Chapter V

RESULTS
5.1 MOLECULAR ANALYSIS OF BKM-ASSOCIATED GENOMIC CLONES, CS314 AND CS316

5.1.1 Restriction analysis, Subcloning and Restriction mapping of CS314 and CS316

5.1.1.1 Restriction analysis of CS314 and CS316

The genomic clones CS314 and CS316 upon digestion with EcoRI enzyme release, as expected, left arm (19.5 kb) and right arm (11.0 kb) of Charon4A phage, in addition to the different fragments of the genomic DNA cloned in it (Fig 5.1). The genomic DNA insert of CS314 upon digestion with EcoRI produces 3 fragments of the sizes: 6kb, 4.3kb and 3.8kb; whereas, digestion of CS316 insert gives 6 fragments, 3.9kb, 2.6kb, 2.4kb, 2.4kb, 1.5kb and 0.9kb in size (Fig 5.1). In the case of CS314, the smallest fragment (3.8kb), hybridizes with Bkm. On the other hand, one of the two 2.4kb fragments, is Bkm-positive in CS316 (Fig 5.2).

5.1.1.2 Subcloning of CS314 and CS316

The two clones were subcloned into pGEM3Z using 'Shotgun cloning' method. Equimolar ratios of EcoRI digested CS314 or CS316 and EcoRI digested, dephosphorylated pGEM3Z vector were mixed, after individually purifying the DNA solutions through Phenol/Chloroform method ,and ligated at 16 °C for 16h.

Two of the fragments were subcloned in pUC18 vector, after individually purifying the insert by 'Geneclean method' followed by mixing of the vector and the insert in a molar ratio of 1:3. The ligation was carried out at 16 °C for 16h.

The recombinant white colonies obtained after transformation of the ligated products, were individually screened for the presence of the desired sized inserts. The screening involved isolation of the plasmids by mini-prep method, followed by restriction digestion to check the release of the desired sized insert. All the subclones obtained, were digested with EcoRI and electrophoresed along with their respective EcoRI-digested genomic clones (CS314 and CS316) (Fig 5.3). The DNA inserts released from the subclones matched in size to their, respective EcoRI fragments of the genomic clones.
Fig 5.1: *Eco RI digestion of CS314 and CS316*. 1.5 μg DNA of each of CS314 and CS316 digested separately with *Eco RI*, electrophoresed in 1% agarose gel, stained with Ethidium bromide and photographed under UV.

Lane 1: CS314 DNA digested with *Eco RI*;
Lane 2: CS316 DNA digested with *Eco RI*;
Lane M2: Molecular weight marker (λ-*Eco RI/HindIII* double digest).

Note three fragments, f1-, f2-, & f3- 314 are released from CS314, while CS316 releases six fragments, f1-, f2-, f3-, f3B-, f4-, & f5- 316. The molecular weights of all the fragments are written on the right side. Also note that both f3-316 and f3B-316 are, approximately, of the same size.
Fig 5.2: Southern hybridization of $^{32}$P labeled Bkm-2(8) to EcoRI digested CS314 and CS316. 1.5 μg DNA of each of CS314 and CS316 digested separately with EcoRI, electrophoresed in 1% agarose gel and hybridized with $^{32}$P labeled Bkm-2(8).

(A) Lane M2: λ-EcoRI/HindIII double digest; Lane 1: UV photograph of CS314 digested with EcoRI; Lane 2: Same blot as in Lane 1, hybridized with Bkm-2(8);
(B) Lane 1: UV photograph of CS316 digested with EcoRI; Lane 2: Same blot as in Lane 1, hybridized with Bkm-2(8).

Note 3.8 kb f3-314 is Bkm-positive in CS314, while in CS316, one of the two 2.4 kb fragments, f3B-316, is Bkm-positive.
(A) - 3.8 kb (13-314)

(B) - 2.4 kb (13B-316)
Fig 5.3: *EcoRI* digestion of subclones of CS314 and CS316. All the subclones and their respective clones digested with *EcoRI*, electrophoresed in 1% agarose gel, stained with Ethidium bromide and photographed under UV.

(A) Lane 1: CS314 digested with *EcoRI*; Lanes 2–4: *EcoRI* digested pf1(314), pf2(314) and pf3(314), respectively.

(B) Lanes 1–2 & 4–6: *EcoRI* digested pf1(316), pf2(316), pf3(316), pf4(316) & pf5(316), respectively; Lane 3: CS316 digested with *EcoRI*.

Lane M2: Molecular weight marker (*λ*-EcoRI/HindIII double digest)

Note all the fragments generated from the insert are marked on the right side. The vector of the clones pf4(316) and pf5(316) is pUC18, while for the remaining clones pGEM3Z.
To confirm further that the origin of the inserts in the subclones was from their respective genomic clones, the gel containing EcoRI-digested clones and their respective subclones (Fig 5.3) was vacuum-blotted on to nylon membrane. The blot was divided into two parts, one containing CS314 and its subclones and the other had CS316 and its respective subclones. The two blots were separately hybridized with their respective ³²P-labeled total genomic clones (Fig 5.4). As expected, the inserts of all the subclones and all the fragments from the clones lighted up, confirming the origin of the inserts of the subclones from their respective genomic clones.

One Bkm-positive, 2.4kb fragment of CS316 cloned in EcoRI site of 4kb vector, pACYC184, (Fig 5.5B) was a gift from Dr Lalji Singh (Singh and Jones, 1982).

All the details about the subclones are summarized in Table II.

5.1.1.3 Inserts of pf3(314) and pf3B(316) are Bkm-2(8) positive

To further confirm the cloning of Bkm-positive fragment of CS314, EcoRI digested pf3(314) was hybridized with ³²P-labeled Bkm-2(8) at 65 °C. After washing with 0.5XSSC at 65 °C, the insert of pf3(314), showed the positive signal (Fig 5.5A).

Similarly, pf3B(316) digested with EcoRI upon hybridization with Bkm-2(8) at 60°C followed by washing at 60 °C with 1XSSC, yielded a single band on autoradiogram, corresponding to its insert (Fig 5.5B). The stringency was kept low in order to detect the homology due to the repeat component i.e. GATA repeats.

5.1.1.4 Restriction map of CS314

To place the three EcoRI fragments of CS314, relative to each other, CS314 DNA was partially digested by adding 1U EcoRI/μg of DNA. The aliquots were taken at increasing time intervals and reaction was stopped quickly after each incubation by the addition of EDTA and quickly chilling it. Gel pattern obtained after electrophoretic separation of different aliquots showed the completion of digestion within 15 min of incubation (Lane 4 of Fig 5.6A). First five lanes of the gel corresponding to the aliquots taken at 0, 1, 5, 15, and 30 min, respectively were vacuum-blotted and the membrane containing DNA was hybridized with the 4.3kb f2-314. The analysis of the band pattern obtained showed f2-314 to be
Table II

Description of the subclones of CS314 and CS316

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the subclone</th>
<th>Size (and name) of the insert</th>
<th>Vector used for cloning</th>
<th>Genomic clone of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pf1(314)</td>
<td>6kb (f1-314)</td>
<td>pGEM3Z</td>
<td>CS314</td>
</tr>
<tr>
<td>2</td>
<td>pf2(314)</td>
<td>4.3kb (f2-314)</td>
<td>pGEM3Z</td>
<td>CS314</td>
</tr>
<tr>
<td>3</td>
<td>pf3(314)</td>
<td>3.8kb (f3-314)</td>
<td>pGEM3Z</td>
<td>CS314</td>
</tr>
<tr>
<td>4</td>
<td>pf1(316)</td>
<td>3.9kb (f1-316)</td>
<td>pGEM3Z</td>
<td>CS316</td>
</tr>
<tr>
<td>5</td>
<td>pf2(316)</td>
<td>2.6kb (f2-316)</td>
<td>pGEM3Z</td>
<td>CS316</td>
</tr>
<tr>
<td>6</td>
<td>pf3(316)</td>
<td>2.4kb (f3-316)</td>
<td>pGEM3Z</td>
<td>CS316</td>
</tr>
<tr>
<td>7</td>
<td>pf3B(316)</td>
<td>2.4kb (f3B-316)</td>
<td>pACYC184</td>
<td>CS316</td>
</tr>
<tr>
<td>8</td>
<td>pf4(316)</td>
<td>1.5kb (f4-316)</td>
<td>pUC18</td>
<td>CS316</td>
</tr>
<tr>
<td>9</td>
<td>pf5(316)</td>
<td>0.9kb (f5-316)</td>
<td>pUC18</td>
<td>CS316</td>
</tr>
</tbody>
</table>
Fig 5.4: Southern hybridization of $^{32}$P labeled CS314 and CS316 to their respective EcoRI digested subclones.

(A) Same blot as in Fig 5.3(A) hybridized with $^{32}$P labeled CS314
(B) Same blot as in Fig 5.3(B) hybridized with $^{32}$P labeled CS316
All the resulting fragments are marked on the right side. See legends of Fig 5.3 for further details.

Note that inserts of all subclones hybridize and the hybridizing bands correspond in size to the bands hybridizing in the parent clone. This confirms the genuineness of the subclones. Since the probe includes vector also, hybridization is seen with the arms of Charon 4A and with few of the molecular weight marker fragments ($\lambda$-EcoRI/HindIII double digest). In two lanes (lanes 2 & 4 of B), weak bands of linearised/circular subclone, are visible in addition to the completely digested actual insert bands.
Fig 5.5: The inserts of the subclones pf3(314) and pf3B(316) are Bkm-positive. pf3(314) and pf3B(316) digested with EcoRI, electrophoresed in 1% agarose gel and hybridized with $^{32}$P labeled Bkm-2(8).

(A) Lane 1: EcoRI digested pf3(314) hybridized with $^{32}$P labeled Bkm-2(8); Lane 2: pf3(314) digested with EcoRI; Lane M2: Molecular weight marker ($\lambda$-EcoRI/HindIII double digest).

(B) Lane M4: Molecular weight marker (Marker X from Boehringer Mannheim, Germany); Lane 1: pf3B(316) digested with EcoRI; Lane 2: EcoRI digested pf3B(316) hybridized with $^{32}$P labeled Bkm-2(8).

Note that the inserts of both pf3(314) and pf3B(316), marked as f3-314 and f3B-316, respectively, hybridize with Bkm-2(8), while the plasmid vectors, pGEM3Z and pACYC184, do not hybridize.
Fig 5.6: Southern hybridization of $^{32}$P labeled f2-314 to partial EcoRI digest of CS314.

CS314 DNA partially digested with EcoRI (1U/μg), electrophoresed in 1% agarose gel and hybridized with $^{32}$P labeled f2-314.

(A) Lane M4: Molecular weight marker (Marker X from Boehringer Mannheim, Germany); Lanes 1–5: Aliquots taken at 0, 1, 5, 15 and 30 min respectively after the addition of the enzyme,

(B) Same blot as in (A) hybridized with $^{32}$P labeled f2-314. Only lanes 1-5 are shown.

Note that besides hybridization to completely digested f2-314 fragment (4.3 kb), other fragments of 8.1 kb, 10.3 kb and 14.1 kb are also hybridized. This is due to association of f2-314 with f3-314, f1-314 and both f1-314 & f2-314, respectively. Thus, f2-314 is the middle fragment of CS314. Fragment 'H' corresponds to complete CS314.
the middle fragment of CS314 (Fig 5.6B). The following bands were obtained in the partial digests:

1. a 4.3 kb band (corresponding to the completely digested insert);
2. a 8.1 kb band (where it associates with 3.8kb f3-314);
3. a 10.3 kb band (where it associates with 6kb f1-314);
4. a 14.1kb band (consisting of full insert; without vector).

In addition, higher size bands were obtained due to the association of the two ends with the arms of the phage vector. If either of the other two fragments (3.8kb or 6kb fragments) were to be the middle fragment, one or more bands, obtained upon hybridization, with f2-314 would not have appeared.

There are two restriction sites for HindIII in f1-314. Upon digestion of pf1(314) with HindIII, three fragments of 6.7kb, 1.2kb and 0.7kb in size are released since, pGEM3Z has one HindIII site in its MCS (Lane 3 of Fig 5.7). f3-314 and f2-314 do not have any site for HindIII in them (Lanes 1 and 2 of Fig 5.7, respectively). Double-digestion of pf1(314) with EcoRI/HindIII separates vector pGEM3Z (2.7kb in size), from 4kb insert (in 6.7kb HindIII fragment), without making any visible change in the size of the other fragments (Lane 2 of Fig 5.8). The release of 0.7kb fragment (and, not of 4kb or 1.2kb fragment) upon digestion of CS314 with HindIII confirmed that in f1-314, 0.7kb fragment is the middle fragment flanked by 4kb and 1.2 kb fragments (Lane 4 of Fig 5.7).

Southern hybridization of HindIII digest of CS314, gives a fragment of 9.3 kb when 4kb f1-314 is used as a probe(Lanes 4 and 5 of Fig 5.9B). This band results from the joining of the 4kb fragment (through its EcoRI site) with 5.3 kb EcoRI/HindIII fragment of the right arm of the phage DNA (See Fig 5.12 for the complete restriction map of CS314). This shows that the 4kb fragment is attached to the "right arm" through its EcoRI site (Fig 5.7 and Fig 5.9). Thus making the order of sequences as:

Left arm of charon phage—3.8kb f3-314—4.3kb f2-314—6kb insert of f1-314—Right arm of charon phage.

where, '—' symbolises joining through EcoRI site.

The subclone pf2(314) upon digestion with PstI releases two fragments: (A) a 2.7kb fragment (from the insert) and (B) a 4.3 kb fragment resulting from 1.6 kb of
**Fig 5.7: HindIII digestion of CS314 and its subclones.** CS314 and its three subclones digested with *HindIII*, electrophoresed in 1% agarose gel, stained with ethidium bromide and photographed under UV.

Lanes 1–3: *HindIII* digested pf1(314), pf2(314) and pf3(314) respectively; Lane 4: 2 μg of *HindIII* digested CS314; Lane 5: 800 ng of *HindIII* digested CS314; Lanes M1, M3 & M4: DNA molecular weight markers, λ-*HindIII* digest, λ-BstEII digest, and Marker X (from Boehringer Mannheim, Germany), respectively.

Note that 5.7 kb 'RR' fragment is released upon digestion of the right arm of Charon phage with *HindIII* (See restriction map of CS314 in Fig 5.12).
Fig 5.8: *Eco RI and HindIII double digestion of pf1(314).* pf1(314) DNA digested with *Eco RI* and *HindIII*, electrophoresed in 1% agarose gel, stained with ethidium bromide and UV photographed.

Lane 1: pf1(314) digested with *HindIII*;
Lane 2: pf1(314) double-digest with *Eco RI* and *HindIII*;
Lane M3: DNA molecular weight marker (*λ*-BstEII digest).

Note that the 4 kb insert and the vector are separated from 6.7 kb *HindIII* fragment in *Eco RI/HindIII* double digest.
Fig 5.9: Southern hybridization of $^{32}$P labeled 4 kb $EcoRI/HindIII$ fragment of pf1(314) to $HindIII$ digested CS314 and its subclones.

(A) Same gel photograph as in Fig 5.7;
(B) Blot of the gel in (A) hybridized with the 4 kb fragment of pf1(314).

Note hybridization of CS314 to 9.3 kb fragment containing $EcoRI/HindIII$ portion of the right arm of Charon phage (Lane 4 and 5). Also note hybridization to 6.7 kb fragment of pf1(314) containing 2.7 kb pGEM3Z vector in it (Lane 3).
insert and 2.7 kb of vector (Lane 5 of Fig 5.10a). On the other hand, pf1(314) upon restriction digestion with \textit{Pst}I gives: (A) a 2.6 kb fragment of the insert, and (B) a 4kb fragment resulting from 1.3 kb of insert and 2.7 kb of vector (Lane 4 of Fig 5.10a). Thus, the restriction maps of pf1(314), pf2(314) and pf3(314) resulting from their respective \textit{EcoRI}, \textit{HindIII} and \textit{Pst}I fragments are shown in Fig 5.11.

CS314, upon digestion with \textit{Pst}I gave two bands of 7.7 kb and 2.9 kb when it is hybridized with 4.3kb f2-314 (Lane 2 of Fig 5.10a and 5.10b). The same 4.3kb insert when hybridized to CS314 double-digested with \textit{Pst}I and \textit{HindIII} gave two bands of 3.9 kb and 2.9 kb (Lane 1 of Fig 5.10a and 5.10b). Thus, one \textit{Pst}I site is located 2.7 kb away (in the 3.8kb \textit{EcoRI} fragment), while the second site is 5.6 kb away (in the 4.3 kb \textit{EcoRI} fragment) from the left arm, as shown in the final restriction map of CS314 (Fig 5.12).

5.1.1.5 \textit{Restriction map of CS316}

The restriction map of CS316 was provided by Dr Lalji Singh (unpublished data) (Fig 5.13).

5.1.2 \textit{Molecular characterization of Subclones of CS314 and CS316}

5.1.2.1 \textit{Chromosomal localization of different subclones}

In order to find out, if there exists any differential cytological localization of Bkm-related and Bkm-associated sequences, $^{33}\text{P}$-d\text{ATP} labeled genomic insert of Bkm-positive and Bkm-negative subclones were \textit{in situ} hybridized with the salivary gland polytene chromosomes of the third instar larvae of CS strain of \textit{Drosophila melanogaster}.

The Bkm-negative fragments of CS314 i.e. f1-314 and f2-314 localized at 38B region of the 2L arm of the polytene chromosomes (Fig 5.14 and Fig 5.15). No hybridization was detected at the base of the X chromosome. Interestingly, Bkm-positive fragment, f3-314 hybridized both at 38B, and at the base of the X chromosome (Figs 5.16A & B).

Similarly, in the case of CS316, the Bkm-positive fragment, f3B-316, hybridized at two places, i.e. at 11E as well as at the base of the X chromosome (Figs 5.17A & B). On the other hand, as expected, all the five Bkm-negative fragments, (f1 to f5)-
Fig 5.10: Southern hybridization of $^{32}$P labeled f2-314 to PstI digested and PstI/ HindIII digested CS314 and its subclones.

(A) UV gel photograph showing: Lane 1: CS314 digested with HindIII/PstI; Lane 2: CS314 digested with PstI; Lane 3: CS314 digested with EcoRI (a control); Lane 4: pf1(314) digested with PstI; Lane 5: pf2(314) digested with PstI; Lane 6: pf3(314) digested with PstI. Lanes M2, M3 and M4: DNA molecular weight markers, λ-EcoRI/HindIII double-digest, λ-BsrEII digest, and Marker X (from Boehringer Mannheim, Germany), respectively.

(B) The blot of the gel shown in (A) hybridized with $^{32}$P-labeled 4.3 kb f2-314.

Note that the Southern hybridization pattern, obtained upon hybridizing PstI alone (lane 2) and HindIII/PstI double-digested (lane 1) CS314 with the middle fragment f2-314, can be obtained only in one orientation of f2-314 with respect to f1-314 and f3-314.
Fig 5.11: Restriction maps of pf1(314), pf2 (314) and pf3(314)
Fig 5.12: Restriction map of CS314
Fig 5.13: Restriction map of CS316
Fig 5.14: In situ hybridization of $^{33}$P labeled f1-314 fragment to *Drosophila* salivary gland chromosomes. Polytene chromosomes of third instar larvae of *Drosophila melanogaster* (Canton-S) hybridized with $^{33}$P labeled f1-314 at 62 °C and exposed for ten days. Note concentration of grains around 38B region (marked with arrow) on 2L arm.

Fig 5.15: In situ hybridization of $^{33}$P labeled f2-314 fragment to *Drosophila* salivary gland chromosomes. Polytene chromosomes of third instar larvae hybridized with $^{33}$P labeled f2-314 fragment at 62 °C and exposed for one week. Note concentration of grains at 38B (marked with arrow) on 2L arm.
Fig 5.16: *In situ* hybridization of $^{33}$P labeled f$_3$-314 fragment to *Drosophila* salivary gland chromosomes. Polytene chromosomes of third instar larvae hybridized with $^{33}$P labeled f$_3$-314 fragment at 60 °C and exposed for one week.

(A) Note concentration of grains at 38B (marked with arrow) on 2L arm and at the base of the X chromosome, at 19F-20AB region (marked with arrowhead);

(B) A low magnification of a similar hybridization with f$_3$-314 fragment showing the concentration of grains at the two sites in many plates.
**Fig 5.17 (A & B):** *In situ* hybridization of $^{33}$P labeled Bkm-positive f3B-316 fragment to *Drosophila* salivary gland chromosomes. Two plates of polytene chromosomes of third instar larvae hybridized with $^3$H labeled f3B-316 fragment at 60 °C and exposed for two weeks. Note concentration of grains at 11E (marked with arrow) on the X chromosome. Also note another signal at the base of the X chromosome, at 19F-20AB region (marked with arrowhead).
316 hybridized at only one place, i.e. 11E on the X chromosome (Figs 5.18 to 5.22). Again, no hybridization was detected at the base of X chromosome, just as in the case of CS314.

5.1.2.2 Genomic organization of different subclones

To find out the developmental changes, if any, in the organization of Bkm and associated sequences in the *Drosophila* genome, Southern hybridization was carried out. Embryonic, third instar larval and adult DNAs were digested with PstI enzyme and probed with ³²P labeled Bkm-2(8) at 60 °C. The subsequent washings were done at 60 °C in 2XSSC. Three bands (marked with arrows) of 2.7kb, 4kb and 5.1kb in size, respectively, were observed in the larvae and the adults, but were missing in the embryos (Fig 5.23).

This, differential hybridization of Bkm-related sequences observed with the PstI digested DNAs from different stages of development prompted us to look for the hybridization pattern with different subclones of CS314 and CS316. The hybridization patterns of PstI digested embryonic, larval and adult DNAs with different subclones of CS314 and CS316 are shown in the Figs 5.24–5.32. All the hybridizations were carried out at high stringency (except for the hybridization with pf3B(316) insert), i.e. at 65 °C. Post-hybridization washing of the blot was also carried out at the same temperature i.e. 65 °C in 0.1XSSC.

5.1.2.2(A) CS314

PstI digested embryonic, larval (third instar), salivary gland from third instar larvae and adult DNAs, upon hybridization with the ³²P labeled f1-314 fragment showed a single band at 7.6kb in all the three lanes (Fig 5.24). The same size band was present when the same blot was hybridized with f2-314 fragment (Fig 5.25). This was expected as this is the common PstI fragment between the two fragments, f1 and f2 of CS314 (See restriction map of CS314, Fig 5.12). In addition, f2-314 fragment detected two bands of 2.9kb and 2.7kb (Fig 5.25). The 2.9kb band was present in all the three digests. On the other hand, the band at 2.7 kb was visible in larval and adult DNAs, but was absent from embryonic DNA.

Hybridization of ³²P labeled Bkm-positive fragment of CS314, i.e. f3-314 insert, with embryonic, larval (third instar), and adult DNAs showed two strongly hybridizing bands (since PstI has one site in f3-314 insert; See Fig 5.12) of 3.5kb and 2.9kb. Both these bands were present in the DNAs of all the three stages (Fig
Fig 5.18: *In situ* hybridization of $^{33}$P labeled f1-316 fragment to *Drosophila* salivary gland chromosomes. Polytene chromosomes of third instar larvae hybridized with $^{33}$P labeled f1-316 fragment at 62 °C and exposed for one week. Note concentration of grains at 11E (marked with arrow) on the X chromosome.

Fig 5.19: *In situ* hybridization of $^{33}$P labeled f2-316 fragment to *Drosophila* salivary gland chromosomes. Polytene chromosomes of third instar larvae hybridized with $^{33}$P labeled f2-316 fragment at 62 °C and exposed for one week. Note concentration of grains at 11E (marked with arrow) on the X chromosome.
Fig 5.20: *In situ* hybridization of $^{33}$P labeled f3-316 fragment to *Drosophila* salivary gland chromosomes. Polytenes of third instar larvae hybridized with $^{33}$P labeled f3-316 fragment at 62°C and exposed for one week. Note concentration of grains at 11E (marked with arrow) on the X chromosome.

Fig 5.21: *In situ* hybridization of $^{33}$P labeled f4-316 fragment to *Drosophila* salivary gland chromosomes. Polytenes of third instar larvae hybridized with $^{33}$P labeled f4-316 fragment at 62°C and exposed for one week. Note concentration of grains at 11E (marked with arrow) on the X chromosome.
Fig 5.22: *In situ* hybridization of $^{33}$P labeled f5-316 fragment to *Drosophila* salivary gland chromosomes. A composite plate (reconstructed from chromosomes of the same plate) showing polytene chromosomes of third instar larvae hybridized with $^{33}$P labeled f5-316 fragment at 62 °C and exposed for ten days. Note concentration of grains at 11E (marked with thin arrow) on the X chromosome. Also note the presence of another, unexpected signal at 38B (marked with thick, curved arrow).
Fig 5.23: Southern hybridization of $^{32}$P labeled Bkm-2(8) to PstI digested genomic DNA of embryos, larvae and adults of *Drosophila*.

1 μg genomic DNA of embryos, third instar larvae and adults separately restricted with *PstI*, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled Bkm-2(8) at low stringency (at 60 °C in hybridization buffer containing 0.5M Phosphate buffer and 7% SDS). Post hybridization washing was at 60 °C in 2 x SSC.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane 1: Embryonic DNA;
Lane 2: Third instar larval DNA;
Lane 3: Adult DNA.

Note three additional bands of 5.1 kb, 4.0 kb and 2.7 kb (marked with arrows) are present in larval and adult DNA, but are absent in embryonic DNA.
Fig 5.24: Southern hybridization of ³²P labeled f1-314 fragment to \textit{PstI} digested genomic DNA of embryos, larvae, larval salivary glands and adults of \textit{Drosophila}.

2 µg genomic DNA of embryos, third instar larvae, salivary glands and adults separately restriction digested with \textit{PstI} was electrophoresed in a 1\% agarose gel and hybridized with ³²P labeled f1-314 fragment at high stringency (at 65 °C in hybridization buffer containing 0.5M Phosphate buffer and 7\% SDS) with post-hybridization washing at 65 °C in 0.1 x SSC.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval DNA;
Lane SG: Salivary gland DNA of third instar larvae;
Lane A: Adult DNA.

Note the presence of 7.6 kb band in all the lanes. Also note that salivary gland DNA gives same band pattern as larval DNA.
Fig 5.25: Southern hybridization of $^{32}$P labeled f2-314 fragment to PstI digested genomic DNA of embryos, larvae, larval salivary glands and adults of *Drosophila*.

Same blot as in Fig 5.24, deprobed with 0.4N NaOH and hybridized with $^{32}$P labeled f2-314 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval;
Lane SG Salivary gland DNA of third instar larvae;
Lane A: Adult DNA.

Note 2.7 kb additional band (marked with arrow) is present in larval and adult DNA, but is absent in embryonic DNA. Also note the presence of 7.6 kb band, same as in the blot hybridized to f1-314, in all the lanes. Similarly, 2.9kb band is also present in all the four lanes. Moreover, salivary gland DNA gives same band pattern as larval DNA.
5.26A). In addition, there were weaker bands recognized by this probe. One relatively weak band of 5.9kb was the third common band between the three stages. The interesting feature was that just like pf2 insert a weak 2.7kb band was absent from embryonic DNA even in this blot, while it was present in larval and adult DNA (Fig 5.26A). One more band of 5.1 kb was, similarly, differentially absent from embryos.

When the blot used for hybridization with f1-314 and f2-314 fragments was reprobed (after deprobing the previous probe) with 32P labeled f3-314 fragment, all the common bands observed in the DNA of the three stages in Fig 5.26A were present in the salivary gland DNA, as well (Fig 5.26B). One more common band (of 4.8 kb) observed in this blot was not present in other blots. An interesting feature was that the same size additional bands of 2.7 kb and 5.1 kb, observed in larval and adult DNA (shown in Fig 5.26A) were also present in the salivary gland DNA (Fig 5.26B). As there was no difference in the hybridization pattern of salivary gland and total larval DNA, for many blots hybridized with the fragments of CS316, salivary gland DNA was omitted.

5.1.2.2(B) CS316

Just like CS314, different EcoRI fragments of CS316 were labeled with 32P-dATP and hybridized with the PstI digested genomic DNAs of the three stages, embryo, larva and adult. The results obtained are presented as the successive hybridization patterns resulting from the hybridization of the genomic DNAs with 32P labeled EcoRI fragments of CS316 increasingly distant from the left arm of the phage vector of CS316 (See Fig 5.13 for the restriction map of CS316).

When the fragments f2-316 and f3-316 were hybridized, each of them produced a single band of 10.3kb (Figs 5.27 and 5.28), while the f4-316 upon hybridization produced a single 1.5kb band (Fig 5.29). The fragment f1 is contiguous with the fragment f4 in CS316 (See Fig 5.13 for the restriction map of CS316). Four bands of 1.6kb, 1.1 kb, 0.9 kb and 0.6 kb (Fig 5.30) were seen after hybridization of the labeled f1 fragment with a similar genomic blot containing the DNAs from the three stages. The total of the sizes of the four bands is very close to the size of the f1-316 fragment. Thus, most probably, the overlap of the PstI fragment at the two ends with adjacent subclones is very little. Moreover, it shows that there is a PstI site in f3-316 fragment close to its right end which joins with f4-316. The 1.1 kb
Fig 5.26A: Southern hybridization of Bkm-positive $^{32}$P labeled f3-314 fragment to
PstI digested genomic DNA of embryos, larvae and adults of *Drosophila*.

1 μg genomic DNA of embryos, third instar larvae and adults separately restricted with
PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f3-314
fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim,
Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval DNA;
Lane A: Adult DNA.

Note two additional bands of 5.1 kb and 2.7 kb (marked with arrows) are present in larval
and adult DNA, but are absent in embryonic DNA.
Fig 5.26B: Southern hybridization of Bkm-positive $^{32}$P labeled f3-314 fragment to $PstI$ digested genomic DNA of embryos, larvae, larval salivary glands and adults of *Drosophila*.

Same blot as in Fig 5.25, deprobed with 0.4N NaOH and hybridized with $^{32}$P labeled f3-314 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval DNA;
Lane SG: Salivary gland DNA of third instar larvae;
Lane A: Adult DNA.

Note that the band pattern is the same as in Fig 5.26A, with two additional bands of 5.1 kb and 2.7 kb present in larval, salivary glands and adult DNA, which are absent in embryonic DNA. This is the same blot which was used for hybridization with f1-314 and f2-314, both of which show no partial digestion of DNA in any of the lanes. Thus, the possibility of additional bands resulting from partial digestion is ruled out. Also note that salivary gland DNA gives same band pattern as larval DNA.
Fig 5.27: Southern hybridization of $^{32}$P labeled f2-316 fragment to PstI digested genomic DNA of embryos, larvae, adults and other larval tissues of *Drosophila*.

2 μg genomic DNA of third instar larvae, adults, larvae (without salivary glands), salivary glands, brains and 1 μg genomic DNA of embryos were separately restricted with PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f2-316 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval DNA;
Lane A: Adult DNA;
Lane L-SG: DNA of third instar larvae without salivary glands
Lane SG: Salivary gland DNA of third instar larvae;
Lane Br*: Cephalic complex (brain, ventral ganglia and eye-antennal imaginal discs) DNA.

Same size band (10.3 kb) is present in all the lanes. Note that salivary gland DNA gives the same band pattern as larval DNA.
Fig 5.28: Southern hybridization of $^{32}$P labeled f3-316 fragment to $Pst$I digested genomic DNA of embryos, larvae, adults and larvae (without salivary glands) of *Drosophila*.

1 μg genomic DNA of embryos, third instar larvae, adults and larvae (without salivary glands) separately restricted with $Pst$I, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f3-314 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval DNA;
Lane A: Adult DNA;
Lane L-SG: DNA of third instar larvae without salivary glands.

Same size 10.3 kb band is present in all the lanes, just as obtained upon hybridization of f2-316. So, this is a common $Pst$I band between f2-316 and f3-316.
Fig 5.29: Southern hybridization of $^{32}$P labeled f4-316 fragment to PstI digested genomic DNA of embryos, larvae and adults of *Drosophila*.

1 µg genomic DNA of embryos, third instar larvae and adults separately restricted with PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f4-316 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane 1: Embryonic DNA;
Lane 2: Third instar larval DNA;
Lane 3: Adult DNA.

Note that the same size band (1.5 kb) is present in all the three lanes.
Fig 5.30: Southern hybridization of $^{32}$P labeled f1-316 fragment to PstI digested genomic DNA of embryos, larvae and adults of *Drosophila*.

1 µg genomic DNA of embryos, third instar larvae and adults separately restricted with PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f1-316 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane 1: Embryonic DNA;
Lane 2: Third instar larval DNA;
Lane 3: Adult DNA.

Note that all the four bands (1.6 kb, 1.1 kb, 0.9 kb and 0.6 kb) are common between all the three lanes.
band is the common band between the f1-316 and f5-316 and is the only band detected, when hybridized with the fragment f5-316 (Fig 5.31).

Just as in the case of f3-314, the hybridization pattern of Bkm-positive fragment, f3B-316, was obtained by hybridizing at 60 °C followed by washing at low stringency in 2XSSC at 60 °C. The low stringency was used in order to detect the bands not only at the desired locus, but also all the bands due to the hybridization of the repeat component, GATA, in the fragment. Out of the many bands obtained, two bands, of 4kb and 2.7 kb were present in larval and adult DNAs, but were absent in the embryonic DNA (Fig 5.32).

5.1.2.3 Differential arrangement of DNA is detected by various enzymes

To check if the differential pattern obtained with larval and adult DNA, as compared to that of embryos by using PstI enzyme was not a case of specific mutation at or modification of PstI sites in the genome, the DNAs of the three stages were digested with various enzymes and probed with the Bkm-positive fragment, f3-314. First of all, pf3(314) was digested with many restriction enzymes to pick up those, which cut the genomic insert of this subclone at least once. The digests were separated on 1% agarose gel, Southern blotted and probed with the f3-314 (Fig 5.33). All the enzymes, except those which showed only one band of hybridization corresponding to the linearised subclone, were used for digesting the genomic DNA. Thus finally, the enzymes used were: PstI, HpaII, MspI, AluI, HaeIII, HindII, EcoRI, SacI and Smal (Figs 5.34A and 5.34B). EcoRI was chosen to know the size of the genomic fragment from which the fragment f3-314 has been derived. With all the restriction enzymes, except for Smal and AluI, the pattern obtained, showed one or two additional bands in the larval and adult DNAs, as compared to that in the embryo. Presence of additional band(s) in larval and adult DNAs with so many enzymes is very unlikely to have resulted from modification of the restriction sites of all these enzymes. Deletion or addition or rearrangement of sequences is more likely explanation.

5.1.2.4 Distribution of the additional bands in different larval tissues

To find out whether the appearance of additional bands is a common feature of all the larval tissues or there exists tissue-specific variation, DNA from different larval tissues was compared with that of embryonic and adult DNA. DNA was
Fig 5.31: Southern hybridization of $^{32}$P labeled f5-316 fragment to PstI digested genomic DNA of embryos, larvae and adults of *Drosophila*.

1 µg genomic DNA of embryos, third instar larvae and adults separately restricted with PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f5-316 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane 1: Embryonic DNA;
Lane 2: Third instar larval DNA;
Lane 3: Adult DNA.

Note the presence of a common band (1.1 kb) in all the three lanes.
Fig 5.32: Southern hybridization of Bkm-positive $^{32}$P labeled f3B-316 fragment to PstI digested genomic DNA of embryos, larvae and adults of Drosophila.

1 μg genomic DNA of embryos, third instar larvae and adults separately restricted with PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f3B-316 at low stringency (at 60 °C in hybridization buffer containing 0.5M Phosphate buffer and 7% SDS). Post hybridization washing was at 60 °C in 2 x SSC.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane 1: Embryonic DNA;
Lane 2: Third instar larval DNA;
Lane 3: Adult DNA.

Note two additional bands of 4 kb and 2.7 kb (marked with arrows) present in larval and adult DNA, but absent in embryonic DNA. Also note that overall hybridization pattern of this blot is very similar to that of Bkm-2(8).
Empirically calculated amounts of pf3(314) digested with different restriction enzymes electrophoresed in 1% agarose gel and hybridized with $^{32}$P labeled f3-314. Different restriction enzymes used were:

Lane 1: EcoRI; Lane 2: HindIII; Lane 3: PstI; Lane 4: BamHI; Lane 5: HinfI; Lane 6: HpaII; Lane 7:MspI; Lane 8: AluI; Lane 9: HaeIII; Lane 10: SacI; Lane 11: KpnI; Lane 12: Smal; Lane 13: Smal (in this lane 5 times more DNA compared to lane 12 was loaded); Lane 14: SalI; Lane 15: XhoI.

Lane M4: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane 16: Undigested pf3(314) DNA.

(A) Photograph of the ethidium bromide stained gel under UV;
(B) Photograph of the autorad obtained after hybridization with f3-314 fragment.

Note that the following enzymes yielded more than one band after hybridization of the restricted fragments with f3-314: PstI, HinfI, HpaII, MspI, AluI, HaeIII, SacI, Smal. These enzymes, therefore, were chosen for the restriction digestion of the genomic DNA.
Fig 5.34: Southern hybridization of Bkm-positive $^{32}$P labeled f3-314 fragment to genomic DNA of embryos, larvae and adults of *Drosophila*, digested with various enzymes.

1 µg genomic DNA of embryos, third instar larvae and adults separately restricted with different enzymes, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f3-314 fragment at high stringency.

Lane M4: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany); Lane E: Embryonic DNA; Lane L: Third instar larval DNA; Lane A: Adult DNA.

Different enzymes used are indicated at the top of each set of genomic DNA.

(A) Genomic DNA digested with *PstI*; *EcoRI*; *Hinfl*; *Saci*; *SmaI*.

(B) Genomic DNA digested with *PstI*; *HpaII*; *MspI*; *AluI*; *HaeIII* (shown on the next page).

The additional bands present in larval and adult DNA and absent in embryonic DNA are indicated by arrows. Note that *MspI* and *HpaII* digested DNA give identical pattern.
Fig 5.34 (A)
Fig 5.34 (B)
isolated from total larvae, larvae without salivary glands, brain (the cephalic complex consisting of eye-antennal discs, Supraoesophageal ganglia and ventral ganglia was collected), imaginal discs, embryos and adults. PstI digested DNAs, upon hybridization with Bkm-positive fragment of CS314, f3-314, showed no obvious difference in the number and position of different bands among different larval tissues which, of course, was different from the pattern of embryonic DNA (Fig 5.35). Thus, all the larval tissues undergo the rearrangement. On the other hand, yeast DNA (isolated from dried yeast grains which were used for making Drosophila food) digested with PstI showed no hybridization with f3-314 (lane Y of Fig 5.35), thus ruling out the possibility that additional bands may arise due to contamination from yeast DNA in larval and adult DNA.

5.1.3 Nucleotide sequencing of f3-314 and f5-316

A subclone of CS316, surprisingly, showed its chromosomal localization at 38B, a site where all the subclones of CS314 localize (Fig 5.22), in addition to its localization at 11E (where all the remaining subclones of CS316 hybridize). In order to find out, if there exists any common sequence between the two subclones, which may hint at the common site of recombination used for rearrangement by the two clones, CS314 and CS316, both the subclones were sequenced.

5.1.3.1 Nucleotide sequencing of the insert of pf3(314)

The size of the Bkm-positive EcoRI fragment of CS314 i.e. f3-314 was estimated to be approximately 3.8kb (Fig 5.1). To further reduce the size of the fragment for sequencing, pf3(314) was double-digested with EcoRI & PstI. The mix was kept for re-ligation without the addition of vector. The two subclones, ligated to pGEM3Z vector, contained inserts of 1.3kb and 2.5kb, respectively, as can be seen by double-digestion with EcoRI and PstI (Fig 5.36; and See Fig 5.12 for the restriction map of CS314). pUC/M13 forward and reverse primers were the starting points for sequencing both these subfragments. As a maximum of 750 nucleotides per lane can be sequenced using the automated sequencer, oligonucleotides were designed from the sequences obtained to further/confirm the sequence, using either pf3(314) or either of the two subfragments (1.3kb or 2.5kb) containing subclones as templates. The nucleotide sequence thus obtained
Fig 5.35: Southern hybridization of Bkm-positive $^{32}$P labeled fragment, f3-314 to PstI digested genomic DNA of embryos, larvae, adults and other larval tissues of Drosophila.

1 µg genomic DNA of different developmental stages and third instar larval tissues separately restricted with PstI, electrophoresed in a 1% agarose gel and hybridized with $^{32}$P labeled f3-314 fragment at high stringency.

Lane M: DNA molecular weight marker (Marker X from Boehringer Mannheim, Germany)
Lane E: Embryonic DNA;
Lane L: Third instar larval DNA;
Lane A: Adult DNA;
Lane L-SG: DNA of third instar larvae without salivary glands;
Lane Br+: Cephalic complex (brain, ventral ganglia and eye-antennal imaginal discs) DNA;
Lane ID: DNA of different Imaginal discs:
Lane Y: Yeast DNA
'-' denotes empty lane.

Note two additional bands of 5.1 kb and 2.7 kb (marked with arrows) are present in the larval and adult DNA, and DNA of different tissues of third instar larvae, but are absent in embryonic DNA. Also note that yeast DNA does not show hybridization with f3-314.
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- 5.9 kb
- 5.1 kb
- 3.5 kb
- 2.9 kb
- 2.7 kb
Fig 5.36: Subcloning of pf3(314):

The two subclones of pf3(314) double-digested with EcoRI and PstI, electrophoresed in 1% agarose gel, stained with Ethidium bromide and photographed under UV.

Lane M4: Molecular weight marker (Marker X from Boehringer Mannheim, Germany);
Lane 1 and 2: The two subclones double digested with EcoRI and PstI.

Note that the two subclones yield inserts of 1.3kb and 2.5kb respectively.
from pf3(314) and the two subfragments using different primers was collated and the discrepancies in the overlapping regions were resolved to get a complete consensus sequence.

5.1.3.2 Sequence analysis of f3-314

The actual size of the fragment, f3-314, thus, was 3828 bp(Fig 5.37A). It is an AT-rich sequence containing 59.64% AT. Sequence analysis revealed the presence of 79 GATA repeats, 69 of which were concentrated in 426 bp long stretch, between bps 2571 and 2996. In this cluster, there are five uninterrupted stretches of 13, 8, 6, 6 and 5 repeats of GATA, respectively. The GATA rich region is flanked, on both the sides, by several copies of sequences homologous to Scaffold Associated Region (SAR). These SAR sequences are imbeded in the pool of AT-rich sequences (90% or more).

Additionally, f3-314 contains 10 direct repeats (10bp or longer), 15 inverted repeats (10bp or longer) and 6 palindromic sequences. There are two potential hairpin loops, one centered on base 195 and the other on base 3025.

A representative electropherogram showing a portion of the nucleotide sequence of f3-314 is shown as Fig 5.37B.

5.1.3.3 Nucleotide sequence analysis of f5-316

The size of f5 fragment of CS316 was estimated to be 0.9 kb (Fig 5.1). It was cloned in pUC18. The sequencing was carried out using forward and reverse primers of M13. The sequence analysis showed that the actual size of f5-316 fragment was 830bp (Fig 5.38A). It contains 7 direct repeats (8bp or larger), 4 inverted repeats (8bp or larger) and 4 palindromic sequences. Interestingly, it contains six tandem repeats of hexanucleotide, CCCATG, from base 693 to base 728, as well.

The electropherogram showing the complete nucleotide sequence of f5-316 is shown in Fig 5.38B.
Fig 5.37 (A): Nucleotide sequence of f3-314. Bkm-positive fragment of CS314, f3-314, is 3828 bp long. It has 79 repeats of tetranucleotide GATA (shown by open bars below the sequence) of which 69 repeats are concentrated in 426 nucleotide long region (between nucleotide no. 2571-2996). It has 4 copies of SAR sequences (shown by solid bars) flanking GATA repeats. In addition, it has 10 pairs of direct repeats numbered I to X shown by broken arrows; 15 pairs of inverted repeats numbered IR 1-15 shown by solid arrows; 6 palindromic repeats numbered P 1-6 shown by double-headed arrows; and two potential hairpin loop forming sequences numbered HL1 & HL2 shown by brackets above the sequence.
Fig 5.37 A (Page 1)
GAAACAATGCGTCGTCGTCATTACTGGCGTGCCCAAAACTCGCCTTGGGG 1250
TCAAGGTCGGGTTTGGACCTGCGGTTGGATCGGTTTCTGACTTCTCCG 1300
GCTTGACGGGGTCCATTGACGCCAGCCGAGCGCTTGGCGGGGCTCACT 1350
TAAAACTTAATAGATATTAAATCTGTGTTGACTTCAATGGAATCTCATT 1400
CTCAAATGGGAAGCGTTCCAAATGTTCAAAAAACGTGTTTAAAGCCTGAAT 1500
GGAAAAATAATCTTATCATAAGAGGAAAATACTTCTAATATCTTTTTACTTT 1550
TCTACTAACATTTGACAGTAAAAATATGAGTTAAACAGGAAAACATCAAAT 1600
AAACTTGGGTTCATGAATACGACAAAGAAATTGGGCTCCTACACACAAGAA 1650
GGCACCTGTGAACCACACCGATGTGCTATATTAAATACCTACATCTCA 1800
ATCTCAACCATAATCAAAATATTACACCTTCTCCGCTCCCTCTTCTCAGA 1900
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CAATTGTAAATACAAATAGAAAACCTGGCCGAAAGCAGTGGAGCAAAAGG 2000
TAACATTTACTATAAATAACAAAACCACGGTTAGCTGGAAATCTTCCG 2050
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GGTATGCGACTGCAAATGAGCCTTGCTTTTTCTGCCCCTTCGGTG 2300
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GCAGCTTGTTTTGATTCGGTTTTAACAAGGTGATGTCTTACAAATGT 2400
GACGGGTCCGGTTTTATTTGCTGACCTTCTATATGAAATTTGCACGCTGCTCTTTCG 2450

Fig S.37A (Page 2)
Fig 5.37 A (Page 3)
Fig 5.37 A (Page 4)
Fig 5.37 (B): An electropherogram showing a portion of the nucleotide sequence of f3-314.
Fig 5.38 (A): Nucleotide sequence of f5-316. Bkm-negative fragment of CS316, f5-316, is 830 bp long. Note that it does not contain the conserved component of Bkm, i.e., long stretches of GATA repeats. It has 7 pairs of direct repeats numbered I-VII shown by broken arrows; 4 pairs of inverted repeats numbered IR 1-4 shown by solid arrows; 5 palindromic sequences numbered P 1-5. In addition, it has 6 tandem repeats of a hexanucleotide CCCATG in the region spanning nucleotide no. 694-722 (5 nucleotides are identical in all the repeats, while first nucleotide is same 4 out of 6 repeats).
Fig 5.38 A
Fig 5.38 (B): An electropherogram showing the complete nucleotide sequence of f5-316.
5.1.3.4 Comparison of nucleotide sequences of f3-314 and f5-316

Alignment of the nucleotide sequences of f3-314 and f5-316, using 'NALIGN' program of PC/GENE, revealed approximately 40% homology of f3-314 with both of the orientations of pf5-316 used, after inserting 5 or 6 gaps in the sequence of f5-316. No long stretch of homology was detected between the two sequences, when 'Comparative Align' of SeqEd v1.03 software, available with automated sequencer, was used.

5.2 SEX AND TISSUE-SPECIFIC BKM-BINDING PROTEIN IN DROSOPHILA MELANOGASTER

5.2.1 Detection of Bkm-binding Proteins

Water soluble proteins isolated from different tissues of male and female adult flies were transferred onto nitrocellulose membrane using a slot blot apparatus and probed with an end-labeled, GATA rich, double-stranded Bkm-2(8) insert. Adult ovaries showed a strong signal, which increased with the increase in the amount of proteins (Fig 5.39). Thus, *Drosophila* Bkm-binding protein(s), dBbP, are present predominantly in the ovaries. The signal was also seen in the slots containing proteins from total females and testis, but was extremely low.

Since in the slot-blot assay there is a high chance of detecting a non-specific binding to the proteins, electrophoretic mobility-shift assay was undertaken. Here the complex formed between DNA and proteins can be checked more stringently for its specificity. Moreover, since the proteins used are in the native form, the results obtained will be true representative of *in vivo* situation. As mentioned earlier, the main conserved component of Bkm is the repeats of tetranucleotide, GATA. To look for proteins binding exclusively to the repeats of GATA, nuclear proteins from the adult ovary were used in a mobility-shift assay. Chemically synthesized oligonucleotides containing 16 tandem repeats of GATA cloned into pUC18 in *Smal* site, was excised by double-digestion with *EcoRI* and *HindIII*. The 124 bp DNA fragment thus obtained was called (GATA)_{16} and was used as a representative of conserved component of Bkm for mobility-shift assays. End-labeled (GATA)_{16} was allowed to bind to increasing amounts of ovary nuclear protein extracts and the resulting complexes were separated electrophoretically. Three DNA-protein complexes X1, X2 and X3, were detected (Fig 5.40). Complex X2 is a relatively abundant complex as compared to the other two.
Fig 5.39: Detection of dBBP in *Drosophila* ovary extracts by slot-blot assay.

10, 25 and 50 µg of total protein extracts isolated from different tissues of male and female adult flies were vacuum transferred onto nitrocellulose membrane and probed with $^{32}$P end-labeled Bkm-2(8) in the presence of 50µg/ml of sheared *E. coli* DNA at room temperature and autoradiographed.

Note predominant signal with ovarian tissue, which increases with the increasing amounts of protein. Also note absence of signal with whole body (male) and with the negative control, BSA.
Amount of protein per slot

- Whole body (female)
- Testis
- Ovary
- Head (male)
- Head (female)
- Gonadless (male)
- Gonadless (female)
- Whole body (male)
- BSA
Fig 5.40: Identification of dBBP in *Drosophila* nuclear extract by mobility-shift assay.

Mobility shift assays with $^{32}$P end-labeled $(GATA)_6$ as a probe and 2 μg of sheared *E. coli* DNA as a non-specific competitor performed with increasing amounts of *Drosophila* ovary nuclear extracts. The complexes were resolved in 6% polyacrylamide gel and autoradiographed.

Lane 1: End-labeled probe without protein;  
Lanes 2-4: 5, 10 and 20 μg of *Drosophila* ovary nuclear extracts, respectively.  
F—Free probe.

Note the presence of three complexes $X_1$, $X_2$ and $X_3$. $X_2$ complex shows the strongest signal, while $X_3$ complex shows the least signal.
5.2.2 Binding of dBBP is affected by Salt concentration

The interaction between DNA and proteins is known to get affected by changes in the salt concentrations of their immediate environment. To look at the effect of salt concentration on the binding of (GATA)$_{16}$ with dBBP, mobility-shift assays were carried out with ovary nuclear protein extracts in the buffers containing increasing amount of salts. The intensity of signal for X2 complex increased slowly with the increase in the salt concentration from a feeble signal obtained at 50mM NaCl concentration to a maximum at 350mM NaCl (Fig 5.41). The signal intensity decreased, as the salt concentration was increased to 400mM of NaCl or above. Thus, X2 complex shows optimum binding at 350mM NaCl. It was notable that the X1 complex didn't get affected appreciably by the change in the salt concentration. The higher molecular weight complex X3 was detectable as a weak band only around 350mM NaCl concentration, the optimal salt concentration for X2 complex, and disappeared at lower or higher salt concentrations.

5.2.3 Sequence specificity of dBBP

To find out if the interaction between GATA repeats and dBBP is specific for GATA repeats, mobility-shift assay with ovary nuclear protein extracts were carried out in the presence of increasing amounts of $E. coli$ DNA, (GATA)$_{16}$ and Bkm-2(8) insert. $E. coli$ DNA doesn't hybridize with Bkm-2(8) insert, indicating the absence of long stretches of the tetranucleotide, GATA, in it. More than 5µg of $E. coli$ DNA is required to completely abolish the signal obtained from binding interaction between 1ng of probe, (GATA)$_{16}$, and 5µg of ovary nuclear protein extract(Fig 5.42). On the other hand, 90 ng of (GATA)$_{16}$ is sufficient to completely abolish the signal. Bkm-2(8) insert was as effective as (GATA)$_{16}$, since 90 ng of Bkm-2(8) insert was sufficient to compete out the binding.

5.2.4 Effect of EDTA on the binding of dBBP

Many GATA-binding proteins are known to recognize and bind to one or two (GATA) in a specific context of flanking sequences, through their zinc-finger motifs. To check whether binding of dBBP to GATA repeats requires zinc ions,
Fig 5.41: Effect of salt concentration on the binding of dBBP to (GATA)$_{16}$.

Mobility shift assays with 1ng of $^{32}$P end-labeled (GATA)$_{16}$ probe and 2 μg of sheared *E. coli* DNA, as non-specific competitor, performed with 5 μg of *Drosophila* ovary nuclear extracts containing increasing concentrations of NaCl, resolved in 6% polyacrylamide gel and autoradiographed.

Lane 1: End-labeled probe without protein;
Lane 2: A typical assay with 5 μg of ovary nuclear protein in 350 mM NaCl;
Lanes 3-10: 5 μg of protein in 50, 100, 150, 200, 300, 350, 400 and 500 mM NaCl, respectively.
F— Free probe.

Note that optimum binding is observed at 350 mM NaCl concentration,
Fig 5.42: Sequence specificity of dBBP binding to (GATA)$_{16}$.

Increasing amounts of cold (GATA)$_{16}$, Bkm-2(8) insert containing 66 copies of GATA repeats, and E. coli DNA added to mobility shift assay reaction mixes containing 1ng of $^{32}$P end-labeled (GATA)$_{16}$ probe and 5 µg of Drosophila ovary nuclear protein extracts, resolved in 6% polyacrylamide gel and autoradiographed.

Lane 1: End-labeled probe without protein and cold competitor;
Lanes 2-5: 5 µg of Drosophila ovary nuclear extract with 1, 2, 5 and 10 µg of sheared E. coli DNA, respectively;
Lanes 7-10: 5 µg of Drosophila ovary nuclear extract with 8, 16, 40 and 90 ng of (GATA)$_{16}$ insert, respectively;
Lanes 12-15: 5 µg of Drosophila ovary nuclear extract with 8, 16, 40 and 90 ng of Bkm-2(8) insert, respectively;
Lanes 6 and 11: Empty lanes.

Note that the signal is completely abolished with 90 ng each of (GATA)$_{16}$ insert and Bkm-2(8) insert, while 10 µg of E. coli DNA is needed to abolish the signal.
the effect of EDTA on the binding of dBBP to (GATA)\textsubscript{16} was looked into. Preincubation of ovary nuclear protein extract with or without 35mM EDTA was carried out before adding \textsuperscript{32}P labeled (GATA)\textsubscript{16} to it. No difference in the binding efficiency of (GATA)\textsubscript{16} was observed in the presence of EDTA (Fig 5.43). Rather, EDTA prevented non-specific degradation of probe, probably, by hindering the nucleases present in tissue proteins from acting.

5.2.5 Tissue-specific expression of dBBP

To confirm the earlier results of a predominantly ovarian expression of dBBP, nuclear protein extracts were made from different tissues of the male and female adult flies. Nuclear protein extracts isolated from equal number of different body tissues of the adult flies were used for DNA-binding assay with (GATA)\textsubscript{16}. The ovaries showed an intense signal, while the signal was absent from the other tissues of the female as well as all the tissues of male flies (Figs 5.44A and 5.44B). Upon overexposing, the faint signal observed in the lane containing proteins from gonad-less female (data not shown), could be due to the small amounts of ovarian tissue remaining with the female body while dissecting away the ovary from those females. Thus, the Bkm-binding protein in \textit{Drosophila melanogaster} is sex- and tissue-specific.
Fig 5.43: Effect of addition of EDTA on binding of dBBP to (GATA)\textsubscript{16}.

Mobility shift assays containing 1ng of $^{32}\text{P}$ end-labeled (GATA)\textsubscript{16} probe and 2 μg of sheared E. coli DNA, as non-specific competitor, performed using 5 μg of Drosophila ovary nuclear protein extracts with or without the addition of EDTA, resolved in 6% polyacrylamide gel and autoradiographed.

Lane 1: End-labeled probe without protein;
Lane 2: 5 μg ovary nuclear extract with 35mM EDTA;
Lane 3: Empty lane;
Lane 4: 5 μg ovary nuclear extract without EDTA.
F– Free probe.

Note that the presence of EDTA has no effect on binding of dBBP to (GATA)\textsubscript{16}.
Fig 5.44: Sex and tissue specific expression of dBBP.

Nuclear protein extracts isolated from equal number of different tissues of male and female flies used in mobility shift assays containing 1ng of \(^{32}\text{P}\) end-labeled \((\text{GATA})_{16}\) probe and 2 \(\mu\)g of sheared \(E.\ coli\) DNA as non-specific competitor, resolved in 6% polyacrylamide gel and autoradiographed.

(A)
Lane 1: End-labeled probe without protein;
Lane 2: Male heads; Lane 3: Male headless; Lane 4: Female heads; Lane 5: Female headless; Lane 6: Ovary; Lane 7: Testis.

(B): Figure shown on the next page

Lane 1: End-labeled probe without protein;
Lane 2: Male gonadless; Lane 3: Testis; Lane 4: Male (whole body); Lane 5: Female (whole body); Lane 6: Female gonadless; Lane 7: Ovary.

F– Free probe.

Note the intense signal of complex \(X_2\) with ovary nuclear extracts (Lane 6 of A and Lane 7 of B). Signal is also observed with headless female (Lane 5 of A) and whole body female (Lane 5 of B) nuclear protein extracts.
Fig 5.44 (A)
Fig 5.44 (B)