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The arflike gene encodes an essential GTP-binding protein in *Drosophila*

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ABSTRACT **We have identified a *Drosophila* gene (arflike, *arl*) encoding a protein that is structurally related (≈55% identity) to the ADP-ribosylation factors (ARFs) of yeast and mammals. Biochemical analyses of purified recombinant *arl*-encoded protein revealed properties similar to the ARF proteins, including the ability to bind and hydrolyze GTP. Clear functional differences between arl and ARF proteins, including a complete lack of ARF activity, suggest that arl is not a functional homolog of ARF. A recessive lethal *arl* mutation was recovered, demonstrating that the *arl* locus is an essential gene. We conclude that the *arl* locus encodes an essential member of the ARF subfamily of small GTP-binding proteins in *Drosophila*.**

GTP-binding proteins (G proteins) participate in a wide range of biological processes, including signal transduction, protein synthesis and secretion, and cellular proliferation. One family of this group of proteins, the small (≈20 kDa) GTP-binding proteins (SGBPs), has received considerable attention because it includes the oncogenic p21 ras proteins (1). Although dozens of SGBPs have been identified, relatively little is known about their biochemical or biological properties in any multicellular organism.

Within the SGBP family, four subfamilies of structurally related proteins have been identified: the ras and related proteins (including the R-ras, rap, and ral proteins), the rho proteins (including the rac and CDC42 proteins), the SEC4/YPT1 proteins (including the rab proteins), and the ADP-ribosylation factor (ARF) proteins. ARF is unique among SGBPs in that it was originally purified from animal tissues based on a specific biochemical assay: ARF serves as cofactor in the cholera toxin-dependent ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase, G_s (ref. 2; reviewed in refs. 3 and 4). In each organism examined to date, multiple ARF genes encode highly conserved 21-kDa proteins that exhibit structural similarities to both the p21 ras-related proteins and the α (GTP-binding) subunits of the trimeric G-protein family (5). ARFs bind guanine nucleotides but possess low GTPase activities relative to other SGBPs. Genetic studies in yeast have shown that a number of SGBPs, including ARFs, regulate protein secretion (6–8). Consistent with this newly discovered role for ARF proteins in the secretory process, ARF is highly concentrated in the Golgi apparatus of mammalian cells (6).

Elucidating the exact cellular functions of SGBPs, including ARFs, in higher eukaryotic organisms has proven to be a difficult task. *Drosophila melanogaster*, with its extensively characterized genome and available genetic and molecular techniques, offers a powerful system for the analysis of SGBP function in a multicellular organism. Three *Drosophila* SGBP genes (*Ras1*, *Ras2*, and *Ras3*) have been previously identified by low-stringency hybridization with mammalian *ras*-derived probes (9), but little is known about the biochemical and functional properties of their protein products in flies. The protein encoded by the *Drosophila Ras2* gene has been altered *in vitro* and reintroduced into flies by P-element-mediated transformation. Expression of this altered protein under the control of a heat-inducible promoter has been shown to cause a variety of tissue abnormalities (10). Mutations in the three wild-type *Drosophila ras*-related genes have not been reported.

This report describes the genetic, molecular, and biochemical characterization of a SGBP in *D. melanogaster* encoded by the arflike (*arl*) locus.** The *arl*-encoded SGBP is a member of the ARF subfamily of SGBPs, based on structural analysis, but appears to have a different biological function. Genetic studies of the *arl* locus are presented that provide a clear demonstration of an essential role for a SGBP in a multicellular eukaryote. As a result, we conclude that *arl* is a member of the family of SGBPs with potentially unique functions in cellular physiology.

MATERIALS AND METHODS

Construction of a *Drosophila* cDNA Library. RNA for the construction of the cDNA library was isolated (11) from 0- to 22-hr (at 25°C) embryos of an isogenic strain (iso-1). The cDNA library was constructed in the phage vector λgt11 by a modification (12) of the method of Gubler and Hoffman (13). Approximately 600,000 independent recombinants were amplified, resulting in a library that is >70% recombinant. To isolate an *arl* cDNA clone, standard techniques were used to screen 100,000 independent recombinants with a 7-kilobase (kb) genomic *Bam*HI fragment spanning the sequence shown in Fig. 1.

DNA Sequencing. DNA fragments were subcloned into plasmid vectors and directional deletions were generated by using the Erase-a-Base kit (Promega Biotec). Double-stranded plasmid DNA was prepared by the alkaline lysis method (14), purified by adsorption to glass (15), and sequenced by the chain-termination method (16) using the Sequenase kit (United States Biochemical). The sequences of both strands of DNA were determined for all reported sequences.

Abbreviations: G protein, GTP-binding protein; SGBP, small G protein; ARF, ADP-ribosylation factor; ORF, open reading frame; IPTG, isopropyl β-D-thiogalactopyranoside; GTP[γ-^{35}S], guanosine 5′-[γ-[^{35}S]thio]triphosphate.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. M61127).

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Expression and Purification of arl Protein. An *Nde* I site was engineered into the *arl* cDNA at the initiating methionine and a *Bam*HI site was added 6 base pairs (bp) downstream of the stop codon by using synthetic primers and the *arl* cDNA as template in a polymerase chain reaction. The amplified 550-bp *Nde* I/*Bam*HI fragment containing the complete coding sequence for *arl* was ligated into the *Nde* I and *Bam*HI sites of the pET3C expression vector putting the open reading frame (ORF) under the control of the T7 promoter (17, 18). BL21(DE3) cells, which carry the T7 polymerase under control of the *lacZ* promoter, were transfected with pJCD1-6 and grown to a density of A_{600} = 0.3–1.0 in LB with 50 μg of ampicillin per ml. Expression of *arl* was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 120 min in IPTG, cells were harvested by centrifugation at 10,000 × *g* and stored at −80°C until used. The pET3C vector and BL21(DE3) cells were the generous gift of William Studier (Brookhaven National Laboratory, Upton, NY). The recombinant protein was purified from bacterial lysates as described for the purification of recombinant bovine ARF1 (19).

Analysis of Bound Nucleotides. The amounts and types of noncovalently bound nucleotides that copurify with the recombinant protein were determined as described (19). After denaturation of protein at 95°C for 10 min, the proteins were removed by ultrafiltration and released nucleotides were analyzed by HPLC. The binding of radiolabeled nucleotides to *arl* was determined by nitrocellulose filter trapping (20). The nucleotide exchange reaction was performed for 60 min at 30°C in a buffer containing 25 mM Tris·HCl (pH 7.5), 100 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 3 mM dimyristoyl-L-α-phosphatidylcholine, 0.1% sodium cholate, and 1 μM GTP[γ-^{35}S] (10^4–10^5 cpm/pmol). GTP[γ-^{35}S] was purchased from New England Nuclear.

Mutagenesis. Males homozygous for a third chromosome from the isogenic strain iso-1 were fed ethyl methanesulfonate (21). Fertile sons with mutagenized third chromosomes were individually crossed to females carrying a deficiency for the *arl* region [*Df(3L)th102* (22)] and newly induced lethal and visible mutations were identified. To determine which of the mutations were in the *arl* gene, a genomic restriction fragment spanning the *arl* gene was reintroduced into the genome by P-element-mediated transformation (23) using the CaSpeR vector of Pirrotta (24). Plasmid DNA was coinjected with a transposase helper plasmid, pπ25.7wc (25), into embryos from a *y Df(1)w67c2* strain (22). Transformants were isolated and tested for their ability to rescue mutations in the region.

Miscellaneous Methods. ARF assays (20) were performed with purified recombinant $G_{s\alpha}$ (the generous gift of Michael Graziano, Merck, Sharp & Dohme) as substrate. Protein determinations (26), GTPase assays (20), immunoblotting (27), and polyacrylamide (13%) gel electrophoresis (28) were performed as described. Cholic acid was purchased from Fluka; other reagents, including DMPC, were obtained from Sigma.

RESULTS

Identification and Molecular Characterization of the *Drosophila arl* Gene. The *Drosophila* gene encoding a member of the family of SGBPs was fortuitously identified during studies of the brahma locus, a trans-activator of homeotic gene expression (29). To clone the brahma gene, we conducted a chromosome walk in salivary gland polytene chromosome region 72AB. RNA blot hybridizations with probes derived from our chromosome walk revealed the presence of a transcription unit encoding a 1.05-kb mRNA immediately proximal to the brahma gene. To learn more about this gene and its products, we sequenced the region of our chromosome walk that hybridized to the 1.05-kb mRNA on RNA blots (Fig. 1). In

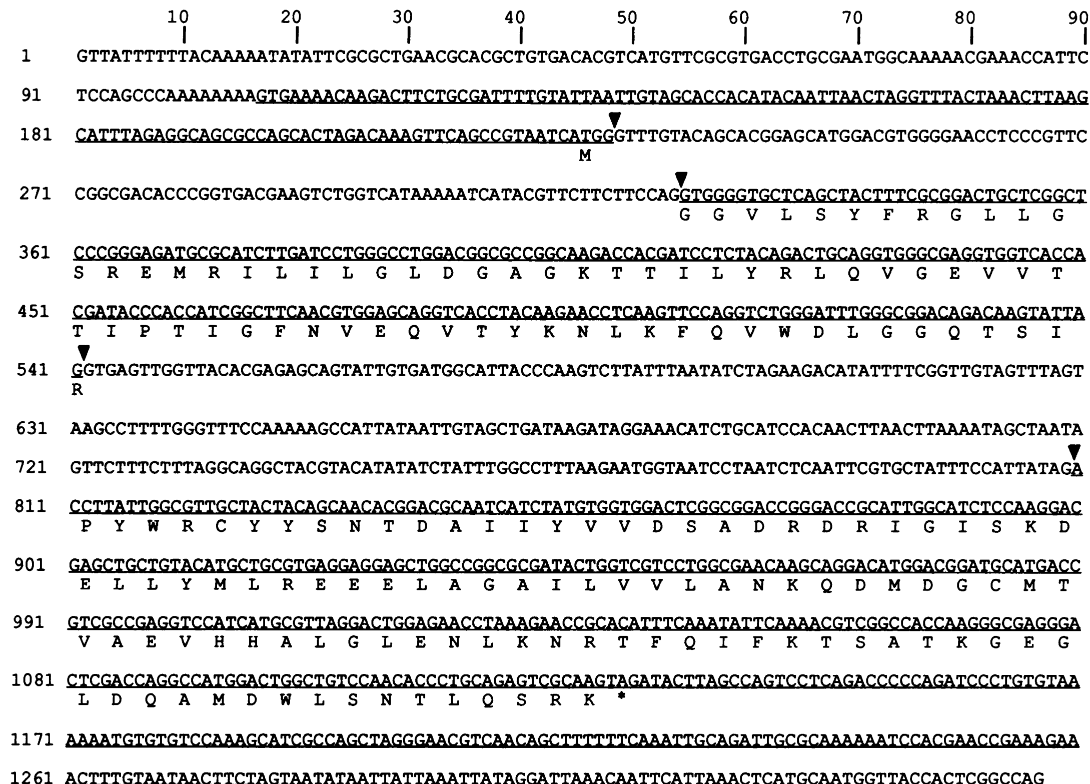

FIG. 1. Sequence of the *arl* gene and mRNA. The sequence of the region of the genome encoding the mRNA represented by cDNA-1 is shown in a centromere distal to proximal orientation. The complete sequence of cDNA-1 is underlined and the positions of exon boundaries are marked by arrowheads. The translation of the long ORF in cDNA-1 is shown by the single-letter amino acid code. The termination codon at the end of this ORF is marked with an asterisk.

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bARF1:  MGNIFANLFKGLFGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKNIS  62
yARF1:  MGLFASKLFSNLFGNKEMRILMVGLDGAGKTTVLYKLKLGEVITTIPTIGFNVETVQYKNIS  62
  arl:  MGGVLS YFRGLLGSREMRILILGLDGAGKTTILYRLQVGEVVTTIPTIGFNVEQVTYKNLK  61
        MG      F  L G *EMRIL**GLD AGKTT*LY*L *GE**TTIPTIGFNVE V YKN*

bARF1:  FTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERVNEAREELMRMLAEDELRDAVLLVF 124
yARF1:  FTVWDVGGQDRIRSLWRHYYRNTEGVIFVVDSNDRSRIGEAREVMQRMLNEDELRNAAWLVF 124
  arl:  FQVWDLGGQTSIRPYWRCYYSNTDAIIYVVDSADRDRIGISKDELLYMLREEELAGAILVVL 123
        F VWD*GGQ  IR  WR Y* NT  *I*VVDS DR R*   ** *  ML E*EL  A  *V

bARF1:  ANKQDLPNAMNAAEITDKLGLHSLRHRNWYIQATCATSGDGLYEGLDWLSNQLRNQK      150
yARF1:  ANKQDLPEAMSAAEITEKLGLHSIRNRPWFIQATCATSGEGLYEGLEWLSNSLKNST      150
  arl:  ANKQDMDGCMVTAEVHHALGLENLKNRTFQIFKTSATKGEGLDQAMDWLSNTLQSRK      149
        ANKQD*   M  AE*   LGL  ** R * I  S AT G*GL   **WLSN L
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FIG. 2. Similarities between the arl protein encoded by cDNA-1 and ARFs from yeast and mammals. The complete sequences of yeast ARF1 [yARF1 (5)] and bovine ARF1 [bARF1 (5)] are aligned with the complete sequence of the *Drosophila* arl protein. Amino acids common to all three forms are listed below the sequences, while conserved residues (I/L/V/M, S/T, R/K, F/W/Y, N/Q, and E/D) are marked with an asterisk.

addition, we isolated a cDNA clone corresponding to this transcript by screening a *Drosophila* embryonic cDNA library with a restriction fragment from our chromosome walk that spans the sequence presented in Fig. 1. From 100,000 independent recombinants, one cDNA (cDNA-1) was isolated that corresponds to the 1.05-kb mRNA, as evidenced by hybridization to this transcript on blots of *Drosophila* RNA isolated from various stages of development (data not shown). The hybridization signal seen on RNA blots indicates that the level of expression of the 1.05-kb mRNA is fairly constant throughout development. By probing RNA blots with strand-specific RNA probes derived from cDNA-1, the direction of transcription of the 1.05-kb mRNA was determined to be distal to proximal—i.e., from the telomere toward the centromere (data not shown).

The sequence of the insert contained in cDNA-1 was determined on both strands (Fig. 1). The insert is 791 nucleotides long and can be perfectly matched to the corresponding genomic sequence with interruptions by introns 96 and 278 nucleotides long. The sequence at each of the exon/intron boundaries matches the consensus sequences for 5′ and 3′ splice sites (30, 31). Assuming an average poly(A) tail length of 100–200 bases, the cDNA-1 insert [which lacks a poly(A) tail] is nearly full length.

A 540-base ORF consistent with the direction of transcription was found in the sequence of cDNA-1 (Fig. 1). Two potential initiation codons (at nucleotides 225 and 369) are preceded by a termination codon (at nucleotide 177) upstream of the first potential initiation codon, precluding the use of an initiation codon further upstream. The sequence surrounding the first initiation codon exhibits a better match to the initiation codon context for *Drosophila* (32) than does the sequence surrounding the second potential initiation codon. Furthermore, though indirect, support for the use of the first AUG for the initiation of translation is derived from the similarity of the predicted translation product to previously identified proteins (see below). If the AUG at nucleotide 225 is used to initiate translation, the predicted product of the 1.05-kb mRNA is a protein of 180 amino acids with a molecular weight of 20,250 (Figs. 1 and 2). A search of the GenBank and EMBL protein sequence data bases revealed a significant degree of similarity with a number of members of the family of small molecular weight G proteins as well as members of the family of trimeric G proteins. These sequence similarities are essentially restricted to the previously defined consensus GTP-binding domains (33). For the 20-kDa G proteins, the presence of guanine nucleotide-binding domains results in 20–25% sequence identities. In contrast, a much greater degree of similarity appears (≈55% identity, ≈70% conservation) when the *arl* sequence is aligned with members of the ARF subfamily (Fig. 2). This alignment reveals an extensive degree of sequence conservation throughout the length of the protein. In addition, a consensus sequence for amino-terminal myristoylation (34) is present in the *Drosophila* protein, as in most ARF proteins. Given these similarities, we have named the locus encoding this member of the family of SGBPs arflike (*arl*). The high degree of similarity between *arl* and ARFs led us to investigate whether *arl* is a functional homolog of ARF in *Drosophila* or a newly discovered type of SGBP with distinct cellular functions.

Purification of the *Drosophila arl* Gene Product. To allow the biochemical characterization of the product of the *arl* gene, the ORF of cDNA-1 was inserted into the pET3C expression vector (17, 18). Upon addition of IPTG to bacteria carrying this plasmid, pJCD1-6, a single protein band with an apparent molecular mass of ≈21 kDa, was induced in a time-dependent manner to an estimated 5–10% (after 120 min) of Coomassie blue-stained material on SDS gels (Fig. 3, lane 2). Sequential ion-exchange and gel-filtration chromatographies resulted in a protein that is at least 85% pure, as determined by laser densitometry of Coomassie blue-stainable material (lane 5). The induced 21-kDa band in cell extracts, as well as the purified protein, reacted strongly on immunoblots (data not shown) with an affinity-purified antibody generated against a synthetic peptide from a well-conserved region of ARF proteins (35) that is present in *arl* (residues 22–35).

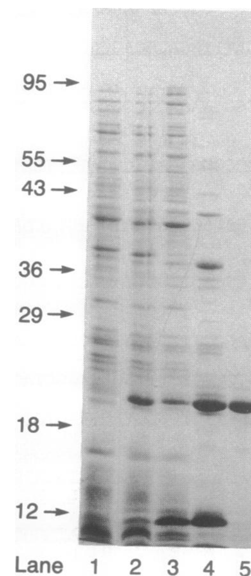

FIG. 3. Expression and purification of *arl* in bacteria. Cell lysates from BL21 cells containing the expression plasmid pJCD1-6 at $t = 0$ (lane 1) or after 120 min of induction (lane 2) are shown. The prominent band running at 21 kDa was purified as described. Samples from different stages of purification are shown: lane 3, cell extract after lysozyme and Triton X-100 lysis (added lysozyme is seen running at 12 kDa); lane 4, DEAE-Sephacel pool; lane 5, Ultrogel AcA 54 pool. Lanes 1–4 were each loaded with 10 μg of total protein and lane 5 has 3 μg of protein.

arl Protein Binds Guanine Nucleotides and Hydrolyzes GTP. Like many of the previously characterized G proteins (20, 36, 37), purified *arl* preparations were found to contain bound guanine nucleotides. Three different preparations of *arl* were examined for the presence of bound guanine nucleotides. In each case, only guanine nucleotides (0.91 ± 0.21 mol of guanine nucleotide per mol of protein; $n = 3$) were detected. In contrast to ARF proteins (20), both GDP (55% ± 9.9%) and GTP were found in ultrafiltrates from *arl* preparations. These results indicate that all, or nearly all, of the recombinant protein is capable of binding guanine nucleotides.

A radioligand exchange assay was used to characterize the requirements and kinetics of guanine-nucleotide binding to *arl*. Optimal conditions for the binding of guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[γ-^{35}S]) were determined at 30°C to be 25 mM Hepes, pH 7.5/100 mM NaCl/0.5 mM $MgCl_2$/1 mM EDTA/3 mM DMPC/0.1% sodium cholate. Free magnesium concentration in this mixture is estimated at 1–10 nM (19). Under these conditions, *arl* was found to release GDP with a $t_{1/2}$ of 5.5 min (data not shown). This value, and the rate of association of GTP[γS] (Fig. 4), is consistent with release of bound GDP being the rate-limiting step in binding of activating ligands, as has been demonstrated for a number of G proteins (37, 38). Omission of lipid and cholate results in the loss of ≈50% of maximal binding (Fig. 4). This effect was found to result from a decrease in the extent of binding rather than the rate. Higher levels of magnesium (10 mM) or sodium chloride (0.8 M) were also found to decrease binding of GTP[γ-^{35}S] to *arl* by dramatically decreasing the rate of release of bound GDP. Similar effects of salt and magnesium ions have been reported for recombinant bovine ARF1 (19).

The relative affinities of GDP, GTP, and GTP[γS] for binding arl were determined by the competition for GTP[γ-^{35}S] binding. Protein (5 nM) was incubated at 30°C with 30 nM (1 μCi; 1 Ci = 37 GBq) GTP[γ-^{35}S] and various amounts of each of the nucleotides. At steady state (60 min), binding of GTP[γ-^{35}S] was determined as described in *Materials and Methods*. Fifty percent of maximum binding was observed at 85, 680, and 235 nM, for GDP, GTP, and GTP[γS], respectively.

The rate of GTP hydrolysis catalyzed by arl was determined and found to be constant for at least the first 10 min and then decreased. An estimate of the rate of GTPase activity was derived by normalizing the initial rate of GTP hydrolysis to the initial rate of GTP[γS] binding under identical conditions. The derived rate, 0.05 min^{-1}, represents the lower limit of the actual rate of hydrolysis of GTP catalyzed by arl and is similar to values reported for a number of G proteins (38, 39).

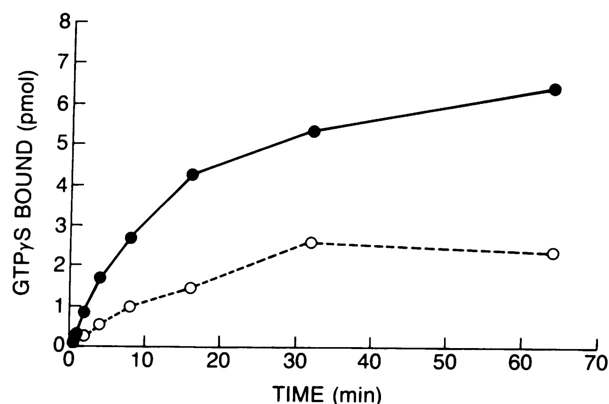

FIG. 4. Time course of the binding of GTP[γ-^{35}S] to recombinant *arl* at 30°C. Binding of the radioligand to *arl* was determined either in standard conditions (●) or in the absence of lipid and cholate (○). At each time point, there was 1.5 μg of purified *arl* in a 20-μl mixture.

***Drosophila* Tissue Contains an ARF Activity Lacking from the *arl* Gene Product.** The ability of arl to serve as cofactor in the cholera toxin-dependent ADP-ribosylation of $G_{s\alpha}$ was determined. This is a sensitive and specific assay for ARF function in crude cell lysates or purified protein preparations, allowing detection of subpicomole amounts of ARF protein with no activity detected for any other proteins including other members of the family of SGBPs. Under the conditions of this assay, purified recombinant bovine ARF1 and bovine brain ARF have identical specific activities (19). Purified recombinant ARF proteins from four different species were examined: bovine ARF1p, human ARF4p, yeast ARF1p, and arl. All but arl had easily detectable ARF activities [0.3–20 pmol per min per pmol of ARF (40)]. Thus, while ARF proteins from diverse species all have activity in the ARF assay, arl is devoid of any such activity. In contrast, the S100 from 1% cholate extracts of homogenized whole flies was found to have significant ARF activity (0.2 fmol per min per μg of extract protein), indicating the presence in flies of a functional ARF homolog. This suggests that arl has a role in cell physiology that is distinct from the ARF protein(s).

Isolation and Identification of an *arl* Mutant. Although several SGBPs have been shown to be essential in yeast, none has been shown to be essential in any multicellular organism. Genetic studies were therefore conducted to determine whether *arl* mutations have an identifiable phenotype. The *arl* locus is located between the distal breakpoints of *Df(3L)th102* and *Df(3L)st-f13* (22). From 3350 mutagenized third chromosomes, 27 zygotic-lethal mutations in this region were recovered that identify five essential genes (an average of 5.4 alleles per gene). To determine which, if any, of the five essential genes represents the *arl* locus, we introduced a genomic DNA fragment spanning the *arl* gene into the germ-line of *Drosophila* by P-element-mediated transformation and assayed its ability to rescue our newly identified mutations in the region. The genomic DNA fragment used in this assay was a 2.5-kb *Pst* I fragment that begins 484 nucleotides distal and terminates ≈700 nucleotides proximal to the 1347-nucleotide sequence shown in Fig. 1. The absence of other intact genes within this genomic fragment was demonstrated by probing blots of RNA isolated from various stages of *Drosophila* development with the 2.5-kb *Pst* I fragment. With the exception of extremely weak hybridization to 5.5- and 3.5-kb transcripts encoded by neighboring genes, no additional transcripts were detected (data not shown). An insertion of this fragment into the second chromosome was tested for its ability to rescue mutations in the five essential genes in the region. One tested mutation (*arl^1*) was completely rescued by the *arl* genomic DNA, demonstrating an essential function for the *arl* gene in *Drosophila*.

DISCUSSION

The *arl* locus of *D. melanogaster* is an essential gene that encodes a newly discovered member of the family of SGBPs. Purification of the recombinant protein from bacteria allowed the direct demonstration that *arl* encodes a G protein. We have described a number of properties for the recombinant protein that appear common to all of the SGBPs, including copurification with bound guanine nucleotides, high-affinity guanine-nucleotide binding, a slow and magnesium-sensitive release of bound GDP, and low GTPase activities ($k_{cat} \geq 0.05$ min^{-1}). We have also identified two characteristics of the nucleotide exchange reaction that are similar to those previously reported as associated with ARF proteins: a partial dependence on lipids and a greater affinity for GDP than for GTP. While nucleotide binding, exchange, and hydrolysis are functions of all the SGBPs, including ARF, the ability to serve as cofactor in the cholera toxin-catalyzed ADP ribosylation of $G_{s\alpha}$ is unique to ARF proteins. We have failed to

detect any ARF activity for the recombinant arl protein in the *in vitro* cofactor assay, even though this activity is readily detectable in all eukaryotes examined to date, including *Drosophila*. Furthermore, unlike the mammalian ARF genes, expression of *arl* has proven incapable of suppressing ARF mutations in yeast (R.A.K., unpublished observation). Our data thus strongly support the hypothesis that arl represents a previously unknown type of SGBP of as yet unknown function, while the functional *Drosophila* homolog of ARF is encoded by a distinct locus.

Although the precise role of *arl* in cellular physiology is unknown, the zygotic lethality of an *arl* mutation clearly demonstrates the essential nature of this SGBP in *Drosophila*. The genetic studies presented here demonstrate an essential role for a SGBP in a multicellular eukaryotic organism. The isolation of the arl^1 mutation and the ability to rescue the mutant phenotype by reintroducing the gene provides a sensitive *in vivo* assay for testing the function of engineered arl proteins. Such studies of engineered arl proteins should provide insights into the function of SGBPs in a complex, multicellular eukaryote.

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