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F1 and *F2*: Two similar genes regulated differently during development of *Drosophila melanogaster*

(gene isolation/gene organization/developmental expression)

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ABSTRACT Screening a cDNA library of 2- to 3-day-old flies with poly(A)$^+$ RNA from male and female flies, we were able to isolate a small number of clones hybridizing preferentially with RNA from female flies. Four cDNA clones, derived from a single mRNA species, proved to be highly abundant in female flies. When we screened a genomic library with the longest cDNA, we obtained two genomic clones, *F1* and *F2*; *F1* was a direct copy of the cDNA and *F2* was obtained by cross-hybridization. A detailed analysis of these genomic clones revealed two independent genes coding for proteins of 50 kDa that are >90% homologous. RNA analysis with gene-specific probes from the 3′ untranslated region showed an expression of *F1* in all stages of development with a 5- to 10-fold overexpression of this RNA in female flies compared with males. In contrast to *F1*, *F2* is mainly expressed in late pupae and is expressed only at low levels in adult flies.

Genes involved in the production of eggs or sperm are expected to be expressed in a female- or male-specific manner, and they represent a class of genes that are regulated specifically at certain stages of development. A group of hormone-inducible female-specific genes (*YP1–YP3*) encode vitellogenin, the major egg yolk protein (1, 2). Yolk proteins have been isolated by virtue of their hybridization with poly(A)$^+$ RNA isolated from female flies but not with RNA from males. In the original screen prepared in this laboratory for the isolation of vitellogenin genes (2), we have obtained several cDNA clones sharing identical sequences and thus representing independent isolates of the same and probably rather abundant message. Using the largest cDNA clone as a probe, we obtained several independent isolates of genomic clones from a phage λ library of *Drosophila* DNA, which by restriction nuclease mapping were found to belong to two different genes, *F1* and *F2*, mapping at 48D and 100E and coding for putative proteins of similar amino acid sequences. In this paper, we summarize the sequence information on these genes and their putative proteins and present data on the expression of *F1* and *F2* during development.

MATERIALS AND METHODS

General Methods. Isolation of DNA and RNA, restriction analysis, S1 nuclease mapping, sequencing reactions, blotting, and hybridization procedures were done following the standard protocol given by Maniatis *et al.* (3).

Electron Microscopy. DNA heteroduplexes were formed according to the procedure given by Davis *et al.* (4) with slight modifications (5).

***In Situ* Hybridization.** Chromosomes were dissected and prepared for hybridization according to the method of Spradling *et al.* (6). Additional acetylation of the squashed chromosome preparations was performed to reduce background labeling (7). [^{3}H]DNA with a specific activity of 1×10^7 cpm/μg was prepared by nick-translation according to Rigby *et al.* (8) using all four [^{3}H]deoxynucleoside triphosphates; 1×10^5 cpm was used per slide in a hybridization mixture containing 0.01 M Pipes (pH 6.8), 3× NaCl/Cit (1× Nacl/Cit = 0.15 M NaCl/0.015 M Na citrate), 1× Denhardt's solution (9), 50% formamide, and 2 μg of sonicated carrier DNA. The hybridization was performed for 16 hr at 38°C.

Dot Blots. Total RNA was pelleted by centrifugation through 5.7 M CsCl in 100 mM EDTA for 16 hr at 40,000 rpm in an SW 60 rotor. The RNA pellet was dissolved in water and precipitated. Dot blots were done according to White and Bancroft (10).

RESULTS

In a screen for yolk protein genes, we obtained several cDNA clones, which, although unrelated to yolk protein genes, still hybridize preferentially to RNA from female flies. Four of these clones shared common sequences, indicating that the corresponding poly(A)$^+$ RNA is quite abundant.

The largest cDNA clone of this group was chosen to select genomic clones from two libraries of *Drosophila melanogaster* DNA (11, 12). Restriction endonuclease mapping of a number of such genomic clones indicated that these fell into two groups of overlapping sequences. The clones of the two homology groups were named F1 and F2. The entire set of overlapping F1 and F2 clones obtained from genomic libraries of either Canton S or Oregon R flies is shown in Fig. 1 together with the positions of the two largest of the cDNA clones, cDm 49 and cDm 28. All four cDNA clones isolated appear to be derived from F1, as they all possess restriction sites at the same positions relative to the oligo(dT) priming end of the cDNA, which are also represented in the genomic *F1* DNA. They all cross-hybridize with each other under stringent hybridization conditions and cross-hybridize with the genomic *F2* clones (data not shown), sharing variable portions of a region of homology, probably the coding region. Detailed restriction mapping and cross-hybridization of cDm 49 to various fragments in the region of the 4.0-kilobase (kb) *Eco*RI fragment in λDm 2 (indicated as pDm 2 below the F2 restriction map) revealed that the region of homology must be ≈2 kb (data not shown). As shown below, *F1* and *F2* messages only possess a coding capacity for 463 and 462 amino acids, respectively, suggesting that either the 3′ untranslated regions must be very similar or *F2* must have introns that were not detectable with our hybridization experiments.

To determine the length of homology and to establish the relative orientation of the two coding regions, heteroduplex

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Abbreviations: kb, kilobase(s); bp, base pair(s).

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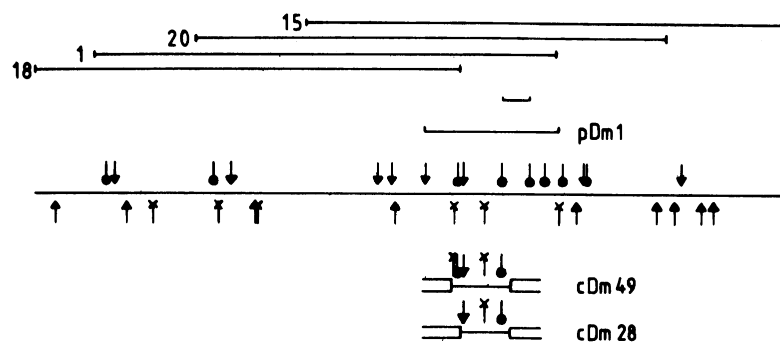

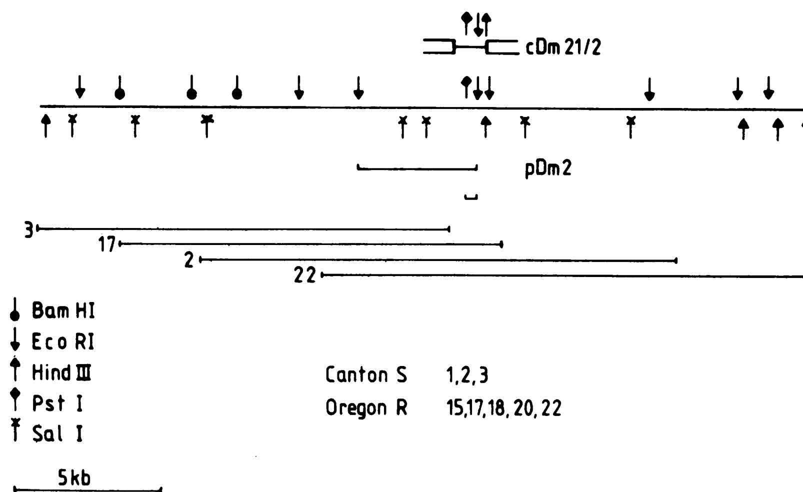

FIG. 1. Chromosomal organization of *F1* and *F2* gene loci. λ phages containing overlapping DNA fragments from both gene loci were isolated either from a library of Canton S DNA (11) or Oregon R DNA (12) and are indicated as horizontal lines on top and at the bottom. The origin of the corresponding DNA is explained below the F2 map. Restriction sites are shown for *Bam*HI (↓), *Eco*RI (↓), *Hin*dIII (↑), and *Sal* I (↑). The only *Pst* I site (↑) shown was used for the isolation of a 3′ specific probe for the *F2* gene. The longest two cDNA clones for the *F1* gene, cDm-49 and cDm-28, and a cDNA clone for the *F2* gene, cDm-21/2, are indicated between the restriction maps of genomic DNA. Recloned genomic DNA fragments containing the *F1* and *F2* genes are shown above the restriction map for the *F1* gene locus and below the restriction map for the *F2* gene locus, respectively. Restriction fragments used as gene-specific probes for *F1* and *F2* are represented by horizontal bars above and below pDm-1 and pDm-2, respectively.

analysis between λDm-1 and λDm-2 λ clones was performed (Fig. 2). A homologous region of 1.5 kb is interrupted by a loop of 0.5 kb, later identified as an intron. First the 5′–3′ orientation in λDm-1 was established by nuclease S1 protection experiments (Fig. 3) with the 0.95-kb *Bam*HI fragment from λDm-1, later used as a gene-specific probe for *F1* (see Fig. 1). The protected fragment of ≈305 base pairs (bp) fits well to the expected 3′ end for F1 deduced from the cDm-49 sequence (data not shown). By measuring the distances from the heteroduplexes to the borders of the genomic *Drosophila* DNA within the phage λDm-2, it was also possible to determine the 5′–3′ orientation in the map of the genomic *Drosophila* DNA of *F2*.

To establish the chromosomal location of *F1* and *F2*, *in situ* hybridizations were carried out with λDm-15 and λDm-17 DNA (Fig. 4). λDm-15, a λ clone containing the *F1* gene region, maps at 48D on the right arm of the second chromosome; λDm-17, a λ clone of the *F2* gene region maps at 100E on chromosome 3R on the cytogenetic map of *D. melanogaster*.

To identify specific probes from the 3′ nonhomologous part of the recloned gene regions, we selected the 0.95-kb *Bam*HI fragment in pDm-1 and the 0.34-kb *Eco*RI/*Pst* I fragment in pDm-2 (indicated as bars in Fig. 1). These probes were used to determine the copy number by genomic Southern analysis. It was found that both regions consist of single-copy sequences in the *D. melanogaster* genome (data not shown).

The 3′ specific probes were also used to assay for the presence of F1- and F2-specific mRNAs in embryos, larvae, pupae, and flies. RNA was extracted at different times of development and assayed by dot blot and RNA blot analysis for relative abundance of F1- and F2-specific sequences. As can be seen in Fig. 5*a*, the F1-specific probe hybridizes most strongly with RNA from embryos, somewhat less but nevertheless significantly with RNA from larvae, pupae, and flies, whereas the F2-specific probe produced a hybridization signal only with RNA from the pupal stages but not from embryos or larvae (Fig. 5*b*). Traces of F2 RNA are also found in adult flies. The complete absence of 3′ termini of F2 RNA in embryonic and larval tissues suggests that only the *F1* gene is expressed during these stages of development. On the basis of the original cDNA screen, the *F1* gene appears to be expressed preferentially but not exclusively in female flies. It was of interest to see whether the F2 RNA is expressed likewise in a sex-specific manner and to examine the length of both mRNAs. As shown in Fig. 5*d*, the F2-specific probe reacts with an RNA of ≈2.5 kb; the F2-nonspecific probe (Fig. 5*c*) hybridizes, in addition, to an RNA band of 2.0 kb, the *F1* gene product. In contrast to the *F1* message, *F2* appears to be expressed at least equally well in males as in females. Thus, the two genes appear to be regulated differently during development. When we compared the relative quantities of *F1* and *F2* messages on the same filter using the nonspecific probe of F2, we observed at least 50 times more *F1* than *F2* message in female flies.

Regions homologous to the largest cDNA clone as well as the sequences immediately flanking these regions in *F1* and *F2* were sequenced by the Maxam and Gilbert technique and

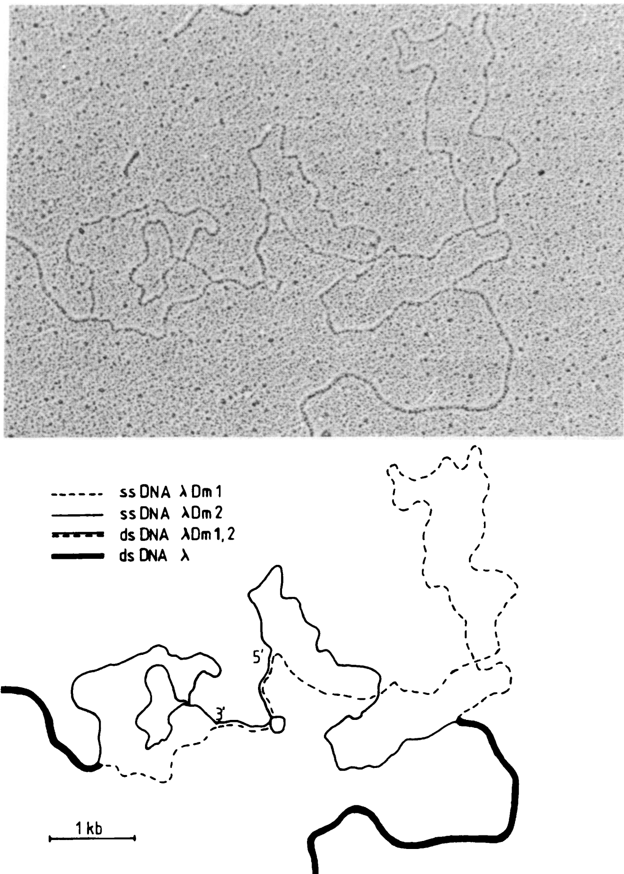

FIG. 2. Heteroduplex formation between λ phages λDm-1 and λDm-2. Heteroduplexes were formed between the λ clones λDm-1 and λDm-2, which contain DNA from the two different chromosomal loci. Single-stranded (ss) regions are shown by thin lines, dashed line represents λDm-1 DNA, and continuous line represents λDm-2 DNA. Thick lines display double-stranded (ds) DNA regions of the λ phage vector arms, the homologous region between λDm-1 and λDm-2 is indicated by a dashed and a continuous line. Length measurements (n = 30) were done with pBR322 and phage fd DNA molecules as internal markers for double- and single-stranded DNA, respectively. Bar represents the length of 1 kb of double-stranded DNA deduced from the size of the internal marker molecules (data not shown).

the sequence of *F1* is shown in Fig. 6. *F1* specifies an RNA ≈2000 nucleotides long, with an untranslated 5′ end of 24 nucleotides, an open reading frame coding for 463 amino acids followed by an untranslated 3′ end of some 600

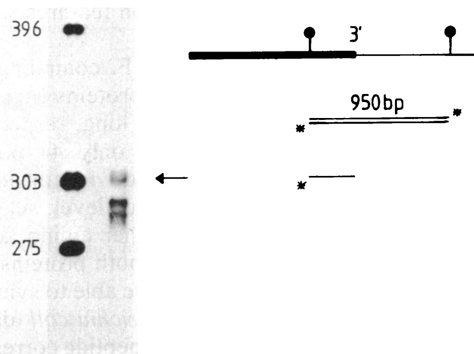

FIG. 3. Determination of the 3′ end of the *F1* gene transcript. The 0.95-kb *Bam*HI fragment from phage λDm-1, indicated in Fig. 1 as *F1*-specific probe, is shown below a schematic map of part of the *F1* gene region. Asterisks represent labeled 3′ ends. To the left, the S1 nuclease-protected fragment, separated on a 6% denaturing acrylamide gel, is indicated next to molecular weight markers ($M_r \times 10^{-3}$.)

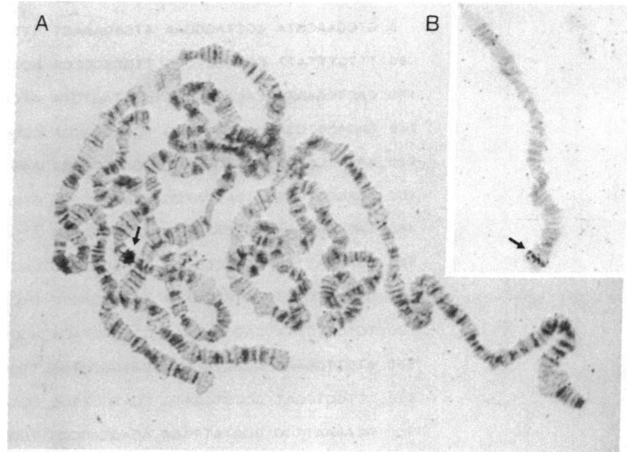

FIG. 4. *In situ* hybridization of λDm-15 and λDm-17 DNA to chromosomal DNA. [^{3}H]DNA (specific activity, 1×10^7 dpm/μg) of λDm-15 and λDm-17 was hybridized *in situ* to squashed preparations of polytene chromosomes from Oregon R third instar larvae. Exposure time, 14 days. (*A*) Hybridization probe is λDm-15 DNA. (*B*) Hybridization probe is λDm-17 DNA.

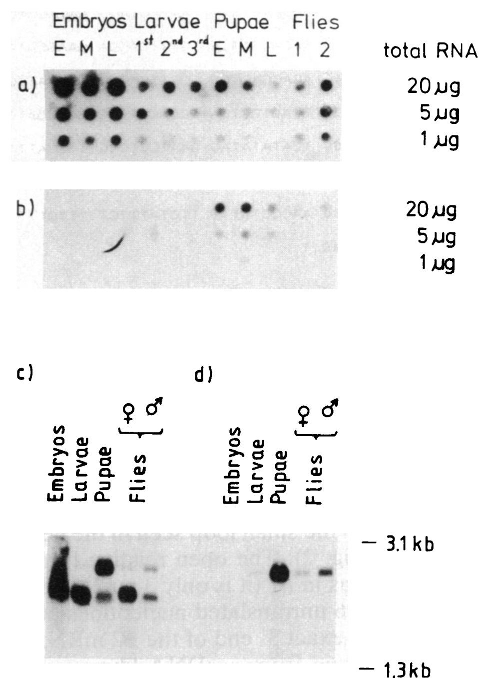

FIG. 5. RNA blot analysis of *F1* and *F2* gene transcripts. (*a* and *b*) Different amounts of total RNA were spotted on nitrocellulose filters and hybridized with specific probes from the 3′ untranslated regions of *F1* (*a*) and *F2* (*b*). RNAs, isolated at different times of development, are spotted according to age, from left to right: Embryos: E, 0–4; hr, M, 10–12 hr; L, 20–22 hr. Larvae: 1st, 2nd, and 3rd instar larvae. Pupae: E, 1- to 2-day-old pupae; M, 3- to 4-day-old pupae; L, 5-day-old pupae. Flies: 1, 1-day-old flies; 2, 2-day-old flies. (*c* and *d*) Five micrograms of poly(A)$^+$ RNA from embryos, larvae, pupae, and 2- to 3-day-old female (♀) and male (♂) flies (Oregon R), respectively, was transferred to nitrocellulose after electrophoresis on a formaldehyde/agarose gel (13, 14). Two identical filters containing the same RNA preparations were hybridized to different nick-translated probes. (*c*) Hybridization with the 4.0-kb *Eco*RI fragment of pDm-2 (nonspecific probe). (*d*) Hybridization with the 3′ specific probe of *F2*. 5′ end-labeled restriction fragments were used as standard for size determination of the RNAs. Specific activities of the probes used were 1–2 × 10^8 dpm/μg.

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               10         20         30         40         50         60         70         80
   0 GTCGAACGTA CGCTAGGGAA ATGAGAAAGT GTTATACCCA CTAATAATTG TAGTTGTAAT CCCACCGAAT TGTTTTACCC
  80 TTTGTTTATT CCAACCTCTC TTGCTCGCCA ACCCGCCGAA CCCTGCAACC TTCCAATGTT CCAACGTTCC GTTAATCCAA
                                                *
 160 CACTCGAATA CACACAACAG CCATAGTGTA ATCATCCAAC ATGGGCAAGG AAAAGATTCA CATTAACATT GTCGTGATCG
 240 GACACGTCGA TTCCGGTAAG TCGACCACCA CCGGACACTT GATCTACAAG TGCGGTGGTA TCGACAAGCG TACCATCGAG
 320 AAGTTCGAGA AGGAGGCCCA GGAGATGGGA AAGGGATCCT TCAAGTACGC CTGGGTTTTG GATAAGTTGA AGGCTGAGCG
 400 CGAGCGTGGT ATCACCATCG ATATCGCCCT GTGGAAGTTC GAAACTGCCA AGTACTACGT GACCATCATT GATGCCCCCG
 480 GACACAGGGA TTTCATCAAG AACATGATCA CTGGTACCTC GCAGGCCGAT TGCGCCGTGC AGATTGACGC CGCCGGAACC
 560 GGAGAATTCG AGGCCGGTAT CTCGAAGAAC GACCAGACCC GCGAGCACGC CCTGCTCGCC TTCACCCTGG GTGTGAAGCA
 640 GCTGATCGTT GGTGTGAACA AGATGGACTC CTCCGAGCCA CCATACAGCG AGGCCCGTTA TGAGGAAATC AAGAAGGAAG
 720 TGTCCTCTTA CATCAAGAAG GTCGGCTACA ACCCAGCCGC CGTTGCCTTC GTGCCCATTT CCGGATGGCA CGGCGACAAC
 800 ATGTTGGAAC CCTCTACCAA CATGCCCTGG TTCAAGGGAT GGGAAGTGGG ACGCAAGGAG GGTAACGCTG ACGGCAAGAC
 880 CCTGGTCGAT GCCCTCGATG CCATCCTTCC CCCAGCCCGT CCCACCGACA AGGCCCTGCG TCTGCCCCTG CAGGATGTGT
                                                                      ↓
 960 ACAAAATTGG CGGTATTGGA ACAGTACCCG TGGGTCGTGT GGAGACTGGT GTGCTGAAGC CCGGTACCGT TGTGGTCTTC
1040 GCCCCTGCTA ACATCACCAC TGAGGTCAAG TCCGTGGAGA TGCACCACGA GGCCCTGCAG GAGGCCGTTC CCGGAGACAA
1120 CGTTGGCTTC AACGTCAAGA ACGTGTCCGT GAAGGAGCTG CGTCGTGGCT ACGTTGCCGG TGACTCCAAG GCTAACCCCC
                                ↓
1200 CCAAGGGAGC CGCCGACTTC ACCGCCCAGG TCATCGTGCT GAACCACCCC GGTCAGATTG CCAACGGCTA CACCCCAGTG
1280 TTGGATTGCC ACACCGCTCA CATTGCTTGC AAGTTCGCTG AGATCTTGGA GAAGGTCGAC CGTCGTTCCG GCAAGACCAC
1360 CGAGGAGAAC CCCAAGTTCA TCAAGTCTGG CGATGCTGCC ATCGTCAACC TGGTGCCCTC TAAGCCCCTG TGCGTGGAGG
1440 CCTTCCAGGA GTTCCCCCCT CTGGGTCGCT TCGCTGTGCG TGACATGAGG CAGACCGTGG CTGTCGGTGT CATTAAGGCT
                                                                                 ▼
1520 GTCAACTTCA AGGATGCCTC CGGTGGCAAG GTCACCAAGG CCGCCGAGAA GGCCACCAAG GGCAAGAAGT AGCTGGTTTG
1600 CTTCCACTCA ACAACAACAA CAACACGCAG TAGTAGCAGC AACAACAAGC ATATAACCAA CATCATAATG CAGCCAACAA
1680 CACCACTCAA TAATACCAGC AACAGCAGCA GCGAACACAA TAGTAGTATA ACACCAACAC CTGTCCTGCG CAAGATGACC
1760 GATAAGATGA TGTTTCAGCA GAAGCATAAG TTTAATTTCT TCCATCGAAA GGAGTTTCGA CGGATACGAA TGCTAAATGC
1840 AGACGAGGCC GCCTTCACTG GGAAATCGGT GGATCCCAAG GATAAGAGTG CACACTGGGA AAACACTTGC ATTTATGCAT
1920 CCACTCCTCA TCCACTTCCC CGTCGATCTT TAGTTTACTA AATATGGTAT GATGCACGCA GTTGACTTCG TTTTATCATA
2000 TCATATATAG GAATCCTCTG TAGCATTTAT GATATCGTTT AAATTAACCT TTATACTTTG ATATGTATCA TTTATCTTAC
2080 CCTACTTTTG CACACACTAC TTTGTACACA AGAAAAGAAC CAGAATAGAA GCGATAAACT ATATTTACAA AAAAAATAAA
                                                                                    ------
2160 AACCCTATTT TTGTATTTCT TTTGTTTTTA CCACCCAGCC CGTAAAAGAG CACTCTCTTT TTGGTTGTTG CCTCCCGATT
                 ↑
2240 T
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FIG. 6. Sequence of the *F1* region. * denotes the translation initiation codon, ▼ denotes the translation termination codon, and ↑ denotes the polyadenylylation site. Positions of the intervening sequences in the homologous *F2* gene are indicated (↓). The polyadenylylation signal (AATAAA) is underlined.

nucleotides (15). There is no intron in *F1*. Sequence analysis of the 5′ end of cDM-49 and comparison with the genomic sequence revealed that this cDNA clone is almost a full-length copy of the F1 mRNA, missing only 10 bp at the 5′ end. *F2* is interrupted by two introns of 456 and 78 bp, the larger one corresponding to the small loop seen in the heteroduplex photomicrograph (Fig. 2). The open reading frame in *F2* is about the same size as in *F1* (it is only 3 nucleotides shorter) with a sequence of 36 untranslated nucleotides at the 5′ end (15). To look for the exact 3′ end of the F2 mRNA, we have isolated a cDNA clone from a cDNA library of 1-day-old pupae (kindly provided by M. Goldschmidt-Clermont). This cDNA clone (Fig. 1) maps at the 3′ end of the F2 mRNA near the *Hin*dIII site within the 0.4-kb *Eco*RI fragment neighboring the 4.0-kb *Eco*RI fragment of λDm-2 (Fig. 1). Thus, the 3′ untranslated region of the F2 mRNA must be ≈1.05 kb long.

Comparison of the *F1* and *F2* sequences (15) shows that the coding sequence itself has an average homology of ≈80%, whereas the 5′ control regions as well as the 3′ untranslated messenger sequences are completely nonhomologous—i.e., not exceeding a percentage of homology that is expected for random sequences. To search for sequence homologies between different parts of the untranslated regions, we have used a computer program blotting the two sequences against each other. With this blot, we could identify a region ≈100 bp long located 20 bp behind the stop codon in *F1*, which is to some extent >50% homologous to a region in *F2* located 40 bp behind the stop codon. This is in good agreement with the heteroduplex data where ≈650 bp after the intron in λDm-2 are homologous but only 550 bp belong to the translated region. The fact that this short homologous region has different distances from the stop codons in *F1* and *F2* cannot be explained by the existence of an additional intron in *F2*, because restriction fragments that derive from this region in *F2* are of identical length in both the genomic and the cDNA clone. These homologous sequences in the 3′ untranslated regions of F1 and F2 have some common features with the *opa* sequence family (16).

Fig. 7 shows the organization of F1 and F2 compiling all the sequence information. The hypothetical proteins encoded by *F1* and *F2* are 463 and 462 amino acids long, respectively. Their amino acid sequences differ in only 44 positions corresponding to 90% homology, which is considerably higher than the homology at the nucleotide level, suggesting a strong selection pressure against further amino acid exchanges—i.e., a biological function for both proteins.

By using an expression vector, we were able to synthesize a fusion protein of the *F1* gene in *Escherichia coli* (data not shown). As the size of the observed polypeptide corresponds rather well with the size of the hypothetical hybrid protein, we conclude that (*i*) the DNA sequence, as determined, is correct and (*ii*) the protein encoded by the *F1* gene can, at least in bacteria, assume a configuration that confers stability and solubility in a cellular environment.

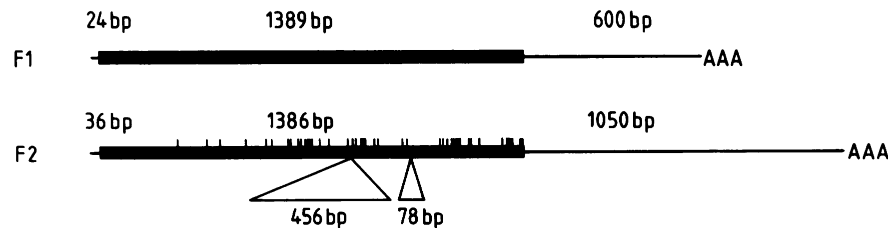

FIG. 7. Organization of *F1* and *F2* genes. Solid bars indicate the coding regions and thin bars represent the untranslated regions at the 5′ or 3′ end. Introns in the *F2* gene are indicated with their lengths given in base pairs at their positions within the coding region. Vertical bars in the *F2* coding region represent amino acid exchanges in *F2* compared with *F1*.

DISCUSSION

In the original screen for cDNA clones, we have obtained four independent isolates homologous to most parts of the *F1* gene. Hybridizing the largest of the cDNA clones to genomic λ DNA clones, we isolated several λ phages containing *Drosophila* DNA of two types of sequences, *F1* and *F2*. The two putative genes are unlinked but probably encode two proteins almost identical in size and very similar in sequence. In contrast to *F1*, the *F2* gene does not show preferential expression in females and it is maximally expressed only during pupal stages of development. The difference in sex specificity and in the time of expression between *F1* and *F2* suggests that the two genes might be expressed in different tissues. We can test this by hybridizing *F1*- and *F2*-specific probes *in situ* to RNA in tissue sections (17).

A sequence comparison of the *F1* and *F2* genes and their flanking regions yields interesting information on the evolutionary divergence of the two genes. The fact that the two proteins are homologous suggests a common or related function. The homology at the nucleotide level, as expected, is much less. Judging from third base exchange frequencies (>50%), it appears that there exists a strong selection pressure against further amino acid exchanges. Also, no selection pressure at all apparently existed on the 3′ untranslated sequence, which is not only nearly devoid of homologies but also of different length. The fact that the homology is strictly confined to the coding region of the two genes led us first to the interpretation that *F1* originated from *F2* (loss of introns) via reverse transcription and that the 3′ untranslated sequences arose after duplication by mutations of transcriptional stop or processing signals. The extensive divergence of third base positions of the amino acid codons raises the possibility that even some flanking sequences were duplicated before extensive base-pair exchanges took place during evolution of *Drosophila*. In any case, it is clear that the duplication must have occurred very early during evolution.

So far we have not shown that the F1 and F2 RNAs are translated into functional proteins in *Drosophila*. The finding of a fusion protein of the expected size in *E. coli* is suggestive but no proof. We therefore need to raise antibodies to F1- and F2-specific peptides in order to identify the products of both genes individually in *Drosophila* extracts or tissue sections. The obviously strong selection pressure operating on the two genes suggests that the DNA sequence encodes proteins of an important function.

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