene *Pø2*, which is evolutionarily highly conserved in eukaryotes, from humans to mouse, snakes, birds and *B.mori* is predominantly expressed in the testis in all of them, irrespective of their heterogametic sex (male heterogamety in mammals, XYÓ/XXQ and female heterogamety in snakes, birds and *Bombyx mori*, ZZÓ/ZWQ). In mouse and humans, *Pø2* gene is located on the X-chromosome and it is highly significant that *Bmpø2,* a homologue of *P02* is located on the Z-chromosome, two copies of which are present in males and only one in females. Since *P®2* is present on the X-chromosome in both the sexes but expressed predominantly in males in the presence of Y-chromosome, it may require presence of Y-chromosome for its transcriptional activation. In the case of *B. mori,* it appears that *Bmp02* is expressed constitutively in the male testis but presence of W-chromosome in females may repress its transcription in the female, which remains unaffected in males. If the gene knock out experiments in mouse involving *Pø2* gene, which is in progress (Singh and Colleagues, in progress) confirms the predicted role of this gene in the organization of testis, it would also demonstrate the molecular basis of the origin of the female heterogamety. Origin of a regulatory gene present on the W-chromosome, which can repress the expression of testis organizing gene on the Z-chromosome may lead to development of ovary and therefore female sex.

In conclusion, *BmPø2* by virtue of its location on Z-chromosome, gonad specific and sexually dimorphic expression profile during embryogenesis and later on during development, homology to Drosophila gene having RNA binding motif is a good candidate gene for male determining factor located on Z-chromosome. In other words, the available data suggests that *BmPø2* probably plays an important role in complex pathway of sex-determination and differentiation. Further experiments involving knock out of *BmPø2* in silkworm genome is necessary for establishing the role of *Bmpø2* in sex-determination and spermatogenesis.

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