Chapter IV

MATERIALS AND METHODS
EXPERIMENTAL MATERIALS

4.1 BACTERIAL STRAINS

(a) \textit{E.coli} DH5\(\alpha\) \(\lambda^-, \text{rec A}1 \text{ end A}1 hsd \text{ R}17(\text{r}_{\text{k}}^-, \text{m}_{\text{k}}^-)\)
\(\delta(\text{lac ZYA-arg F}) \text{ UI69} (\text{f80 lacZ dM15})\)
\text{sup E44 thi-1 gyrA96 relA1} \ (\text{BRL, 1986})

This strain was used for all routine transformations, plasmid isolations, selection of recombinant plasmids etc

(b) \text{LE392} \ F^-, \text{hsd R}574 (\text{r}_{\text{k}}^-, \text{m}_{\text{k}}^+), \text{sup E44, sup F58},
\text{lac Y}1, \text{or d(lac 1ZY)}6, \text{galK2, gal T22, met B}1,
\text{trp R}55, \text{t}^- \ (\text{Murray et al., 1977}).

4.2 PLASMIDS

pUC18 and pGEM3Z, high copy number plasmids derived from pBR322 (Messing, 1983; Yanisch-Perron et al., 1985), were used in this study.

4.3 FLY STRAIN AND CULTURE CONDITIONS USED

Wild type strain of \textit{Drosophila melanogaster}, Canton-Special (CS), was used for the present study. Flies were maintained in milk bottles or glass vials on standard medium containing agar, maize powder, sugar and yeast at 22 ± 1°C. For the collection of third instar larvae, the cultures were grown in uncrowded culture conditions and were provided with additional yeast suspension.

4.4 CLONES/PROBES USED

a) \textbf{Bkm-2(8)}: A BamHI/ PstI insert of the \textit{Drosophila} clone CS314 containing 545 bp insert having 66 copies of tetranucleotide repeat GATA \ (Singh et al., 1981)has been cloned into pUC18 for easier handling. The insert can, now, be
taken out with the help of EcoRI and HindIII, both the enzyme sites being outside the BamHI and PstI, on the two sides.

b) pUC18-(GATA)$_{16}$: Oligos containing poly-GATA and poly-CTAT were synthesised, annealed, kinased and cloned into SmaI site of pUC18. For use in Electrophoretic Mobility-Shift assays, the insert was taken out by digesting with EcoRI and HindIII, the two restriction sites flanking SmaI. The insert is ~120bp long and has 16 repeats of tetranucleotide GATA. (Singh et al., 1994b).

4.5 BACTERIAL MEDIA, ANTIBIOTICS AND COMMONLY USED SOLUTIONS

1. Ampicillin: 100 mg/ml stock in double distilled water was filter-sterilized through 0.22μ Millipore membrane filter and stored in -20°C.

2. Denaturing Solution: 0.5N sodium hydroxide, 1.5M sodium chloride, 1mM EDTA, adjusted pH to 7.2 with 1N HCl.

3. DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF and 50% glycerol in water. Stored frozen at -20°C.

4. Drosophila Ringer: 7.5g NaCl, 0.35g KCl, 0.21g CaCl$_2$, dissolved in 1L of water (Ephrussi and Beadle, 1936).

5. IPTG: 1M stock of IPTG in double distilled water was filter-sterilized through 0.22μ Millipore membrane filter.

6. LB: 1% bacto-tryptone, 1% sodium chloride, 0.5% bacto-yeast extract, pH adjusted to 7.0 with 0.1N sodium hydroxide.

7. LB agar (bottom): LB containing 1.5%(w/v) bactoagar.

8. SOB: 2% bacto-tryptone, 0.05% sodium chloride; 0.5% bacto-yeast extract and 2.5 ml of 1M KCl; pH adjusted to 7.0 with 0.1N sodium hydroxide. After autoclaving and just before the use added 5ml of 2M MgCl$_2$ per litre of solution.

9. SOC: Added filter-sterilized 1M glucose solution to the autoclaved SOB to a final concentration of 20mM.

10. Top agar: LB containing 0.7%(w/v) bactoagar.
11. Neutralizing Solution: 0.5M tris base, 1.5M sodium chloride, 1mM EDTA, adjusted pH to 7.2 with 1N HCl.

12. Hybridization Solution: 0.5M phosphate buffer, pH7.5 and 7% SDS.

13. RFI: 100 mM rubidium chloride, 50 mM MnCl₂.4H₂O, 10 mM CaCl₂.2H₂O, 30 mM potassium acetate, glycerol 15%, pH adjusted to 5.8 with 0.2M acetic acid.

14. RFII: 10 mM MOPS (3-(N-morpholine)propanesulfonic acid)), pH6.8, 75 mM calcium chloride, 10 mM rubidium chloride, 15%(w/v) glycerol, pH was adjusted to 6.8.

15. SM: 0.58% NaCl, 0.2% MgSO₄, 50 mM Tris-HCl (pH 7.5) and 10 mg gelatin in 100 ml double distilled water.

16. 20XSSC: 3M sodium chloride and 0.3M sodium citrate, pH7.0.

17. 10XTAE: 0.9M Tris base, 10Mm EDTA, pH adjusted to 8.0 with glacial acetic acid.

18. 10XTBE: 0.9M Tris base, 10Mm EDTA, pH adjusted to 8.0 with boric acid.

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

20. 25XTPE: 2.25M Tris base, 50mM EDTA, adjusted pH to 8.0 with 85% phosphoric acid (1.679g/ml).

21. X-gal: 20 mg/ml of X-gal in dimethyl formamide was stored at -20°C.

4.6 CHEMICALS

The different sources of the fine chemicals used in this study are listed below:

AGFA-Gevart AG- 25ASA B/W negative Panchromatic film for microphotography.

Amersham- Hybond-N, Hybond-N⁺, T4 DNA ligase, LM1 emulsion, ³H-dATP, ³H-dTTP

Bangalore Genei- DNA molecular weight markers, e.g. λ- HindIII digest, λ-EcoRI/HindIII double digest
BARC, India- Nick translation kit, Random Primer kit, $\alpha$-$^{32}$P-dATP, dephosphorylation kit.

BDH- acrylamide, bis-acrylamide, sodium acetate, TEMED, Gurr's Giemsa stain, low melting agarose

Bio 101- Gene clean kit

Boehringer Mannheim- calf intestinal phosphatase, IPTG, X-Gal, DNA molecular weight markers- II, III, V, X.

Difco- Bacto-Agar, Bacto-Tryptone, Bacto-Yeast extract

DuPont- $\alpha$-$^{33}$P-dATP, Intensifying screens (for X-ray film cassettes).

Fluka- potassium acetate

HiMedia- Bacto-Agar, Bacto-Tryptone, Bacto-Yeast extract

Ilford- K2 emulsion for micro-autoradiography

Kodak- X-ray films, 400ASA color negative film

Konica corporation- X-ray films

Millipore- Nitrocellulose filter discs of 0.45µ and 0.22µ pore sizes

New England Biolabs- All restriction enzymes and their buffers, T4 DNA ligase and its buffer

Oswel DNA service- Custom-made oligonucleotides

Pharmacia- Ficoll, Sephadex G-25, ligase

Promega- M13/pUC primers

Schleicher and Schuell- nitrocellulose filter and slot blot apparatus

Oncor Inc- FISH detection kit

Serva- acrylamide, bis-acrylamide

Sigma-agarose (TypeII), $\beta$-mercaptoethanol, BSA (Fraction V), caesium chloride, calcium chloride, DTT, ethidium bromide, formamide, heparin, lysozyme, proteinase K, SDS, rubidium chloride, tris base, RNaseA and RNaseT1.
Whatman International Ltd.—Whatman filter papers-1MM and 3MM

All other chemicals were obtained from local manufacturers and were of Analytical grade.

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4.7 Sterilization

All glasswares and plastic-wares were sterilized by autoclaving at 15 lb per square inch (psi) pressure for 20-30 min or by baking at 180 °C for 12 h, before using for the experiments. All solutions were prepared in double distilled water or milli Q water and then filtered through 0.45μ nitro-cellulose filter. Sterilization of the solutions, including bacterial growth media was done by autoclaving at 15 psi for 15-20 min. The antibiotics were sterilized by filtration through 0.22μ filters.

4.8 Siliconization

The glassware and plastic-wares needed for small quantities of nucleic acids were siliconized in a 5% solution of dichlorodimethylsilane (about 2% in 1,1,1-trichloroethane) diluted in chloroform. The siliconized lab-ware were heated at 80°C for 2 h and autoclaved after rinsing several times with double distilled water.

4.9 Preparation of frozen competent cells

Competent cells were prepared according to the method described by (1985), with some modifications. A single colony of E. coli DH5α was inoculated from an LB agar plate into 5 ml LB medium and incubated at 37°C in a shaking incubator at 150rpm for overnight. One ml from this was inoculated into 50 ml of LB medium in a conical flask, and incubated at 37°C in a shaking incubator at 150 rpm till its absorbance at 600 nm reached between 0.45 and 0.55 (the cell density being 4-5 x 10^7 cells/ml). The culture was chilled on ice. The cells were collected by centrifuging 35 ml of culture at 3,000 rpm for 15 min at 4°C in HB-4 rotor in Sorval RC 5B centrifuge. The supernatant was completely drained, and the pellet
was suspended in 10 ml of RF1 buffer (RbCl 100 mM, MnCl₂·4H₂O 50 mM, CaCl₂·2H₂O 10 mM, potassium acetate 30 mM, glycerol 15% pH 5.8). The cell suspension was incubated on ice for 15 min and the cells were centrifuged as above. The pellet was resuspended in 2.4 ml of RF2 (MOPS 10 mM, RbCl 10 mM, CaCl₂·2H₂O 75 mM, glycerol 15%; pH 6.8). The cell suspension was again incubated on ice for 15 min and distributed into sterile, pre-chilled microfuge tubes in 100 µl aliquots. The aliquots were flash frozen in liquid nitrogen and stored at -70 °C till use.

4.10 TRANSFORMATION OF THE COMPETENT CELLS

The frozen competent cells were allowed to thaw at room temperature. The tubes were immediately placed on ice and 2-5 µl of DNA sample (5-100 ng) was added to 100 µl of competent cells and mixed by gentle tapping. The transformation mixture was then incubated on ice for 40 min. The cells were given a heat shock at 42°C for 90 sec. The tubes were chilled by returning them immediately to ice and were kept in ice for 5 min. LB medium (500 µl) was added and the transformed cells were incubated at 37°C for 1 h before plating them.

The transformed bacteria were plated on 90 mm LB agar plates containing 100 µg/ml ampicillin, 1 mM IPTG and 50 µg/ml X-GAL, for pUC based vectors. The plates were incubated at 37°C for 12-16 h to allow the bacteria to grow. The plates were left at 4°C from 4 hs to overnight for blue color development. The white colonies were checked for the presence of the desired recombinant plasmids by screening using miniprep for the plasmid or by colony hybridization.

4.11 SMALL SCALE ISOLATION (MINIPREP) OF PLASMID DNA

The protocol of Alkaline lysis method (Birnboim and Doly, 1979 and Horowicz and Burke, 1981) as described by Sambrook et al. (1989) was used for miniprep of plasmid with minor modifications. Centrifuged 1.5 ml of bacterial culture, grown overnight at 37°C (in appropriate antibiotic), at 6000 x g for 5 min at 4 °C. The pellet was resuspended in 0.5 ml of STE (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA, pH 8.0) and centrifuged again at 6000 x g for 5 min at 4°C. Resuspended the pellet in 100µl of ice-cold Solution I (50mM Glucose, 25mM Tris.Cl, 10mM EDTA, pH 8.0) by vigorous shaking. 200µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added and mixed by inverting the tube rapidly five times.
Then 150μl of Solution III (3M potassium acetate, pH adjusted to 4.8 with glacial acetic acid) was mixed by vortexing the tube, gently, in inverted position, incubated on ice for 5 min and centrifuged at 12000 × g for 5 min at 4°C. The solution was extracted with phenol:chloroform mixture, centrifuged at 12000 × g for 5 min at 15°C. The aqueous phase was extracted with chloroform and centrifuged, as written earlier. Ethanol (2.5 volumes) was added to the aqueous phase. After 2 min at room temperature, the mixture was centrifuged at 12000 × g for 5 min at 4°C. The pellet was washed thrice with 70% ethanol at RT. The dried pellet was dissolved in 50μl of TE, pH8.0. If required, the plasmid solution was treated with RNase at a final concentration of 50μg/ml, followed by phenol:chloroform purification, ethanol precipitation, drying and redissolving in TE.

4.12 LARGE SCALE ISOLATION OF PLASMID DNA

The procedure used was similar to that used for small scale isolation, except for some changes made for scaling up. 5 ml of overnight grown culture (containing appropriate antibiotic) was inoculated into 500 ml of LB, with antibiotic. The culture was grown at 37°C for 12-16 h with vigorous shaking (200-250rpm) in a rotary shaker. The cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C in a Sorvall RC5B GSA rotor, washed the pellet in 100 ml of ice-cold STE (0.1M NaCl, 10mM Tris.Cl, 1mM EDTA, pH 8.0). The pellet was resuspended in 18 ml of solution I (50mM glucose, 25mM Tris.Cl, 10 mM EDTA, pH 8.0). Freshly prepared 2 ml lysozyme solution (10mg/ml in solution I) was added and incubation was carried out at room temperature for 10 min after mixing it thoroughly. 40 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was then added and the contents were mixed by gently inverting the tube several times. After incubation at room temperature for 5-10 min, 40 ml ice-cold solution III (3M potassium acetate, pH adjusted to 4.8 with glacial acetic acid) was added and mixed thoroughly. The mix was kept on ice for 10 min and centrifuged at 10,000 rpm for 1 h at 4°C in a GSA rotor. To the supernatant 0.6 volumes of isopropanol was added and stored at room temperature for 15 min. Centrifuged at 10,000 rpm for 30 min in GSA rotor at room temperature. The pellet was washed thrice in 70% ethanol at room temperature. The pellet was air-dried and dissolved in 3 ml of TE. The plasmid was further purified by equilibrium density centrifugation in CsCl-ethidium bromide equilibrium density gradient centrifugation.
4.13 Puriﬁcation of the Plasmid DNA

The procedure described here is adapted from Sambrook et al. (1989) with some modifications. To 8 ml of plasmid solution (in TE, pH8.0), 8g of CsCl and 0.4 ml of ethidium bromide solution (10mg/ml in water) were added. After mixing, the solution was centrifuged at 72K rpm for 5.5 h at 20°C in a VTi80 rotor in Beckman preparative ultracentriguge. Out of the two bands visible, the lower band was collected. Ethidium bromide was removed from the DNA solution by partitioning it into water-saturated butanol. The resulting solution was dialysed against TE (pH8.0) with many changes at 4°C for 12-16h. The DNA, so obtained was treated with RNase, only if used for sequencing. The solution was immediately extracted with phenol: chloroform and precipitated by adding 1/10 volumes of 3M sodium acetate, pH 5.5, and 2.5 volumes of ethanol. The pellet obtained after centrifugation at 4°C was washed with 70% alcohol, air dried and ﬁnally dissolved in TE, pH8.0.

4.14 Estimation of Concentration and Purity of DNA

The quantity of nucleic acids present in the samples was estimated by gel electrophoresis, followed by staining in ethidium bromide. The intensity of the band was visually compared with that of standard DNA of known concentration e.g. λ DNA or λ-HindIII/RI digest or λ -BstEII digest.

The purity of DNA was checked by taking a ratio of O.D. at 260 nm versus that at 280nm. The ratio of 1.75-1.9 was taken as a measure of the quality of DNA.

4.15 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 upto 20 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 with ﬁnal concentration of 2mM was also added. Most of the digestions were stopped by incubating at 65°C for 15 min and only if the need arose, phenol:chloroform puriﬁcation was performed.
4.16 **Purification of insert DNA from agarose gel**

The procedure used was adapted from Vogelstein and Gillespie (1979). The DNA fragments were purified using Geneclean kit. The procedure followed is the same as supplied by the manufacturers with some modifications. The agarose gel piece having the DNA fragment was dissolved in three volumes of sodium iodide (w/v) at 55 °C. 5-10 μl of glassmilk supplied with the kit was added to the solution and was incubated on ice for 10 min with occasional mixing. The DNA bound to glassmilk was washed three times with new wash buffer (Tris, NaCl, EDTA in ethanol). The DNA was eluted in TE (pH 8.0) by incubation at 50-55 °C for 2 min.

4.17 **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 linearised vector was dephosphorylated using Calf Intestinal Phosphatase (CIP), as per the protocol of Tabor (1989). To 1-2 μg of linearised vector, added CIP dephosphorylation buffer (composition of 1 x CIP dephosphorylation buffer is 1mM ZnCl₂, 1mM MgCl₂ and 10mM Tris pH8.3), 0.1 unit of CIP and incubated at 37 °C for 30 min. CIP was inactivated by heating at 75°C for 10 min in the presence of 5mM 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 re-dissolved in TE (pH 8.0).

4.18 **Subcloning and ligation**

Subcloning of genomic clones, CS314 and CS316, was carried out mainly by using shotgun method. DNA was digested with EcoRI, extracted with phenol chloroform, precipitated and dissolved in TE buffer. Ligation was carried out at 16 °C for 16h in 10 μl reaction volume containing ligase buffer (50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP), 20 ng of dephosphorylated
vector DNA, 50-100 ng of DNA solution containing a mixture of inserts to be subcloned and 1.25 units of T4 DNA ligase (Revie et al., 1988).

Few fragments were subcloned individually directly into the vectors. Liguations were carried out with dephosphorylated vector and three molar excess of purified, compatible DNA fragments of choice.

After ligation, 2-4 \( \mu l \) of reaction mixture was used for transformation, as per protocol described earlier. The white colonies were checked by miniprep for the presence of the plasmid, followed by restriction digestion to look for the expected pattern.

4.19 RESTRICTION MAPPING

For determining the restriction map of CS314, first partial digestion with EcoRI was carried out to orient the three fragments with respect to each other. This was followed by complete digestion analysis with one or two enzymes (EcoRI, HindIII and PstI) using the procedures given in Maniatis et al. (1982) to determine the orientation of each fragment. The digested fragments were analysed on agarose gels with appropriate markers to calculate their molecular weight.

4.20 CHROMOSOME PREPARATION

The salivary glands of the third instar larvae were dissected in Drosophila Ringer (Ephrussi and Beadle, 1936). The two pairs of salivary glands were fixed in Fixative solution (methanol: acetic Acid = 3:1) for 1 min before putting those in 50\% Acetic Acid. A squash preparation of the chromosomes was made and the cover glass was removed after quick-freezing the slide on dry ice. The slides were dehydrated by passing through 2 changes of 95\% ethanol and air dried.

4.21 IN SITU HYBRIDIZATION

The procedure described by Jones (1973), Singh et al. (1977) and Singh et al. (1984) was used with minor modifications. Chromosome preparations were treated with RNase A (50 \( \mu g/ml \)) and RNase T1 (2 \( \text{units/ml} \)) in 2\( \times \)SSC at 37°C for 1 h washed in 2\( \times \)SSC at room temperature (3 changes, 10 min each), dehydrated
through ascending grades of alcohol (50%, 70%, 90%, and absolute alcohol) and air dried. The chromosomes were heat denatured by dipping the slides in a simmering solution of 10 mM Tris-HCl pH 7.4, 5mM MgCl$_2$ for 1 min followed by dehydration in ice cold 70%, 90% and absolute alcohol for 2 min each and air dried.

15μl of hybridization mix was used for each slide covering a 22x22 mm$^2$ area. Hybridization mix contained $4\times$SET (1xSET is 0.15M NaCl, 0.03M Tris, pH 7.4, 2mM EDTA), 10% dextran sulphate, 50μg/ml sheared and denatured E. coli DNA, 500μg/ml heparin and 5x10$^4$ cpm/μl of $^3$H labeled probe (specific activity 2-5x10$^7$ cpm/μg) or $^{33}$P labeled probe (specific activity 1-8x10$^8$ cpm/μg). Hybridizations were carried out at 53°C for 16 h. The coverslips were removed and the hybridized slides were washed twice for 10 min each in 2 x SSC at room temperature, followed by twice for 30 min each in 2xSSC at 55°C. An additional washing step in 0.5 x SSC at 62°C for 30 min was carried out for high stringency washes. Finally, slides were washed in 2xSSC at 4°C overnight. Next day, the slides were dehydrated through ascending series of alcohol and air dried. Hybridized slides were coated with Ilford K2 nuclear emulsion or Amersham LM1 emulsion and exposed for 1-2 weeks (for slides hybridized with $^{33}$P labeled probes) or 6-8 weeks (for slides hybridized with $^3$H labeled probes) at 4°C. The slides were developed in ice-cold Kodak D19b developer [2.2g metol (or Elan), 72g sodium sulphite, 8.8g hydroquinone, 48g sodium carbonate (anhydrous), 4g potassium bromide added to 1L water] for 12 min and fixed for 5 min in ice-cold high speed fixer. The fixer was washed thoroughly with several changes of water. Slides were stained in Giemsa stain (2 ml Giemsa in 50ml of 0.1M Phosphate buffer, pH 6.8) for 20-30 min, rinsed in phosphate buffer pH 6.8 (or in water) and air dried. Photographs were taken on a slow speed (25ASA) 35 mm negative film using a Zeiss Research microscope with attached camera.

4.22 EMBRYO COLLECTION

For embryonic genomic DNA isolation, the embryos were collected on the egg collection medium (1% Sucrose and 2% Agar) poured in the bacterial culture plates after boiling. A drop of yeast paste was put on one corner after the medium had solidified and the plates were kept in a collection cage. The plates were changed every 6 h for a fresh batch of collection. The embryos were then dechorionated with 5% solution of Sodium Hypochlorite, then washed
thoroughly with water. The dechorionated embryos, so obtained were flash-frozen in liquid nitrogen and kept in -70°C, till they were used for DNA isolation.

4.23 COLLECTION OF THIRD INSTAR LARVAE

The milk bottles in which 5% of larvae have pupated, were used for the collection of the larvae. The larvae were collected by washing off from the bottles with water. Thus wandering larvae and those on the surface of the medium were collected in a tall measuring cylinder (250 ml). The larvae along with lot of debris sank to the bottom. The supernatant was poured off. This step of washing with water was repeated till the supernatant was clear. Now, the cylinder was filled with 3M NaCl. The larvae along with some pieces of medium started floating. The dilution was done with water until the medium sank and larvae remained floating. The larvae were collected by pouring off the top layer in a sieve. Larvae were washed free of salt with the help of water. Any contamination of other stages was removed by hand-picking.

4.24 COLLECTION OF SALIVARY GLANDS, IMAGINAL DISCS AND BRAIN OF THIRD INSTAR LARVA

All the three tissues were dissected out individually from the larvae in Drosophila Ringer. Care was taken to dissect salivary glands free of even closely attached fat tissue.

Different imaginal discs were collected together. No attempt was made to collect one specific kind of disc.

Brains (Supraoesophageal ganglia) were collected along with ventral neural ganglia and attached imaginal discs. No special attempt was made to separate the brain from these tissues.

After isolation of salivary glands, the remaining carcasses of the larvae were also collected and designated as larvae without salivary glands (L-SG).
4.25 EXTRACTION OF GENOMIC DNA FROM EMBRYOS, LARVAE AND FLIES

Wild type CS adult flies or embryos or third instar larvae (1 g each) were homogenized in 10 ml of extraction buffer (0.1 M Tris-HCl pH 8.0, 50 mM NaCl and 10 mM EDTA) in a glass homogenizer at 4 °C. The contents were spun at 4 °C for 10 min at 5,000 rpm. The contents were incubated at 37 °C for 3 h with intermittent mixing, extracted twice with Tris-saturated phenol (pH 8.0), once with phenol: chloroform: isoamylalcohol (25: 24: 1) and then finally with chloroform: isoamylalcohol (24: 1). The aqueous phase was finally collected in a fresh tube and 1/10th volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of chilled ethanol were added to precipitate DNA. The precipitated DNA was centrifuged at 12,000 rpm for 15 min, and washed with 70% ethanol, air-dried and dissolved in appropriate amount of TE (pH 8.0).

4.26 ISOLATION OF GENOMIC DNA FROM SALIVARY GLANDS, IMAGINAL DISCS AND BRAIN OF THIRD INSTAR LARVA

The three tissues isolated in Drosophila Ringer were collected in the 'Quick Fly' extraction buffer (0.1 M Tris pH 9.1, 0.1 M NaCl, 0.2 M Sucrose and 0.05 M EDTA containing 1% SDS and 100 μg Proteinase K/ ml). The DNA was isolated using a procedure described by Bender et al. (1983) with minor modifications. To break the tissues, a collection from 100 animals was passed gently through 23 G needle four times. The contents were incubated at 37 °C for 3 h. A phenol: chloroform purification of DNA was performed as described above for other genomic DNAs. The DNA was ethanol precipitated, washed three times with 70% ethanol, dried and dissolved in TE (pH 8.0).

4.27 RNASE TREATMENT OF DNA

Removal of RNA from genomic and plasmid DNA was accomplished by treating with DNase free RNase A (final concentration 50 μg/ml) at 37 °C for 1-3 h. The RNase was extracted with equal volume of phenol, phenol : chloroform : isoamylalcohol (25 : 24 : 1) and chloroform : isoamylalcohol (24 : 1). The DNA was precipitated, washed three times with 70% ethanol, dried and dissolved in TE (pH 7.5).
4.28 AGAROSE GEL ELECTROPHORESIS AND SOUTHERN TRANSFER

Agarose gels were used for restriction digest analysis and for checking the quality of DNA samples. 1-2 μg of genomic DNA was digested to completion with 20 units/μg of appropriate restriction enzyme at the recommended temperature for 16-20 h. 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 (15 mM Tris-HCl pH 7.2, 18 mM sodium dihydrogen phosphate, 2 mM EDTA) at 2-5 V/cm. After ethidium bromide staining (0.5 μg ethidium bromide /ml of TPE), the gel was visualised under UV, photographed and depurinated with 0.25N HCl for 15-20 min. Southern transfer (Southern, 1975) was done by the vacuum blotting procedure of Olszewska and Jones (1988). The DNA was transferred to Hybond-N+ membrane at a constant vacuum of 70 mm Hg using a vacuum blotting assembly. Transfer was carried out first in denaturing solution (0.5 N NaOH, 1.5 M sodium chloride) for 40 min, followed by neutralising solution (1.5 M sodium chloride, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) for 2 h. The Hybond N+ membrane was used directly for hybridization after air drying, without baking.

4.29 PREPARATION OF RADIOLABELED PROBES

4.29.1 Nick translation

Plasmid or phage DNAs were radiolabeled by nick translation as described by Rigby et al. (1977). The reaction was carried out in 50μl volume containing 200 ng - 1 μg of DNA, 0.5 mM of each unlabeled dNTP, 30-40 μCi of α-32P-dATP (specific activity 3000 Ci/mmol), 50 mM Tris-HCl pH 7.2, 10mM magnesium chloride, 0.1 mM DTT, 2.5 μg nuclease free BSA, 50 pg DNase I and 5 units of E.coli DNA Polymerase I. After 90 min of incubation at 15 °C the reaction was stopped by adding EDTA to a final concentration of 10 mM. 10 μg of sheared and denatured E.coli DNA was added as carrier and the DNA precipitated at -20 °C for 2 h. Subsequently, the probe was pelleted by centrifugation at 10,000 rpm for 15 min. 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).
4.29.2 Multiprime labeling

50-200 ng of double stranded DNA was denatured in a boiling water bath for 10 min, followed by chilling on ice. 75 ng of random hexanucleotides, 5μl of 10 x reaction buffer (900 mM HEPES pH 6.6, 10 mM magnesium chloride and 40 mM DTT), 5mM each of unlabeled dNTPs, 50 μCi of α-32P-dATP (specific activity 3000 Ci/mmol) and 5 units of Klenow enzyme (E.coli DNA Polymerase I– large fragment) were added in a total volume of 50 μl. The samples were incubated at 20-24 °C for 6 h. (Feinberg and Vogelstein, 1983, 1984). The reaction was stopped and the probe was precipitated, washed as written in the section 'Nick translation'.

4.29.3 3H labeling of probes

1 μg of DNA was radiolabeled by nick translation as described earlier. Equimolar amounts (15-20 μM) of 3H labeled dNTPs (3H-dATP, specific activity 70Ci/mmol. 3H-dTTP, specific activity 60Ci/mmol), unlabeled dGTP and dCTP were used for each reaction. The specific activity of the probe ranged from 2-5 x 10^7 cpm/μg of DNA.

4.29.4 33P labeling of probes

100 ng of double stranded DNA insert of different subclones was radiolabeled with 50μCi of α-33P-dATP (specific activity 2000Ci/mmol) by multiprime labeling reaction as described earlier. The specific activity of the probe ranged from 1-8 x 10^8 cpn/μg of DNA.

4.29.5 End-labeling of DNA

End-labeling of double stranded DNA was carried out as described by Maniatis et al. (1982). End labeled DNA was used for mobility shift assays and slot-blot binding experiments.
4.29.5.1 End-labeling of (GATA)$_{16}$ and Bkm-2(8)

The 124 bp (GATA)$_{16}$ fragment used in the mobility shift experiments was end labeled by filling-in the enzyme site overhangs with Klenow enzyme (E.coli DNA Polymerase I - large fragment). 10 µg of DNA of the clone containing (GATA)$_{16}$ in pUC18 was double digested with EcoRI and HindIII restriction enzymes in EcoRI buffer for 3-4 h at 37 °C. The digest was end-labeled in a 50 µl reaction volume containing 5 µl of 10 x Klenow buffer, 6 µl of A mix (10 mM each of dGTP, dCTP and dTTP), 2 µl of DTT (1.0 M), 30 µCi of α-32P-dATP (specific activity 3000 Ci/mmol) and 2 units of Klenow enzyme. The incubation was carried out at 37 °C for 1.5 h and was terminated by the addition of 3 µl of 0.5 M EDTA. The labeled DNA fragments were precipitated by the addition of 10 µg of sheared E.coli DNA, 5 µl of 3M sodium acetate pH 5.5) and 150 µl of isopropanol at 0 °C overnight. The precipitate was washed in absolute alcohol, dried and redissolved in 20 µl of sterile water.

The clone containing 545 bp Bkm-2(8) insert, used in slot blot binding assay, was similarly end-labeled after digesting the clone (in pUC 18) with EcoRI and HindIII, as described above.

The labeled vector and inserts fragments, so obtained, were size fractionated in 10% polyacrylamide gel in 1 x TBE (at 100-120 V). The DNA of interest was located by autoradiography. The insert band was excised and the labeled DNA was recovered by crush and soak method.

4.29.5.2 "Crush and Soak" method

(Sambrook et al., 1989): The excised piece of polyacrylamide gel having the labeled insert was crushed by passing through a 1 ml plastic syringe. The crushed pieces were soaked in 1 ml of TE and incubated at 37 °C overnight. The samples were centrifuged in microfuge at 12,000rpm for 10 min. The supernatant recovered was concentrated with the help of n-butanol to about 150 µl and was purified through Sephadex G50 spun column chromatography to remove minute acrylamide pieces.
4.29.5.3 Sephadex G50 column chromatography

A sterile 2 ml disposable plastic syringe (Steriware), plugged with sterile glass wool was filled with autoclaved slurry of Sephadex G50 in TE (pH 8.0). The column was centrifuged at 3,000 rpm for 4 min at room temperature in HB4 rotor. The centrifugation was repeated twice under the same conditions after loading 150 µl of TE (pH 8.0) each time. By now, the volume of the eluate was equal to the volume loaded i.e. 150 µl. The labeled DNA sample (150 µl) was then loaded onto the column and centrifuged under the same conditions. The purified DNA so obtained was stored at 4 °C. Spun columns were used for purifying labeled probe from free nucleotides or for purifying any DNA sample from impurities.

4.30 Measurement of radioactivity in nucleic acids

2 µl of radiolabeled probe was spotted on a piece of nylon membrane, dried and washed in ice cold 10% trichloroacetic acid for 10 min. Subsequently, the filter was rinsed in ice cold ethanol for 10 min. 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. Nick translation resulted in probes with a specific activity of 1-5 x 10^7 cpm/µg while the probes prepared by multiprime labeling had ten fold higher specific activity.

4.31 Southern hybridization

Blots were prehybridized in a solution of 0.5 M sodium phosphate buffer (pH 7.5) and 7% SDS at 60-65 °C for 2h. 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 10^8-10^9 cpm/µg) with a probe concentration of 1-5 x 10^6 cpm/ml at 60-65 °C (depending on the sequence composition and stringency of hybridization) for 16-24 h.
4.32 **POST-HYBRIDIZATION WASHING AND AUTORADIOGRAPHY**

Low stringency wash: The blots were washed twice in 2 x SSC, 0.1% SDS at 60 °C for 30 min each.

High stringency wash: The blots were washed thrice in 0.1 x SSC, 0.1% SDS at 65 °C for 30 min each.

After washes, the blots were rinsed thrice in 2 x SSC at room temperature for 5 min 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.

4.33 **COLLECTION OF TISSUES FOR PROTEIN ISOLATION**

Ovaries and testis were dissected out individually from the adult fly in *Drosophila* Ringer and stored in protein homogenization buffer (50mM Tris-HCl pH7.5, 0.25M Sucrose, 25mM KCl, 5 mM MgCl₂, 2mM DTT and 1mM PMSF). The carcasses without the gonads were also collected and were designated as gonadless female and male, respectively. Heads and headless adults were obtained by flash freezing the adult flies in liquid nitrogen, vortexing the eppendorf containing flies to break the fragile neck. The heads thus broke off and were separated from headless body by passing through a sieve. Cross contaminants were removed by hand-picking.

All the tissues were flash frozen in liquid nitrogen and kept in -70 °C until used for protein isolation.

4.34 **QUANTITATION OF PROTEINS**

For accurate estimation of proteins concentrations, Lowry's method (Lowry *et al.*, 1951) was used. The following solution was prepared fresh every time:

2 ml of 2% (w/v) CuSO₄ solution + 2 ml of 4% (w/v) K⁺ tartarate was added to 96 ml of 3% (w/v) Na₂CO₃ in 0.1N NaOH. Phenol reagent was diluted to 50% by adding equal volume of water.

10 μl aliquots of protein were diluted to 500μl by the addition of water. To this 5 ml of CuSO₄-tartarate solution was added and left for 10 min at room temperature, following which 0.5 ml of diluted phenol reagent was added and
mixed vigorously on a cyclomixer. The samples were left for 30 min at room temperature for the color development. The O.D. of the samples were recorded at 650 nm.

4.35 Preparation of Total Protein Extract

Total protein extract from whole body, heads, gonads, headless and gonadless body of male and female were isolated by homogenization in protein homogenization buffer (50mM Tris pH7.5, 0.25M Sucrose, 25mM KCl, 5 mM MgCl₂, 2mM DTT and 1mM PMSF) in a glass homogenizer at 4 °C. The homogenate was centrifuged at 10,000 rpm at 4 °C in a microfuge. The supernatant containing water soluble proteins was used for slot-blot assay after estimation by Lowry method (Lowry et al., 1951).

4.36 Slot Blot-Binding-Assay

10, 25 and 50 μg respectively of water soluble proteins of different tissues were transferred onto the nitrocellulose membrane by a Slot blot apparatus (Schleicher and Schuell). The vacuum was applied to get fast and efficient transfer. The membrane was air dried and the binding assay was performed using end labeled Bkm-2(8), as described by Majumdar et al. (1996).

The blot was preincubated in Standard binding buffer (SBB; 10 mM Tris-HCl pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.02% BSA, 0.02% PVP and 0.02% Ficoll) for 1 h at room temperature in the presence of 100 μg/ml of sheared E.coli DNA. ³²P-end-labeled Bkm-2(8) probe (10⁵ cpm/ml) was added to the fresh SSB and the blot was incubated at room temperature for 2-3 h. Washing of the blot was carried out in SBB at room temperature. In order to increase the stringency of washing NaCl concentration was increased slowly upto 0.5 N. The blots were autoradiographed by exposing to X-ray film.

4.37 Isolation of Nuclei from Drosophila Tissues

Equal number (150) heads, headless body, pairs of gonads, gonadless body of Drosophila were homogenized separately in 0.5 ml of STM buffer (0.25 M Sucrose, 50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 1 mM EDTA and 1 mM PMSF) in
a glass homogenizer (20 strokes). The procedure used was modified from Gerace and Blobel (1980). The homogenate was centrifuged in a Sorvall SS-34 rotor at 3,000 rpm at 4°C for 10 min. The pellet was washed by resuspending in STM buffer and centrifuging in a SS-34 rotor at 3,000 rpm at 4°C for 10 min. The pellet was now resuspended in 400 µl of DS buffer (50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 1 mM EDTA and 1 mM PMSF) containing 2.1 M sucrose. This was layered on 400 µl of DS buffer and centrifuged for 1 h at 35,000 rpm in a Beckman tabletop ultracentrifuge using TLS 55 rotor at 4°C. The nuclei pelleted at the bottom of the tube were resuspended in STM buffer.

4.38 Preparation of Nuclear Extracts

The procedure used for nuclear extracts preparation was modified from Andrews and Faller (1992). The purified nuclei prepared, as described above were resuspended in STM buffer. The nuclei were centrifuged at 6,000 rpm for 10 min at 4°C in Sorvall SS34 rotor. The pellet was resuspended in lysis buffer (10 mM Tris pH 7.5, 0.1 M NaCl and 1 mM PMSF) and left on ice for 10-15 min. The samples were vortexed for 10 seconds. The lysed nuclei were pelleted and resuspended in extraction buffer (10 mM Tris-HCl pH 7.5, 0.42 M NaCl, 10% Glycerol and 1 mM PMSF). The suspension was further incubated on ice for 20 min. The chromatin was separated by centrifugation at 14,000 rpm in a sorvall SS-34 rotor at 4°C for 30'. The supernatant was used in mobility shift assays.

4.39 Electrophoretic Mobility Shift Assay

The mobility shift assays were performed to detect specific DNA-protein complexes (Fried and Crothers, 1981). To approximately 5 µg of nuclear protein extract in 50 mM Tris-HCl pH 8.0, 350 mM NaCl, 50 mM DTT, 5 mM MgCl₂, 10% Glycerol, 0.1% NP-40 and 1 mM PMSF, 2 µg of sheared E. coli DNA was added and incubated at room temperature for 10 min. To this, approximately 50,000 cpm of end-labeled (GATA)₁₆ DNA was added and further incubation was done at room temperature for 30 min. Following incubation, 6 x gel loading dye (0.025% bromophenolblue, 0.025% xylene-cyanol, 40% glycerol in TE) was added to the samples and the mixture was directly loaded onto a 6% non-denaturing polyacrylamide gel. The electrophoresis was carried out in 1x TBE buffer at 100 V till the Bromophenol blue dye just migrated out of the gel. The gel was dried and autoradiographed.
4.40 DNA SEQUENCING

Nucleotide sequencing was performed using the dideoxy-termination method of Sanger (Sanger et al., 1977) modified by Chen and Seeburg (1985). Cycle sequencing was carried out with the help of universal pUC/M13 primers and custom-made oligonucleotides. Sequencing was carried out with the ultrapurified supercoiled plasmid DNA. Dye terminator cycle sequencing kit (Perkin Elmer) provided the reaction mix containing all the dNTPs and the differentially labeled, four dideoxynucleotides along with Taq DNA polymerase in the sequencing buffer. Four dideoxy nucleotides were labeled with four different fluorescent dyes.

The reagent mix was made by mixing 8 μl of Terminator Ready Reaction Mix (Perkin Elmer sequencing manual) and 3.2 pmoles of primer to 500 ng of plasmid DNA in a final reaction volume of 20 μl. Cycle sequencing was carried out in thermal cycler, GeneAmp PCR System 9600, by first giving a rapid thermal ramp to 96 °C and holding at this temperature for 30 sec. This step was followed by rapid thermal ramp to 50 °C and holding for 15 sec. Last step was rapid thermal ramp to 60 °C and holding for 4 min at it. The cycle consisting of these three steps was repeated 30 times. After 30 cycles, the reaction was rapidly brought to 4 °C by a rapid thermal ramp. The unincorporated dye terminators were removed by ethanol precipitation.

The pellet was resuspended in 4.5 μl of loading buffer (deionized formamide and 25 mM EDTA pH 8.0, in a ratio of 5: 1). The sample was vortexed, spun, heated at 90 °C for 2 min to denature and placed immediately on ice. The samples were loaded in a pre-electrophoresed 4% polyacrylamide gel. Electrophoresis was carried out for 10 h at a voltage of 2kV and at the current of 50 mA. the temperature was maintained at 51 °C.

4.41 SEQUENCE ANALYSIS

Raw data collected after electrophoresis were analysed using sequence analysis and autoassembler softwares (Perkin Elmer).

4.42 PRIMERS USED

The different primers used for sequencing are listed in Table I.
### Table I

**List of primers used for sequencing**

**(A)** M13/pUC primers used were obtained from Promega. The forward and reverse primers are listed below:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>pUC/M13 Primers</th>
<th>Size (length)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward (1-40)</td>
<td>17mer</td>
<td>GTTTTCCCAGTCACGAC</td>
</tr>
<tr>
<td>2</td>
<td>Forward (1-47)</td>
<td>24mer</td>
<td>CGCCAGGGTTTTCCCAGTCACGAC</td>
</tr>
<tr>
<td>3</td>
<td>Reverse</td>
<td>17mer</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>4</td>
<td>Reverse</td>
<td>22mer</td>
<td>TCACACAGGAAACAGCTATGAC</td>
</tr>
</tbody>
</table>

**(B)** For sequencing f3-314, the following oligonucleotides were custom-made from Oswel DNA service:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sequence of the primer</th>
<th>Location of the primer (nucleotide no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACACGGAAACTAAATTTCCCTCTGT</td>
<td>R C of 3438-3462</td>
</tr>
<tr>
<td>2</td>
<td>TTCTAGAAAGAGCTCACAACC</td>
<td>R C of 3264-3284</td>
</tr>
<tr>
<td>3</td>
<td>GCTTAAAGCAGCGTTCTCAAAT</td>
<td>1436-1459</td>
</tr>
<tr>
<td>4</td>
<td>CCCATTGAGAAACGCTGTTTAAGC</td>
<td>R C of 1436-1460</td>
</tr>
<tr>
<td>5</td>
<td>GGATTCTGCCCAGGGGAATCC</td>
<td>751-771</td>
</tr>
<tr>
<td>6</td>
<td>GGATTCCCCCTGGGCAAGAATCC</td>
<td>R C of 751-771</td>
</tr>
<tr>
<td>7</td>
<td>GGCTGCGCGGTACGCAGTCAGA</td>
<td>543-563</td>
</tr>
</tbody>
</table>

Note: RC denotes reverse complement of the sequence range mentioned.