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Structural analysis of the three vitellogenin genes in Drosophila melanogaster

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ABSTRACT

Genomic fragments coding for sequences expressed as abundant mRNA in female Drosophila melanogaster were isolated from a lambda library. Hybridization of these clones to polytene chromosomes, in situ, identified four which mapped to X chromosomal region 9A to 9B, the locus for yolk proteins 1 and 2 (Yp1,2) and two which mapped to 12A6-7 to 12D3, the locus for Yp3. These clones were mapped with restriction enzymes, and the coding regions and regions of homology determined by Southern blots probed with cDNA, 5'-end-labelled RNA and nick-translated DNA. Heteroduplex and R-loop mapping confirmed that three of the clones carried two genes separated by about 1.4 kb and oriented in opposite directions. Southern blots probed with cDNA made from alkali-hydrolyzed RNA showed that these genes had their 5' ends next to each other. All 3 genes show homology to each other and have a main coding region of about 1.35 kb, the approximate size for the mRNAs.

INTRODUCTION

The major egg yolk proteins of insects, the vitellins, can be classed on a basis of size, both of the native molecule and of the polypeptide subunits, and by hormonal control of synthesis. In the great majority of insects, the precursor to vitellin is a high molecular weight polypeptide of 200,000 - 250,000 daltons[1]. This precursor polypeptide undergoes several post-translational modifications, the major one being cleavage into two fragments, usually one of 140,000 - 180,000 daltons and one of 45,000 to 60,000 daltons [2-4]. The expression of these vitellogenin genes is controlled by juvenile hormone which acts on the fat body to stimulate synthesis and also on the ovary to promote uptake.

In the higher Diptera, as exemplified by Drosophila melanogaster[5,6] and Calliphora erythrocephala[7] vitellin is different, being entirely composed of polypeptides of about 50,000 daltons. These subunit polypeptides which are very similar in size to the primary translation products, are synthesized in the ovary as well as the fat body[8] and both juvenile hormone and ecdysone are involved in control of their synthesis[9].

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Recent work by Postlethwait & Jowett[10] has localized the vitellogenin genes by mapping electrophoretic variants. Genes for yolk proteins (Yp) 1 and 2 behave in a very closely linked manner, mapping to X chromosomal region 8E to 9B1; and YP3 maps to X chromosomal region 12A6-7 to 12D3. These three genes have also been isolated and localized by _in situ_ hybridization of cRNA to the polytene chromosomes[11].

The vitellins are rapidly evolving proteins and, in spite of functional identity, there is very little immunological cross-reactivity, even at the family level[12]. The question arises: are the higher dipteran vitellins homologous to the small polypeptide fragments of the other insects or have they evolved from completely different genes? Examination of this question requires analysis of parallel systems at the gene level. It was decided to isolate chromosomal DNA carrying the three vitellogenin genes of _D. melanogaster_ from a genomic lambda library and to use these fragments to study the structure of the genes. The initial screening of the library was based on the knowledge that the mRNA for vitellogenin is abundant and female specific.

MATERIALS & METHODS

Isotopes were purchased from New England Nuclear, Corp.. Restriction enzymes and other nucleic acid modifying enzymes were obtained from Bethesda Research Labs. and Boehringer-Mannheim, Canada. The 1-[(m-nitrobenzyloxy)-methyl]-pyridinium chloride was obtained from Pierce Chemical Co., and the oligo-$(dT)_{18}$-cellulose was from Collaborative Research. The reverse transcriptase was a gift from Dr. J. W. Beard, Life Sciences, Inc., St. Petersburg, Florida.

(a) _Fly rearing_ - Wild type (Samarkand) and two mutant strains of _Drosophila melanogaster_ were used. All flies were raised at 22°C on medium containing 10% yeast extract, 10% sucrose and 1.5% agarose. Adult females (shi^{ts1}:XY) were harvested at 4-8 days after emergence, while adult males (E6:=/Y^{bb1} x In(1)w^{vg}/Ybbl) were harvested as needed.

(b) _Nucleic Acid Extractions_ - RNA was extracted from whole flies by a modification of the phenol-chloroform method[13]. Immediately after deproteinization, the solution was centrifuged at 100,000 g for 30 min at 4°C to remove glycogen. The RNA was then ethanol precipitated, redissolved at a concentration of 5 mg/ml and digested with 500 ug/ml proteinase K for 2 hours at 37°C[14], phenol-chloroform extracted, adjusted to 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5) and passed over an oligo-(dT)-cellulose column at 4°C. Poly (A^{+}) RNA was collected by elution with H_2O.

Supercoiled plasmid DNA was prepared by CsCl-ethidium bromide gradient centrifugation[15] followed by a phenol-chloroform extraction. DNA was prepared by the method of Maniatis et al.[16].

(c) Gel Electrophoresis - RNA was electrophoresed under denaturing conditions on 1.5% agarose gels containing 6mM methylmercuric hydroxide[17]. Markers used in determining the message size were restriction fragments from a Hae III digest of ØX174 DNA. Gels were stained in 0.5 M sodium acetate containing 2 µg/ml ethidium bromide for 20-30 min. DNA was electrophoresed on 0.8%, 1% or 2% agarose gels in 40 mM Tris-HCl (pH 8.3), 33 mM NaOAc and 1mM EDTA. Staining was with ethidium bromide.

(d) Nucleic Acid Labelling - Complementary DNA was synthesized under the conditions of Buell et al.[18]. The template RNA was either intact or had been alkali hydrolyzed to a mean length of 150-200 nucleotides. The alkali treatment was 50 mM NaOH at 22°C for 5 min, followed by neutralization with acetic acid. Nick-translation followed a modification of the procedure of Maniatis et al.[19]. The cold dNTP concentration was increased to 18 µM and the reaction was allowed to proceed for 3 hours at 15°C. The specific activity of the DNA was 0.5-30 x 10^7 cpm/µg. Alkali-hydrolyzed RNA was 5' end-labelled with ^{32}P according to Donis-Keller et al.[20].

(e) Screening of Genomic Library and Subclones - The bacteriophage lambda library of Drosophila melanogaster genomic DNA was that prepared by Maniatis et al.[16]. The library was screened by in situ plaque hybridization using ^{32}P-labelled cDNA (8 x 10^8 cpm/µg) made from RNA of vitellogenic females[21]. Subclones were generated by insertion of Eco RI fragments from the lambda clones into the Eco RI site of the plasmid vector pAT153. The recombinant plasmid was then cloned into E. coli HB101. These subclones were screened by in situ colony hybridization[14] using the same probe, thereby selecting for those subclones which carried coding sequences. These subclones were subsequently used for restriction mapping and for comparative and genomic Southern blots.

(f) Hybridizations - In situ hybridizations to the polytene chromosomes were performed by the method of Pardue & Gall[22]. The specific activity of the probes, nick-translated with [^{3}H]-dCTP, varied from 5-10 x 10^6cpm/µg DNA and 2 - 4 x 10^5 cpm in 20 µl were used in each hybridization. For Northern blots, diazobenzyloxymethyl paper was prepared and the RNA blotted according to Alwine et al.[23]. The blotted RNA was prehybridized

in 5 x SSC[24], 1% glycine, 2 x Denhardt's solution[25], 300 µg/ml sonicated, denatured calf thymus DNA and 50% deionized formamide for 12-24 hours at 42°C. Hybridization was in 3-5 ml of the same buffered solution (without glycine) for 15 hours at 42°C, with 8-10 x 10^6 cpm of ^{32}P-labelled DNA/filter. Hybridized filters were washed by the method of Smith et al.[26]. Southern Blots were performed essentially as described by Southern[27].

(g) Electron Microscopy - Heteroduplex mapping was performed as described by Davis et al.[28], as modified by Kidd & Glover[29]. Markers used were open circular SV40 DNA as a double-stranded length (5.24 kb) and a 6.62 kb hairpin loop formed by snapback after Eco RI cleavage of ckDM103B as a single-stranded length marker[29]. R-loop hybridizations followed the technique of White & Hogness[30]. Open circular SV40 was used as a double-stranded length marker.

(h) One Dimensional S1 Mapping - S1 mapping was performed as outlined by Berk & Sharp[31] except that the DNA was denatured at 85°C for 20 min and the DNA-RNA annealing was at 52°C for 4 hours. The nuclease digestion was at 37°C for 30 min.

RESULTS

(a) Initial Screening of Lambda Library and Subclones

The method chosen for the initial screening of the lambda library was based on the assumption that, since the adult female is synthesizing large quantities of vitellogenin[32], then the mRNAs for these polypeptides should be present at a high frequency. This is substantiated by the fact that when total RNA from females is translated in a wheat germ cell-free translation system, the vitellogenin polypeptides are predominant[33]. The in situ plaque hybridization conditions were such that sequences expressed at high frequency could be specifically detected following hybridization with cDNA to female RNA. Thirty plaques were selected, purified and the DNA isolated. Following restriction of fourteen of these clones with Eco RI, a Southern blot was made and probed with cDNA to female and male poly (A^+) RNA to identify those restriction fragments which carried coding sequences (Fig. 1).

Only clones 1, 7, 19, 25 and 29 showed complete specificity to female cDNA, while most of the others showed weak hybridization to male cDNA. Clone 11 is clearly complementary to abundant mRNA in both males and females.

Eco RI fragments of these lambda clones were then subcloned into pAT153

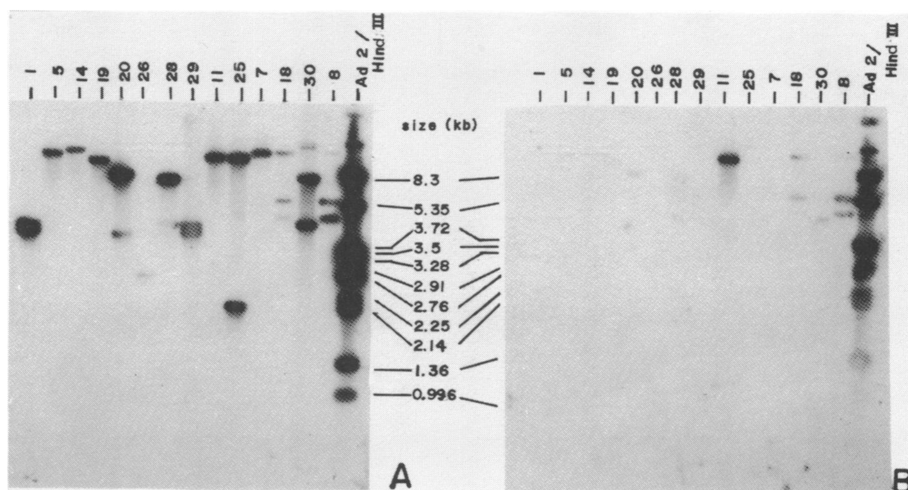

Figure 1: Preliminary screening of the Drosophila genomic lambda library. Complementary DNA to either female or male RNA was hybridized to an Eco RI digest of lambda clones carrying sequences expressed at high frequency in female RNA. (A) Southern blot probed with cDNA to female RNA; (B) Southern blot probed with cDNA to male RNA.

and an additional screening with cDNA to female RNA allowed selection of those subclones carrying coding sequences.

(b) Selection of Putative Vitellogenin Clones

The genomic clones were then labelled with [^{3}H]-dCTP by nick-translation and hybridized, *in situ*, to polytene chromosomes. Clones 1,19,25 and 29 hybridized to bands 9A to 9B, the location of genes for Yp 1 and Yp 2, while clones 5 and 14 hybridized to bands 12A6-7 to 12D3, the site for Yp 3[10,11] (Fig. 2). On the basis of their specific hybridization to chromosomal regions known to contain the vitellogenin genes, these six clones were selected for further analysis.

Comparative Southern blots of these lambda clones probed with subclones carrying coding regions confirmed that clones 1, 19, 25 and 29 were overlapping segments of the same chromosomal region. Clones 5 and 14 also overlapped with each other. These clones were therefore named according to their chromosomal region; so that clone 1 is termed λDm(Can S)Yp1/2:1 and its Eco RI subclones are pDmYp1/2:1R^{x}.

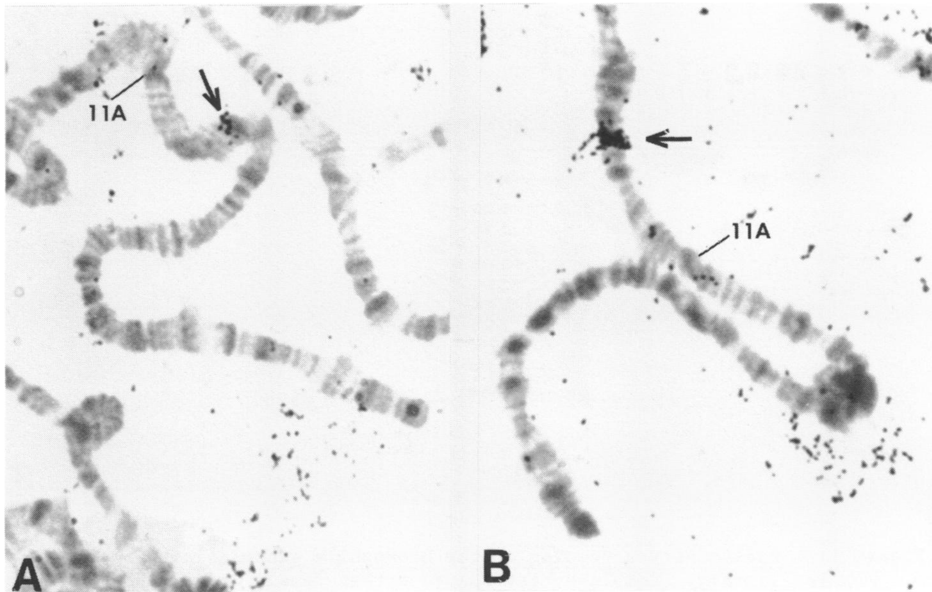

Figure 2: Identification of clones hybridizing to chromosomal regions carrying genes for the yolk proteins. Clones carrying sequences expressed in a high frequency, female specific manner were hybridized, in situ, to polytene chromosomes. Representative results are shown. (A) Hybridization of clone 1 to the X chromosomal region 9A to 9B, which codes for Yp1 and Yp2, (B) Hybridization of clone 5 to X chromosomal region 12A6-7 to 12D3, which codes for Yp3. The arrows point to regions of hybridization. Band 11A is indicated as a reference band.

(c) Restriction Maps of Clones and Genomic Southern Blots

The lambda clones and plasmid subclones were mapped by standard techniques. The relationships of the lamdba clones to each other and to the respective subclones are shown in Fig. 3. It was shown that the restriction maps derived from these clones are identical to those determined by Barnett et al.[11] for the vitellogenin gene regions.

Genomic Southern blots were probed with pDmYp1/2:1R^a, pDmYp1/2:1R^b and pDmYp3:2R^a (Fig. 4). A comparison of the restriction patterns derived from these blots with maps of the lambda clones showed that there had been no rearrangement during cloning. It also indicated the presence of a 0.1 kb Eco RI fragment between pDmYp1/2:1R^a and pDmYp1/2:1R^b which contained a Bam HI site.

The genomic Southern blot probed with pDmYp3:2R^a generated several minor

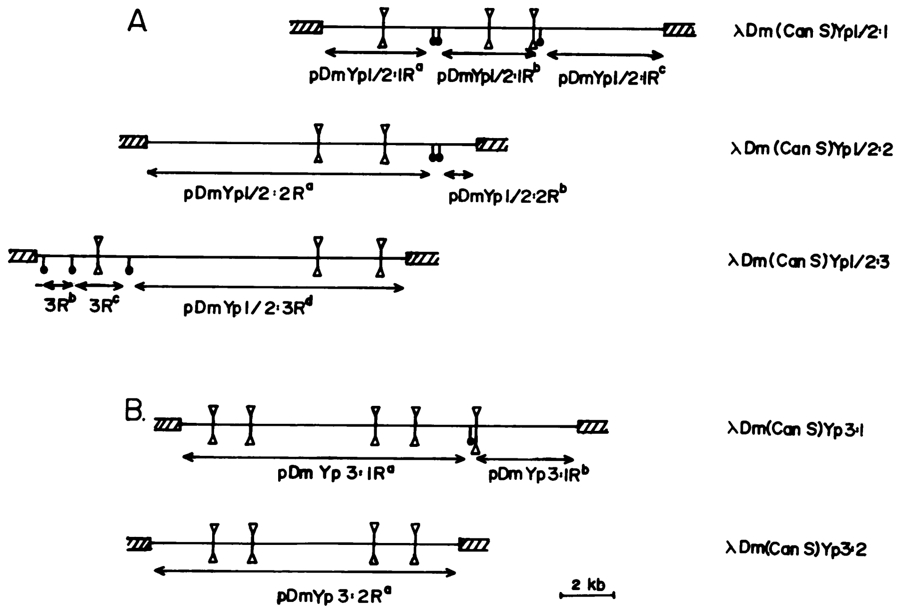

Figure 3: Comparison of lambda clones carrying genes for yolk proteins. (A) Clones from X chromosomal region 9A to 9B. (B) Clones from X chromosomal region 12A6-7 to 12D3. Hatched regions indicate vector DNA; thin line represents inserted DNA. ↓ - Eco RI· Hind III sites. The arrows indicate Eco RI fragments contained in each subclone.

bands which could not be explained by the restriction maps alone. They could indicate the presence of a moderately repeated sequence in this chromosomal region or polymorphisms in the genomic DNA.

(d) Positioning the Coding Regions Within the Cloned Fragments

The positions of the coding regions of the clones were determined by hydridizing 5'-end-labelled RNA from female flies to Southern blots of restriction digests of the lambda clones. The RNA was alkali-hydrolyzed to give an average length of 150-200 nucleotides and end-labelled. In pDmYpl/2:1R^{a}, the 1.0 kb and 0.35 kb Pst I fragments, the 0.5 kb Pst I/HindIII fragment and the 0.3 kb Pst I/Eco RI fragment hybridized to the labelled RNA (Fig. 5, fragments a,b,c and d, respectively), while the 1.2 kb HindIII/Pst I fragment showed little hybridization (Fig. 5, fragment e). In pDmYpl/2:1R^{b}, the 0.25 kb Pst I/Eco RI fragment and the 1.9 Pst I/HindIII fragment (Fig. 5, fragments f and g) showed a limited amount of

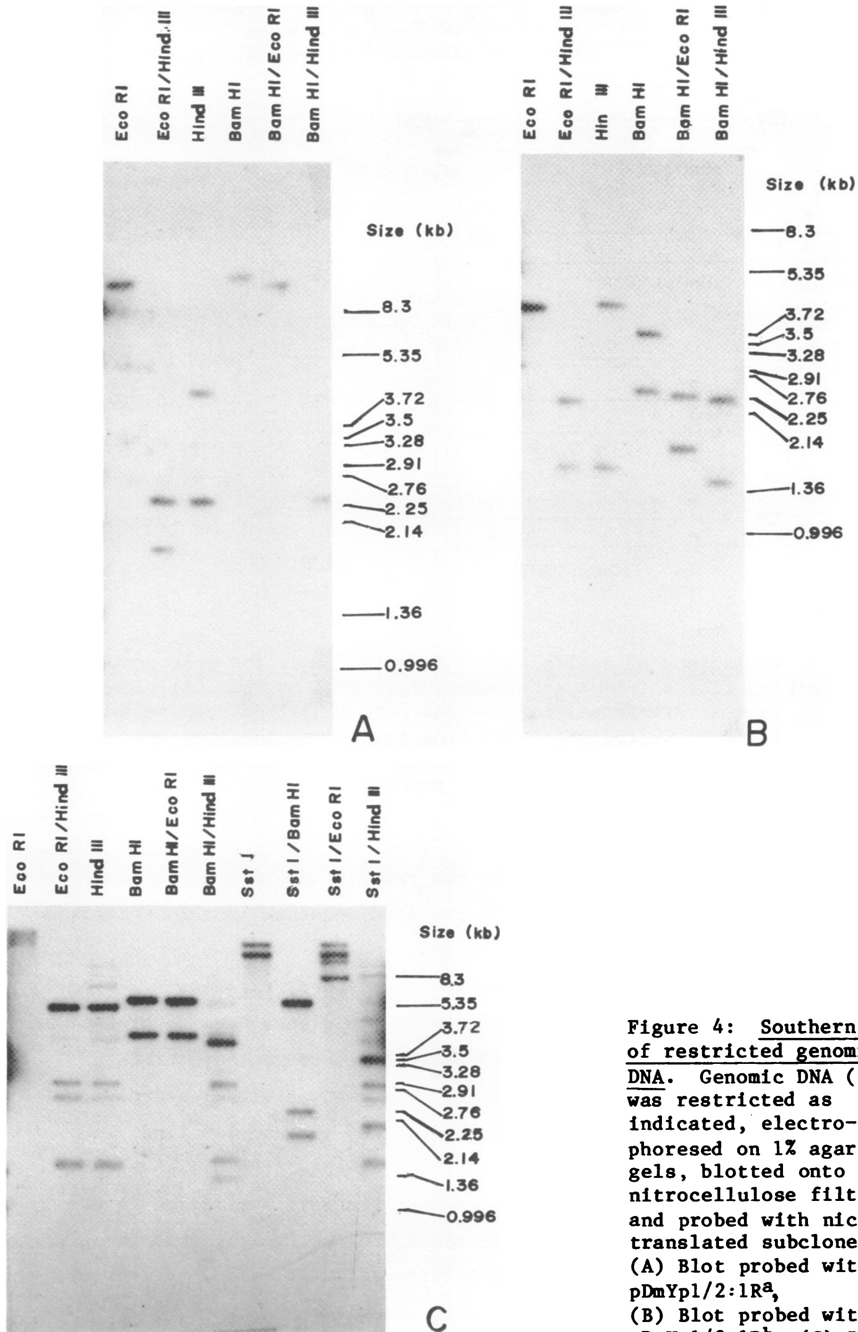

Figure 4: Southern blots of restricted genomic DNA. Genomic DNA (1μg) was restricted as indicated, electrophoresed on 1% agarose gels, blotted onto nitrocellulose filters and probed with nick-translated subcloned DNA. (A) Blot probed with pDmYp1/2:1R[a], (B) Blot probed with pDmYp1/2:1R[b], (C) Blot probed with pDmYp3:2R[a].

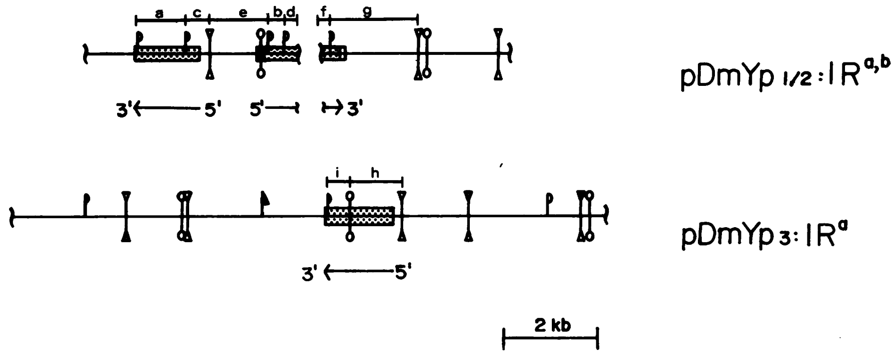

Figure 5: Restriction maps and coding regions of subclones carrying yolk protein genes. Restriction maps were developed by standard techniques, using enzymes Eco RI( ), Pst I( ), Hind III( ), Bam HI( ) and SstI( ). Positions and limits of coding regions (stippled boxes) and direction of transcription were determined as outlined in text.

hybridization to the labelled RNA. Only the 1.09 Bam HI/HindIII fragment and the 0.35 kb Bam HI/Pst I fragments of pDmYp3:1R^{a} hybridized to the RNA (Fig. 5, fragments h and i).

The direction of transcription was determined by comparing hybridization patterns of cDNA made from intact RNA and from alkali-hydrolyzed RNA to various restriction digests. The latter cDNA preparation would preferentially locate the 3' ends of the coding regions. In pDmYp1/2:1R^{a} this comparison, using Pst I, Hind III/Pst I and PstI/Eco RI digests, showed preferential hybridization to the 1.0 kb Pst I fragment and the 0.3 kb Pst I/Eco RI fragments and none to the 0.5 kb and 1.2 kb Hind III/Pst I fragment (Fig 5, fragments a, d, c and e respectively). The 0.25 kb Pst I/Eco RI fragments and the 1.9 kb Pst I/ Hind III fragment of pDmYp1/2:1R^{b} hybridized equally to both cDNA preparations (Fig. 5, fragments f and g). It can be seen at this point that there are two coding regions in λDm(CanS)Yp1/2:1 and that these regions are transcribed in opposite directions, with their 5' ends close to each other. In pDmYp3:1R^{a}, it was shown that the 3' end of the coding region lies in, or very near, the 0.35 kb Bam HI/Pst I fragment (Fig. 5, fragment i).

Limits of the coding regions were determined by one dimensional SI mapping (Fig. 6). This suggested that the major structural portions of the genes were 1.35 kb and that, if any intervening sequences were present, they must lie very close to the ends of the coding regions.

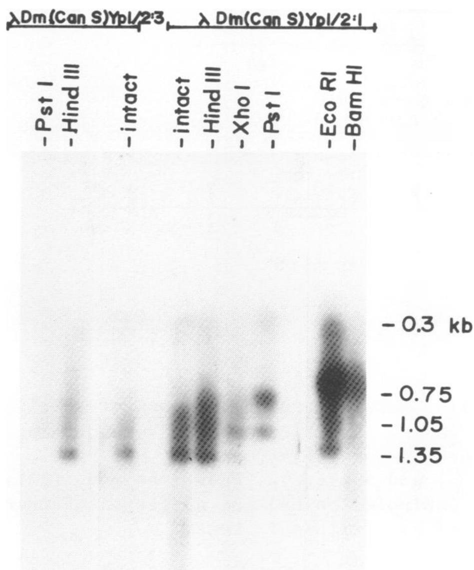

Figure 6: Positioning of coding regions by one dimensional SI maping. Poly (A^+) RNA was hybridized to restricted or unrestricted DNA from λDm(Can S)Ypl/2:1 and λDm(Can S)Ypl/2:3. The hybrids were digested with SI nuclease, electrophoresed on alkaline agarose gels, blotted and probed with the homologous plasmid DNAs.

Recent work by Wensink[34] has indicated the presence of small intervening sequences near the ends of the genes.

A comparison of the restriction patterns obtained from S1 mapping of λDm(Can S)Ypl/2:1 and λDm(Can S)Ypl/2:3 indicated that the common gene had 2 PstI sites, 1.05 kb apart and no Hind III sites, while the second gene had 3 PstI sites, generating 2 large fragments of 0.75 kb and 0.3 kb, and 2 small fragments, one at each end of the gene. This second gene also contains the double Eco RI sites, giving 2 fragments of 0.94 kb and 0.47 kb, and one small fragment, bordered by the Eco RI sites. Detailed restriction maps of the subclones, and the positions of the coding regions with direction of transcription are shown in Fig. 5.

(d) Electron Microscopy

Cloned genomic DNA was spread under conditions maintaining hetero-

duplex formation. A frequently occurring inverted repeat structure was observed in DNA from λDm(Can S)Ypl/2:1 (Fig. 7A), indicating the presence of an essentially palindromic sequence with 2.17 kb of single-stranded DNA at its center. Within the complementary region there is a 0.35 kb region of nonhomology. By comparison with the equivalent structure in Eco RI fragments of pDM Ypl/2:1R[a], it was shown that this small region of non-homology contains the double Eco RI sites of the genomic clone (Fig. 7B). Heteroduplexes of λDm(Can S)Ypl/2:1 and λDm(Can S)Yp3:1 resulted in one of two structures. One structure contained the hairpin loop and enabled it to be oriented with respect to the vector arms (Fig. 8A), the other structure showed a 1 kb region of homology located 3.8 kb from the insertion point of the cloned DNA of λDm(Can S)Ypl/2:1 into the short arm of the vector (Fig. 8B).

R-loops formed between total female RNA and λDm(Can S)Ypl/2:1 showed

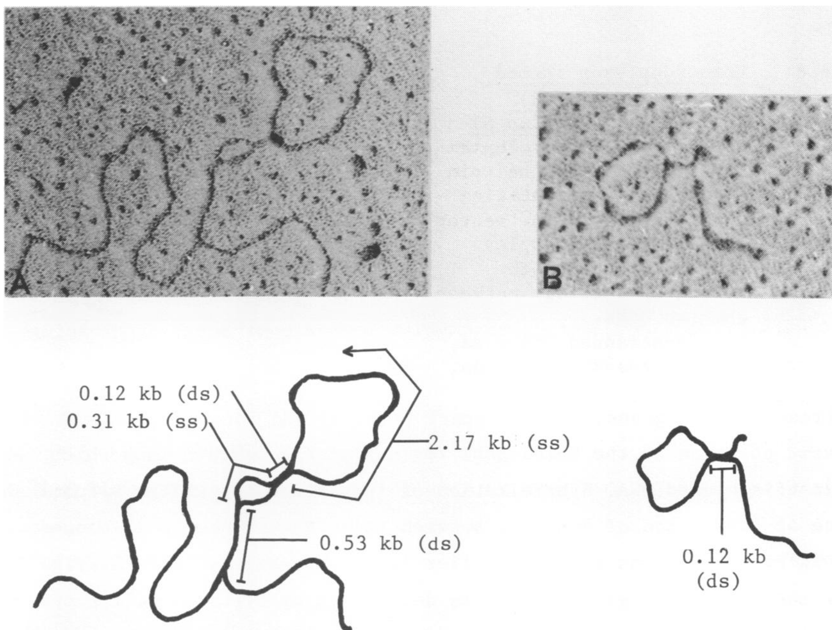

Figure 7: Electron microscopy of duplex structures in DNA from λDm(Can S)Ypl/2:1 and pDm Ypl/2:1R[a]. (A) Hairpin loop formed within λDm(Can S)Ypl/2:1 following complete denaturation. (B) Hairpin loop formed in pDm Ypl/2:1R[a] which had been denatured following restriction with Eco RI. ss - single-stranded DNA; ds - double-stranded DNA.

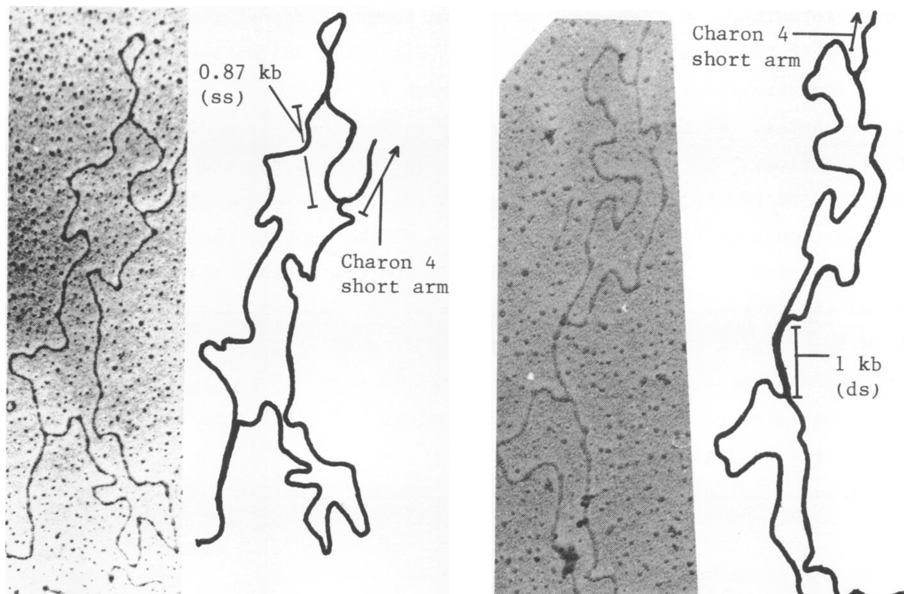

Figure 8: Heteorduplexes formed between λDm(Can S) Ypl/2:1 and λDm(Can S) Yp 3:1. (A) Heteroduplex structure showing hairpin loop and its orientation with respect to the vector arms. (B) Heteroduplex structure showing the region of homology between the two DNAs. single-stranded DNA - ss, double-stranded DNA - ds.

the presence of 2 genes, 1.42 kb apart (Fig. 9) and located in essentially the same position as the homologous regions of the hairpin loop which forms within this cloned DNA. Hybridization of female RNA to λDm(Can S)Yp3:1 showed a gene at the region of homology between this DNA and the Ypl/2 clones. All RNA-DNA hybrid regions were of similar length, averaging 1.16 kb. Fig. 10 shows the homologous gene regions as determined by electron microscopy and compares them to the coding regions determined by hybridization studies.

DISCUSSION

Six clones carrying three coding sequences which are expressed at high frequency in adult female *Drosophila melanogaster* have been isolated from a lambda library of genomic DNA. Based on their chromosomal locations of bands

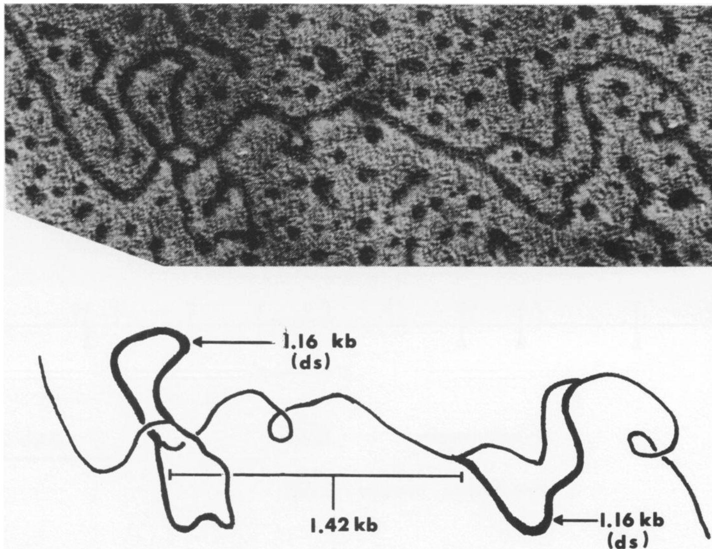

Figure 9: R-loop mapping of λDm(Can S)Yp1/2:1. The hybridization of total poly (A^+) female RNA to λDm(Can S)Yp1/2:1 resulted in the formation of double R-loop structures. RNA-DNA hybrids are indicated as double-stranded (ds) and the displaced DNA as single-stranded (ss).

9A to 9B, the site for Yp1 and 2, and bands 12A6-7 to 12D3, the site for Yp3[10,11], and also on their restriction patterns which are identical to those determined for the vitellogenin gene regions by Barnett *et al.*[11], it is concluded that these six clones carry sequences for the three vitellogenin genes.

Three of the clones carry both Yp1 and Yp2 sequences which form snapback structures when visualized under the electron microscope (Fig. 7A). Heteroduplex analysis, R-loop mapping and analysis of coding sequences with cDNA probes have shown the 5' ends to be about 1.4 kb apart. This head to head arrangement of genes is not unique to the vitellogenin genes, but has been reported for silk-moth chorion genes[35], *Drosophila* histone genes[36] and the *Drosophila* heat shock locus, 87A7[37]. The possible consequence of such an organization would be to allow control of expression from a single site between the two genes. This would be of particular advantage in any system where two or more genes are coordinately expressed.

The ability to generate heteroduplexes between genes for Yp1 and 2,

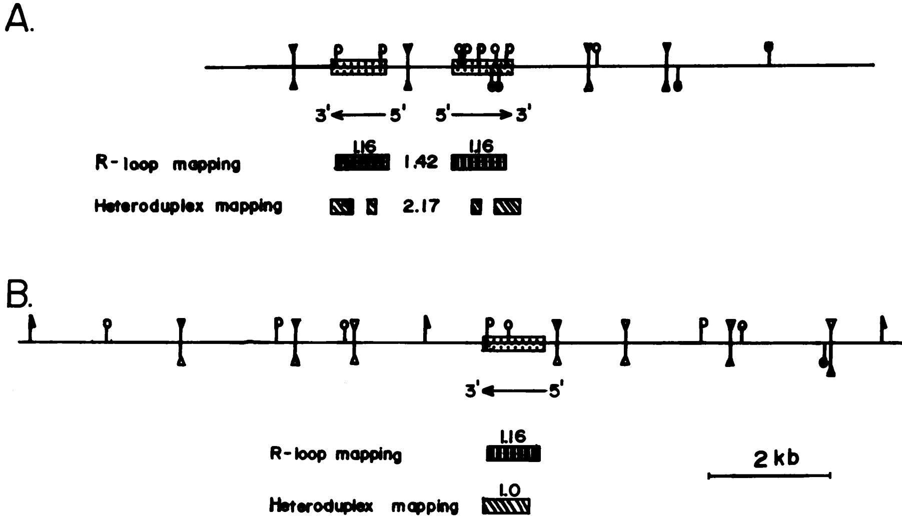

Figure 10: Summary of the structure of the vitellogenin genes. Results from heteroduplex mapping and R-loop mapping are shown in context with restriction maps of the genomic regions and the structural regions of the genes. (A) Genomic region of genes for Yp1 and Yp2, (B) Genomic region of the gene for Yp3. - EcoRI; - HindIII; -Pst I; - Sst I; - Bam HI. Stippled boxes represent coding regions.

and the gene for Yp3 does indicate at least partial sequence homology, even though this is not indicated by analysis of the gene products[38]. Considering the rapid evolution of the insect vitellins, it is reasonable to suppose that the 3 vitellogenin genes originated from one ancestral gene by gene duplications. The physical separation of Yp1 and 2 from YP3 could be accounted for by the presence of repeated sequences in the genomic region of the gene for Yp3 which would facilitate both transposition and unequal crossing over.

In order to compare the higher dipteran vitellogenin genes with those of other insects, it will be necessary to have probes from a variety of insects. Libraries of genomic DNA from Locusta migratoria and Aedes aegyptii have been constructed and parallel studies on these libraries are underway. Since, in many insects, there is more than one primary vitellogenin transcript and therefore more than one gene, the cloned DNA can be used to directly compare different genes within an organism, as well as to make interspecific comparisons. The cloned sequences can also

be used for in vitro transcription experiments to analyze and compare the different modes of regulation found in the different insects.

ACKNOWLEDGEMENTS

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