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Regulation of *Drosophila yolk protein* Genes by an Ovary-Specific GATA Factor

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The divergently transcribed *yolk protein* genes (*Yp1* and *Yp2*) of *Drosophila melanogaster* are expressed only in adult females, in fat body tissue and in ovarian follicle cells. Using an in vitro transcription assay, we have identified a single 12-bp DNA element that activates transcription from the promoters of both *Yp* genes. In vivo, this regulatory element is tissue specific: it activates transcription of *Yp1* and *Yp2* reporter genes in follicle cells but has no detectable effect in fat body or other tissues. The sequence of the element consists of two recognition sites for the GATA family of transcription factors. We show that among the *Drosophila* genes known to encode GATA factors, only *dGATAb* is expressed in ovaries. The single transcript that we detect in ovaries is alternatively spliced or initiated to produce an ovary-specific isoform of the protein. Bacterially expressed dGATAb binds to the 12-bp element; a similar binding activity is also present in the Kc0 nuclear extracts used for in vitro transcription assays. These in vitro and in vivo results lead us to propose that *dGATAb* makes several developmentally regulated products, one of which is a follicle cell-specific protein activating transcription of *Yp1* and *Yp2* from a known regulatory element.

Members of the GATA family of transcription factors share one or, more commonly, two copies of a highly conserved zinc finger domain that binds DNA sites with the core sequence GATA. The GATA proteins are found in organisms ranging from fungi to humans and are likely to play important roles in the development and differentiation of all eukaryotic organisms. The vertebrate protein GATA-1, for example, is necessary to activate transcription of globin and other erythroid-cell-specific genes in human, murine, and avian experimental systems. It is also required for erythropoiesis in transgenic mice and cultured embryonic stem cells (reviewed in references 10, 48, and 55). Other vertebrate GATA proteins are involved in the development and terminal differentiation of the immune system, optic tectum, placenta, kidneys, and heart (e.g., see references 6, 32, 36, 39, 60, 68, and 70).

In *Drosophila melanogaster*, an organism particularly well suited for the analysis of developmental functions, three GATA transcription factors have been identified. One factor, dGATA-2, is expressed in a highly restricted pattern during late embryogenesis but is otherwise uncharacterized (64). Another factor, dGATAa, is encoded by the *pannier* gene (51, 67). *pannier* is required at least twice: for development of the dorsal region of the embryo and for development of the sensory bristles. In the latter process, it appears to regulate two key proneural genes, *achaete* and *scute* (51).

The third factor, dGATAb, acts in the development of embryonic and larval fat body tissues (1). Two lines of evidence support this contention. First, by mid-embryogenesis, expression of the *dGATAb* gene is restricted to apparent precursor cells and then to maturing and fully differentiated fat body tissue (1). Second, dGATAb protein binds to a DNA element that is necessary for larval fat body-specific transcription of *alcohol dehydrogenase-1* (*Adh-1*) and activates transcription through this DNA element in cultured-cell cotransfection assays. dGATAb is therefore likely to regulate *Adh-1* and other genes expressed in embryonic and larval fat body tissues (1, 20).

We propose that dGATAb, in addition to its role in fat body development, also plays a role in oogenesis. In this study, we found that the *dGATAb* gene is expressed in the ovaries of adult flies, where it produces an ovary-specific protein isoform. We also show that the dGATAb protein binds to a 12-bp, ovarian follicle cell-specific regulatory element located between the divergently transcribed *yolk protein* genes, *Yp1* and *Yp2*. These genes, which encode abundant constituents of mature oocytes (reviewed in reference 59), are expressed coordinately with a third member of the family, *Yp3*, in two cell types of the adult female: the fat body tissue dispersed throughout the body and the follicle cells surrounding each developing oocyte (8, 12, 23, 26, 40, 41, 53). We find that the 12-bp element activates both in vitro and in vivo transcription of *Yp1* and *Yp2*. Activation in vivo is observed only in the follicle cells. Our results suggest that *dGATAb* encodes several developmentally regulated products, one of which is a follicle cell-specific protein that activates *Yp* transcription through a defined binding site.

MATERIALS AND METHODS

Templates for in vitro transcription. Unless indicated otherwise, nucleotide positions are relative to *Yp1* (33). A 1,866-bp *Bst*EII fragment including 126 bp of *Yp2*, the 1,226-bp intergenic region, and 514 bp of *Yp1* was subcloned into pUC19 and pUC18 by standard methods (7). Serial deletions 5′ towards *Yp1* (pΔ2 series, 3′ end at +514) or *Yp2* (pΔ1 series, 3′ end at +126 relative to *Yp2*) were made in these parental plasmids with *Bal* 31 nuclease (Double-Stranded Nested Deletion Kit; Pharmacia). For internal controls, a linker was inserted into the *Ava*II site (+36) in pΔ2[−320]; products from this template, pΨ3, are 11 nucleotides longer than those of other *Yp1* templates. For substitutions within the intergenic region, the *Bcl*I site at −323 in pΔ2[−323] was changed to *Sal*I, yielding pΔ2wt. Fragments incorporating substitutions 1 to 5 were made by PCR and introduced into pΔ2wt to yield pΔ2sub1 to pΔ2sub5.

IR and IRsub3 were inserted at −160 relative to *Yp2* in two steps: first, a fragment of *Yp2* from *Hga*I (−125 relative to *Yp2*) to *Afl*III (in vector) was subcloned into pUC18; then, fragments of pΔ2wt and pΔ2sub3 from *Acc*I to *Stu*I (−181 to −92) were inserted into the pUC18 polylinker.

In vitro transcription reactions. Nuclear extracts for in vitro transcription

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were prepared from *Drosophila* Kc0 cells (30). *Drosophila* embryo extract was a gift from K. O'Donnell (Brandeis University).

In vitro transcription conditions for supercoiled templates (30) were modified. For reasons discussed elsewhere (47), the template concentration was limiting at 1.25 nM (0.25 nM pΨ3 plus 1 nM test template), and nontemplate DNA (supercoiled pUC18) was added to a final concentration of 30 μg/ml of reaction mixture. Transcription reactions were performed in a volume of 10 μl for 45 min at 21°C and included, in addition to DNA, 0.5 mM nucleoside triphosphates, 2.5 to 5 μl of protein extract, $MgCl_2$ adjusted to 7 mM, KCl adjusted to 60 mM, and HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), pH 7.5, adjusted to 40 mM. Reaction mixtures were digested for 15 min at 37°C in 100 μl of stop solution (1% sodium dodecyl sulfate, 20 mM EDTA, 200 mM NaCl) with 15 μg of proteinase K, extracted with phenol-chloroform, and precipitated with ethanol.

Transcription products were detected by primer extension assays (57). Each pellet was resuspended in 5 μl of 100 mM KCl with 200 fmol (10^5 cpm) of ^{32}P-labeled primer and incubated for 30 min at 55°C. The RNA-primer hybrids were extended for 30 min at 37°C in a final volume of 10 μl with 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate, 4 mM sodium PP_i, and 5 to 10 U of avian myeloblastosis virus reverse transcriptase (Life Sciences). Extension reactions were stopped with 10 μl of sequencing load buffer and fractionated on sequencing gels.

The primer used against *Yp1* products hybridizes from positions +67 to +89; the primer used against *Yp2* products hybridizes from positions +58 to +80 relative to *Yp2*. Results were quantitated by densitometry and normalized against pΨ3.

Constructions for germ line transformation. Constructions for germ line transformation are based on the P element vector cp20.1. pCR1 (constructed by M. Lipson, Brandeis University) includes *Yp1* fused to *Drosophila alcohol dehydrogenase* (*Adh*), the *Yp1-Yp2* intergenic region, and *Yp2* fused to the β-galactosidase gene (*lacZ*) from *Escherichia coli*. It is identical to pCR2 (41), except that *Yp2* is fused in frame with *lacZ* at +65 relative to *Yp2* rather than at +105. pCR1sub3 was constructed by the general procedure used for pCR2.

P element-mediated germ line transformation and genetic crosses. Fusion gene constructions and the helper plasmid pπ25.7wc were injected into *Drosophila* ry^{506} embryos by standard procedures (58). Independently transformed lines were balanced with *CyO*/*Sco*; ry^{506}/ry^{506} or *TM2*, *Ubx*, ry^{506}/*MKRS*, *Sb*, ry^{506} strains or crossed into an alcohol dehydrogenase (ADH)-null background (20). The number of insertions was determined by Southern blotting, and only single-copy transformants were retained.

β-Galactosidase histochemical and spectrophotometric assays. Prior to all assays, 0- to 3-day-old flies, either all heterozygous or all homozygous, were kept on food supplemented with live yeast paste for 2 to 3 days. The flies were dissected in Ringer's solution with 0.1% Triton X-100 and stained for β-galactosidase activity as previously described (28).

Spectrophotometric assays were carried out essentially as previously described (15, 54), except that the flies were dissected and at least 10 pairs of ovaries or five remaining carcasses (taken as representative of fat body tissue) were used to make each 250-μl extract; the buffer was either 50 mM KPO_4 (pH 7.5)–1 mM $MgCl_2$–1 mM AEBSF or TB1 (11) with 1 mM AEBSF; and the change in A_{574} was recorded continuously for 1 to 10 min. Each transformant line was assayed two to five times. Endogenous β-galactosidase activity was negligible. Activity was calculated as the change in A_{574} (× 10 for fat body tissue and × 100 for ovaries) per minute per microgram of protein.

ADH histochemical and spectrophotometric assays. The flies were fed and dissected as described above and stained for ADH activity as previously described (11, 41). Spectrophotometric assays were performed essentially as previously described (11, 41), except that extracts were made from dissected flies as described above; final assay volume was 500 μl, including 30 μl of 50-mg/ml β-NAD and 15 μl of 2-butanol; and the change in A_{340} was recorded continuously for 1 to 10 min. Each transformant line was assayed two to three times. Endogenous ADH activity in ADH-null flies was negligible. Activity was measured as the change in A_{340} (× 100 for fat body and × 1,000 for ovaries) per min per μg of protein.

Northern (RNA) blots and RNA mapping. The flies were fed and dissected as described above. Total RNA was prepared as previously described through the ethanol precipitation step (8). Staged embryonic, larval, and cell line RNAs were kindly provided by D. Jacoby (Brandeis University) and T. Abel (Harvard University). RNA samples (5 to 15 μg) and DNA markers (restriction digest of *rp49* [46]) were denatured with glyoxal and dimethyl sulfoxide, fractionated on 1% agarose–10 mM $NaPO_4$ (pH 7.0) gels, and blotted onto a Nytran membrane (Schleicher and Schuell). The blots were hybridized to ^{32}P-labeled, nick translated DNA and then washed under stringent conditions, exposed, and stripped according to the manufacturer's protocols.

For RNA mapping experiments (7), a fragment including the 5′ end of the *dGATAb* embryonic cDNA (to *Ava*I at +565 [1]) was subcloned into pSP72 (Promega), yielding pSP-Gb5′. A 585-nucleotide uniformly ^{32}P-labeled probe was prepared for RNase protection assays by in vitro transcription of pSP-Gb5′ with SP6 RNA polymerase.

Mobility shift and DNase I protection assays. For mobility shift experiments, gel-purified oligonucleotides were annealed in Tris-EDTA–50 mM NaCl and ^{32}P-labeled with T4 polynucleotide kinase. For DNase I protection, DNA fragments (from −229 to −81) were made by PCR with either pΔ2wt or pΔ2sub3 as the template (31).

To eliminate background nuclease activity, extract from Kc0 cells was adjusted to 100 mM KCl, heated at 90°C for 10 min, and then cleared by centrifugation for 5 min. Extracts from *E. coli* transformed with *dGATAb* in the sense and antisense orientations were a gift from T. Abel (Harvard University).

Each binding reaction mixture (15 min) for mobility shift assays (7) contained 50 mM KCl, 30 mM potassium glutamate, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM β-mercaptoethanol, 4% glycerol, 1 μg of reannealed poly(dI-dC) · poly(dI-dC), 2 ng (25,000 cpm) of ^{32}P-labeled oligonucleotides, and 1 to 2 μl of heat-treated Kc0 or 0.5 to 1 μl of bacterial protein extract in a 15-μl volume. Unlabeled competitor oligonucleotides were added at a 100-fold molar excess over the ^{32}P-labeled probe.

The top strand of each double-stranded oligonucleotide used in these assays is given below. GATA-TATC sequences are underlined. The IRwt oligonucleotide includes IR and a few flanking bases; mutations in IRa through IRe are shown in boldface type; IRd mutations are identical to IRsub3 (see Fig. 3A). Oligonucleotide CD8α includes two GATA sites of the murine CD8α gene; Ctrl is an unrelated sequence. The oligonucleotides were as follows: IRwt, 5′-GATCTTC CGCTATCGATAGCATATG-3′; IRa, 5′-**TCGAC**TCCGCTATCGATAGCAT A**AC**-3′; IRb, 5′-GATCTTCCGCTATC**GTC**AGCATATG-3′; IRc, 5′-GATCT TCCGC**CATGCCG**AGCATATG-3′; IRd, 5′-GATCTTCC**CGC**AT**GCCGAC** CATATG-3′; IRe, 5′-GATCTTCCGC**TGAC**GATAGCATATG-3′; CD8α, 5′-TCGAGTGATAGAATAGATAGCAG-3′; and Ctrl, 5′-GTTACCCGTAGCA TTCTTAATAACC-3′.

With bacterial extracts, each binding reaction mixture for DNase I protection (7) contained 125 mM NaCl, 4 mM Tris-HCl (pH 7.5), 1.33% polyvinyl alcohol, 1.5 μg of reannealed poly(dI-dC) · poly(dI-dC), 5×10^4 cpm (1 to 2 ng) of ^{32}P-labeled fragment, and 2 μl of protein extract in a 15-μl volume. With heat-treated Kc0 extract, each binding reaction mixture contained 2% polyvinyl alcohol, 1 μg of reannealed poly(dI-dC) · poly(dI-dC), 10^5 cpm (2 to 4 ng) of ^{32}P-labeled fragment, and 25 μl of protein extract in a 50-μl volume. After 15 to 25 min, the reaction mixtures were digested for 1 min in 2 volumes of 10 mM $CaCl_2$–1 mM $MgCl_2$ with 5 to 50 ng of DNase I, stopped, and fractionated as previously described (30).

RESULTS

Identification of a new *Yp* regulatory element. We used in vitro transcription assays to identify new DNA elements regulating the divergently transcribed *Yp1* and *Yp2* genes. In preliminary experiments, we showed that transcription of *Yp1* and *Yp2* in nuclear extracts of *Drosophila* Kc0 cells required RNA polymerase II and initiated at the same closely spaced nucleotides as in vivo. We also established limiting template conditions, under which small changes in activation and repression can be observed (see Materials and Methods) (47). We then surveyed three series of templates with deletions in increments of 10 to 20 bp, the approximate length of single protein binding sites (Fig. 1A). One series approaches the *Yp2* promoter from the 5′ upstream side (Fig. 1B). The other two series approach the *Yp1* promoter from the 5′ upstream side or the 3′ downstream side (Fig. 1C).

Two known regulatory regions of *Yp1* influenced transcription in vitro (Fig. 1C). One is the fat body enhancer (−320 to −193; all numbers are relative to the major *Yp1* initiation site) previously identified by its in vivo regulatory properties in germ line transformation experiments (24). Deletions within this region had modest but reproducible effects, suggesting the presence of multiple activator and repressor binding sites. This is consistent with the complex organization of the fat body enhancer reported recently (4, 5). The second known region is the TATAA box (−36 to −31), recognized by its homology to the consensus and its position relative to the initiation sites. A deletion (−38 to −25) including the TATAA box abolished transcription, presumably reflecting a requirement for the core transcription factor, TFIID (13).

Three other *Yp1* regions also affected transcription in vitro. First, a region downstream of the initiation sites, from +89 to +112, had a threefold positive effect. Deleting this region changes the structure of the transcripts, so that stability rather than synthesis of RNA may have been altered. However, prolonged incubation of the reaction mixtures after the addition of

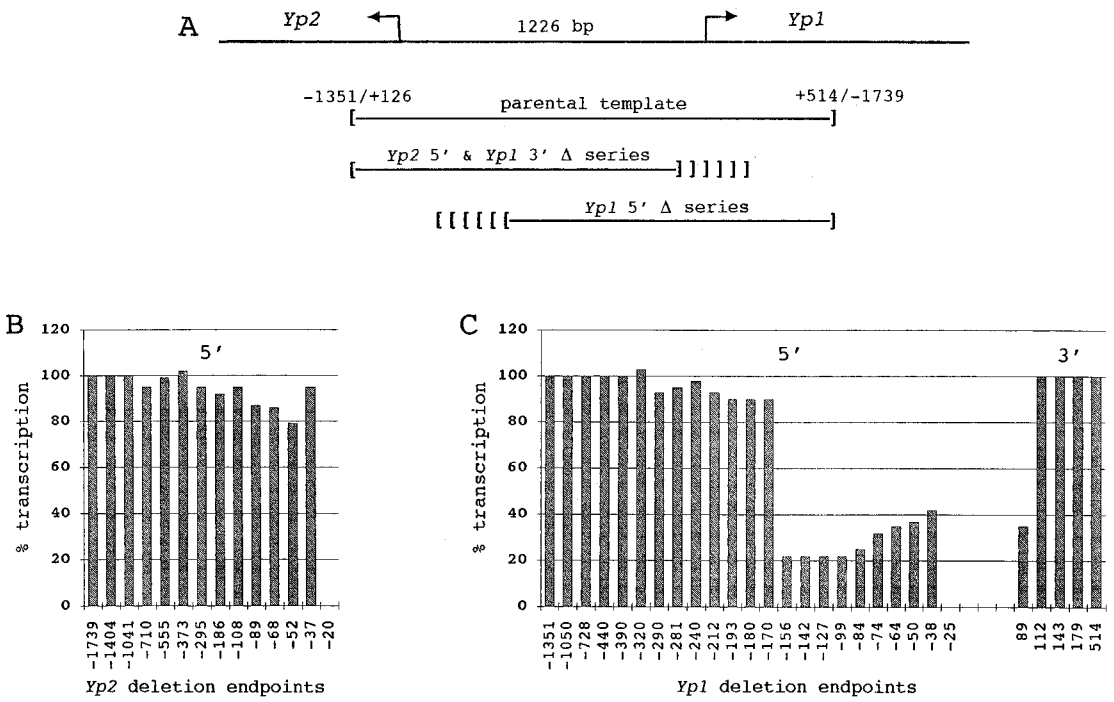

FIG. 1. In vitro transcription of *Yp1* and *Yp2*. (A) Structures of *Yp1* and *Yp2*, the parental template, and the nested deletion (Δ) series. Endpoints of the parental template relative to *Yp1* and *Yp2* are shown. (B and C) Transcription of the *Yp2* and *Yp1* deletion templates, respectively. Each bar represents the average transcriptional activity from four experiments.

α-amanitin did not affect the level of these transcripts relative to that of wild-type transcripts (data not shown). Several imperfect repeats of a 7-bp sequence [(G/C)CTTGG(G/C)] occur within the region and at similar positions downstream of *Yp2* and *Yp3* (25), suggesting a conserved function. Downstream transcriptional regulatory elements are also found in other *Drosophila* genes (e.g., see references 57 and 62).

A second *Yp1* region, from −99 to −38, had a twofold negative effect on transcription. The incremental effect of deletions in this region suggests that it contains several repressor binding sites. Finally, the region from −170 to −156 had at least a fourfold positive effect. This region includes most of a perfect 12-bp inverted repeat, termed IR, a potential binding site for a transcriptional activator (Fig. 2). IR is the focus of the remainder of this study.

The 5′ deletion analysis of *Yp2* (Fig. 1B) yielded no single element as effective as IR.

In vitro regulatory activity of IR. We used directed mutagenesis to show that the positive regulatory effect of the IR region is limited to IR itself and therefore likely to be mediated by a single activator protein. A *Yp1* template in which 8 of the 12 bp in IR are changed gave at least a fivefold-lower level of transcription; however, 5-bp substitutions upstream or downstream of IR had no effect (Fig. 3A and B). A similar result was obtained with nuclear extract from *Drosophila* embryos, indicating that a factor activating transcription from IR also occurs in embryos (data not shown).

Other mutations were used to demonstrate that transcriptional regulation by IR does not depend on the spacing between IR and the *Yp1* promoter. To alter this spacing by half- or full-helical turns, we either inserted or deleted 5- to 6-bp blocks of DNA at a restriction site (at −92) within a region that had no influence on transcription in the 5′ deletion analysis (Fig. 1C). Neither the insertion of up to 4 nor the deletion of up to 10 half-helical turns of DNA (+21 bp and −54 bp, respectively) had any significant effects on in vitro transcription of *Yp1* (data not shown).

A number of enhancers located between *Yp1* and *Yp2* are known to regulate both genes (2, 23, 40, 41, 53, 61). In the initial survey, deletion of a region including IR had no effect on transcription from the *Yp2* promoter located 1,070 bp away (Fig. 1B, template −1404 versus template −1041). However, in this type of in vitro assay, regulatory elements generally do not function further than 200 bp from a promoter (38). We therefore inserted a DNA fragment with IR at −160 relative to *Yp2*. The template with IR was transcribed at fivefold-higher levels than either the parental template or the same template with IR mutated (Fig. 3C).

We conclude that IR has a fivefold positive effect on in vitro transcription from both *Yp* promoters. Over short distances, the mechanism by which it exerts this effect appears to be largely insensitive to distance and helical-face orientation rel-

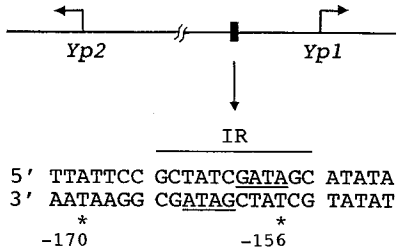

FIG. 2. Sequence of the IR region. The diagram shows *Yp1* and *Yp2* and IR (filled box). Below is the sequence of the IR region, with IR separated from flanking DNA by spaces and the core GATA sequences underlined. Endpoints of *Yp1* deletion templates are marked by a number and a star.

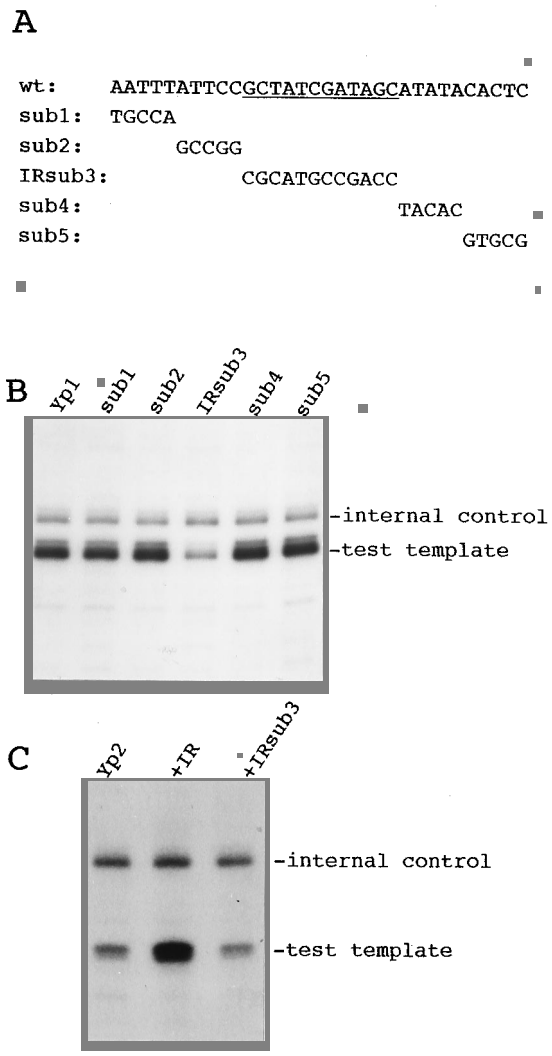

FIG. 3. Effect of IR on in vitro transcription of *Yp1* and *Yp2*. (A) Substitutions in the IR region. The wild-type (wt) sequence is shown at the top with IR underlined. IRsub3 targets the 12 bp of IR. (B) Primer extension assays of in vitro transcription from *Yp1* templates. (C) Primer extension assays of in vitro transcription from *Yp2* templates with or without IR or IRsub3 inserted at −160 relative to *Yp2*.

ative to these promoters. Also, IR must be recognized by a transcriptional activator protein(s) found in both Kc0 cells and embryos.

IR is a follicle cell-specific activator of *Yp* expression in vivo. The *Yp* genes are normally expressed only in adult females, in fat body tissue and in ovarian follicle cells at middle stages of oogenesis. We investigated the role of IR in directing these developmental specificities by introducing the in vitro-characterized IR mutation into a reporter construct, pCR1. The pCR1 construct is a germ line transformation vector in which the *alcohol dehydrogenase* gene (*Adh*) of *Drosophila melanogaster* and the β-galactosidase gene (*lacZ*) of *E. coli* are fused to *Yp1* and *Yp2*, respectively (Fig. 4A). The resulting fusion proteins can be assayed independently either by quantitative spectrophotometric assays, which accurately reflect transcript levels (41), or by histochemical staining. Like *Yp1* and *Yp2*, the fusion proteins are expressed only in the fat body tissue and follicle cells of adult females.

Mutation of IR affected the level of follicle cell-specific expression of both reporter genes. Histochemical staining of dissected ovaries and spectrophotometric assays performed on ovary extracts showed a threefold reduction in ADH and β-galactosidase activity in the mutant relative to the parental transformed flies (Fig. 4A and B). However, neither the spatial nor the temporal pattern of fusion gene expression was altered: follicle cells stained in the IR mutant and parental transformed flies were of the same subtype and at the same developmental stages (β-galactosidase staining shown in Fig. 4A).

In contrast, mutation of IR had no effect on fat body expression of either fusion gene or on expression of those genes in any other tissue. Neither histochemical staining (data not shown) nor spectrophotometric assays of extracts made from dissected flies revealed any significant difference in timing, distribution, or level of activity of the fusion gene products (Fig. 4C).

To confirm that IR has no significant effect on fat body expression, we introduced the IR mutation into another reporter construct truncated at −322 relative to *Yp1*. This second reporter construct fuses the first codon of *Yp1* to *lacZ* and is expressed only in female fat body tissue, because unlike pCR1, it does not include the ovarian enhancers OE1 and OE2 (40, 41). As before, there was no detectable difference in fat body expression between IR mutant and parental transformed flies, either by histochemical staining or by spectrophotometric assays (data not shown).

Taken together, these results demonstrate that IR is a follicle cell-specific element that positively regulates transcription from both the *Yp1* and *Yp2* promoters. IR may have no role in fat body expression of *Yp* genes, or its loss may be compensated by redundant regulatory circuits.

***dGATAb* is expressed in ovaries and is predicted to encode an ovary-specific protein.** IR is a perfect inverted repeat with no apparent homology to any protein binding site in sequence databases (including TFD [27]). However, IR can also be read as two identical overlapping sites, CGATAG (Fig. 2), which resemble the consensus binding site, (A/T)GATA(A/G), derived for the GATA family of transcription factors (reviewed in reference 48). Building on this observation, we found that in mobility shift assays, IR competed with a natural target site in the CD8α gene for binding of the murine GATA-3 factor (reference 37 and data not shown). A *Drosophila* GATA factor that binds IR is therefore likely to be expressed in ovarian follicle cells.

To identify a GATA transcription factor expressed in ovaries, we used three recently characterized *Drosophila* embryonic cDNA clones, *dGATAa* (51, 67), *dGATAb* (1), and *dGATA-2* (64), to probe RNA in Northern blots. Of the three, only *dGATAb* gave a detectable signal against ovarian RNA. Comparable *dGATAb* signals were detected in RNA from embryos and the embryonically derived Kc0 and SL2 cell lines. Much-lower-level signals were obtained with RNA from whole adult males and females minus ovaries (Fig. 5A).

Because they are first detected approximately 3 h after fertilization, *dGATAb* transcripts in the embryo are zygotic in origin (1). Hence, in ovaries, *dGATAb* is most likely expressed in the follicle cells (59). This cell type specificity is confirmed by in situ hybridization to whole-mount ovaries (42).

The Northern blots revealed at least three *dGATAb* transcripts differing by approximately 250 nucleotides in length. Only the smallest was detected in ovaries, and only the middle one was detected in embryos. In RNase (Fig. 5B) and S1 nuclease protection experiments (data not shown) with a radiolabeled probe from the 5′ end of the embryonic cDNA, the major protected species in ovary RNA was significantly shorter than that detected in embryo RNA. The 5′ end of this pro-

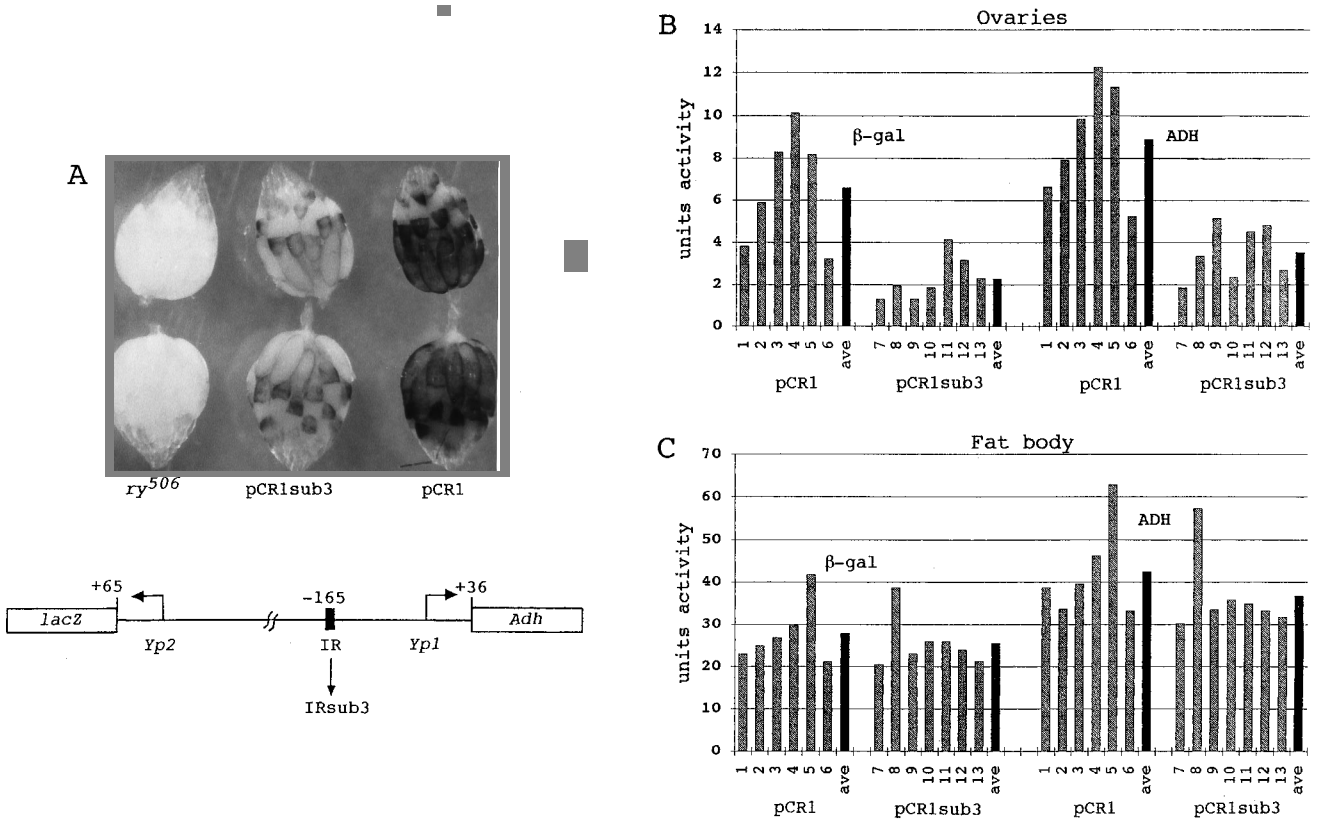

FIG. 4. Effect of IR on *Yp* reporter gene expression in vivo. (A) Diagrams of pCR1 and pCR1sub3 (not to scale) and photo of ovaries from untransformed and pCR1- or pCR1sub3-transformed adult females, stained with the substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Magnification, ×20. The dark pattern results from β-galactosidase activity from the *Yp2-lacZ* moiety of pCR1 or pCR1sub3. (B) Spectrophotometric assays of β-galactosidase and ADH activities in ovarian extracts. Shaded bars represent the average of at least two β-galactosidase or ADH activity assays of a transformed line. Each black bar represents the average β-galactosidase or ADH activity for a set of transformed lines. Background ADH and β-galactosidase levels were negligible. Lines 1 to 6 are transformed with pCR1, and lines 7 to 13 are transformed with pCR1sub3. (C) β-galactosidase and ADH activity in extracts made from female flies with ovaries removed, representative of fat body tissues.

tected species mapped within the open reading frame, at approximately the 20th codon of the embryonic cDNA (1). Either this is the genuine 5′ end of the ovarian transcript and represents an alternative site of transcription initiation or there is an alternative ovary-specific 5′ exon, not detected with the embryonic cDNA probe. The 5′ end of the major protected species in Kc0 RNA also differed from that in the embryonic RNA but mapped in the noncoding leader.

These results show that *dGATAb* is the only known GATA gene whose expression pattern is consistent with a role in regulating follicle cell-specific transcription. Furthermore, *dGATAb* encodes at least two different proteins, one found predominantly in embryos and the other found predominantly in ovaries. The two proteins share a putative DNA binding domain, as central portions of the transcripts including the GATA zinc finger sequences (1) are identical (data not shown); however, they may have other distinct properties, for example, at the level of protein-protein interactions (see Discussion).

Bacterially expressed dGATAb binds to IR. To determine whether dGATAb binds to IR, we used bacterially expressed protein for mobility shift and DNase I protection experiments. In mobility shift assays (Fig. 6A), a radiolabeled, double-stranded oligonucleotide including IR was strongly shifted in an extract from *E. coli* expressing dGATAb in the sense, but not the antisense, orientation (the multiple shifts are due to degradation of the dGATAb protein [1]). The radiolabeled complexes were inhibited by a 100-fold excess of unlabeled, double-stranded oligonucleotides either identical to the probe (IRwt), mutated outside of IR (IRa), mutated in only one of the two GATA motifs (IRb), or carrying the CD8α binding site for murine GATA-3 (CD8α). They were not inhibited by oligonucleotides carrying different mutations in IR (IRc and IRd) or in the CD8α site (data not shown) or by an unrelated oligonucleotide (Ctrl). At lower concentrations of competitor, where other competitors were still fully effective, the oligonucleotide with one intact GATA motif (IRb) only partially inhibited the complexes (data not shown). We conclude that bacterially expressed dGATAb binds to IR in a specific way and also that it can recognize a single GATA motif in IR but at a lower efficiency (see below).

In further confirmation of this interaction, bacterially expressed dGATAb protected IR, but not mutated IR, from DNase I digestion (Fig. 6B). The protected region included at least 10 of the 12 bp of IR (labeled noncoding strand) and extended no further than IR (labeled coding strand).

A protein in Kc0 extract has properties similar to those of dGATAb. The dGATAb transcription factor is probably active in Kc0 cells, since IR was identified by in vitro transcription with extracts from these cells (Fig. 1 and 3) and they produce *dGATAb* but not *dGATAa* or *dGATA-2* transcripts (Fig. 5 and data not shown). In mobility shift experiments, results with

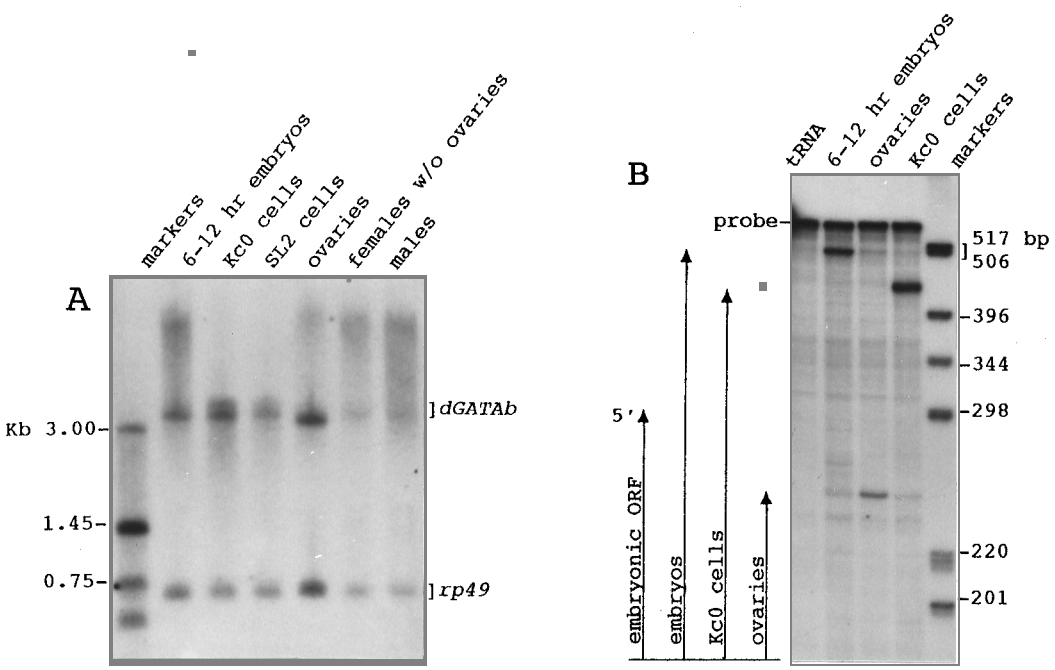

FIG. 5. *dGATAb* transcript analysis. (A) Northern blot probed with a ^{32}P-labeled fragment of *dGATAb* and *rp49* (46). Approximately 12 μg of total RNA from the indicated sources was loaded in each lane. Markers are restriction-digested *rp49* DNA. (B) RNase protection experiment with a uniformly labeled probe corresponding to the 5′ end of *dGATAb*. RNA sources are marked above each lane. The diagram to the left indicates homologous portions of the different transcripts relative to the open reading frame (ORF) at the 5′ end of the embryonic cDNA.

heat-treated Kc0 extract (Fig. 7A) were indistinguishable from those obtained with bacterially expressed dGATAb (Fig. 6A), except that only one slowly migrating complex was detected, even after long exposure of the autoradiogram or at higher protein concentrations (data not shown). Furthermore, DNase I protections with Kc0 extract (Fig. 7B and data not shown) and bacterially expressed dGATAb (Fig. 6B) were indistinguishable over IR. Adjacent protections seen with Kc0 extract were due to proteins binding independently of IR (Fig. 7B).

These results suggest that the IR-binding protein detected in Kc0 nuclear extract is dGATAb; this protein is probably responsible for transcriptional activation from IR in vitro.

dGATAb binds to single GATA motifs of IR. IR is an atypical GATA-binding site in that it is a perfect inverted repeat (Fig. 2). Most GATA-binding sites contain a single GATA motif, and less frequently, they contain two. The two-motif sites are thought to be functionally distinct (44). The motifs can occur in all possible orientations but are generally asymmetric and separated by 3 to 30 bp (reviewed in reference 48).

The unusual structure of IR led us to investigate whether the motifs are equivalent and whether IR binds one or two molecules of dGATAb (e.g., see references 17, 18, 44, and 65). We therefore mutated each GATA motif in IR singly, while leaving the other intact. Whether they had one or the other GATA motif, or both as in wild-type IR, radiolabeled oligonucleotides shifted to the same respective positions (Fig. 8). This indicates that only one molecule of dGATAb at a time can occupy IR and that both GATA motifs can be recognized. Also, at the same specific activity, oligonucleotides with one GATA motif shifted at approximately half the efficiency of IR or less, consistent with mobility shift competition results.

Since DNase I protection extended over the whole of IR, the two GATA motifs appear to be filled with equal frequency under these binding conditions (Fig. 6B and 7B). The bidirectional activity of IR on *Yp1* and *Yp2* may then depend on alternate occupancy of the motifs; alternatively, perhaps the motifs are equivalent in function.

DISCUSSION

Role of dGATAb in follicle cell-specific transcription of *Yp* genes. Using an in vitro transcription assay, we have identified IR, a 12-bp DNA element in the intergenic region of *Yp1* and *Yp2*. In vivo, this single element activates both *Yp1* and *Yp2* reporter gene expression, but only in ovarian follicle cells; it has no detectable effect in fat body or any other tissue.

We have further shown that IR is a binding site for GATA transcription factors and that dGATAb is likely to be the member of this family relevant to follicle cell-specific expression of *Yp* genes. First, of three known *Drosophila* GATA factors, only *dGATAb* is expressed in ovaries. The ovarian *dGATAb* transcript is uniquely spliced and/or initiated, with the major embryonic and ovarian transcripts encoding different isoforms of the dGATAb protein. Second, bacterially expressed dGATAb protein binds IR specifically. Finally, a DNA-binding protein with properties similar to those of bacterially expressed dGATAb is also detected in the Kc0 nuclear extracts used to demonstrate the transcriptional activity of IR.

Broader role of dGATAb. The complex pattern of expression of *dGATAb* in follicle cells (42) and the recent isolation of a *Bombyx mori* GATA gene which encodes a follicle cell-specific factor binding to chorion genes (16, 56) suggest a general role for GATA factors in the development of insect follicle cells during oogenesis. We anticipate that dGATAb will regulate not only *Yp* genes but also other genes expressed in the broader program of follicle cell differentiation. These may include, for example, the genes encoding chorion, vitelline membrane proteins, and dorso-ventral markers (reviewed in reference 59).

However, identifying binding sites for dGATAb in other

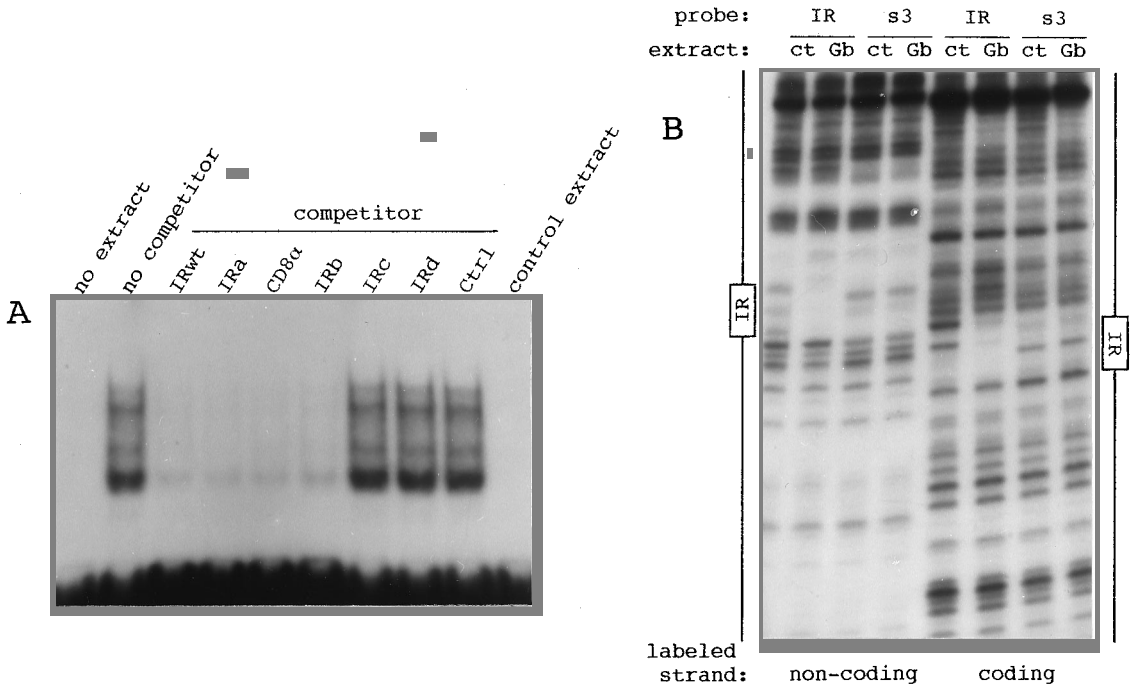

FIG. 6. Bacterially expressed dGATAb binds to IR. (A) Mobility shift and competition assays with bacterially expressed dGATAb. No extract was added in the first lane, and control antisense dGATAb extract was used in the last lane. The ^{32}P-labeled probe was IRwt, a double-stranded oligonucleotide with wild-type sequence; unlabeled competitor, as indicated above each lane, was added at a 100-fold molar excess over the labeled probe. Sequences are given in Materials and Methods. (B) DNase I protection of IR and IRsub3. Four 148-bp fragments including IR or IRsub3 were made by PCR. The noncoding strand of the IR and IRsub3 (s3) fragments was labeled with ^{32}P in the left lanes; the coding strand was labeled in the right lanes. The position of IR is marked by a box. The same binding and DNase I digestion (5 ng) conditions were used for all lanes. As indicated, one lane of control antisense bacterial extract (ct) alternates with one lane of dGATAb extract (Gb).

follicle cell-specific genes is complicated by the observation that GATA factors bind to a much wider range of sequences than suggested by the original (A/T)GATA(A/G) consensus. In random site selection assays, many of the sites bound at high affinity by GATA-1, -2, and -3 did not conform to this consensus, and some did not include the core motif GATA (34, 45, 66). Within the *Yp* intergenic region, at least one GATA motif outside of IR was not recognized by bacterially expressed dGATAb (35).

Adult fat body tissue is descended from founder populations of embryonic cells similar to imaginal disc cells (reviewed in reference 52). Given its proposed role in the development of embryonic and larval fat body, dGATAb might also be expected to function in adult tissue. However, mutation of IR had no discernible effect on fat body expression of three different *Yp* reporter genes. One possibility is that dGATAb can function through alternate binding sites or that its function at IR is fully compensated by redundant proteins. Another possibility is that different combinatorial interactions (63) determine embryonic, larval, and adult fat body-specificity. The *Adh* genes, for example, are organized as two separate genes (*Drosophila mulleri* [19]) or have alternate promoters independently regulated by larval and adult enhancers (*D. melanogaster* and other species [14]). In this case, either dGATAb is not present or it does not interact productively with the spectrum of proteins that control expression in adult fat body tissue.

Mechanism of transcriptional activation by dGATAb. In vertebrates, GATA-binding sites are remarkable for the variety of positions which they occupy relative to the genes that they regulate. The sites are found in locus control regions (chromosomal position effect insulators), enhancers, or upstream promoter regions or in place of the canonical TATAA box (reviewed in references 10 and 48). This is matched by an apparent diversity of mechanisms, examined most thoroughly for GATA-1. In some contexts, GATA-1 directly activates transcription (e.g., see references 18 and 44); in others, it mediates the activity of DNA-bound factors (e.g., see references 18, 22, and 29), contributes to the establishment of an open chromatin structure (e.g., see references 9 and 43), or displaces a repressor (50). GATA-1 can also negatively regulate transcription, by interfering with the activity of another factor (21) or by excluding basal factors (3). These observations suggest a capacity for multiple protein-protein interactions: GATA-1 is indeed detected in multiprotein complexes (49) and has several activation domains (44, 50, 69).

Like other members of the GATA family, dGATAb is probably a versatile protein with more than one mode of action. In cultured cells, in common with all of the GATA factors, dGATAb activates transcription when cotransfected with a minimal reporter construct carrying binding sites for the protein (1). No other DNA-bound factors are required, although coactivators present in the cultured cells may be needed.

In the organism, by contrast, dGATAb does not appear to function by itself, even from multiple binding sites (reference 20 and data not shown). Instead, analysis of *Adh-1* regulation suggests that dGATAb requires a partner activator protein. For *Adh-1*, this protein binds to the regulatory element Box B: both Box B and a small element including the dGATAb-binding site are necessary, but neither alone is sufficient to activate transcription from the natural *Adh-1* promoter. However, Box B alone can activate expression in fat body tissue from a heterologous promoter; adding the dGATAb element then activates expression in other tissues (1, 20).

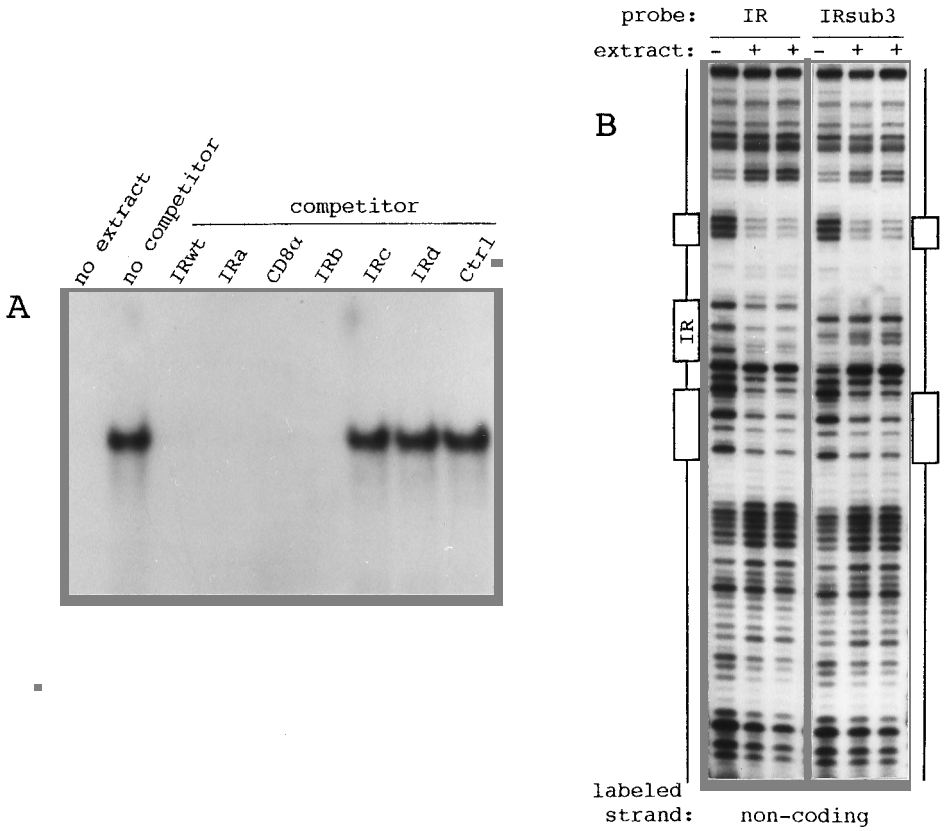

FIG. 7. An activity in Kc0 nuclear extract binds to IR. (A) Mobility shift and competition assays with heat-treated Kc0 nuclear extract. No extract was added in the first lane. Probe and competitors are as described in the legend to Fig. 6A. (B) DNase I protection of the IR and IRsub3 fragments was as described in the legend to Fig. 6B. Only the noncoding strand is shown. IR is marked by a box; additional protections upstream and downstream of IR are also marked by boxes. Lanes with no extract were digested with 33 ng of DNase I; other lanes were digested with 33 and 50 ng of DNase I, respectively.

dGATAb probably fills a similar mechanistic role in regulating the *Yp* genes in vivo. Thus, for *Yp1* and *Yp2*, we surmise that a partner element for IR equivalent to Box B must lie in the sequences included with *Yp* reporter genes, perhaps within the previously described follicle cell-specific enhancers OE1 and OE2 (40, 41).

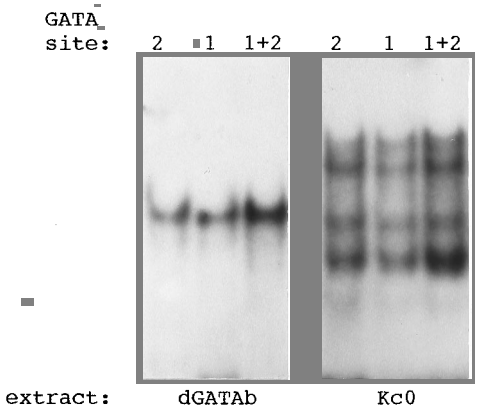

FIG. 8. dGATAb binds one or the other GATA motif but not both. Mobility shift assays. ^{32}P-labeled probes are IRwt with two intact GATA motifs (sites 1+2), IRb with one 5′ motif (site 1), and IRe with one 3′ motif (site 2). Bacterially expressed dGATAb was used for the left gel and generates multiple band shifts due to protein degradation, as in Fig. 6A. Heat-treated Kc0 nuclear extract was used for the right gel, as in Fig. 7A. Band positions in the two gels are not directly comparable.

Summary. We have identified an element activating follicle cell-specific expression from the *Yp1* and *Yp2* promoters. This element binds the transcription factor dGATAb, which occurs as distinct isoforms in ovaries and in embryos. We suggest that dGATAb is involved in at least two major developmental pathways: development of the embryonic and larval fat body tissues, in which it regulates *Adh* expression (1), and differentiation of the follicle cells during oogenesis, in which it regulates *Yp* expression.

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