Molecular Characterization of a Conserved, Guanine Nucleotide-Dependent ADP-Ribosylation Factor in *Drosophila melanogaster*†

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ABSTRACT: ADP-Ribosylation factors (ARFs) are ubiquitous ~20-kDa guanine nucleotide-binding proteins that stimulate cholera toxin-catalyzed ADP-ribosylation in vitro. Because the functional role(s) of ARF in mammalian systems is (are) elusive, we looked for ARF in *Drosophila melanogaster*, and report the partial purification and molecular cloning of an ARF from *Drosophila*. We cloned the *Drosophila* ARF 1 gene without library screening by a combination of 5 polymerase chain reactions (PCRs), yielding a 546-base open reading frame encoding 182 amino acids, which are >93% identical to those of mammalian class I ARFs. This ARF gene maps to 79F3-6 in the proximal region of the left arm of *Drosophila* chromosome 3. The *Drosophila* ARF 1 gene structure, including placement of introns, is highly conserved relative to mammalian class 1 ARF genes. A single ARF mRNA species of 1.8 kb was abundant in all *Drosophila* body segments. Recombinant *Drosphila* ARF 1 synthesized in *Escherichia coli* had biochemical and immunochemical activities similar to those of mammalian ARF. The similarities of sequence and biochemical properties between *Drosophila* and mammalian ARFs contrast with their differences from *Drosophila arl* (ARF-like protein), which does not stimulate cholera toxin-catalyzed ADP-ribosylation, and is only ~52–56% identical in amino acid sequence to mammalian ARFs.

Guanine nucleotide-binding proteins participate in diverse eukaryotic physiological processes including transmembrane signaling, cellular differentiation, and neoplastic transformation. They are often considered in two general categories: (1) the heterotrimeric transmembrane signaling G proteins[1] (Casey & Gilman, 1988; Birnbaumer et al., 1990); (2) the family of smaller (~20 kDa) monomeric proteins related to the *ras* oncogene product (Barbacid, 1987; Hall, 1990; Takai et al., 1990). Some of these *ras*-like proteins, including *sec4*, *ypt1*, and the *rab* family gene products, have been implicated in intracellular protein trafficking, secretion, and exocytosis (Salminen & Novick, 1987; Segev et al., 1988). The ADP-ribosylation factors (ARFs)[1] are a multigene family of ~20-kDa GTP-binding proteins (Bobak et al., 1990) that have been identified as components of the Golgi system and may participate in vesicular protein transport (Stearns et al., 1990a,b). ARF was initially isolated as a guanine nucleotide-dependent activator of cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ (Kahn & Gilman, 1984). It was later shown that ARF enhances all cholera toxin-catalyzed reactions, consistent with other evidence that ARFs are allosteric activators of the toxin catalytic subunit (Tsai et al., 1987, 1988; Noda et al., 1990). Molecular cloning has revealed three classes of mammalian ARFs (Tsuchiya et al., 1991): class I (ARFs 1, 2, and 3), class II (ARFs 4 and 5), and class III (ARF 6). ARFs are highly conserved in eukaryotes, and immunoreactive ARF protein has been found in mammals, chicken, frog, yeast, and slime mold (Kahn et al., 1988; Tsai et al., 1991a).

Earlier screening of chromosomal DNA and embryonic *Drosophila* cDNA libraries for ARF 1 resulted in cloning of an ARF-like cDNA (*arl*), which has an open reading frame of 180 amino acids which are only 52–56% identical to mammalian ARFs (Tamkun et al., 1991). The ~20-kDa recombinant protein expressed from this cDNA bound GTP, but did not stimulate cholera toxin ADP-ribosyltransferase activity. Since insects and other invertebrate animals are more closely related to mammals than are yeast and protozoans (species from which highly conserved ARFs have been cloned), the relatively limited sequence similarity to known ARFs and lack of toxin-activating activity of the *arl* gene product were surprising. If *arl* were the only *Drosophila* gene with homology to mammalian ARFs, this insect would be an exception to the emerging consensus that ARFs are essential to eukaryotes.

To avoid difficulties that perhaps interfered with the isolation of *Drosophila* ARF cDNA by plaque hybridization screening of phage libraries, we used specialized applications of the polymerase chain reaction (PCR) and describe here the cloning of a full-length cDNA for *Drosophila* ARF 1.

EXPERIMENTAL PROCEDURES

Materials. GTP, NAD, thymidine, chicken ovalbumin, sodium cholate, dimyristoylphosphatidylcholine, and cardi-

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[1] Abbreviations: ARF, ADP-ribosylation factor; sARF II, soluble ARF from bovine brain; *arl*, ARF-like; CS, Canton-S wild-type flies; G protein, guanine nucleotide-binding protein; G_i, inhibitory G protein of adenylyl cyclase; G_s, stimulatory G protein of adenylyl cyclase; PCR, polymerase chain reaction; CTA, cholera toxin A subunit; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; dARF, *Drosophila* ARF; DTT, dithiothreitol; DMPC, dimyristoylphosphatidylcholine.

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Degenerate ARF amplification primers

XARF-F: GC clamp-ACIGTITGGGA$^{T}_{C}$GTIGGIGGICA$^{A}_{G}$GA

XARF-R: ccgtctagacc$^{A}_{G}$GTCICCI$^{G}_{C}$T$_{A}$IGTIGC$^{A}_{G}$CAIGTIGC$^{T}_{C}$TG

Drosophila ARF-specific amplification primers

DFP1	ggcctggttccgcggATGGGAAACGTATTCGCGA	(1 → 19)
DFP2	ctgcgcctcgctccATTTTAGTGTTTAATTAGCG	(835 ←854)
DAF	gggaagaggattcGGTAAAACCACAATTCTG	(85 →102)
DAR	cgcgggaggatccTTCTTCAATTGGTTGGAC	(810 ←827)
D4	TTACTAATCTTCGCAAACAAACAG	(553 →576)
D5	CTTATCTTCGTCGTGGACAGCAAT	(455 →478)
D7	GACAGCCAGTCGAGTCCCTC	(733 ←752)
D8	AGGGGACGAATTTTGTCTTG	(211 ←230)
D9	GAGGGACTCGACTGGCTGTC	(793 →812)
D10	GACAGCCAGTCGAGTCCCTC	(733 →752)

Amplification primers specific to Vector pNB40cDV

DV1	ACTATAGGGAGACCGGAATT	(31 ← 50)
DV2	CAGAATAAACGCTCAACTTT	(2451 →2470)

Amplification primer specific to λgt11

gt11-F GGTGGCGACGACTCCTGGAGCCCG

FIGURE 1: Oligonucleotides used as primers and probes. Oligonucleotides (left to right, 5′ to 3′) were synthesized on an Applied Biosystems 380B DNA synthesizer and desalted on G-50 Sephadex (Pharmacia). Bases that are not part of the targeted sequence (lower case letters) were included at the 5′ ends to facilitate cloning. A 33-base "GC clamp" (GCCGAATTCCCGCCCGCCGCGCCCC-GCGCCGCC) was attached to the 5′ end of XARF-F to increase the melting temperature and permit annealing at high temperatures (Sheffield et al., 1989). This appears to increase the specificity of mixed oligonucleotide-primed amplification of ARFs (Monaco et al., 1990). Inosine was used in positions where the alternative was four different bases. *Drosophila* ARF 1-specific primers were used to obtain segments of ARF cDNA. Numbers within parentheses indicate the position of the oligonucleotides in *Drosophila* ARF 1 as shown in Figure 5. The direction of the arrow indicates whether an oligonucleotide is sense (→) or antisense (←) relative to the recorded sequence. Vector-specific primers DV1 and DV2 flank the cDNA insertion site of vector pNB40cDV. Primer gt11-F is complementary to the sequence immediately upstream of the *Eco*RI insertion site in λgt11 (i.e., identical to oligonucleotide 1218 from New England Biolabs).

olipin were purchased from Sigma Chemical Co. (St. Louis, MO); dithiothreitol (DTT) was from ICN Biochemicals (Richmond, CA); cholera toxin A subunit (CTA) was from List Biological Laboratories (Campbell, CA); Ultrogel AcA 54 was from IBF Biotechnics (Columbia, MD); Centritip-10 and Centricon-10 was from Amicon Division, Grace Company (Beverly, MA); urea, protein standards for SDS–polyacrylamide gels, and Tris were from Bethesda Research Laboratories/Life Technologies, Inc. (Gaithersburg, MD); *Thermus aquaticus* DNA polymerase (*Taq* polymerase), polymerase chain reaction (PCR) buffer, and deoxynucleotides were purchased from Perkin-Elmer Cetus (Norwalk, CT); [α-^{32}P]-ATP (6000 Ci/mmol), [*adenylate*-^{32}P]nicotinamide adenine dinucleotide (30 Ci/mmol), and [α-^{35}S]dATP (1000 Ci/mmol) were from New England Nuclear (Boston, MA); Sequenase kits for DNA sequencing were from United States Biochemicals (Cleveland, OH).

Oligonucleotides were made by automated phosphoramidite chemistry on a 380B DNA synthesizer (Applied Biosystems, Foster City, CA) and desalted on Sephadex G-50 (Pharmacia, Piscataway, NJ). Some oligonucleotides were biotinylated at the 5′ terminus using a biotinylated phosphoramidite (Clontech, Palo Alto, CA).

Drosophila Culture and Isolation of Body Parts. Canton-S wild-type adult flies were grown on standard cornmeal agar medium (Lewis, 1960) at 21 °C. Larvae were grown at 18 °C for preparation of salivary gland squashes. Heads, bodies, and legs were isolated by freezing whole flies at −76 °C and

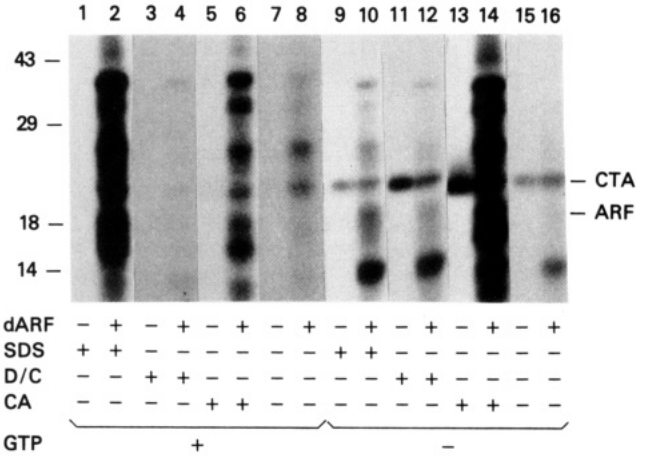

FIGURE 2: Effect of soluble *Drosophila* proteins on CTA-catalyzed ADP-ribosylation. Assays as noted under Experimental Procedures with the indicated additions including 100 μM GTP, 0.003% SDS, 0.9 mM DMPC/0.06% sodium cholate (D/C), 1 mg/mL cardiolipin (CA), and concentrated proteins from Ultrogel AcA 54 column fractions (dARF: 51 μg in 60 μL) or elution buffer B (60 μL) as indicated in a total volume of 100 μL were incubated at 30 °C for 4 h before addition of 50% trichloroacetic acid followed by ovalbumin. After precipitation at 4 °C overnight and centrifugation, pellets were dissolved (see Experimental Procedures) and subjected to SDS–PAGE in 14% gels. Autoradiograms of lanes 1–8 were exposed for 3 h to Kodak XAR film, whereas lanes 9–16 were exposed for 64 h. Molecular weight markers (×10^{-3}) are on the left.

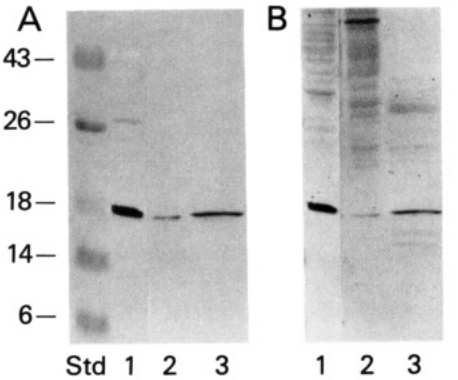

FIGURE 3: Immunoreactive ARF in soluble proteins from *Drosophila* heads. Soluble proteins were separated by electrophoresis in a 16% gel and electrophoretically transferred to nitrocellulose. (A) Blot incubated with anti-sARF II IgG fraction, 2 μg/mL. (B) Blot stained with Ponceau S (1 min, 1 mg/mL in 1% acetic acid, destained with 1% acetic acid). Lane 1, bovine brain supernatant (50 μg); lane 2, *Drosophila* head supernatant (100 μg); lane 3, *Drosophila* soluble proteins from Ultrogel AcA 54 eluate (51 μg).

separating body parts through prechilled standard sieves. Thoraces and abdomens were collected on the 25-mesh sieve, heads on the 40, and legs in the bottom pan.

Partial Purification of ARF. Drosophila heads were homogenized in buffer A (0.2 M sucrose/10 mM EDTA/10 mM EGTA/0.02% sodium azide/20 mM HEPES, pH 7.0) with a Teflon pestle/glass homogenizer. The homogenate was centrifuged (10000 *g*, 10 min). The supernatant was concentrated using a Centriprep-10, and centrifuged (140000 *g*, 60 min). The supernatant (~30 mg of protein in 5 mL) was applied to a column (1.7 × 116 cm) of Ultrogel AcA 54, which was eluted with buffer B [20 mM Tris HCl (pH 8.0)/0.25 M sucrose/20 mM DTT/1 mM EDTA/1 mM sodium azide/1 mM phenylmethanesulfonyl fluoride/1 mM benzamidine/0.1 M NaCl]. Samples of fractions (2.5 mL) were assayed for protein. Fractions that eluted between 115 and 175 mL (containing proteins of 15–28 kDa) were pooled and

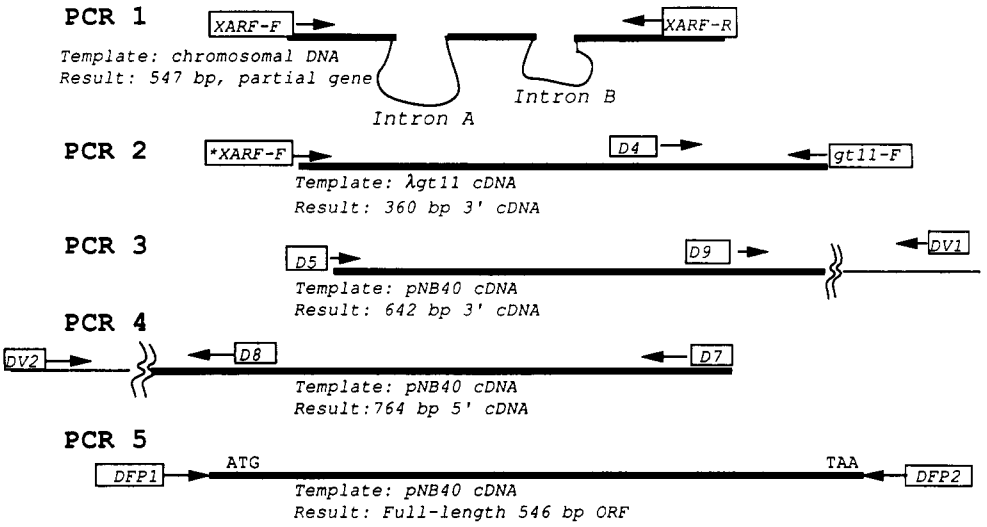

FIGURE 4: PCR cloning strategy used to obtain full-length *Drosophila* ARF 1 cDNA and partial gene structure. Five PCRs were used to obtain segments of ARF and to assemble a composite sequence of the full-length coding region (shown in Figure 5). Relative locations of oligonucleotides used in PCR or in sequencing dARF are shown in boxes, with arrows indicating that the oligonucleotides are sense (→) or antisense (←). Sequences of the oligonucleotides are as given in Figure 1. In each case, DNA coding for protein is shown as a boldface line (not to scale); introns are curved thin lines; untranslated regions are thin straight lines. PCR 1: cloning of the 547 bp central segment of the *Drosophila* ARF 1 gene that links regions coding for two highly conserved GTP-binding sequences. Genomic DNA (300 ng) was amplified by the degenerate oligonucleotides XARF-F and XARF-R. PCR 2: cloning of a partial 3′ ARF cDNA end amplified from a *Drosophila* cDNA library in λgt11. Biotinylated primer XARF-F (biotin at the oligonucleotide 5′ is indicated by an asterisk) was paired with the vector-specific primer gt11-F (complementary to the sequence flanking the *Eco*RI cDNA insertion sites in λgt11) for 20 cycles of PCR. The product was concentrated and purified using M-280 streptavidin beads (Hultman et al., 1991; Lew & Kemp, 1989; Rosenthal & Jones, 1990) and reamplified using primers D4 and gt11-F. A second *Drosophila* cDNA library (in plasmid vector pN540cDV) was used as template in PCRs 3–5. PCR 3: amplifications of the more distal 3′ sequence using vector-specific primer DV1 and ARF-specific primer D5. After 30 cycles, the product was reamplified with primers DV1 and D9. PCR 4: primers D8 and D7 paired with vector-specific DV2. This yielded the 5′ end of *Drosophila* ARF 1. PCR 5: amplification of a 546 bp complete coding region cDNA directly from the cDNA library with primers DFF1 and DFP2.

Table I: Exon/Intron Organization of *Drosophila* and Human ARF Genes[a]

Species	5′ Boundary	Intron Size	3′ Boundary
	F Q N T Q	Intron A	G L I F V
dARF 1	TTCCAGAATACACAAgtaagtgtgatt	(192 bp)	tttgaatcgttacagGGTCTTATCTTCGTC
hARF 1	TTCCAGAACACACAAGgtaagtggctg	(138 bp)	actggctgcccggcagGCCTGATCTTCGTG
hARF 3	TTCCAGAACACCCAAGgtatgctcagg	(217 bp)	tgctctgccttcctagGGTTGATATTTGTG
	F A N K Q	Intron B	D L P N A
dARF 1	TTCGCAAACAAACAGgtaggtccaagc	(98 bp)	ctgggatcattttagGATCTGCCAAATGCA
hARF 1	TTCGCCAACAAGCAGgtaggcgcccgg	(136 bp)	ccttccttcccccagGACCTCCCCAACGCC
hARF 3	TTTGCAAACAAACAGgtgagacttctt	(543 bp)	tcccacgtgctccagGATCTGCCTAATGCT

[a] Exon/intron organization of *Drosophila* and human ARF genes. dARF 1, *Drosophila* ARF; hARF 1, human ARF 1 (Lee et al., 1992); hARF 3, human ARF 3 (Tsai et al., 1991b). Positions of the intron/exon boundary differed by a single base in intron A, and not at all in intron B.

concentrated by Centritip-10 and Centricon-10 to 1.2 mL (~1 mg of protein).

Assays for ARF stimulation of cholera toxin contained 50 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 3 mM DTT, 20 mM thymidine, 1–3 μM [^{32}P]NAD (4 μCi), and the indicated additions (total volume = 0.1 mL). Incubation at 30 °C for 4 h was followed by precipitation with 25 μL of ice-cold 50% trichloroacetic acid and 10 μL of 1% chicken ovalbumin. Proteins dissolved in 60 mM Tris/10% glycerol/5% 2-mercaptoethanol/3% SDS/0.006% bromophenol blue were separated by SDS–PAGE in 14% gels. Kodak X-Omat AR film was used for autoradiography.

Immunodetection. Following SDS–PAGE in 16% gels, proteins were transferred to Immobilon, which was incubated with a rabbit polyclonal antibody raised against bovine ARF (sARF II; Tsai et al., 1991a), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG and detection with 4-chloro-1-naphthol and hydrogen peroxidase (Halpern et al., 1986). Bovine brain soluble ARF was used as a reference for ARF immunodetection (Bobak et al., 1990; Tsai et al., 1988).

Polymerase Chain Reaction. Unless otherwise specified, the protocol (Perkin-Elmer Cetus TC1 thermal cycler) for PCR amplification was 35 cycles of 95 °C/30 s, 55 °C/45

s, 72 °C/60 s, followed by extension at 72 °C for 7 min (Saiki et al., 1988). The PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, a 200 mM aliquot of each dNTP, 0.1% Tween, amplification primers (each 20 pmol), and *Taq* polymerase, 1–2.5 units (total volume 50 μL). Magnesium concentrations were not varied. Samples of reaction mixtures were analyzed by electrophoresis in a 1.3% agarose gel.

PCR with one specific primer for ARF, similar to RACE-PCR, the rapid amplification of cDNA ends (Frohman et al., 1988), with modifications (Lew & Kemp, 1989; Rosenthal & Jones, 1990), was used sequentially to isolate the extreme 3′ and 5′ ends of *Drosophila* ARF 1 cDNA, and thereby obtain the complete *Drosophila* ARF 1 cDNA sequence. Samples (1 μL) of two different *Drosophila* cDNA libraries (a λgt11 *Drosophila* cDNA library from Stratagene, La Jolla, CA, and a pNB40 *Drosophila* cDNA library constructed by Dr. Nicholas Brown, Harvard University) served as templates in the one-side-specific PCR used to capture 3′ and 5′ ends.

Expression of Recombinant ARF in Escherichia coli. A *Drosophila* ARF–glutathione *S*-transferase fusion protein cDNA was constructed and expressed in *Escherichia coli* as described (Guan & Dixon, 1991; Haun & Moss, 1992; Smith & Johnson, 1988). The full-length *Drosophila* ARF 1 coding region cDNA was generated by PCR with primers DFP1 and DFP2 using the pNB40 *Drosophila* cDNA library as template. The PCR-generated DNA was purified and cloned into expression vector pGEX-5G/LIC, resulting in pGDARF, and transformed into *E. coli* (DH5α) (Haun & Moss, 1992).

Samples (1 mL) of overnight cultures containing pGDARF were inoculated into 50 mL of LB medium containing ampicillin, and growth was continued for 90 min at 200 rpm, 37 °C. Isopropyl β-D-thiogalactopyranoside was added (final concentration 0.5 mM), and cultures were grown for an additional 2 h. Cells were pelleted and disrupted by sonification. Samples of the lysate were tested for their ability to stimulate cholera toxin-catalyzed auto-ADP-ribosylation of cholera toxin A1 protein in reaction mixtures containing 50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 20 mM thymidine, 0.1 mM GTP, 0.003% SDS, and 10 μM [^{32}P]NAD (2 μCi) (total volume = 0.1 mL) (Tsai et al., 1987, 1988). Mixtures were incubated with cholera toxin A subunit (CTA, 1 μg, preincubated 30 °C for 10 min in 30 mM DTT/75 mM glycine, pH 8.0) at 30 °C for 1 h. Reactions were terminated by the addition of 1.0 mL of ice-cold 7.5% trichloroacetic acid. After precipitation overnight at 4 °C, and centrifugation, proteins were dissolved in 60 mM Tris (pH 6.8)/10% glycerol/5% 2-mercaptoethanol/3% SDS/0.006% bromophenol blue (10 min, 65 °C) and subjected to SDS–PAGE in 16% gels, before transfer to nitrocellulose. Blots were incubated with antibody against bovine sARF II (Tsai et al., 1991a) and CTA_1 and developed as described (Halpern et al., 1986).

Chromosome Mapping. The gene encoding *Drosophila* ARF 1 was mapped by *in situ* hybridization to larval salivary gland polytene chromosomes using 2 different cDNA probes: (1) a gel-purified 483-base ARF cDNA produced by PCR from the *Drosophila* λgt11 cDNA library using primers DAR and DAF (Figure 1); and (2) the full-length 546-base dARF cloned in pGEX-5G/LIC (plasmid pGDARF, see above). Probes were biotinylated by nick-translation using biotin-14-dATP and the bioNick labeling system (BRL catalog no. 8247SA). Hybridization to *Drosophila* larvae salivary gland chromosome squashes was conducted as described (Engels et al., 1985). Hybridization was detected by a streptavidin–alkaline phosphatase conjugate (BluGene detection system, catalog no. 8279SA).

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                                                                    TAT -241
TCGCAATTCGGTGTGTGGTGTGTTTGGCAG CATAGAAAATTCAATCAAAGAAAAGTCCAC -181
GAAAAAAGGCAGAATAATCGATTCTCCCAC TCCATGCACACAAGTGTCAGTTTGCATATT -121
CTGATCGGTATAGCAACTCGGATTTGAACT TGTCATAATTCCAGCCAGCAGACAGCGAGC  -61
AGGCCAACCAGAGATCCAACTTCCTACGAC TACCGAGTATCCGAAAGACAAACAGCAGCC   -1

ATGGGAAACGTATTCGCGAACTTATTCAAA GGCCTATTCGGCAAAAAGGAAATGAGGATA   60
M  G  N  V  F  A  N  L  F  K   G  L  F  G  K  K  E  M  R  I    20

TTGATGGTCGGTTTGGATGCCGCTGGTAAA ACCACAATTCTGTACAAACTCAAATTAGGC  120
L  M  V  G  L  D  A  A  G  K    T  T  I  L  Y  K  L  K  L  G    40

GAAATTGTTACAACGATACCTACCATTGGT TTCAATGTGGAGACTGTAGAATACAAGAAT  180
E  I  V  T  T  I  P  T  I  G    F  N  V  E  T  V  E  Y  K  N    60

ATTAGCTTTACAGTGTGGGATGTGGGTGGC CAAGACAAAATTCGTCCCCTGTGGAGGCAC  240
I  S  F  T  V  W  D  V  G  G    Q  D  K  I  R  P  L  W  R  H    80

TACTTCCAGAATACACAAgtaagtgtgatt tcagcttttccacagattccagaaaattct  300
Y  F  Q  N  T  Q                                                86

atatgatgttaacagcttgtaatgattatg cattgtgattattcaagtttattttgtttc  360
aagacgggactgactatattatcttagaga tctatcttctacgattcaagccaggacaat  420
tggaataaagttttcatttgaatcgttaca gGGTCTTATCTTCGTCGTGGACAGCAATGA  480
                                G  L  I  F  V  V  D  S  N  D    96

CCGAGAGCGTATCGGTGAGGCGAGAGAGGA GTTGATGCGCATGCTGGCAGAGGACGAGCT  540
R  E  R  I  G  E  A  R  E  E    L  M  R  M  L  A  E  D  E  L   116

GAGGGATGCAGTCTTACTAATCTTCGCAAA CAAACAGgtaggtccaagcaaaaggttgtcg  600
R  D  A  V  L  L  I  F  A  N  K  Q                             128

ttatcagtagccagttgctcggtccttata ttgtcatactaatattgcactcgctctttt  660
ctgggatcattttagGATCTGCCAAATGCA ATGAACGCGGCCGAAATCACCGACAAGCTC  720
                D  L  P  N  A    M  N  A  A  E  I  T  D  K  L   143

GGCTTGCACTCACTCAGAAACCGCAACTGG TACATCCAGGCGACGTGTGCAACTAGCGGT  780
G  L  H  S  L  R  N  R  N  W    Y  I  Q  A  T  C  A  T  S  G   163

GATGGGCTGTACGAGGGACTCGACTGGCTG TCCAACCAATTGAAGAACGCTAATCGCTAA  840
D  G  L  Y  E  G  L  D  W  L    S  N  Q  L  K  N  A  N  R  ***  182

TTAAACACTAAAATTAAAGCAACAATATGA ATACAACTAACTCTGATTTCAAGCGAGCCG  900
AAGCAGTTAACCAGCAACCAGAACCATAGA AAGAGCGGCTGAAGGACATCCAACATCCAG  960
CGGTAAACGGTGGAAATCGACATCAGAAGG AATTTTCACAAAACAGCAGCAGCGGCACCA 1020
ACACCAACAACAAGAGCAGCAGATCTTCGC ATAACTTAAAGGCGTCCGCATGTCCGTATT 1080
TCCAAACAAGAAACTGAAATTGTTTATTGA ATTGCAAGAACATAACCTTTCAAGCAGCAT 1140
ATAAAGAACGCTATAATATAATTTAAATTC GTATACATAATTATTGGATGTTTAAAAGCA 1200
AATCGAGTAAAAGAAAAGCCCAACCAATCA CACAAGAAAGGAAACTATAGTATATGGTAT 1260
AAAAATGTCTTTTTTAAGTTATTTTTGTCC TCTCCCTACCCCTCATCAAAATATATATTT 1320
CTTTTTAATTTTATAATATTAAAGTAATAT TAATGGGAAAAAACTGTAATTGTAATTGCT 1380
CTTTAAAAATACAACAAATACCATTTTAAA AAAAAAAAAAAAAAAAAAAAAAAA       1434
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FIGURE 5: Nucleotide and deduced amino acid sequences of *Drosophila* ARF 1 cDNA with partial gene structure: deduced amino acid sequence above the corresponding nucleotide sequence (numbered from A of the initiation codon). Locations of the degenerate oligonucleotide primers used in PCR 1 to obtain the underlined gene sequence are marked with arrows (XARF-F by →; XARF-R by ←) beneath the corresponding deduced amino acid sequence. Exon nucleotides are in upper case letters and intron nucleotides in lower case letters. Sequence not underlined was obtained from amplification of cDNA. The *Drosophila* gene may contain introns in these regions. Consensus amino acid sequences believed to be involved with nucleotide binding and hydrolysis are boxed; the termination codon is starred. The position of a potential adenylation signal AACAAA is underlined twice.

Southern Blots. Genomic DNAs (3–5 μg/lane) isolated from wild-type Canton-S flies (Jowett, 1986) were digested with restriction enzymes and hybridized with the same 483-base dARF probe. Membranes were prehybridized for 6–10 h at 65 °C in 5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate)/5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/0.2% SDS. Denatured ^{32}P-labeled DNA probe was added (final concentration 10^6 cpm/mL) together with denatured, sonicated salmon sperm (100 μg/mL) DNA and incubated at 65 °C for 16–20 h followed by washing in 2× SSC/0.1% SDS and twice in 0.2× SSC/0.1% SDS at 65 °C for 30 min. Blots were exposed to X-ray film at −70 °C in the presence of intensifying screens for 1–3 days.

Northern Blots. Total RNA was isolated from wild-type flies by the acid/guanidinium thiocyanate/phenol/chloroform

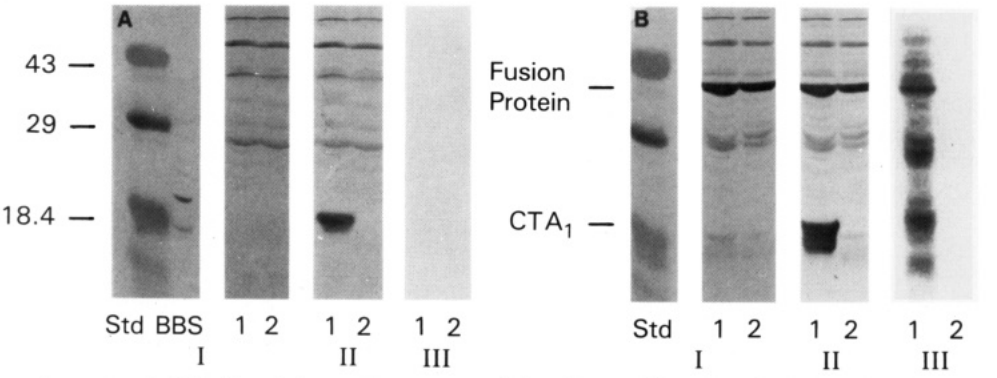

FIGURE 6: Cholera toxin-catalyzed ADP-ribosylation and immunoreactivity of *Drosophila* ARF 1 fusion protein. Sonified cell pellet proteins (10 μg) from *E. coli* DH5α (A) or DH5α transfected with glutathione *S*-transferase–*Drosophila* ARF 1 fusion protein (B) were incubated with (1) or without (2) cholera toxin A subunit (1 μg) and other additions as described under Experimental Procedures, separated by electrophoresis in 16% gels with SDS, and transferred to nitrocellulose. (I) Blot reacted with sARF II polyclonal antibodies (2 μg/mL); BBS, bovine brain supernatant. (II) Blot reacted with anti-sARF II and anti-CTA_1 antibodies (Tsai et al., 1991a; Chang et al., unpublished results). (III) Autoradiogram exposed ~4 h.

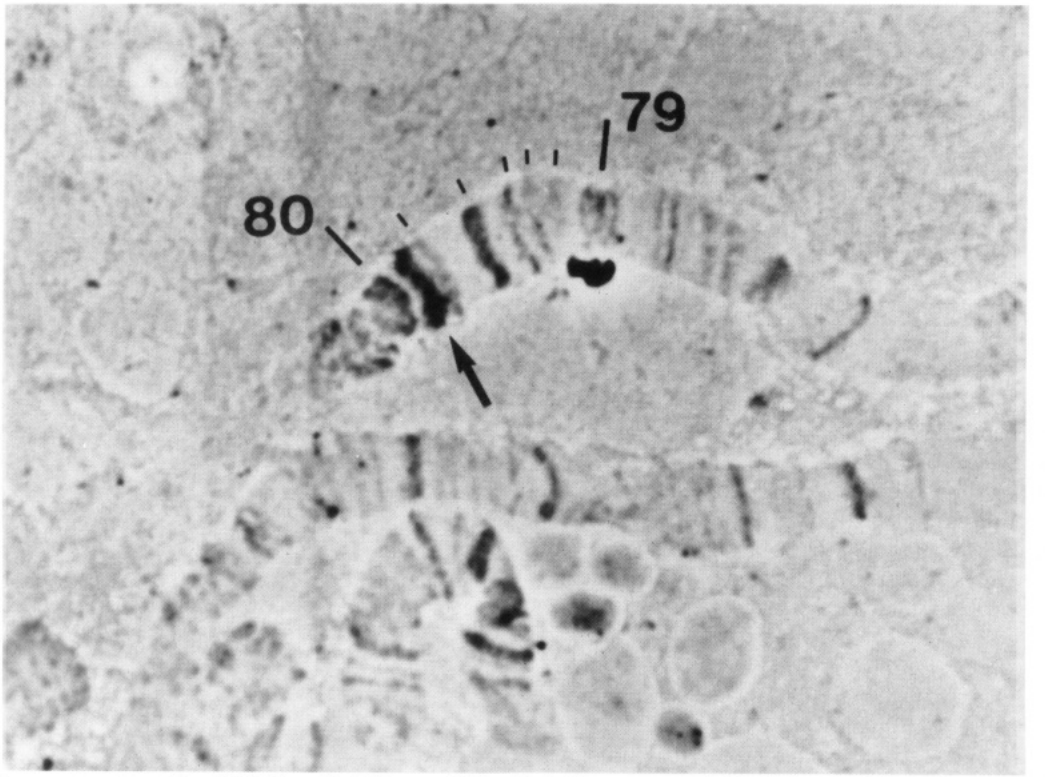

FIGURE 7: ARF gene maps to region 79F3-6 on the left arm of the third chromosome. A biotinylated 483-base fragment of ARF cDNA was hybridized to *Drosophila* salivary gland polytene chromosomes. The arrow indicates the site of hybridization detected by alkaline phosphatase reaction product. Long tics mark the starts of numbered areas (79, 80); shorter tics indicate starts of the lettered areas (B–F).

extraction method (Chomczynski & Sacchi, 1987). Poly(A)$^+$ RNA was selected on oligo(dT)–cellulose (Collaborative Research Inc., Bedford, MA). Blots were hybridized to the 483-base dARF probe as described for the Southern blot experiments.

DNA Sequence Analysis. DNA sequences were analyzed using a PC Gene software package (IntelliGenetics, Mountain View, CA) with ktup = 1 and gap penalties = 10. Ktup or Ktuple value specifies the sensitivity of the data base search; the lower the Ktup, the more sensitive the search. Multiple protein alignments were made using the Gene Works software package (IntelliGenetics).

RESULTS

Biochemical and Immunochemical Characterization of Drosophila ARF 1. Drosophila soluble proteins from the Ultrogel AcA 54 column (average size 20–30 kDa), in the presence of GTP and detergent, stimulated cholera toxin-catalyzed ADP-ribosylation, consistent with the presence of active GTP-dependent ARF protein (Figure 2). A single immunoreactive band was seen after electrophoresis of proteins from the Ultrogel AcA 54 fraction described above (Figure 3). Its mobility was perhaps slightly greater than that of the faster moving component (ARF 1) of the bovine brain-soluble ARFs.

Isolation of a Segment of the Drosophila ARF Gene and of the Full-Length ARF cDNA. The PCR cloning strategy took advantage of the high degree of amino acid sequence conservation in known ARF GTP-binding domains (Figure 4). An initial PCR amplified a 547 bp segment of the *Drosophila* ARF gene (including portions of three coding exons and two introns) from chromosomal DNA using mixed

oligonucleotides targeted to conserved GTP-binding domains of ARF (Figures 1 and 5).

Positions of intron/exon boundaries differed by only one nucleotide from those of human ARF 1 and ARF 3 (Table I). Amplification of these gene segments was followed by three PCRs to amplify overlapping 3′ and 5′ segments of *Drosophila* ARF cDNA, to obtain the sequence of the complete coding region. The composite of overlapping sequences revealed an open reading frame of 546 nucleotides encoding 182 amino acids. This coding region cDNA was 93–95% identical to those of class I mammalian ARFs, and included four consensus sequences for guanine nucleotide-binding and hydrolysis (boxed in Figure 5).

As further confirmation of the composite sequence of dARF 1, a fifth PCR was used to amplify a full-length coding region cDNA (Figure 4). Its sequence matched that of the composite derived from the segmental PCRs. The dARF 1 protein expressed as a recombinant glutathione *S*-transferase fusion protein in *E. coli* has biochemical and immunological properties similar to those of the ARF in soluble extracts from *Drosophila* heads, consistent with the similarity of the cDNA and gene to those of class I mammalian ARFs (Figure 6).

Nucleic Acid Hybridizations. (*A*) *Chromosome Mapping.* Hybridization of a biotinylated dARF 1 probe to salivary gland chromosomes identified a single site at 79F3-6 in the proximal region of the left arm of the third chromosome (Figure 7). Very few genes and/or mutations have been localized to this region, although a number of chromosomal aberrations and transposon insertions with breakpoints are found nearby. If any of these disrupts the ARF message, it could be useful in defining the physiological role of the ARF protein.

(*B*) *Genomic Southern Analysis.* To determine whether the PCR-amplified ARF fragment represents a single copy gene, genomic DNA was isolated from Canton-S wild-type flies, digested with four different restriction enzymes, and subjected to Southern blot analysis after agarose gel electrophoresis. In each digest the 483-base ARF probe hybridized with a single fragment (Figure 8). The sizes of the fragments after digestion with the indicated enzyme were as follows: *Sac*I, 6 kb; *Pst*I, 4.5 kb; *Eco*RI, 10 kb; *Cla*I, 2 kb.

(*C*) *Northern Analysis.* Poly(A)$^+$ mRNA was isolated from bodies, heads, and legs of wild-type flies. In each preparation, a single mRNA of 1.8 kb hybridized with the ARF probe (Figure 9). The blot was stripped and reprobed for ribosomal protein rp49 mRNA (O'Connell & Rosbash, 1984) which is expressed uniformly throughout *Drosophila* tissues. ARF mRNA appeared highest in legs (which contain mostly muscle and neurons) and lowest in heads (which are enriched in neuronal tissue). Overall, there was relatively little variation among the body parts, consistent with ubiquitous expression of this message in adult flies.

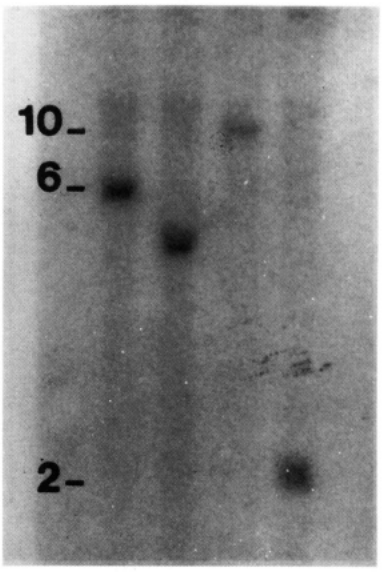

FIGURE 8: Southern blot analysis of genomic DNA from wild-type flies. Genomic DNA was digested with *Sac*I (S), *Pst*I (P), *Eco*RI (E), or *Cla*I (C) restriction enzymes, and fragments were separated by electrophoresis in a 0.7% (w/v) agarose gel. The blot was incubated with the ^{32}P-labeled 483 bp ARF fragment. Positions of size markers (in kilobases) are indicated on the left.

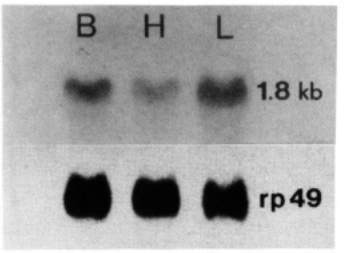

FIGURE 9: Northern blot analysis of poly(A)$^+$ RNA from wild-type flies. RNA (~20 μg) from bodies (B), heads (H), and legs (L) was loaded into each lane, subjected to electrophoresis, and transferred. The blot was incubated first with the ^{32}P-labeled 483 bp ARF fragment (upper panel). Following quantification (Betascope) and autoradiography, the blot was stripped and incubated with ^{32}P-labeled rp49 to assess the amount of RNA loaded per lane.

DISCUSSION

The *Drosophila* ARF 1 cDNA described here is very similar to mammalian class I ARFs, with >93% amino acids identical to bovine and human ARFs 1–3 (Figure 10). Identities of *Drosophila* ARF 1 are 82% and 79%, respectively, to ARFs 4 and 5 (class II), and 71% to ARF 6 (class III). Although GTP-binding proteins are highly conserved in many species, it is remarkable to find an amino acid sequence so extensively conserved between mammals and insects. The degree of mammalian–insect interspecies identity is greater than that of other guanine nucleotide-binding proteins previously compared. The most conserved *Drosophila* heterotrimeric G protein α subunits, $G_{o\alpha}$ and G_s, are ~81% and ~71% identical, respectively, to their mammalian counterparts (Quan et al., 1989), and *Drosophila ras-1* is 75% identical to mammalian *H-ras*. The >93% identity of *Drosophila* ARF 1 with mammalian class I ARF thus exceeds even the remarkable evolutionary conservation among other guanine nucleotide-binding proteins, possibly reflecting a central role for ARF in the evolution of guanine nucleotide-binding proteins.

ARFs have structural and sequence similarities to 40-kDa G_α proteins that are not shared by other 20-kDa monomeric guanine nucleotide-binding proteins. Two domains in ARFs and G_α subunits display striking identity, a five-amino acid sequence in a GTP-binding domain (Asp-Val-Gly-Gly-Gln, DVGGQ) that is absolutely conserved in all ARFs and all G_α subunits identified to date and a four amino acid sequence (Thr-Cys-Ala-Thr, TCAT) that is somewhat less conserved. These signature sequences have been found only in proteins of the ARF and G_α families. In addition, G_α subunits and ARFs are myristoylated at the N-terminus (Kahn et al., 1988). Structural conservation among G_αs and ARFs has both practical and theoretical consequences. In our work, nucleotides that encode the conserved signature sequences were incorporated into the mixed-oligonucleotide primers used for

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                    10         20         30         40         50         60         70
dARF1         1 MGnvfanLFk gLFGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
darl          1 MGgvlsy FR gLlGsrEMRI LilGLDgAGK TTILYKLqvG EvVTTIPTIG FNVEqVtYKN lkFqVWDlGG
hARF1         1 MGnifanLFk gLFGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
bARF2         1 MGnvfekLFk sLFGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
hARF3         1 MGnifgnLlk sLiGKKEMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IsFTVWDVGG
hARF4         1 MGltissLFs rLFGKKqMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IcFTVWDVGG
hARF5         1 MGltvsaLFs riFGKKqMRI LMVGLDAAGK TTILYKLKLG EiVTTIPTIG FNVETVEYKN IcFTVWDVGG
hARF6         1 MGkvlsk    iFGnKEMRI LMlGLDAAGK TTILYKLKLG qsVTTIPTvG FNVETVtYKN vkFnVWDVGG
yarf1         1 MGlfaskLFs nLFGnKEMRI LMVGLDgAGK TTvLYKLKLG EviTTIPTIG FNVETVqYKN IsFTVWDVGG
yarf2         1 MGlyaskLFs nLFGnKEMRI LMVGLDAAGK TTvLYKLKLG EviTTIPTIG FNVETVqYKN IsFTVWDVGG
gARF          1 MGqgaskiFg kLFsKKEvRI LMVGLDAAGK TTILYKLmLG EvVTTvPTIG lNVETVEYKN InFTVWDVGG
CONSENSUS       MG                RI L  GLD AGK TT LYKL  G    TT PT G  NVE V YKN   F VWD GG

                   80         90        100        110        120        130        140
dARF1        71 QDkIRPLWRH YfQNTqGLIF VVDSNDRE  Ri gEAReELmrM LaEDELRDAv LLiFANKQDL PnAMnaaEiT
darl         70 QtsIRPyWRc YysNTdaiIy VVDSaDRd  Ri giskdELlyM LrEeELagAi LvVlANKQDm dgcMtvaEvh
hARF1        71 QDkIRPLWRH YfQNTqGLIF VVDSNDRE  Rv nEAReELmrM LaEDELRDAv LLVFANKQDL PnAMnaaEiT
bARF2        71 QDkIRPLWRH YfQNTqGLIF VVDSNDRE  Rv nEAReELtrM LaEDELRDAv LLVFvNKQDL PnAMnaaEiT
hARF3        71 QDkIRPLWRH YfQNTqGLIF VVDSNDRE  Rv nEAReELmrM LaEDELRDAv LLVFANKQDL PnAMnaaEiT
hARF4        71 QDrIRPLWkH YfQNTqGLIF VVDSNDRE  Ri qEvadELqkM LlvDELRDAv LLlFANKQDL PnAMaisEmT
hARF5        71 QDkIRPLWRH YfQNTqGLIF VVDSNDRE  Rv qEsadELqkM LqEDELRDAv LLVFANKQDm PnAMpvsElT
hARF6        67 QDkIRPLWRH YytgTqGLIF VVDcaDRd  Ri dEARqELhri indrEmRDAi iLiFANKQDL PdAMkphEiq
yarf1        71 QDrIRsLWRH YyrNTeGvIF VVDSNDR  sRi gEARevmqrM LnEDELRnAa wLVFANKQDL PeAMsaaEiT
yarf2        71 QDrIRsLWRH YyrNTeGvIF ViDSNDR  sRi gEARevmqrM LnEDELRnAv wLVFANKQDL PeAMsaaEiT
gARF         71 QDsIRPLWRH YyQNTdaLIy ViDSaDlEpkRi edARnELhtl LgEDELRDAa LLVFANKQDL PkAMsttdlT
CONSENSUS       Q  IR  WR  Y   T   I  V D  D    R                   E   A       NKQD     M

                  150        160        170            180
dARF1       141 dKLGLHSLRn RnWYIQATCA TSGDGLYEGL DWL SN  qL KNanr      182
darl        140 haLGLenLkn RtfqIfkTsA TkGeGLdqam DWL SN  tt qsrK       180
hARF1       141 dKLGLhSLRh RnWYIQaTCA TSGDGLYEGL DWL SN  qL rNqK       181
bARF2       141 dKLGLhSLRq RnWYIQaTCA TSGDGLYEGL DWL SN  qL KNqK       181
hARF3       141 dKLGLhSLRh RnWYIQaTCA TSGDGLYEGL DWL aN  qL KNkK       181
hARF4       141 dKLGLqSLRn RtWYvQaTCA TqGtGLYEGL DWL SN  eLsKr        180
hARF5       141 dKLGLqhLRs RtWYvQaTCA TqGtGLYdGL DWL Sh  eLsKr        180
hARF6       137 eKLGLtriRd RnWYvQpsCA TSGDGLYEGL tWLtSN  y  Ks        175
yarf1       141 eKLGLhSiRn RpWfIQaTCA TSGeGLYEGL eWL SN  sL KNst      181
yarf2       141 eKLGLhSiRn RpWfIQsTCA TSGeGLYEGL eWL SN  nL KNqs      181
gARF        143 erLGLqeLkk RdWYIQpTCA rSGDGLYqGL DWL SNyifdkKNkKkgkkr 191
CONSENSUS         LGL      R        A   G GL      WL
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FIGURE 10: Alignment of ARF-deduced amino acid sequences from *Drosophila* and four other eukaryotes. Amino acids that are identical in at least six of these sequences are indicated in capital letters. Amino acids identical in all known ARFs are on the bottom line. Sources of sequences are the following: darl, *Drosophila* ARF-like protein (Tamkun et al., 1991); hARF1, human ARF 1 (Bobak et al., 1989); bARF2, bovine ARF 2 (Price et al., 1988); hARF3, human ARF 3 (Bobak et al., 1989); hARF4, human ARF 4 (Monaco et al., 1990); hARF5, human ARF 5 (Tsuchiya et al., 1991); hARF6, human ARF 6 (Tsuchiya et al., 1991); yARF1, yeast ARF 1 (Sewell & Kahn, 1988); yARF2, yeast ARF 2 (Stearns et al., 1990a); gARF, *Giardia* ARF (Murtagh et al., 1992).

the initial amplification. From the structural and sequence similarities of ARFs and G_α, a common evolutionary ancestor might be inferred (Botstein et al., 1988; Murtagh et al., 1992). ARFs have maintained a higher degree of sequence conservation between insect and mammalian species than have G_αs. It would thus seem likely that such a common ancestor of these families of guanine nucleotide-binding proteins may have resembled an ARF protein. ARF sequences from *Giardia lamblia*, in which G_α subunits have not been demonstrated, are >60% identical to those of human ARFs (Murtagh et al., 1992).

Levels of ARF mRNA were similar in all *Drosophila* body parts, consistent with previous work showing expression in most types of eukaryotic cells. If anything, ARF expression is higher in legs, which is somewhat different than in mammalian species, in which expression in brain appears to be highest. ARF probes hybridized to single bands on Northern and Southern analyses, and mapped to a single location on the *Drosophila* polytene chromosome. Each of the PCR-generated DNAs yielded a sequence that was present in the single conserved ARF. Although only a single copy of the dARF 1 gene was detected in these experiments, it is unclear whether a single type of ARF exists in insects or, as seems more probable, whether it is part of a larger multigene family, as is the case in mammals.

On the basis of sequence and size, *Drosophila arl* is more similar to ARF than to other guanine nucleotide-binding proteins (Tamkun et al., 1991). However, *arl* has much less similarity to ARF in consensus sequences than does any member of the ARF multigene family (Figure 10), and cannot be assigned to any of the three classes defined for mammalian ARFs. No cross-hybridization was observed with ARF probes under the conditions used for Northern and Southern analyses. Given the differences in sequences of ARF and *arl*, and the inability of *arl* to stimulate cholera toxin-catalyzed ADP-ribosylation or to complement yeast ARF 1/2 mutants, it seems most probable that the two gene products are functionally different.

The cDNA for *Drosophila* ARF 1 demonstrates again the exceptional evolutionary conservation of ARF sequence. It was inferred from studies of yeast ARF 1/2 mutants that ARF might be involved in transport of newly synthesized proteins (Stearns et al., 1990a,b) to the Golgi where ARF has been localized. A detailed genetic analysis of the function of ARF in *Drosophila* may help to define better the role of this highly conserved GTP-binding protein.

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