Evidence for Redundancy But Not *trans* Factor–*cis* Element Coevolution in the Regulation of Drosophila *Yp* Genes

Fabio Piano,*,[1,2] Michael J. Parisi,*,[1,3] Roger Karess†,[4] and Michael P. Kambysellis*

**Department of Biology, New York University, New York, New York 10003 and †Department of Biochemistry, New York University Medical Center, New York, New York 10016*

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ABSTRACT

In *Drosophila melanogaster* and the endemic Hawaiian species *D. grimshawi* three *Yolk protein* (*Yp*) genes are expressed in a similar sex- and tissue-specific pattern. In contrast, DNA sequence comparisons of promoter/enhancer regions show low levels of similarity. We tested the functional significance of these observations by transforming *D. melanogaster* with the genomic region that includes the divergently transcribed *D. grimshawi DgYp1* and *DgYp2* genes; we found that the introduced genes were expressed in female fat body and in ovaries but not in males. Moreover, we found *D. grimshawi* proteins in the hemolymph and accumulating in ovaries. Using reporter constructs we showed that the intergenic region from *D. grimshawi* was sufficient to drive accurate expression, but some low level of ectopic expression was seen in males. Transforming *D. melanogaster* with constructs bearing deletions within the *D. grimshawi* intergenic region revealed only subtle effects in the overall level of expression, suggesting a high level of redundancy. Testing mutants in the sex-specific regulator *doublesex* revealed that it is capable of repressing the *DgYp* genes in males. Together, these data show that *D. melanogaster trans*-acting factors can regulate the *in vivo* pattern of *DgYp* expression and support the notion of a redundant and complex system of *cis*-acting elements.

OOGENESIS in Hawaiian Drosophila provides an exceptional model system to address questions regarding evolution and development by combining the extensive ecological and phylogenetic data on this speciose group with knowledge of developmental programs in the well-studied species *Drosophila melanogaster*. The Hawaiian Drosophila have undergone an explosive adaptive radiation; more than a quarter of the world's known Drosophila species (over 700 species described to date) reside in this geographically isolated archipelago (Carson and Yoon 1982). Most of these species are monophagous, ovipositing in species-specific substrates (Heed 1971). Adaptations to these substrates have been pivotal to the success of the group (Kambysellis *et al.* 1995; Kambysellis and Craddock 1997) and can be readily observed in the diversity of ovary organization (Kambysellis and Heed 1971), chorion morphology (Kambysellis 1993), and yolk protein production (Craddock and Kambysellis 1990). The ability of a species to produce a given number of eggs and the amount of yolk protein per egg are thought to be related to the capacity of the oviposition substrate to sustain hatching larvae (Kambysellis and Heed 1971; Craddock and Kambysellis 1990). Because considerable effort and energy are involved in producing eggs and in producing yolk proteins in particular, the regulation of these processes is presumed to be under strong selection.

During the latter stages of oogenesis, the strictly regulated *Yolk protein* (*Yp*) genes are responsible for producing the most abundant protein products of the mature oocyte. *Yp* genes and their products have been studied extensively in *D. melanogaster* and, to a lesser extent, in the endemic Hawaiian species *D. grimshawi* (reviewed in Wyatt 1991). The genomic organization of these genes is similar in the two species. Both have three X-linked *Yp* genes: two are closely linked and divergently transcribed (*Yp1* and *Yp2*), while the third (*Yp3*) is ~1 Mb away. (To maintain consistency with the *D. melanogaster* literature, we refer to the *D. grimshawi* genes as *DgYp1*, *DgYp2*, and *DgYp3*, rather than their earlier designations *V1*, *V2*, and *V3*). In both species, these genes follow a strict tissue-, sex-, and temporal-specific expression pattern, being transcribed only in adult female fat body and ovarian follicle cells.

Cis-acting elements have been identified in *D. melanogaster* by *P*-element-mediated transformation of different reporter constructs as well as by means of *in vitro* systems (reviewed in Lasko 1994; An and Wensink

Corresponding author: Michael P. Kambysellis, Department of Biology, New York University, 1009 Main Bldg., New York, NY 10003. E-mail: mk2@is2.nyu.edu

[1] These authors contributed equally to this work.

[2] *Present address:* Section of Genetics and Development, Cornell University, Ithaca, NY 14853.

[3] *Present address:* Department of Cell Biology, Duke University Medical Center, Durham, NC 27710.

[4] *Present address:* Centre de Génétique Moleculaire, 91198 Gif-sur-Yvette, France.

1995b; Lossky and Wensink 1995). These experiments have identified sequence elements that are either within the *Yp1-Yp2* intergenic region or are part of the coding region. These elements are sufficient to drive specific expression of both genes in the female fat body [*e.g.*, fat body enhancer (FBE)] and ovary [*e.g.*, ovarian enhancer 1 and 2 (OE1 and OE2)]. More recently, several *trans*-acting proteins have been postulated to be involved in the regulation of *Yp* gene expression by binding to DNA elements (o and r) within the FBE. Of these, the interaction of the *doublesex* (*dsx*) gene product within the FBE has been characterized both genetically and biochemically (Burtis *et al.* 1991; Coschigano and Wensink 1993; An and Wensink 1995a,b).

The *dsx* gene functions as the last component of the genetic hierarchy that determines somatic sexual differentiation in *D. melanogaster*. In the two sexes, it is alternatively spliced to produce male- and female-specific proteins that differ in their carboxy terminus (reviewed in Burtis 1993). *In vitro* studies have shown that both male (DSX^M) and female (DSX^F) forms of the DSX protein, purified from a bacterial expression system, bind specifically to three regions within the FBE. *In vivo*, fat body expression patterns of FBE-reporter constructs have shown that DSX^M acts as a repressor and DSX^F as a coactivator (Burtis *et al.* 1991; Coschigano and Wensink 1993; An and Wensink 1995b).

Once produced, the yolk proteins (YPs) are extensively post-translationally modified. Glycosylation, phosphorylation, and sulfonation have been demonstrated and are postulated to play a role in protein trafficking (Brennan and Mahowald 1982; Minoo and Postlethwait 1985; Saunders and Bownes 1986; Friederich *et al.* 1988). Female-sterile *trans*-dominant temperature-sensitive mutations have been found to map to single point mutations which may disrupt the protein trafficking of all the YPs in the fat body (Butterworth *et al.* 1991). Interestingly, lower copy numbers of these genes do not lead to female sterility, but affect overall egg production (Bownes *et al.* 1991). The major function of the yolk proteins is thought to be in the nutrition of the developing embryo; however, they may play other roles. For example, they have been shown to bind ecdysone and release it upon proteinase treatment (Bownes *et al.* 1988), suggesting a potential function as hormone carriers with release of the hormone during embryogenesis.

Of evolutionary interest is the question of how *D. melanogaster* would regulate the gene expression and trafficking of YPs from a different species. Would the introduced genes be regulated similarly to the host genes, and would their protein products interfere with proper endogenous YP traffic or oogenesis? Studies addressing the evolution of *Yp* gene regulation and of their protein products were initiated by sequence comparisons of the regulatory and coding regions of the *Yp* genes from Hawaiian Drosophila and *D. melanogaster* (Ho 1994; Parisi 1995; Ho *et al.* 1996; M. P. Kambysellis, unpublished observations). In these studies, *D. planitibia*, *D. grimshawi*, and *D. melanogaster* DNA sequences showed a high degree of divergence in the intergenic regulatory sequence. No clear *D. melanogaster*-like enhancer elements could be identified by sequence comparison alone. Some short interspersed stretches of DNA were found to be identical in the interspecific comparisons, but their functional significance (if any) remains unclear. The sequence divergence in the regulatory region, despite similar expression patterns, suggests two possibilities: either the primary DNA sequence is under little constraint with respect to change while still retaining its ability to bind the same *trans*-acting factors, or alternatively, coevolution between the *trans*-acting and *cis*-acting elements has led to the sequence diversity observed. To choose between these competing hypotheses, we initiated functional studies. If *D. grimshawi* genes introduced into *D. melanogaster* are regulated in a tissue- and sex-specific manner, the former hypothesis is supported; alternatively, if the regulation is aberrant then the latter would be supported.

We show here that *D. melanogaster* is capable of controlling, in a sex-, tissue-, and temporal-specific manner, the expression of introduced *D. grimshawi Yp* genes. Once produced, the *D. grimshawi* YPs appear to be post-translationally modified in the same way as in *D. grimshawi*. These proteins are found in hemolymph and oocytes—evidence for correct protein trafficking. We also use this system to begin a dissection of the *cis*-regulatory elements in *D. grimshawi*. By using a dual reporter construct and deletion analysis, we present evidence of a highly redundant regulatory system. Finally, we show that the specific *trans*-acting factor DSX from *D. melanogaster* is genetically involved in regulating *D. grimshawi Yp* genes, despite the apparently low conservation of primary sequence in the putative FBE regions.

MATERIALS AND METHODS

Fly rearing, constructs, and injections: *D. grimshawi* (strain G1) was maintained on the artificial medium of Wheeler and Clayton (1965). All *D. melanogaster* lines were raised on standard glucose/yeast medium supplemented with phosphoric acid and propionic acid as mold inhibitors.

To transform *D. melanogaster* with *D. grimshawi* DNA, the complete *DgYp1-DgYp2* locus and a series of constructs based on a dual reporter gene transformation vector were derived from the pCasPer vector. Standard molecular techniques (Sambrook *et al.* 1989) were used throughout the following cloning experiments except where specifically indicated. The *DgYp1-DgYp2* genes were subcloned from the genomic library clone λgV1V2 (Hatzopoulos and Kambysellis 1987b) by partial digestion with *Sal*I into p-Bluescript (Stratagene, La Jolla, CA) to aid in subsequent steps. The insert was further cut with *Apa*LI and *Nhe*I, modified with *Not*I linkers and subcloned into the *Xho*I site of pCasPer, itself modified with *Not*I linkers to create pG1Yp1Yp2 (Figure 1A). This 6.5-kb genomic fragment contains 570 bp beyond the poly(A) signal of *DgYp1* and 850 bp beyond the poly(A) signal of *DgYp2*.

To assay both the *DgYp1* and *DgYp2* promoters simultaneously, a construct was built that had two promoterless reporter genes, one coding for β-galactosidase (β-gal) and the other coding for chloramphenicol acetyltransferase (CAT), subcloned in opposite orientation (Figure 1B). The *Xba*I-*Bam*HI fragment from pCATbasic vector (Promega, Madison, WI) containing the promoterless *CAT* gene was blunt ended with Klenow fragment and ligated into the blunted *Eco*RI site of pCasPer AUGβ-gal (Thummel *et al.* 1988). The orientation of *CAT* was checked to be divergent from *lacZ*. This plasmid, pCasPer AUGβ-galCAT (abbreviated pCGC in Figure 1B), was used to test the intergenic region of *D. grimshawi*.

The reporter constructs used for transforming *D. melanogaster* were made using the *Bam*HI site in pCGC. The *Eag*I and *Xho*I fragment, containing the *D. grimshawi* intergenic region (from +40 to +10 relative to the transcription initiation sites of *DgYp1* and *DgYp2*, respectively), was subcloned into pCGC via blunt ends. Deletion constructs were made by digesting the plasmid that contains the complete *D. grimshawi* intergenic region (p8BFnuAE) with the restriction enzymes indicated as part of Figure 5 and inserting these deleted constructs in pCGC as above.

P-element-mediated transformation was carried out as previously described (Spradling and Rubin 1982). For the pG1Yp1Yp2 vector the Drosophila strain used for injections was yw^{67C} using pTURBO (Karess and Rubin 1984) as helper plasmid; four independent insertion lines were obtained (as assayed by standard Southern analysis). All four lines showed the same results as those presented here. All the other constructs were injected into *w;Sb e* Δ2-3/TM6 *Ubx* (Robertson *et al.* 1988) without helper plasmid. The number of lines obtained for each construct is shown as part of Figure 5. All quantitative analyses were done in heterozygotes for the introduced transposons.

Protein and RNA analysis: To extract yolk proteins, ovaries were dissected from mature females conditioned on yeast paste and homogenized as previously described (Kambysellis *et al.* 1989). Ovary and fat body cultures of conditioned flies were also performed as described elsewhere (Kambysellis *et al.* 1986). Grace's tissue culture medium (GIBCO, Grand Island, NY) was removed after overnight organ culturing and analyzed for protein. The medium contains newly secreted proteins, which are mostly newly synthesized. Yolk proteins were detected by Coomassie staining and by Western blots (see below). Hemolymph was collected essentially as described previously (Kambysellis 1984). Modifications in the collecting apparatus were made to fit a microfuge. A sealed, cut Pasteur pipette containing Laemmli loading buffer (typically 15–20 μl; Laemmli 1970) and glass wool was placed inside a 0.5-ml Eppendorf tube that had a hole in the bottom. The Pasteur pipette and small Eppendorf were placed in a 1.5-ml Eppendorf tube serving as adapter. The flies (10–15) were anesthetized with CO_2 and their thoraxes punctured with a fine pin, prior to placing them in the Pasteur pipette; centrifugation was typically 8000 rpm for 30–45 sec. All operations were carried out at 4°.

Protein extracts were separated by SDS-PAGE using a Protean II xi cell (Bio-Rad, Richmond, CA) apparatus cooled to 11° essentially as described by Laemmli (1970). A polyacrylamide concentration of either 9% or a gradient from 7 to 12% was used. For the two-dimensional gel electrophoresis, the method of O'Farrell (1975) was used. In the second dimension (SDS-PAGE), tubes were run in pairs on two gels made from the same solution and run on the same apparatus at the same time. This ensured comparison of the migration of the spots in different samples.

Western analysis was done by transferring the proteins to a PVDF membrane (Immobilon-P; Millipore, Bedford, MA) and using standard procedures. Purified *D. grimshawi* anti-yolk protein IgG (Kambysellis *et al.* 1986) was used at 1/20,000 dilution. Detection of protein was performed according to the instructions of the ECL system (Amersham, Arlington Heights, IL).

RNA was extracted from *D. melanogaster* and *D. grimshawi* conditioned adults by standard means and analyzed by Northern blotting. Membranes were hybridized overnight with ^{32}P-labeled *D. grimshawi* probes made from the 3′ untranslated regions to avoid cross-reactivity with the *D. melanogaster Yp1* and *Yp2*. Two 45-min washes were performed at 42° in 50% formamide, 5× SSPE, 0.5% SDS, followed by 20-min washes at 65° in 1× SSPE, 0.5% SDS. Membranes were then exposed to Kodak X-ray film at −70° with intensifying screens. As a control for loading, membranes were hybridized with a *D. melanogaster rp49* probe (O'Connell and Rosbash 1984).

Histochemical and quantitative analysis of β-gal and CAT activity: Conditioned flies were assayed for β-gal activity as described elsewhere (Simon and Lis 1987). Usually, color reaction was observed within minutes and stopped within 1 hr. Stained samples were photographed on a Nikon dissecting microscope with either print film (Gold III; Kodak, Rochester, NY) or slide film (Ektachrome 160T; Kodak). Staining of individual ovarian follicles was done by dissecting ovaries and teasing apart the follicles. For quantification experiments, fly tissues (consisting of males or ovariectomized females or ovaries) were obtained from aged, conditioned flies. The extracts were either assayed immediately or stored at −70°. Duplicate assays showed that frozen material and fresh material gave consistent results. The total protein in the crude extracts was measured using the Bradford Reagent (Bio-Rad) and quantification was determined by reading the OD at 595 nm with a Microplate Autoreader (Bio Tek Instruments) using BSA as standard. The activity assay for CAT was performed using the FLASH-CAT nonradioactive kit (Stratagene) following the procedure suggested by the manufacturer. A 5-μl aliquot of each sample was spotted on a Baker-flex Silica (J. T. Baker, Phillipsburg, NJ) thin layer chromatography (TLC) plate ~1.5 cm from the bottom and spaced 1 cm from adjacent samples. Chromatography was performed by placing the plates in a chromatography tank preequilibrated for 2 hr with an 87:13 (v:v) mixture of chloroform:methanol. The solvent was allowed to reach 1 cm from the top of the plates at which time the TLC plates were removed and air dried, and the results were visualized by exposing the plates to a UV light source. After the results were photographed, the spots were cut from the TLC plate and wetted with methanol. The silica matrix was then removed and placed in 1.0 ml methanol. The samples were centrifuged for 5 min and the supernatant was assayed on a fluorometer. The activity of CAT was measured as the ratio between reacted and unreacted material that was always present to ensure linearity.

To measure β-gal activity, 5 μl of the crude extract preparation was added to 995 μl of a solution containing 100 mm phosphate buffer (pH 7.8) and the chromogenic substrate CPRG at a concentration of 1 mm in disposable spectrophotometric cuvettes. β-Gal activity was measured by spectrophotometric readings at 0.5-hr intervals for 3 hr. Data from these readings were adjusted to reflect the optical density at 574 nm (OD_{574})/mg extract (Simon and Lis 1987).

***dsx* genetics:** The genetic markers and chromosome nomenclature are described in Lindsley and Zimm (1992) unless explained here. The *dsx* alleles used here are the same alleles that have been shown previously to be useful in testing the sex-specific regulation of *Yp* genes in *D. melanogaster* (Coschigano and Wensink 1993; An and Wensink 1995b). The *dsx* lines were crossed to balancer lines to obtain *w*/*Bs*Y; *Cyo*/*Sp*; TM3/dsx^{d+r3} and *w*/*w*; *Cyo*/*Sp*; TM3/dsx^{m+r15}. Only lines bearing the

P element on the second chromosome were used. In the original lines transformed with pG1Yp1Yp2 only one insertion was found to be on the second chromosome (20A2); another line was obtained by jumping the *P* element and selecting for reinsertion in the second chromosome. This was done using the *w;Sb e* Δ2-3/TM6 *Ubx e* line (Robertson *et al.* 1988). The new line (MP85) was checked for integrity of the element and independent location by Southern analysis. Five other lines carrying the dual reporter gene construct including the complete intergenic region in either orientation with respect to the reporter genes (series B and C; Figure 1) were also tested. By crossing to balancer lines with inserted *P*-element constructs, the final sterile flies, which were assayed, had the following genotypes: w/B^SY; p[insert]/+; dsx^{m+r15}/dsx^{d+r3} and w/w; p[insert]/+; dsx^{m+r15}/dsx^{d+r3}. The presence of B^SY indicated the XY configuration. The XX genotype was recognized by the absence of B^SY. Other secondary sexual characteristics were also indicative of the sex chromosome configuration (data not shown).

RESULTS

DNA analysis and constructs: As in *D. melanogaster*, *D. grimshawi Yp* genes *DgYp1* and *DgYp2* are expressed in both the ovaries and fat body of females (Hatzopoulos and Kambysellis 1987a). However, sequencing the *DgYp1-DgYp2* locus showed no significant similarity in the intergenic region between the two species (Parisi 1995; GenBank accession no. AF126373). As seen in other comparisons of *cis*-regulatory sequences (*e.g.*, Ludwig *et al.* 1998), there is a high level of substitutions and indels in this region. This level of sequence diversity makes direct sequence alignments using the whole intergenic region difficult to interpret with a high degree of confidence because the alignments are sensitive to the parameters used and often yield equally parsimonious solutions. To gain more direct insight into the patterns of sequence divergence we also looked for specific DNA elements known to be functional and capable of binding defined transcription factors in *D. melanogaster*. These analyses did reveal potentially meaningful similarities, but the organization of the known *D. melanogaster* elements is different between these two species both in their relative position and in their orientation with respect to the promoters (Table 1). For example, the three DSX binding sites that contain a consensus core sequence can be found, albeit with some differences, in the *D. grimshawi* sequence, but the best putative DSX-C site in *D. grimshawi* is found closer to the *DgYp2* transcription start site and in a reversed orientation. The other putative binding sites follow a similar pattern. These features suggest three possibilities. First, colinearity of enhancers may not be required to maintain proper regulation of the *Yp* genes. Second, these sequence comparisons revealed nonorthologous elements, and other less similar sequences are organized as in *D. melanogaster*. Third, the *Yp* genes from the two species are being regulated by completely different proteins, making this comparison not functionally significant. Thus, any conclusion based on these sequence comparisons alone remains tenuous without functional tests.

To analyze the function of the *D. grimshawi* intergenic region in *D. melanogaster*, we made use of *P*-element-mediated transformation. We designed two different types of constructs. The first, shown in Figure 1A, is a *P* element containing a 6.7-kb genomic fragment from *D. grimshawi* that includes the *DgYp1* and *DgYp2* genes. The complete *DgYp1-DgYp2* locus inserted in this construct includes putative regulatory elements that may be outside the intergenic region. The second construct contains divergently transcribed reporter genes (Figure 1B). This dual reporter construct bearing genes coding for CAT and β-gal can find general use for the study of divergently transcribed genes. Junctions have been made between the transcription start sites of the *D. grimshawi* genes and the translation initiation sites of the bacterial genes (see materials and methods).

Products of introduced *Yp* genes are found in ovary extracts: Using germ-line transformation we obtained *D. melanogaster* lines carrying pG1Yp1Yp2 (see materials and methods). To assay for the presence of *D. grimshawi* YPs within *D. melanogaster*, we analyzed proteins from ovarian extracts. Using these extracts we could resolve four YP bands (Figure 2A, lane 3) by SDS-PAGE. These could be further resolved into five different major proteins by 2-D analysis (Figure 2B). The abundance, location, and morphology of the proteins and satellite spots in the 2-D gel suggest that within the *D. melanogaster* environment, *D. grimshawi Yp* genes are highly expressed and their protein products are post-translationally modified as in their endogenous tissue. Furthermore, because ovarian extracts predominantly contain yolk proteins stored within oocytes, this result strongly suggests that *D. grimshawi* YPs are taken up by the developing *D. melanogaster* oocyte.

Tissue- and sex-specific expression: Having found *D. grimshawi* YPs within *D. melanogaster* ovaries we addressed the question of their sex specificity and tissue of synthesis. In both species, the fat bodies and the follicle cells are capable of producing and secreting YPs within an organ culture system (Kambysellis *et al.* 1986). We used this system to ask if proteins from both species were being secreted in organ cultures from the transformed *D. melanogaster* lines. Western analysis of media from cultures of ovaries and female fat bodies showed the presence of YPs from both species. Moreover, no YPs were detected from culturing male fat bodies (Figure 3A), even though we overloaded the male lanes. Similar sex-specific results were observed when instead of organ cultures, we used hemolymph extracts (see Figure 7A, lanes 3 and 4). We then analyzed RNA from transformed adult females and males and found high levels of *D. grimshawi*-specific *DgYp* transcripts in females but not in males (Figure 3B).

To further examine the pattern of expression of genes driven by *D. grimshawi* DNA within *D. melanogaster* we

TABLE 1

Comparison of selected *D. melanogaster cis*-acting sequences to the *DgYp1-DgYp2* intergenic region of *D. grimshawi*

Element	Sequence and location[a]		
DSX-B			
D. melanogaster	−284 GAGCCTACAAAGTGATTACAAATTAAAATA	−255	
D. grimshawi	−198 GAtgCacCAAAGgGcaTgCAAcTatAtAat	−168	
DSX-C			
D. melanogaster	−240 GGTGCTGCTAAGTCATCA	−223	
D. grimshawi (*Yp2*)	−444 GaaGCTGCTAAcagtatt	−427	(opposite strand)
DSX-A			
D. melanogaster	−308 ACAACTACAATGTTGCAAT	−289	
D. grimshawi	−269 tacACTACAATGTaattga	−287	(opposite strand)
AEF-1-C/EBP			
D. melanogaster	−312 GTGCACAACTACAATGTTGCA	−291	
D. grimshawi	−265 GaattacACTACAATGTaatt	−285	(opposite strand)
D. planitibia	−268 GacttagACTACAATGTaatt	−288	(opposite strand)
BBF-2			
D. melanogaster	−234 GCTAAGTCATC	−224	
D. grimshawi (*Yp2*)	−438 GCTAAcagtat	−428	
D. grimshawi (*Yp2*)	−272 tgTAAtTCtat	−262	

[a] Locations are with respect to the *Yp1* transcription start site except where noted. DSX-A, DSX-B, and DSX-C are DNA elements that bind the DSX transcription factor (Burtis *et al.* 1991) and the consensus regions are shown with a line above them. Other transcription factor binding sequences are from the following: AEF-1-C/EBP (Falb and Maniatis 1992) and BBF-2 (Abel *et al.* 1992).

used flies transformed with the reporter constructs bearing the entire intergenic region in either orientation (B and C series, Figure 1B) and analyzed β-gal activity histochemically. We obtained eight lines that had the *DgYp1* promoter in front of the gene coding for β-gal and the *DgYp2* promoter in front of the gene coding for CAT (B series) and seven other lines that had the promoters reversed (C series). We also tested the plasmid without the intergenic region, and the five lines obtained (series A) showed no expression (Figure 4B).

β-Gal staining patterns in these lines revealed that female fat body and ovaries expressed this reporter gene when driven by either the *DgYp1* or the *DgYp2* promoter (Figure 4A). Furthermore, expression of β-gal in the B and C series was restricted to the somatic follicular epithelial cells surrounding egg chambers that were actively vitellogenic and to the amorphous fat body that lines the cuticle of most of the adult. This spatial and temporal pattern is indistinguishable from that which has been reported for endogenous *D. melanogaster Yp* genes (Lossky and Wensink 1995). These transformants generally displayed the staining within 1 hr (Figure 4A). However, when incubated for prolonged periods, we observed some male expression, which was more pronounced in flies transformed using one orientation (B series; see Figure 5). Potentially, this leakiness could be due to differences in the sex regulation in these two species or, more likely (see below), could arise as a result of the particular way in which the reporter construct was generated. Together, the functional analyses show that *D. melanogaster trans*-acting factors are capable of regulating the expression of *D. grimshawi DgYp1* and *DgYp2* genes, despite the apparent lack of strong sequence similarity between the two species.

Deletions in the intergenic region of *D. grimshawi*: To initiate a study searching for enhancers in the intergenic region of *D. grimshawi*, we made a series of deletions and quantified their effects on reporter gene expression in *D. melanogaster*. Using the dual reporter gene construct allowed monitoring of both the *DgYp1* and the *DgYp2* promoters in the same lines. Quantitative analysis of β-gal or CAT activity levels in transformed flies was performed on extracts prepared from ovaries, ovariectomized females (representing fat bodies), and males. In lines bearing the full-length intergenic region (Figure 5, B and C series), the levels of reporter activity were highest in the fat body when driven by either promoter. As mentioned above, low ectopic male expression was also seen (<10% of female fat body extracts). Activity levels from control flies (A series) were virtually nonexistent. Thus, reporter gene activity was not due to basal level transcription of the constructs alone.

Surprisingly, all of the deletions used to transform *D. melanogaster* showed varying but relatively high activities of β-gal and CAT in both fat body and ovaries, indicating a high level of redundancy in this system. Nine transformed lines (D series) were generated in which a 330-bp fragment adjacent to the *DgYp1* promoter was deleted. The proximity of the deleted sequence to the *DgYp1* promoter of *D. grimshawi* made this a possible target sequence for an element homologous to the *D. melanogaster* FBE. Based purely on position relative to

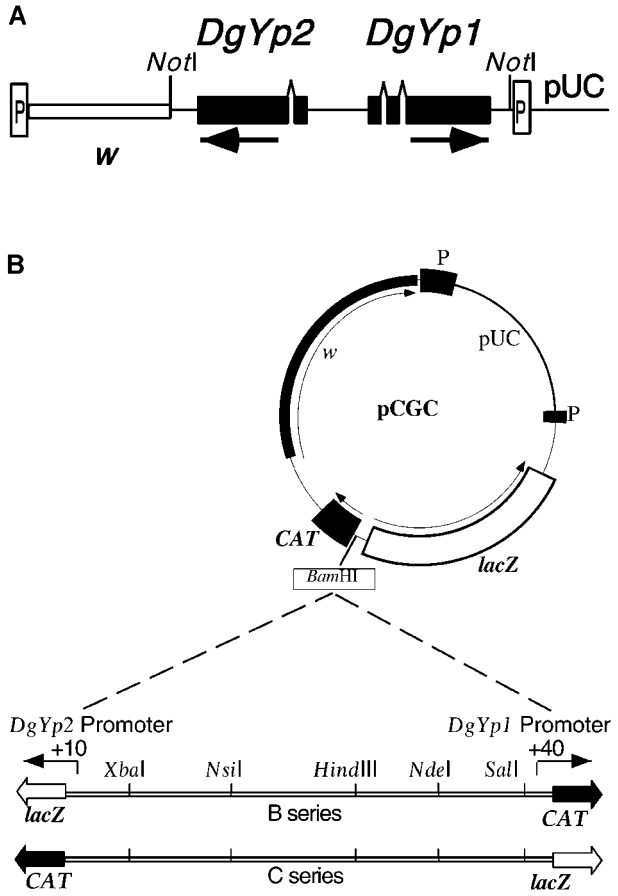

Figure 1.—*P*-element constructs. (A) Map of pG1Yp1Yp2. The *Apa*LI-*Nhe*I genomic fragment containing the *DgYp1-DgYp2* locus cloned into pCasPer (modified with *Not*I linkers). Positions of the introns are shown; arrows indicate direction of transcription. (B) Map of pCGC. Promoterless bacterial *lac-Z* and *CAT* genes are transcribed in opposite orientation (arrows). The unique *Bam*HI site was used to clone the intergenic region of *D. grimshawi* in the middle of the two bacterial genes. The *D. grimshawi* intergenic region cloned in this site is shown below. The ends of the *D. grimshawi* sequence are shown by the arrows and numbers +40 and +10, which indicate base pair distance relative to transcription initiation sites.

the *DgYp1* transcription start site, this deletion would have removed all the potentially orthologous *D. melanogaster* DNA binding elements listed in Table 1. These lines showed reduced activity of both reporter enzymes in the fat body tissue, but only to about one-third of series B levels, with little effect on activities from ovarian extracts. It is worth noting that these data are in agreement with similar deletion analyses in *D. melanogaster* (Abrahamsen *et al.* 1993; Lossky and Wensink 1995).

In *D. melanogaster*, ovarian tissue specificity is regulated by *cis*-acting sequences located in a defined region around the *Yp2* promoter. The ovarian enhancer 1 (OE1) is located at −180 to −320 relative to the *Yp2* cap site, and the ovarian enhancer 2 (OE2) is located within the first *Yp2* exon (Logan and Wensink 1990). Two deletions were made in the *DgYp2*-proximal half of the intergenic region, which could potentially affect the location of an OE1-type enhancer, the E and F series. The E-series lines were missing −240 to −430 relative to the *DgYp2* cap site. Interestingly, this construct produced a reporter-specific effect. Activity of β-gal (*DgYp2* promoter) but not CAT (*DgYp1* promoter) from ovarian extracts was reduced compared to the B-series lines, with little effect seen on fat body extracts. In addition, three of the five lines showed an altered pattern of ovarian expression producing a mottling effect on the stage 9 and 10 egg chambers (Figure 6). In the same region the F series harbored a deletion generated from Exonuclease III digestion deleting a segment from −119 to −389 relative to the *DgYp2* cap site. However, staining patterns showed no discernible qualitative effect (data not shown), and quantitative analysis also revealed only subtle effects. Although the data from the E and F series suggest a putative ovarian enhancer element between −389 to −430, the results below indicate a more complex (context-dependent) scenario.

To attempt to generate more dramatic effects, as much as two-thirds (1.19 kb) of the whole intergenic region was deleted (series G and H). Surprisingly, these lines also showed only subtle effects, regardless of the orientation of the intergenic region with respect to the reporter genes. Moreover, when compared to other deletions a clear pattern did not emerge. For example, none of these lines showed the mottled effect seen in the E series (data not shown). Also, enzymatic activity from fat body extracts was somewhat reduced, despite the presence of the putative FBE region. Quantitative analysis of reporter activity from ovarian extracts did mirror the E series promoter-specific results, albeit slightly less strongly.

Given the complexity and redundant function of the *cis*-elements suggested by these data, it is not surprising that the I series, which removed a small internal segment of the intergenic region, also had little discernible effect. Together these results show that enhancer elements capable of directing *DgYp* expression (within *D. melanogaster*) in a tissue- and sex-specific manner are scattered throughout the intergenic region of *D. grimshawi* and probably have both synergistic and antagonistic roles.

Sex-specific regulation and the role of *dsx*: The above results, demonstrating functional interaction between *D. grimshawi* DNA elements and *D. melanogaster trans*-acting factors, suggest proper DNA-protein recognition between these two divergent taxa despite the low sequence similarity. Moreover, deletions of the DNA regions containing putative binding sites in *D. grimshawi* did not result in drastic alterations in the expression of reporter genes. To further investigate these observations and to address potential differences in sex regulation that arose when using the reporter genes, we used the availability of mutations in the *D. melanogaster dou-*

blesex gene to specifically test the genetic interaction between this *trans*-acting factor and the regulatory DNA of *D. grimshawi Yp* genes. *dsx* has been shown to be genetically involved in repressing *Yp* gene expression in males and to bind directly to sequences within the FBE (see Introduction and Table 1). Thus, we asked if *dsx* was also involved in repressing male expression of *DgYp* genes. For this analysis we assayed dsx^{m+r15} and dsx^{d+r3} *trans*-heterozygous *D. melanogaster* flies bearing *D. grimshawi* DNA (see materials and methods). The XY *dsx* flies exhibit loss of some somatic secondary sexual characteristics and are thus referred to as pseudomales. In these tests we used both the pG1Yp1Yp2 and the reporter constructs (B and C).

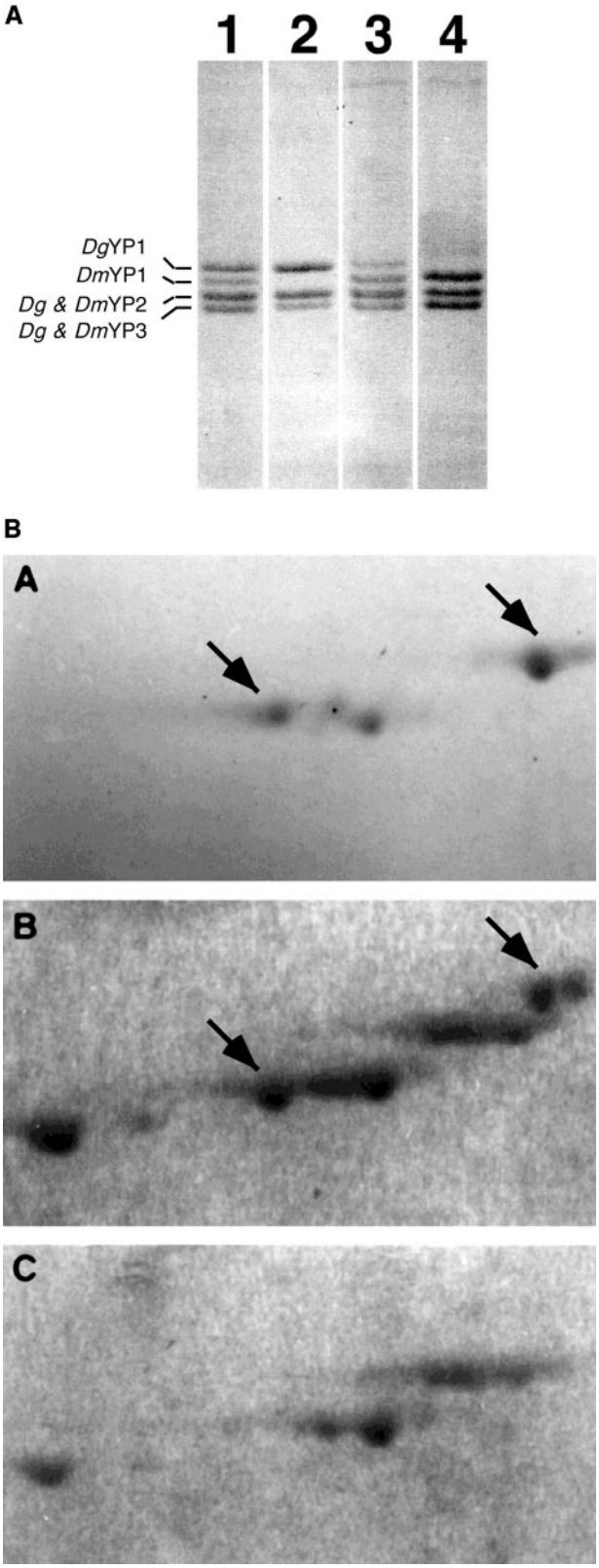

Figure 2.—SDS-PAGE and 2-D analysis of ovary extracts. (A) Ovaries were homogenized and their protein extracts were separated on a gradient 7–12% polyacrylamide gel. In the *D. grimshawi* (lane 2) and *D. melanogaster* (lane 4) samples, their respective YP1, YP2, and YP3 bands (top to bottom) are visualized. When these samples were manually mixed prior to loading (lane 1), four bands were resolved because the YP2 and YP3 proteins from each species comigrate. The sample from *D. melanogaster* transformed with *D. grimshawi Yp1* and *Yp2* genes shows four protein bands (lane 3). Note that these lanes were all from the same gel. (B) Homogenized ovary extracts were separated in an isoelectric focusing (pH range 4–10) tube gel and subsequently on a 9% polyacrylamide gel. *D. melanogaster* transformed with *D. grimshawi Yp1* and *Yp2* genes (middle, B) shows proteins (indicated by arrows) in identical coordinates and with similar patterns to YP1 and YP2 of *D. grimshawi* (top, A). These proteins are missing from the *D. melanogaster* control (bottom, C).

Lines bearing the pG1Yp1Yp2 construct in a *dsx* background were analyzed by hemolymph extracts and Northern blots of adult RNA. Figure 7 shows that *dsx* pseudomales produce *D. grimshawi* and *D. melanogaster* yolk proteins and their protein products are exported to the hemolymph. These data show that once produced by the fat body of pseudomales the yolk proteins can be transported to the hemolymph. However, we were unable to detect yolk proteins from the media of fat body cultures from the same lines (data not shown), raising the possibility that some level of regulation of export is sex specific. When the same crosses were performed using our reporter gene constructs (B and C series) we found that the pseudomales produced β-gal (Figure 8) and CAT (data not shown). These results indicate that the *D. melanogaster dsx*-dependent male repression mechanism can function to repress *D. grimshawi DgYp* genes as well.

DISCUSSION

Evolution of gene regulation has been studied primarily at the level of comparative observations and, more recently, by addressing directly the molecular changes that have led to the different regulatory networks (*e.g.*, Dickinson 1991; Wray 1994; Duboule and Wilkins 1998). Of these examples, only a few have been analyzed within the context of their adaptive significance (Thorpe *et al.* 1993). Increasingly, new data are providing evidence that lends credence to the idea that funda-

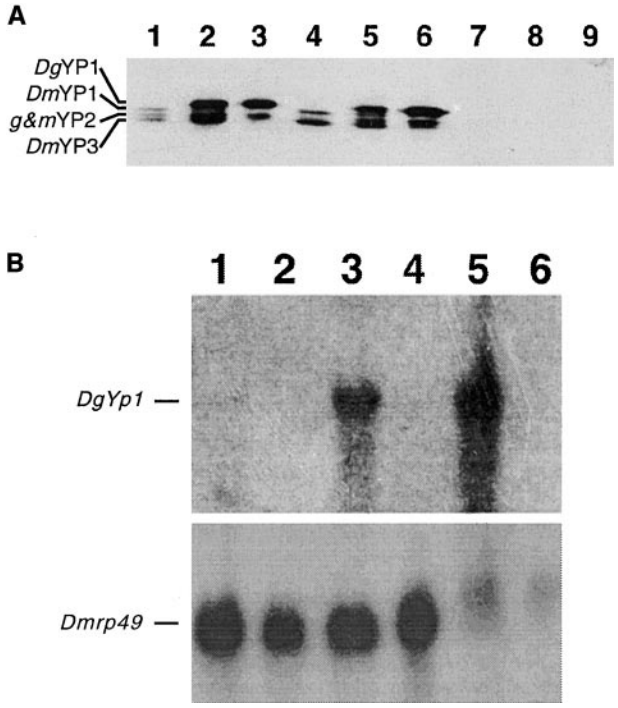

Figure 3.—Western analysis of organ cultures and Northern analysis of adult RNA. (A) Ovaries (lanes 1–3), female fat bodies (lanes 4–6), and male fat bodies (lanes 7–9) were dissected and cultured overnight. The proteins from the culture medium were separated on a 9% polyacrylamide gel and analyzed by Western blotting using polyclonal antibodies raised against YPs (Kambysellis *et al.* 1986). In *D. melanogaster* (lanes 1, 4, and 7) and *D. grimshawi* (lanes 3, 6, and 9) the three YPs are visible only in the media from the female organs. Samples from *D. melanogaster* transformed with pG1Yp1Yp2 (lanes 2, 5, and 8) show an extra YP (*D. grimshawi* YP1) from the female organ cultures. For lanes 7–9 each culture had twice the number of organs, and four times the medium volume was loaded as compared to other lanes, but no male YP production was seen. (B) Total RNA samples from sexed adults were separated through formaldehyde-agarose gels, transferred to nylon, and hybridized to ^{32}P-labeled probes for the indicated genes. The loading control probe was *D. melanogaster rp49* (O'Connell and Rosbash 1984), which migrates slightly differently in *D. melanogaster* (lanes 1–4) and *D. grimshawi* (lanes 5 and 6, females and males, respectively). Lanes 1 (females) and 2 (males) containing untransformed *D. melanogaster* show very faint cross-reactivity between the *D. melanogaster* endogenous *Yp1* and the *D. grimshawi* probe. Transformed *D. melanogaster* females and males (lanes 3 and 4) show female-specific expression of the *D. grimshawi Yp1* gene (lane 3).

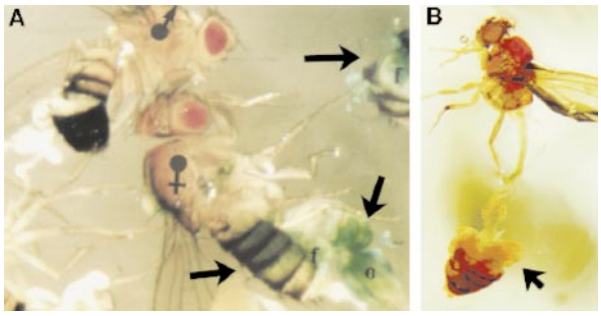

Figure 4.—β-Galactosidase staining. (A) *D. melanogaster* transformed with the B construct. Within 1 hr, the ovaries (o) and the fat bodies (f) of females developed intense staining. Under the same conditions males showed no detectable level of staining. (In longer incubations males will develop the fat body staining; see text for details.) (B) Female *D. melanogaster* transformed with pCGC alone (construct A) exhibit no ovary or fat body staining.

mental morphological and developmental differences are predominantly due to changes in regulatory networks rather than in structural genes. A fundamental question in this area is what role coadaptive changes play in the evolution of the regulatory networks. We have begun to address this issue using the *Yp* genes in Drosophila.

Evolutionary comparisons of developmental systems studied at the molecular level are needed for an understanding of the genetic mechanisms leading to phenotypic diversity. Yet, hypotheses of evolutionary relevance should also include forces postulated to be at work in the fixation of different regulatory patterns observed in the species analyzed. For these reasons, we studied oogenesis in Hawaiian Drosophila as a model to gain insight into the molecular evolution of a developmental system for which considerable ecological data also exist (Kambysellis *et al.* 1995; Kambysellis and Craddock 1997). The phenotypic diversity of ovary organization and egg production in this speciose group is correlated to environmental and ecological factors, which suggests adaptive significance. In this article we have used the power of *D. melanogaster* molecular genetics to conduct an initial functional analysis on the evolution of *Yp* gene regulation.

The production of yolk proteins is under strict regulation in Drosophila (see Introduction), and the Hawaiian endemics exhibit great diversity in the relative amount of each of the YPs found in the hemolymph in the different species (Craddock and Kambysellis 1990). This variation may be related to the breeding substrate to which each species has adapted through the overall control of egg production. When the expression pattern of *Yp* genes from *D. grimshawi* was compared to *D. melanogaster*, some minor differences were observed, but the tissue-, sex-, and temporal-specific expression patterns were shown to be conserved (Hatzopoulos and Kambysellis 1987a; Kambysellis *et al.* 1989). In contrast, when the sequences from these same species were compared, the structural genes were observed to be conserved but the putative regulatory regions were highly divergent. To address the functional significance of these sequence level differences we took advantage of the possibility of transforming *D. melanogaster* and introduced *D. grimshawi* genes and regulatory sequences via *P*-element transformation.

The results presented here show that the basic expression pattern of *DgYp1* and *DgYp2* genes is retained within *D. melanogaster*, demonstrating that the *D. grimshawi*

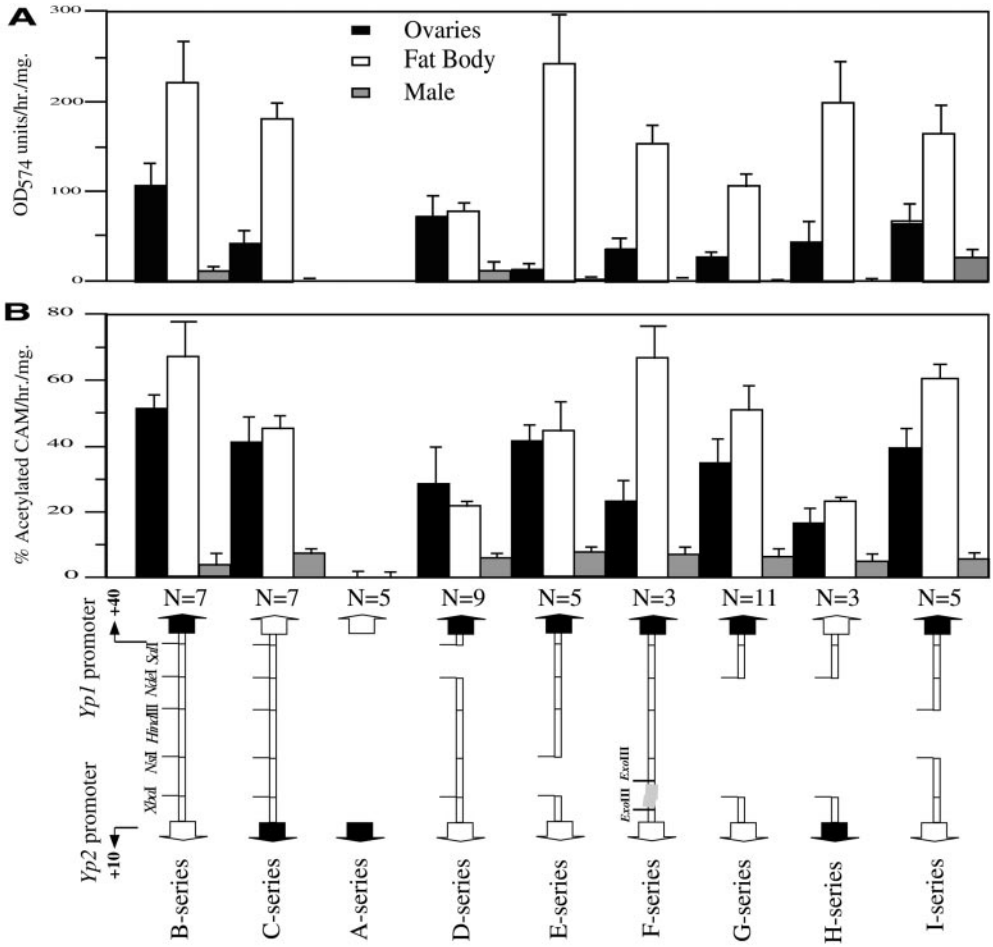

Figure 5.—Quantitative analysis of deletion constructs. Extracts from ovaries (black bars), female fat bodies (white bars), and males (gray bars) from *D. melanogaster* lines transformed with different *D. grimshawi* deletion constructs were assayed for reporter gene activity. The two histograms represent β-gal (A) and CAT (B) activity. The DNA map and name of each construct is shown below the two histograms. *N* represents the number of independent transformed lines that were analyzed for each construct. Each bar represents the average value for all the lines for each construct with the standard deviation shown above each bar. See text for details.

DNA is functionally recognized by *D. melanogaster trans*-acting factors. Thus, these interspecific analyses suggest that the regulation of the *Yp* genes is controlled by a system that can tolerate large mutations in the *cis*-acting elements. Further, these results argue against coevolution between *trans*- and *cis*-acting elements, as that should have resulted in *D. melanogaster trans*-acting factors being unable to specifically regulate *D. grimshawi cis*-elements. This pattern has also been observed in other enhancer systems that have been analyzed functionally (Ludwig *et al.* 1998).

DNA-protein interactions leading to regulated gene expression depend on the use of either weak but multiple binding sites or fewer contact sites with stronger binding affinities. Within *cis*-acting elements a few nucleotides may be absolutely required while the rest may be noncritical. As species diverge, noncontiguous critical nucleotides may be constrained while adjacent nucleotides evolve rapidly, resulting in a complex pattern of element evolution difficult to discern via sequence alignments. Confirmation of the predicted interactions between *D. melanogaster trans*-acting factors and *D. grimshawi cis*-acting elements will require biochemical studies that use, for example, *D. melanogaster* DSX protein and *D. grimshawi* intergenic DNA. In turn, such studies may shed light on the sequence requirements for these interactions over several million years of evolution.

We further found that large deletions in the *DgYp1-DgYp2* intergenic region have only subtle but nonetheless measurable effects on the pattern and quantity of reporter gene expression. A possible interpretation from our expression analysis is that all the major control elements actually lie within the DNA remaining in all of our constructs. However, we know this not to be true because constructs with different portions of the *D. grimshawi* intergenic region ligated with a reporter gene and heterologous promoter can drive correct tissue-, temporal-, and sex-specific expression (Tan 1997). Redundancy in the *D. melanogaster Yp1-Yp2* intergenic region has also been reported (Abrahamsen *et al.* 1993; Lossky and Wensink 1995); moreover, it has been used to argue for a system that allows for fine tuning of yolk protein production in response to variable environmental conditions that the organism encounters (Abrahamsen *et al.* 1993).

Our data lead us to speculate on an evolutionary role for this complexity. During the colonization of new niches by founder populations, the intergenic region between the *Yp1* and *Yp2* genes has undergone extensive rearrangement without completely obliterating expres-

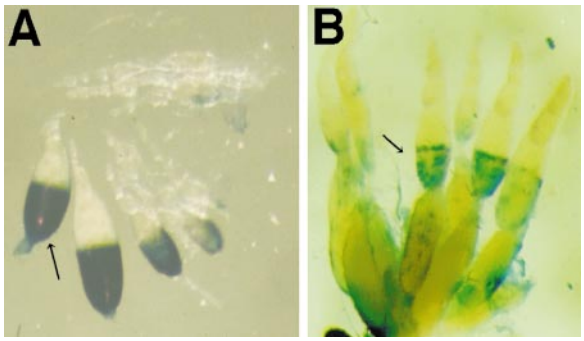

Figure 6.—β-Galactosidase staining. (A) Ovaries from *D. melanogaster* transformed with the entire *D. grimshawi* intergenic region driving expression of *lacZ* reporter showed staining of the follicle cells around vitellogenic oocytes (arrow). (B) In contrast, deletions of a small region of intergenic DNA (E series) gave rise to lower and mottled expression patterns at the same stages (arrow).

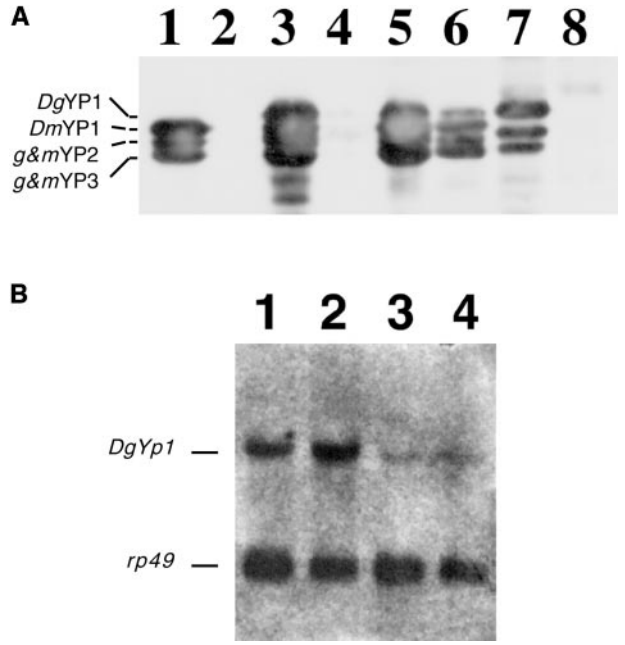

Figure 7.—*D. grimshawi* yolk protein and RNA analysis in *dsx* background. (A) Hemolymph samples extracted from adult females (lanes 1, 3, 5, and 7) and adult males (2, 4, 6, and 8) were separated on a 9% polyacrylamide gel. A Western blot using polyclonal antibodies that recognize all the YPs (Kambysellis *et al.* 1986) is shown. *D. melanogaster* transformed with *D. grimshawi Yp* genes in a *dsx* background shows the presence of YPs from both species in the female (lane 5) and in the male (lane 6). As controls, wild-type *D. melanogaster* (lanes 1 and 2), *D. melanogaster* transformed with *D. grimshawi Yp* genes but not in a *dsx* background (lanes 3 and 4), and *D. grimshawi* (lanes 7 and 8) show YPs only in the females. (B) Total RNA samples from sexed *dsx* adults were separated through formaldehyde-agarose gels, transferred to nylon membrane, and hybridized to ^{32}P-labeled probes for the indicated genes. The *D. grimshawi Yp1* probe shows *D. melanogaster* transformed with *D. grimshawi Yp1* and *Yp2* genes expressing the *Yp1* gene in *dsx* females (lane 1) as well as *dsx* males (lane 2). Nontransformed *dsx* females (lane 3) and *dsx* males (lane 4) were used as controls. A faint cross-reactivity between the *DgYp1* probe and *Yp1* from nontransformed *D. melanogaster* flies can be seen.

sion, instead generating subtle variability in the expression pattern. Natural selection has then played a role in fixing the pattern of *Yp* gene expression best suited for each new environment. This would lead to variable contributions of yolk proteins from the different organs, as well as relative and absolute differences in the amount of yolk proteins produced; such variation in protein production patterns is seen among the Hawaiian Drosophila species (Craddock and Kambysellis 1990). Although these may involve subtle quantitative differences, they may be selectable in the highly competitive environment of the Hawaiian forests. Interestingly, sequence comparisons between the closely related Hawaiian species *D. planitibia* and *D. grimshawi* also show a large number of substitutions and indels in the intergenic region, further supporting the potential for rapid evolution of this region (Ho 1994). This is not to suggest that the intergenic region is somehow targeted for change; rather, that redundant control systems may allow for DNA rearrangements to provide variability in expression pattern (which can be selected) instead of completely obliterating expression that would decrease fecundity (Bownes *et al.* 1991). This suggests that the *cis*-acting elements directly involved in regulating the expression of the *Yp* genes play a major role in the evolution of their regulation, as opposed to more upstream factors.

A prediction of this model is that *trans*-acting factors from distant species such as *D. melanogaster* will interact with *D. grimshawi* DNA, but subtle and quantifiable differences between species will act *cis*-dominantly when inserted in *D. melanogaster*. Testing this hypothesis will require that different Hawaiian species, adapted to different environments, are analyzed in a way similar to the present study. Good candidates for these analyses abound among the Hawaiian endemics; even within the *grimshawi* group there are some very close species adapted to different environments (Piano *et al.* 1997).

The reporter gene constructs used in the quantitative analyses identified two regions that may contain putative enhancer elements acting like the ovarian enhancer and the fat body enhancer identified in *D. melanogaster*, albeit showing only subtle effects. When similar deletions were analyzed in *D. melanogaster*, small effects were also observed (Abrahamsen *et al.* 1993; Lossky and Wensink 1995). Nevertheless, experiments testing the sufficiency of the *D. grimshawi* enhancers here postulated together with DNA binding studies using *D. melanogaster trans*-acting factors may reveal structural requirements for the interaction of specific proteins with the DNA, providing complementary information to the elegant mutagenesis studies on *D. melanogaster* (for example, An and Wensink 1995b).

To begin to probe these questions we tested the ge-

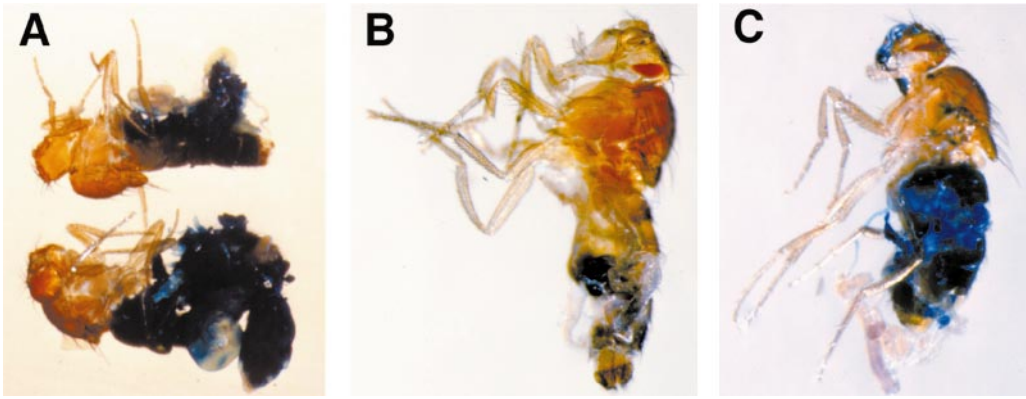

Figure 8.—β-Galactosidase activity in *dsx* flies transformed with *DgYp1-DgYp2* intergenic region driving *lac-Z*. *D. melanogaster* lines expressing *lac-Z* under the control of *D. grimshawi DgYp1-DgYp2* intergenic region (construct B) were crossed to *dsx* flies. Construct C gave the same results. Aged, conditioned adults were dissected and stained for β-galactosidase activity. All the histochemical staining patterns observed here were from simultaneous experiments with the same incubation times. (A) Development and pattern of expression in females that have one copy of the recessive *dsx* allele (lower fly) are indistinguishable from that of wild type. Ovaries are well formed and densely stained. Fat bodies are also heavily stained. Females that are *dsx* mutants do not develop ovaries but still show significant staining in the fat body (upper animal, note that abdominal tergites are darker in *dsx* females). (B) No male staining is observed when either recessive *dsx* allele (dsx^{m+r15} is shown) is tested; the dark parts of the abdomen are from the pigmentation of the tergite. (C) In *dsx* pseudomales the abdominal and thoracic fat body are stained. The bar-shaped eyes are from a Y-linked marker, which confirms the XY genotype.

netic role of a known *trans*-acting factor (DSX) responsible for the sex-specific expression of *Yp*s (see Introduction). Our results mirror those reported in *D. melanogaster*, suggesting that DSX^M acts as a repressor of *Yp* expression. Given these genetic results it is tempting to speculate that the DSX protein from *D. melanogaster* is capable of regulating the *DgYp* genes by direct binding despite the diverse organization and sequence of the putative binding sites. It is noteworthy that when we specifically looked for the putative binding sequences of the DSX protein in the *D. grimshawi* DNA we found that the primary sequence and the arrangement of the putative binding elements have changed. These findings suggest that complex enhancer elements used to regulate genes need not maintain a colinear organization to function.

In addition to selection acting on the regulated expression of the *Yp* genes, strong selection pressures are also acting on the protein products. The sequences for these proteins are unexpectedly well conserved across Diptera, and the *D. grimshawi* proteins were capable of proper trafficking within *D. melanogaster* without giving rise to any phenotypic differences. Furthermore, the 2-D gel results suggest that the proteins are modified in *D. melanogaster* as they would be within *D. grimshawi*, suggesting that the *D. grimshawi* protein signals act *cis*-dominantly. Finding that *D. grimshawi* proteins are abundant in *D. melanogaster* ovaries also suggests that they accumulate in the maturing oocytes because they can interact functionally with the *D. melanogaster* YP receptor, yolkless (Schonbaum *et al.* 1995). The ability of YPs from different species to enter *D. melanogaster* oocytes has also been suggested through injection studies (Martinez and Bownes 1992). Although it is possible that the sequence and functional conservation observed in the YPs may be mostly related to their role as nutritional molecules, it is tempting to speculate that they have other roles in the course of embryogenesis (*e.g.*, Bownes *et al.* 1988) that do not allow mutations to accumulate.

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