Drosophila melanogaster Importin α1 and α3 Can Replace Importin α2 During Spermatogenesis but Not Oogenesis

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Manuscript received August 27, 2001
Accepted for publication December 3, 2001

ABSTRACT

Importin α's mediate the nuclear transport of many classical nuclear localization signal (cNLS)-containing proteins. Multicellular animals contain multiple importin α genes, most of which fall into three conventional phylogenetic clades, here designated α1, α2, and α3. Using degenerate PCR we cloned *Drosophila melanogaster importin α1*, *α2*, and *α3* genes, demonstrating that the complete conventional importin α gene family arose prior to the split between invertebrates and vertebrates. We have begun to analyze the genetic interactions among conventional importin α genes by studying their capacity to rescue the male and female sterility of *importin α2* null flies. The sterility of *α2* null males was rescued to similar extents by *importin α1*, *α2*, and *α3* transgenes, suggesting that all three conventional importin α's are capable of performing the important role of importin α2 during spermatogenesis. In contrast, sterility of *α2* null females was rescued only by *importin α2* transgenes, suggesting that it plays a paralog-specific role in oogenesis. Female infertility was also rescued by a mutant *importin α2* transgene lacking a site that is normally phosphorylated in ovaries. These rescue experiments suggest that male and female gametogenesis have distinct requirements for importin α2.

THE nuclear targeting of proteins is mediated by a number of nuclear localization signal (NLS)-specific receptors called importins or karyopherins (Mattaj and Englmeier 1998; Weis 1998; Görlich and Kutay 1999; Nakielny and Dreyfuss 1999). Importin β-family members bind NLS cargo in the cytoplasm and act as chaperones to facilitate their translocation across the nuclear envelope through serial interactions with nucleoporins arrayed along the central channel of the nuclear pore complex (Rout *et al.* 2000; Rabut and Ellenburg 2001). Importin β1 is unusual in that it functions in conjunction with importin α to mediate the import of "classical" NLS (cNLS) cargo (Nakielny and Dreyfuss 1999), which includes a large variety of nuclear proteins (Michaud and Goldfarb 1993).

Importin α's are composed of 10 tandem armadillo (Arm) repeats, bracketed by shorter N- and C-terminal domains (Peifer *et al.* 1994; Conti *et al.* 1998). Interestingly, importin β-family members contain large domains composed of tandem HEAT motifs, which are related to Arm repeats by a degenerate ~40- to 45-amino-acid consensus sequence (Malik *et al.* 1997; Andrade *et al.* 2001). Arm and HEAT repeats fold into structurally related superhelical rods that serve as selective scaffolds for binding proteins (Huber *et al.* 1997; Conti *et al.* 1998). Importin α's bind cNLS cargo via their Arm domains (Conti *et al.* 1998) and importin β through N-terminal importin β-binding (IBB) sequences (Görlich *et al.* 1996; Weis *et al.* 1996). After cNLS-importin α/β1 ternary complexes enter and dissociate in the nucleus, importin α and β1 are independently recycled back to the cytoplasm (Koepp *et al.* 1996; Percipalle *et al.* 1997). Export of importin α is mediated by another importin β-family member, an exportin called Cse1p in yeast and CAS in higher eukaryotes (Kutay *et al.* 1997; Hood and Silver 1998), which binds within the tenth Arm repeat of importin α (Herold *et al.* 1998).

In contrast to the single importin α gene of *Saccharomyces cerevisiae* (*SRP1*), vertebrates contain as many as eight conventional importin α genes. Phylogenetic analysis has revealed that the majority of importin α genes belong to one of three conserved clades (Köhler *et al.* 1997, 1999; Malik *et al.* 1997), referred to here as α1, α2, and α3. All conventional yeast and plant importin α genes are α1 paralogs. In contrast, metazoan animals typically contain representatives of all three clades. The functional basis for the conservation of multiple importin α genes in animals is not known. However, their occurrence only in metazoan animals suggests they may be involved in tissue differentiation and development. Consistent with this notion, importin α1's, α2's, and α3's show distinct tissue and cell type-specific expression patterns (Prieve *et al.* 1996; Köhler *et al.* 1997; Tsuji *et al.* 1997; Nachury *et al.* 1998; Kamei *et al.* 1999).

In vitro evidence suggests that all conventional im-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AAC26055 and AAC26056.

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portin α's bind a broad class of cNLS sequences, but they do so with different affinities (Prieve *et al.* 1996, 1998; Nadler *et al.* 1997; Miyamoto *et al.* 1997). In permeabilized cell nuclear import assays, representative importin α paralogs show overlapping preferences for different cNLS sequences (Köhler *et al.* 1999). In some cases, specific importin α's show strong preferences for NLS cargo. For example, only an importin α3 mediates the *in vitro* import of RCC1 (Köhler *et al.* 1999) and Ran BP3 (Welch *et al.* 1999), and only an importin α1 imports the Stat 1 transcription factor (Sekimoto *et al.* 1997). In addition, the preference of importin α's for certain NLS cargo is significantly increased when two different substrates are presented together in the import assay (Köhler *et al.* 1999). This latter finding underscores the complexity of the functional interactions between importin α's and different NLS cargo and indicates that *in vivo* studies are needed to unravel the physiological roles of the importin α1, α2, and α3 genes. In this study we exploit the finding that Drosophila *importin α2* is required for male and female fertility to examine functional interactions among the conserved Drosophila importin α genes.

MATERIALS AND METHODS

Cloning of Drosophila importin α genes and phylogenetic analysis: Drosophila importin α genes were cloned using degenerate oligonucleotide-mediated PCR. The 5′ oligonucleotide was made to the conserved amino acid sequence AWALTNIA found in the third helix of arm repeat 2. To facilitate cloning, an *Eco*RI site was added, generating the sequence 5′ ATCGCGAATTCGC/T*I*TGGGC*I*T/CT*I*AC*I*AAT/CATT/C/AGC 3′ (*I* represents inosine). The 3′ oligonucleotide was made to the conserved VGNIVTG sequence in the third helix of arm repeat 6. With the addition of a *Bam*HI site, the nucleotide sequence of the 3′ primer is 5′ GACGTAGGATCCCC*I*GTTACT/G/AATG/ATT*I*CC*I*A 3′. The primers were used to PCR amplify importin α gene fragments [PCR conditions: 1.5 mM $MgCl_2$, 20 mM Tris-Cl pH 8.4, 50 mM KCl, 0.2 μg *Sal*I-digested Drosophila genomic DNA, 2 μM 5′ and 3′ primers, 200 μM dNTPs, and 1 unit of *Taq* DNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD) amplified at 94° for 20 sec, 43° for 20 sec, ramp to 55° for 2 min, 55° for 30 sec, 72° for 30 sec with 50 cycles] from genomic Drosophila DNA. Genomic DNA was digested with *Sal*I to inhibit *importin α2* amplification since PCR with undigested DNA yielded only *importin α2* products. Two distinct PCR products were subcloned into the pGAD 424 vector (CLONTECH, Palo Alto, CA) and sequenced to reveal that they encoded novel *importin α1* and *α3* genes. The two importin α gene fragments were Digoxigenin labeled by PCR (Boehringer Mannheim, Indianapolis) and used to probe an ovary λgt11 cDNA library (Zinn *et al.* 1988) to obtain full-length coding regions (*α1*, accession no. AAC26055; *α3*, accession no. AAC26056). A CLUSTAL V (Higgins *et al.* 1992) alignment of the new Drosophila importin α genes to the importin α-like genes found in GenBank was used to construct a phylogenetic tree by the neighbor-joining method (Saitou and Nei 1987).

Genetic stocks and markers: Flies were kept on standard cornmeal-dextrose media and grown at 25°. The *importin α2* gene was previously referred to as the *oho31* gene and encodes the Pendulin protein (Küssel and Frasch 1995; Török *et al.* 1995). The clean *importin* $\alpha 2^{D14}/y^{+}$ *CyO* and *importin* $\alpha 2^{D3}/y^{+}$ *CyO* stocks were provided by Dr. Istvan Kiss (Hungarian Academy of Sciences, Szeged, Hungary; see Török *et al.* 1995). Deficiency stocks *Df(3L)kto2/TM6B, Tb[1]*, *Df(3R)by416/TM3, Sb[1]*, $Gal4^{Hsp70}/TM6B$, and w^{1118} stocks were obtained from the Bloomington Stock Center. The $Gal4^{nanos\text{-}VP16}$ stock (Rørth 1998) was a gift from Pernille Rørth (EMBL, Heidelberg, Germany).

Fertility assays and testes squashes: Male fertility was assayed by crossing individual males to five w^{1118} virgin females. Female fertility was assayed by mating individual females to three w^{1118} males. Flies were allowed to mate for ~10 days before being discarded. To examine sperm motility, *importin* $\alpha 2^{D14}$ or *importin* $\alpha 2^{D3}$ homozygous and heterozygous testes were dissected from 4-day-old males in cold testis dissecting buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl pH 6.8), gently ruptured under a coverslip, and visualized by dark field microscopy.

Transmission electron microscopy of testes: Testes were dissected in cold testis dissecting buffer from *importin* $\alpha 2^{D14}/$ *importin* $\alpha 2^{D14}$, *importin* $\alpha 2^{D14}/y^{+}$ *CyO*, and w^{1118} 0- to 4-day-old males and prepared for electron microscopy as previously described (Tokuyasu *et al.* 1977). Briefly, samples were fixed in 2.5% glutaraldehyde, postfixed in 1.0% osmium tetroxide, infiltrated in Spurr epoxy resin, and embedded. These samples were then cut into ultra-thin sections, stained with uranyl acetate and lead citrate, and subsequently examined with a Hitachi 7100 transmission electron microscope.

RNA and protein isolation: Total RNA and proteins were isolated from Drosophila tissues with Tri-Reagent LS (Molecular Research Center, Cincinnati; Chomczynski 1993) following the recommended protocols. RNA was quantified by determining the A260, and protein concentration was determined using the Bio-Rad (Richmond, CA) DC protein assay.

Northern and Western blots: To determine the endogenous *importin α1*, *α2*, and *α3* mRNA expression patterns, 20 μg of RNA isolated from adult males, adult females, dissected testes, and dissected ovaries was separated on a 1% agarose, 6% formaldehyde gel; transferred to a nylon membrane; and probed with ^{32}P-random prime-labeled *importin α1*, *α2*, or *α3* probes [GIBCO (Grand Island, NY)/Bethesda Research Laboratories random primers DNA labeling system]. Bands were visualized by phosphoimaging. To examine protein expression patterns, ~10 μg of protein isolated from adult males, adult females, dissected testes, and dissected ovaries was separated on an 8% PAGE gel; transferred to a polyvinylidene fluoride membrane; and blotted with rabbit anti-importin α2 (Török *et al.* 1995) provided by Istvan Török (DKFZ, Heidelberg, Germany) or rabbit anti-importin α3 (Máthé *et al.* 2000) provided by Endre Máthé (University of Cambridge, Cambridge, United Kingdom). Blots were developed using alkaline phosophatase-tagged goat anti-rabbit secondary antibodies. To quantify transgene expression levels RNA and protein were isolated from dissected ovaries from mated w^{1118} or homozygous *importin* $\alpha 2^{D14}$ females expressing UASp importin *α1*, *α2*, *α3*, 2× *α3*, or *α2*S56A transgenes (see next section). Ovarian RNA (15 μg) was tested in a Northern blot using ^{32}P-random prime-labeled *K10* 3′ untranslated region (UTR) and *RP49* probes as described above. Bands were visualized and quantified by phosphoimaging. To examine protein levels 10 μg of protein was tested in a Western blot using rabbit anti-importin α2, rabbit anti-importin α3, or mouse anti-α-tubulin antibodies (Amersham, Arlington Heights, IL) as described above.

Immunofluorescence of testes: Testes were dissected from wild-type or homozygous *importin* $\alpha 2^{D14}$ males in 1× PBS; fixed in 1× PBS, 4% paraformaldehyde; and blocked in PBS-saponin (1× PBS, 0.2% saponin, and 0.3% normal goat serum). Testes were then incubated with rabbit anti-importin α2 (Török *et al.* 1995) diluted 1:50 in PBS-saponin, followed by

a goat anti-rabbit FITC-labeled secondary antibody diluted 1:300 in PBS-saponin. DNA was stained with 10 μM Hoechst in PBS. Confocal microscopy was performed on a Leica TCS NT microscope equipped with UV, Ar, Kr/Ar, and He/Ne lasers, and digital images were processed using Adobe PhotoShop (Adobe Systems, San Jose, CA).

Expression constructs and germline transformations: Importin α transgenic flies were created by cloning *importin α1*, *α2*, and *α3* PCR fragments corresponding to the 1.5-kb coding region of each gene into *Eco*RI and *Not*I sites in the pUASt *P*-element transformation vector (Brand and Perrimon 1993) or into *Kpn*I and *Not*I sites in the UASp *P*-element transformation vector (Rørth 1998). UASp α3 contains an additional 22 nucleotides of its 5′ UTR. The UASp *importin α2* S56A transgene was created by PCR amplifying an *importin α2* 3′ *Ava*II and *Not*I fragment containing a single point mutation that changed the TCG codon for serine-56 to a GCG alanine codon. This PCR fragment was then ligated with a 5′ *Kpn*I and *Ava*II *importin α2* PCR fragment into *Kpn*I and *Not*I sites in UASp. The pUAS$_t$ and UASp *importin α2* and *α3* transgenes contain an additional 7 and 42 nucleotides of their 3′ UTRs, respectively, and UASp *importin α3* contains 22 nucleotides of its 5′ UTR.

Transgenic UASt and UASp *importin α1*, *α2*, *α3*, and UASp *importin α2* S56A lines were created using standard germline transformation procedures (Spradling 1986). The UASt and UASp *importin α1* and UASp *importin α2* inserts used in this study were located on the second chromosome, while the UASt *importin α2*, UASt *importin α3*, UASp *importin α3*, and UASp *importin α2* S56A inserts map to the third chromosome.

Crosses: To examine genetic interactions between the *importin* $\alpha 2^{D14}$ allele and deficiencies that uncover the *α1* or *α3* genes, females of the genotype *importin* $\alpha 2^{D14}/CyO$ were crossed to (1) *importin* $\alpha 1^{Df(3L)kto2}/TM6B$ or (2) *importin* $\alpha 3^{Df(3R)\ by416}/TM3$. Male offspring without balancers are heterozygous for *importin α2* and *importin α1* or *α3*. *CyO* males without *TM3* or *TM6B* balancers serve as heterozygous *importin α1* or *α3* flies in a wild-type *importin α2* background. To test for rescue of *importin* $\alpha 2^{D14}$ male sterility following ectopic *importin α1*, *α2*, or *α3* expression, females of the genotype *importin* $\alpha 2^{D14}/CyO;$ $Gal4^{Hsp70}/TM6B$ were crossed to (1) UASt *importin α1, importin* $\alpha 2^{D14}/CyO$; (2) *importin* $\alpha 2^{D14}/CyO$; UASt *importin α2*/UASt *importin α2*; or (3) *importin* $\alpha 2^{D14}/CyO$; UASt *importin α3*/UASt *importin α3*. Male offspring that are heterozygous for the *importin* $\alpha 2^{D14}$ allele and have the $Gal4^{Hsp70}$ driver and the UASt importin α transgenes were used as the positive control for fertility (*e.g.*, *importin* $\alpha 2^{D14}/CyO;$ UASt *importin α2*/$Gal4^{Hsp70}$); homozygous *importin* $\alpha 2^{D14}$ males that have the $Gal4^{Hsp70}$ driver and the UASt importin α transgenes served as the experimental group (*e.g.*, *importin* $\alpha 2^{D14}/importin\ \alpha 2^{D14};$ UASt *importin α2/*$Gal4^{Hsp70}$); homozygous mutant males that contain the UASt importin α transgene but inherited the *TM6B* balancer instead of $Gal4^{Hsp70}$ were utilized as the negative control (*e.g.*, *importin* $\alpha 2^{D14}/importin\ \alpha 2^{D14};$ UASt *importin α2/TM6B*). Male flies were collected 0–18 hr after eclosion, heat shocked for 2 hr in a 37° air incubator, and then assayed for fertility by mating individually to five w^{1118} virgin females at 25°. To test for rescue of *importin* $\alpha 2^{D14}$ female sterility following ectopic *importin α1*, *α2*, *α3*, or *α2* S56A expression, females of the genotype *importin* $\alpha 2^{D14}/CyO;$ $Gal4^{nanos\text{-}VP16}/TM6B$ were crossed to (1) UASp *importin α1, importin* $\alpha 2^{D14}/CyO$; (2) UASp *importin α2, importin* $\alpha 2^{D14}/CyO$; (3) *importin* $\alpha 2^{D14}/CyO$; UASp *importin α3*/ UASp *importin α3*; or (4) *importin* $\alpha 2^{D14}/CyO$; UASp *importin α2* S56A/ *TM6B*. Female offspring that are heterozygous for the *importin* $\alpha 2^{D14}$ allele and have the $Gal4^{nanos\text{-}VP16}$ driver and the UASp importin α transgenes were used as the positive control for fertility (*e.g.*, *importin* $\alpha 2^{D14}$, UASp *importin α2/CyO;* $Gal4^{nanos\text{-}VP16}/+$); homozygous *importin* $\alpha 2^{D14}$ females that have the $Gal4^{nanos\text{-}VP16}$ driver and the UASp importin α transgenes served as the experimental group (*e.g.*, *importin* $\alpha 2^{D14}/importin\ \alpha 2^{D14}$, *UASp importin α2;* $Gal4^{nanos\text{-}VP16}/+$); homozygous mutant females that contain the UASp importin α transgene but inherited the *TM6B* balancer instead of $Gal4^{nanos\text{-}VP16}$ were utilized as the negative control (*e.g.*, *importin* $\alpha 2^{D14}$, UASp *importin α2/ importin* $\alpha 2^{D14}$; *TM6B/+*). To express two copies of *importin α3* in an *α2* mutant background *importin* $\alpha 2^{D14}/CyO;$/UASp *importin α3*, $Gal4^{nanos\text{-}VP16}/TM6B$ females were crossed to *importin* $\alpha 2^{D14}/CyO$; UASp *importin α3*/UASp *importin α3* and female offspring of the genotype *importin* $\alpha 2^{D14}/importin\ \alpha 2^{D14}$; UASp *importin α3*, $Gal4^{nanos\text{-}VP16}$/UASp *importin α3* were collected. Females of the described genotypes were assayed for fertility as described above or used as a source for RNA and protein isolation from ovaries.

RESULTS

Phylogenetic structure of the Drosophila importin α gene family: Phylogenetic analysis of known importin α gene sequences indicates that metazoan animals contain at least one representative of each of three conserved importin α clades, designated here as conventional α1, α2, and α3 genes (Figure 1; Malik *et al.* 1997; Köhler *et al.* 1997, 1999). For example, the current version of the human genome encodes four importin α1, one importin α2, and three importin α3 genes (Venter *et al.* 2001). Of these three clades, only importin α1 genes have been found in fungi and plant genomes. Nonconventional importin α genes, which contain recognizable IBB domains and conserved cNLS-binding sites, occur in plants, invertebrates, and protozoa. These divergent importin α genes probably arose from the same progenitors that gave rise to extant conventional genes. At the time this project was initiated, Pendulin, an importin α2, was the only known Drosophila importin α (Küssel and Frasch 1995; Török *et al.* 1995).

Prior to the completion of the Drosophila genome sequence, we attempted to clone by PCR the entire Drosophila importin α gene family using degenerate oligonucleotide primers designed against sequences that were well conserved among known importin α genes. Successfully amplified PCR products were then used to screen a Drosophila ovary cDNA library for full-length importin α cDNAs. In addition to recloning the Drosophila *importin α2*, this strategy yielded novel *importin α1* and *α3* genes. The new importin α genes encode Arm domain proteins with consensus importin β and cNLS binding domains. These results demonstrate that the phylogenetic structure of the importin α gene family is conserved between invertebrates and vertebrates (see also Köhler *et al.* 1999). The Drosophila *importin α3* gene was recently recloned by two hybrid screens on the basis of its interactions with DNA polymerase α (Máthé *et al.* 2000) and germ cell-less (Dockendorff *et al.* 1999). In addition, the published Drosophila genome sequence (Adams *et al.* 2000) contains the *importin α1*, *α2*, and *α3* genes, as well as a fourth nonconventional importin α gene [protein identifica-

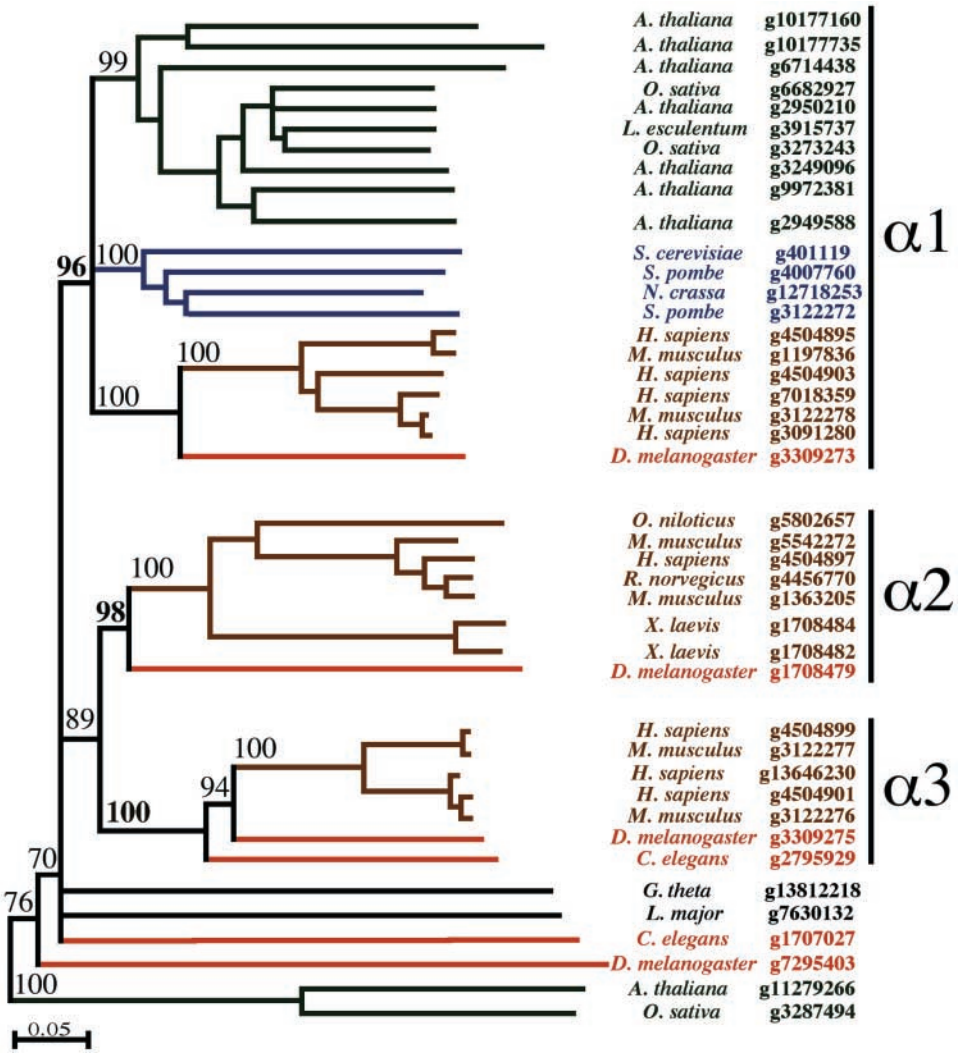

FIGURE 1.—A phylogeny of the importin α's. Alignments of importin α protein sequences were used to construct a phylogeny (MATERIALS AND METHODS). Shown here is the consensus tree of those importin α sequences whose Arm domains are demonstrably homologous (see text). Bootstrap values are represented as a percentage of 1000 replications. Species, protein identification (PID) numbers, and proposed α1, α2, and α3 nomenclature for conventional genes are indicated. Vertebrate genes are shown in brown, invertebrates in red, plants in green, and fungi in blue.

tion (PID) no. g7295403] that may or may not function in cNLS transport.

The phylogeny shown in Figure 1 does not contain all importin α-like sequences found in GenBank. Because importin α's contain tandem Arm repeats, sequence alignments cannot be used in constructing a rigorous phylogeny unless it can be determined that the order of individual repeats has been conserved (MALIK *et al.* 1997). If, for example, shuffling, substitution, or deletion of repeats within an Arm domain had occurred, then the alignment of this altered sequence with other sequences would effectively be comparing nonhomologous sequences. All putative importin α sequences included in the phylogeny shown in Figure 1, including the nonconventional genes, contain virtually the same order of individual Arm repeats, although Arm repeat one is naturally extremely divergent. The *Caenorhabditis elegans* genome contains three putative importin α genes, only two of which pass this homology test and are included on the phylogeny. The Arm repeats of the other *C. elegans* importin α-like gene are dissimilar to other known importin α-like sequences and cannot, therefore, be aligned with confidence, even though it is a functional importin α (GELES and ADAM 2001). This is basically the reason that β-catenins, plakoglobins, the flagellar protein PF16, and other non-importin α Arm domain proteins that share relatively high sequence similarity with importin α's are also left off this tree. The nonconventional *Oryza sativa* (rice) and *Arabidopsis thaliana* importin α sequences are included in the phylogeny because 6 of their 10 Arm repeats are homologous with those of *bona fide* conventional genes. Interestingly, the nonconventional *O. sativa* and *A. thaliana* importin α genes define a distinct clade that arose at least ~75 million years ago, prior to the division between monocotyledonous (*sativa*) and dicotyledonous (*thaliana*) angiosperms.

On the basis of the robust distribution of most known importin α genes into three distinct clades (Figure 1), we propose that the α1, α2, α3 nomenclature be adopted for naming conventional importin α genes. This approach clarifies nonsystematic schemes (*e.g.*,

TABLE 1

Fertility of *importin* α-deficient flies

importin α*2*[a]	*importin* α*1*[b]	*importin* α*3*[c]	% fertile males[d]	% fertile females[e]
+/−	+/+	+/+	100	100
−/−			0	0
+/+	+/−	+/+	90	ND[f]
+/−			30	100
+/+	+/+	+/−	95	ND[f]
+/−			90	90

Fertility of flies carrying different genetic combinations of importin α null alleles was determined. −, null alleles; +, wild-type alleles.

[a] The *importin* α*2* null allele used is *importin* $\alpha 2^{D14}$.

[b] The *importin* α*1* null allele used is *Df(3L)kto2*.

[c] The *importin* α*3* null allele used is *Df(3R)by416*.

[d] The percentage of fertile males was assessed by mating 20 males individually with 5 wild-type virgin females.

[e] The percentage of fertile females was assessed by mating 20 females individually with 3 wild-type males.

[f] ND, no data.

TABLE 2

Rescue of *importin* α*2* null male and female sterility

importin α*2*[a]	Transgene[b]	Gal4 driver[c]	% fertile males[d]	% fertile females[e]
−/−	−	+	5	0
	importin α*1*	−	5	0
		+	60	0
	importin α*2*	−	0	0
		+	60	85
	importin α*3*	−	10	0
		+	50	0
+/−	*importin* α*1*	+	95	85
	importin α*2*	+	100	95
	importin α*3*	+	85	85

The sterility of *importin* α*2* null males and females was used to assay the ability of *importin* α*1*, α*2*, or α*3* transgenes to replace endogenous *importin* α*2*.

[a] The *importin* α*2* null allele used is *importin* $\alpha 2^{D14}$ and is indicated by the −. The wild-type allele is indicated by +.

[b] UASt *importin* α*1*, α*2*, and α*3* transgenes were used to assay male fertility and UASp *importin* α*1*, α*2*, and α*3* transgenes were used for female fertility. − indicates that *importin* α transgenes are not present.

[c] The $Gal4^{Hsp70}$ driver was used to rescue male fertility and the $Gal4^{nanos\text{-}VP16}$ driver was used to rescue female fertility. + indicates that the flies inherited the Gal4 driver and − indicates that the flies did not inherit the driver (MATERIALS AND METHODS).

[d] The percentage of fertile males was assessed by mating 20 males individually with 5 wild-type virgin females.

[e] The percentage of fertile females was assessed by mating 20 females individually with 3 wild-type males.

Qip1, RCH1, etc.) and has the advantage over the previously proposed "S, P, Q" system (TSUJI *et al.* 1997) in that the numerical identifier is incorporated directly into the importin α name (*e.g.*, importin α1). This scheme is not meant to replace the original names given by their discoverers. Rather, as we learn more about the physiological role(s) of individual genes it will be helpful for comparative purposes to know that human Qip1, for example, is an importin α3.

Drosophila *importin* α*2* is required for male and female fertility: Two early studies reported that flies homozygous for a null allele of the *importin* α*2* gene (*oho-31*) die during development while exhibiting an overgrowth of hematopoietic tissues (KÜSSEL and FRASCH 1995; TÖRÖK *et al.* 1995). However, these phenotypes were subsequently found to be due to a second site mutation (I. KISS, personal communication). *Bona fide importin* α*2* null alleles, *importin* $\alpha 2^{D14}$ and *importin* $\alpha 2^{D3}$, generated by imprecise *P*-element mobilization (TÖRÖK *et al.* 1995) and subsequent recombination to remove the *oho31* mutation, were kindly provided to us by I. Kiss (Hungarian Academy of Sciences, Szeged, Hungary). These deletions removed 1.7 kb of 5′ sequence in *importin* $\alpha 2^{D14}$ and 1 kb of 5′ sequence in *importin* $\alpha 2^{D3}$ (TÖRÖK *et al.* 1995). The large amount of coding sequence removed and the absence of *importin* α*2* mRNA (not shown) or protein (TÖRÖK *et al.* 1995) in homozygous *importin* $\alpha 2^{D14}$ flies demonstrate that these mutations are indeed protein null alleles. Consistent with the unpublished observation that the *oho31* phenotype was caused by a second site mutation (I. KISS, personal communication), we do not observe any larval lethality or overgrowth of hematopoietic tissues in flies homozygous for these alleles, despite the fact that Importin α2 protein is absent. Because the *importin* $\alpha 2^{D14}$ and *importin* $\alpha 2^{D3}$ alleles are indistinguishable phenotypically (not shown), we used only the *importin* $\alpha 2^{D14}$ allele in our subsequent experiments.

Males homozygous for *importin* $\alpha 2^{D14}$ were mostly sterile but were otherwise morphologically wild type. Male fertility was quantified by mating individual male flies with five wild-type (w^{1118}) virgin females. In this experiment, all 20 heterozygous *importin* $\alpha 2^{D14}$ males tested were fertile, and all 20 homozygous *importin* $\alpha 2^{D14}$ males were sterile (Table 1). However, in rare cases, homozygous *importin* $\alpha 2^{D14}$ males produced a few offspring when mated with wild-type females (Table 2). Therefore, *importin* α*2* is important but not essential for male fertility. Likewise, homozygous *importin* $\alpha 2^{D14}$ adult females appear morphologically wild type but are sterile (G. ADAM and I. KISS, unpublished results). We independently quantified female fertility by individually mating 20 homozygous *importin* $\alpha 2^{D14}$ ($\alpha 2^{-/-}$) or 20 heterozygous *importin* $\alpha 2^{D14}$ ($\alpha 2^{+/-}$) females with three wild-type males. All $\alpha 2^{+/-}$ females were fertile and all $\alpha 2^{-/-}$ mutant females were sterile (Table 1). In addition, we have repeatedly mated $\alpha 2^{-/-}$ females with wild-type and $\alpha 2^{+/-}$ males during the course of our studies and have never ob-

served any fertile females. Therefore, *importin α2* appears to be essential for female fertility but only important for male fertility.

***Importin α2* is required for normal gametogenesis in males and females:** Because $\alpha 2^{-/-}$ males and females develop and behave indistinguishably from their heterozygous $\alpha 2^{+/-}$ siblings, we focused on the role of *importin α2* in gametogenesis. Intact testes isolated from $\alpha 2^{-/-}$ males appeared normal under the light microscope and contained characteristically large numbers of elongated sperm bundles. However, squashed testes from 4- to 5-day-old $\alpha 2^{-/-}$ males released few, if any, motile sperm (data not shown). In contrast, swarms of motile sperm were released from squashed testes isolated from wild-type and $\alpha 2^{+/-}$ males. In addition, no sperm were observed in the seminal receptacle or spermathecum of wild-type females mated to $\alpha 2^{-/-}$ males (data not shown). Therefore, it is likely that the infertility of males lacking *importin α2* is due to a defect during spermatogenesis that results in defective sperm. The occasional fertility of $\alpha 2^{-/-}$ males is consistent with the occurrence of small numbers of motile sperm in some testes. An analysis of mutant testes by transmission electron microscopy (TEM) indicated that $\alpha 2^{-/-}$ males display a defect in spermatogenesis upon individualization. Prior to individualization, the sperm bundles of homozygous $\alpha 2^{-/-}$ flies were indistinguishable from bundles in heterozygous testes (not shown). Following individualization, most sperm bundles from heterozygous testes contained close to the full complement of 64 properly individualized spermatids (Figure 2A). In contrast, in $\alpha 2^{-/-}$ testes a large number of spermatids failed to individualize and remained syncytial (Figure 2, B and C). This phenotype is not likely to be caused by a defect in the individualization machinery since normal-appearing individualization complexes, visualized with rhodamine-phallodin staining (Fabrizio *et al.* 1998), were observed in $\alpha 2^{-/-}$ testes (not shown). We were curious about the axonemal structure of $\alpha 2^{-/-}$ sperm tails, partly because mutants in a Chlamydomonas importin α-like flagellar protein, PF16, are paralyzed, and detergent-treated mutant axonemes lack central C1 microtubules. PF16 is conserved between *Giardia lamblia* and mice, but appears to be absent from the Drosophila genome (Adams *et al.* 2000). Thus it is possible that an importin α replaces the function of PF16 in Drosophila flagella. However, the organization of the sperm axonemes and the structure of the major and minor mitochondrial derivatives were normal in $\alpha 2^{-/-}$ testes (Figure 2). We conclude that Drosophila Importin α2 probably does not serve a comparable function to PF16 in flagella.

In our hands, the eggs laid by $\alpha 2^{-/-}$ females showed gross morphological defects that corroborate earlier observations (G. Adam and I. Kiss, personal communication). Specifically, eggs laid by $\alpha 2^{-/-}$ mothers were smaller and appeared deflated as if they were deficient in internal contents (not shown). In addition, dorsal appendages were missing, fused together, or otherwise severely deformed, and the chorions appeared thinner than in eggs laid by wild-type females (not shown). These results suggest that the complete sterility of $\alpha 2^{-/-}$ mutant female flies is due to a severe defect in oogenesis.

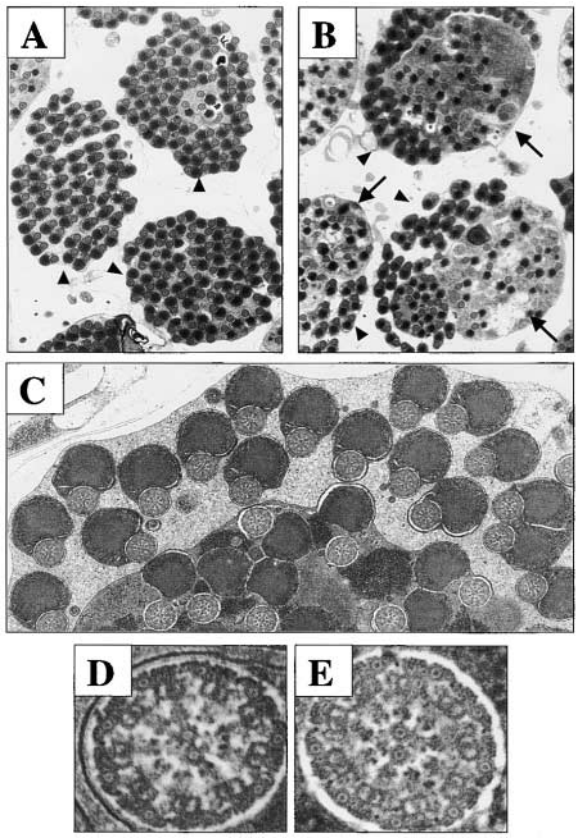

Figure 2.—Ultrastructure of *importin* $\alpha 2^{+/-}$ and $\alpha 2^{-/-}$ testes. (A) Electron microscopic images of mature sperm bundles of $\alpha 2^{+/-}$ males showing uniformly packed and individualized (arrowheads) spermatids; (B and C) $\alpha 2^{-/-}$ testes showing both individualized (arrowheads) and unindividualized syncytial spermatids (arrows). (D) A detail of individualized $\alpha 2^{+/-}$ sperm tails and (E) $\alpha 2^{-/-}$ sperm tails. The axoneme shown in E lacks a membrane (compare to D) and has not, therefore, completed individualization.

***Importin α1*, *α2*, and *α3* are expressed in testes and ovaries:** The results described above indicate that *importin α2* is required for normal gametogenesis. Northern blot analysis was used to begin to assess the particular roles of the three conventional importin α genes in gametogenesis. As shown in Figure 3A, *importin α1*, *α2*, and *α3* mRNAs were all detected in testes, ovaries, and in adult male and female flies. Therefore, as is the case with the three characterized *C. elegans* importin α genes (Geles and Adam 2001), the three conventional Drosophila Importin α genes are all expressed in the germline. *Importin α3* mRNA levels were about equally expressed in ovaries and testes. In contrast, *importin α1* mRNA levels were slightly elevated in the testes compared to ovaries, and *importin α2* mRNA levels were

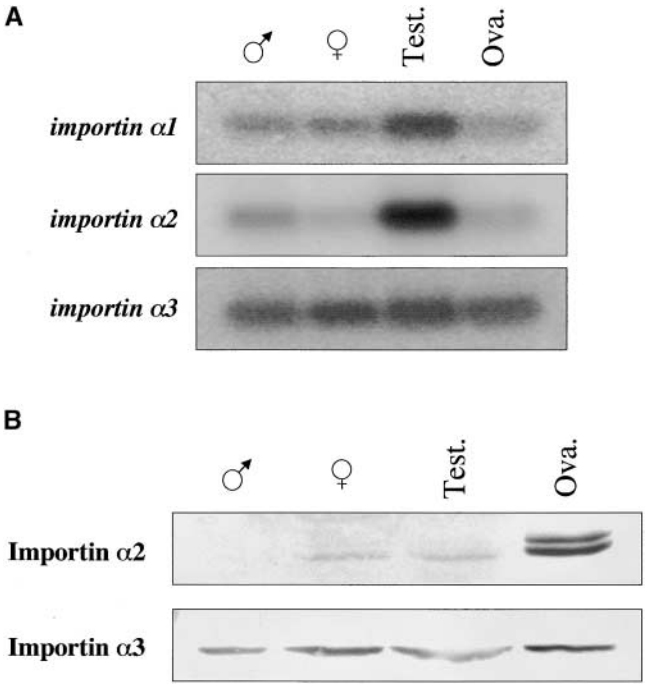

FIGURE 3.—Analysis of *importin* α gene expression. (A) A total of 20 μg of total RNA isolated from adult wild-type males, adult wild-type females, dissected wild-type testes (Test.), and dissected wild-type ovaries (Ova.) was hybridized with *importin* α*1*, α*2*, or α*3*-specific probes (MATERIALS AND METHODS). *Importin* α*1*, α*2*, and α*3* mRNA migrate at ~2.5 kb. (B) A total of 10 μg of total protein isolated from adult wild-type males, adult wild-type females, dissected wild-type testes (Test.), and dissected wild-type ovaries (Ova.) was examined on a Western blot with rabbit anti-Importin α2 or α3 antibodies (MATERIALS AND METHODS).

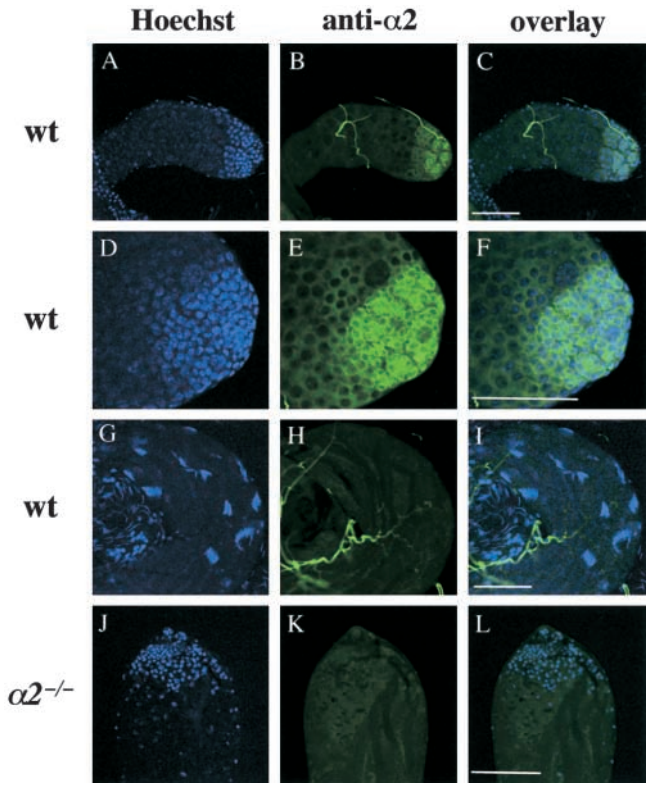

FIGURE 4.—Selective localization of Importin α2 in testes. Testes dissected from wild-type (A–I) or α*2*$^{-/-}$ (J–L) males were stained for DNA with Hoechst (A, D, G, and J) and Importin α2 (B, E, H, and K) with anti-Importin α2 antibodies (MATERIALS AND METHODS). Overlays are shown in C, F, I, and L. Confocal images were taken of the apical tip of wild-type (A–F) and α*2*$^{-/-}$ (J–L) testes and elongated sperm bundles (G–I) from wild-type testes. Bars, ~100 μm.

much higher in the testes than ovaries. Thus *importin* α*2* and, to a lesser extent, *importin* α*1* mRNAs are selectively enriched in testes.

Western blot analyses of Importin α2 and α3 protein levels are shown in Figure 3B. Importin α1 protein levels could not be examined because antibodies are not currently available. The concentration of Importin α3 protein is about the same in testes and ovaries, but somewhat higher in adult females than in males (see also MÁTHÉ *et al.* 2000). This result is consistent with the Northern blot analysis (Figure 3A), which shows similar *importin* α*3* mRNA levels in adult and germ cell extracts. Thus, Importin α3 protein levels appear to be uniformly expressed in somatic and germline tissues in proportion to its mRNA levels. The Importin α2 case is complex. Here, Importin α2 protein is much more highly expressed in ovaries than in total female flies. In males, Importin α2 protein is present at low levels in testes, at about the concentration found in whole adult females, but is undetectable in adult males (see below). In general, then, Importin α2 protein appears to be enriched in ovaries and testes relative to whole flies. However, the very high level of *importin* α*2* mRNA in the testes (Figure 3B) is out of proportion to the relatively low level of Importin α2 protein (see also TÖRÖK *et al.* 1995). These results suggest the possibility that translation of *importin* α*2* mRNA is negatively regulated in the testes.

To further examine Importin α2 expression, the protein was localized by immunofluorescence in wild-type and α*2*$^{-/-}$ testes. These data clearly show that in wild-type testes Importin α2 is highly expressed in immature germ cells located at the apical tip (Figure 4, A–C), where it is predominantly cytoplasmic (Figure 4, D–F), and is undetectable in more mature elongated sperm bundles (Figure 4, G–I). At the apical tip of testes premeitoic stem cells and spermatogonial cells exhibit stronger Hoechst fluorescence than postmeiotic spermatocytes (GÖNCZY *et al.* 1997). On the basis of this criterion, we conclude that Importin α2 is preferentially localized to stem and spermatogonial cells. The observed fluorescence is specific for Importin α2 since it is absent in α*2*$^{-/-}$ testes (Figure 4, J–L). These results suggest that Importin α2 functions during the earliest stages of sperm development.

Rescue of α*2*$^{-/-}$ male sterility with importin α transgenes: The coexpression of *importin* α*1*, α*2*, and α*3* in

the testes raises the possibility that the occurrence of some fertile $\alpha 2^{-/-}$ males could be due to the capacity of endogenous Importin α1 and/or α3 to partially perform the function(s) of the lost Importin α2. If true, then the ectopic expression of *importin α1* and *α3* transgenes in the testes might raise their levels enough to more efficiently rescue the $\alpha 2^{-/-}$ sterility phenotype. Alternatively, if Importin α2 plays a paralog-specific role that is important but not essential in spermatogenesis, then *importin α1* or *α3* transgenes would not rescue male sterility. The capacity of each of the three importin α genes to rescue male sterility was determined using the UASt transgene system (Brand and Perrimon 1993) with a $Gal4^{Hsp70}$ driver to ectopically express *importin α1*, *α2*, or *α3* transgenes (materials and methods). As shown in Table 2, $\alpha 2^{-/-}$ males containing UASt *importin α1*, *α2*, or *α3* transgenes and the $Gal4^{Hsp70}$ driver were significantly more fertile than sibling control males lacking either the transgenes or the driver. These results indicate that the activity(s) of *importin α2* that is important for spermatogenesis can be replaced by elevating the expression levels of *importin α1* or *α3*. Furthermore, testes squashes from 4- to 5-day-old flies demonstrated that each of the three transgenes partially rescued the sperm motility defect of $\alpha 2^{-/-}$ males (data not shown). Specifically, testes from rescued flies contained significantly greater numbers of motile sperm than those from $\alpha 2^{-/-}$ testes. In this experiment, flies received a single 2-hr heat-shock treatment. Repeated heat-shock treatments throughout development increased the rescue efficiency of the transgenes: 100% fertility (six of six males) for *importin α1*, 89% fertility (eight of nine males) for *importin α2*, and 78% fertility (seven of nine males) for *importin α3*. We conclude that the requirement for *importin α2* in spermatogenesis is not due to a unique biochemical property of Importin α2 that is completely lacking from Importin α1 and α3.

Rescue of the $\alpha 2^{-/-}$ female sterility with importin α transgenes: We next investigated the capacity of UASp *importin α1*, *α2*, and *α3* transgenes to rescue the sterility defect in $\alpha 2^{-/-}$ female flies. The $Gal4^{nanos\text{-}VP16}$ driver was used to express UASp importin α transgenes in the female germline (Rørth 1998). The UASp *importin α2* transgene efficiently rescued the $\alpha 2^{-/-}$ sterility defect to the level found in $\alpha 2^{+/-}$ females (Table 2). Eggs laid by *importin α2*-transgene-expressing $\alpha 2^{-/-}$ females were morphologically wild type (not shown). Control sibling $\alpha 2^{-/-}$ flies were sterile and laid defective eggs. As in males, the capacity of an *importin α2* transgene to efficiently rescue the sterility of $\alpha 2^{-/-}$ females is strong evidence that the phenotype is caused solely by a mutation in the *importin α2* gene and not to a second, unlinked mutation. Interestingly, UASp *importin α1* and *α3* transgenes did not rescue the sterility of $\alpha 2^{-/-}$ females (Table 2). Also, the characteristic deflated morphology of eggs laid by $\alpha 2^{-/-}$ flies was unaffected by the ectopic expression of either *importin α1* or *α3* transgenes (data not shown).

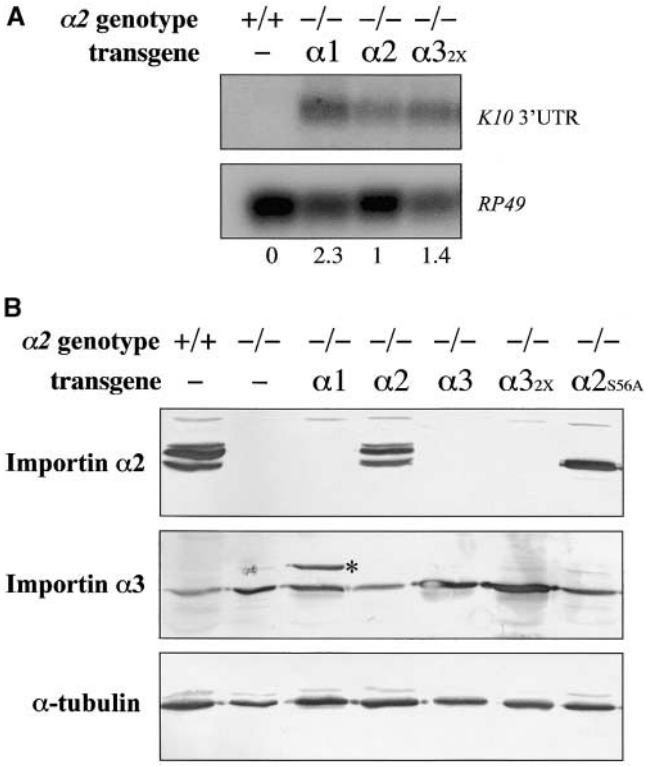

Figure 5.—*Importin α1*, *α2*, and *α3* transgene expression. (A) A total of 15 μg of total RNA isolated from dissected ovaries of the indicated genotype (materials and methods) was hybridized with a *K10* 3′ UTR probe that is common to all UASp-derived transgenes and a *RP49* probe as an internal loading control. Transgene expression levels were determined by normalizing the *K10* 3′ UTR band to the *RP49* band by phosphoimaging and are indicated relative to the expression level of the rescuing *importin α2* transgene. (B) A total of 10 μg of protein isolated from dissected ovaries of the indicated genotype was examined by Western blot with anti-Drosophila Importin α2, α3, or anti-α-tubulin antibodies (materials and methods). * indicates the slower migrating anti-Importin α3-crossreactive band that appears only when α1 is overexpressed. The Importin α2 S56A protein was made by changing the serine at position 56 of α2 to alanine.

To control for transgene expression levels, *importin α1*, *α2*, and *α3* transgene mRNA levels were determined by Northern blot using a *K10* 3′ UTR probe that is common to all UASp-expressed mRNAs (Rørth 1998). Ribosomal protein *RP49* mRNA levels were used as loading controls. Importin α transgene expression levels were quantified and normalized to *RP49* levels by phosphoimaging. As shown in Figure 5A, the *importin α1* transgene was expressed ~2.3 times higher than the rescuing *importin α2* transgene. Initially, we found that single *importin α3* transgenes were expressed at levels slightly less than the *importin α2* transgene. Therefore, flies containing two *importin α3* transgenes were created. The combined expression of both transgenes raised *importin α3* mRNA levels in the ovary to 1.4 times that of the *importin α2* transgene. The $\alpha 2^{-/-}$ females that expressed two copies of *importin α3* were still completely

sterile (0/20 fertility) and laid morphologically defective eggs that never hatched. These results argue that the failure of *importin α1* and α*3* transgenes to rescue the *importin* $\alpha 2^{D14}$ female sterility was not due to poor transcription of the transgenes, which, in both cases, exceeded that of a rescuing *importin α2* transgene.

The levels of importin α proteins in dissected ovaries from transgenic flies were determined by Western blot using available anti-Importin α2 and anti-Importin α3 antisera. As shown in Figure 5B, ovarian Importin α2 migrates as a doublet that is absent from $\alpha 2^{-/-}$ ovaries and is mostly replenished by expression of a UASp *importin α2* transgene. In flies expressing two copies of the *importin α3* transgene, the level of Importin α3 protein increased approximately five times over wild-type levels. The level of Importin α1 could not be directly determined since anti-Importin α1 antibodies are not currently available. However, because the untranslated sequences of the *importin α1* and α*2* transgenes differ by only 7 nucleotides (MATERIALS AND METHODS), it is unlikely that they are differentially translated. In addition, a slower migrating anti-Importin α3-crossreactive band always appeared in extracts from ovaries expressing a UASp *importin α1* transgene (Figure 5B). Because Drosophila Importin α1 is 3300 daltons larger than Importin α3 it is possible that this band is Importin α1. Taken together, these results indicate that *importin α1* and α*3* transgenes are both highly expressed in the ovary. We conclude that Importin α2 has a unique role(s) in oogenesis that cannot be performed *in vivo* by either Importin α1 or α3.

Curiously, it appears that Importin α3 protein levels in the ovary varied in inverse proportion to Importin α2 protein levels. For example, Importin α3 levels were higher in the ovaries of $\alpha 2^{-/-}$ flies than in wild-type flies (Figure 5B). Importin α3 levels decreased in $\alpha 2^{-/-}$ ovaries in flies expressing an *importin α2* transgene (Figure 5B), and the decrease was proportional to the level of transgene expression (not shown). Thus, it is possible that the expression of Importin α3 is influenced by the level of Importin α2.

The conserved Importin α2 phosphorylation site is not required for fertility: Importin α2 isolated from ovaries and preblastoderm embryos is partially phosphorylated and migrates as a doublet on Western blots (Figures 3B and 5B; KÜSSEL and FRASCH 1995; TÖRÖK *et al.* 1995; MÁTHÉ *et al.* 2000). Consistent with this, the upper band disappears upon phosphatase treatment (TÖRÖK *et al.* 1995). The *S. cerevisiae* importin α1 homolog Srp1p is phosphorylated at serine-67 just downstream of the IBB domain. However, mutation of this site resulted in no discernable phenotypes (AZUMA *et al.* 1997). This serine is conserved, but not necessarily phosphorylated, in most conventional importin α's (AZUMA *et al.* 1997), including the three Drosophila importin α's. TÖRÖK *et al.* (1995) speculated that Drosophila Importin α2 was phosphorylated at this site (serine-56) due to its consensus cdc2 phosphorylation sequence. We tested their hypothesis by introducing a mutant *importin α2* transgene containing alanine instead of serine at position 56 (S56A). As shown in Figure 5B, ovarian Importin α2 S56A appeared as a single band that comigrated with the lower of the two wild-type Importin α2 bands. We conclude that serine-56 in Importin α2 is probably phosphorylated; however, we cannot rule out the possibility that serine-56 is required for the phosphorylation of some other residue. Interestingly, an *importin α2* S56A transgene efficiently rescued $\alpha 2^{-/-}$ female sterility. Specifically, 90% (18/20) of $\alpha 2^{-/-}$ females expressing an *importin α2* S56A transgene were fertile, compared with 0% (0/20) of $\alpha 2^{-/-}$ females containing the transgene but not the $Gal4^{nanos\text{-}VP16}$ driver. In the same cross, 90% (18/20) of $\alpha 2^{+/-}$ females expressing *importin α2* S56A were fertile. These results demonstrate that, as in yeast, the phosphorylation of Importin α2 is not required for an essential *in vivo* function. Therefore, the paralog-specific role of Importin α2 in female gametogenesis is not likely due to its phosphorylation.

Effect of importin α gene dosage on fertility: The finding that all three importin α transgenes rescued $\alpha 2^{-/-}$ male sterility is consistent with the notion that a threshold concentration of some combination of the three importin α's normally perform an important function that any single paralog is capable of performing when ectopically expressed. If true, then the partial reduction in the concentration of any two importin α's might also cause sterility. This was tested by simultaneously reducing the gene dosage from two to one for two different *importin α* genes. Because discrete mutations in either *importin α1* or α*3* genes were not available, we employed chromosomal deficiencies in combination with the *importin* $\alpha 2^{D14}$ allele. Males and females heterozygous for both *importin* $\alpha 2^{D14}$ and deficiencies that uncovered either the *importin α1 (Df (3L) kto2)* or the *importin α3 (Df (3R) by416)* genes (analysis not shown) were tested for fertility. Male flies heterozygous for *importin α1* and α*2* were significantly less fertile than males heterozygous for only the *importin* $\alpha 2^{D14}$ allele or the *importin α1* deficiency alone (Table 1). *Importin α1* and α*2* double heterozygotes also produced greatly reduced numbers of motile sperm (not shown). In contrast, males heterozygous for *importin α2* and α*3* genes exhibited wild-type levels of fertility (Table 1). Females heterozygous for the *importin* $\alpha 2^{D14}$ mutation and the *importin α1* or α*3* deficiencies were fully fertile (Table 1) and laid morphologically wild-type eggs. Thus, spermatogenesis appears to be more sensitive than oogenesis to overall importin α levels.

DISCUSSION

Animals contain both conserved (conventional) and organism-specific (nonconventional) importin α genes (Figure 1). Little is known about the individual physiological roles of the various animal importin α's or their functional interplay *in vivo*. Conventional importin α

genes fall into three clades designated α1, α2, and α3 (Figure 1; see also Malik *et al.* 1997; Köhler *et al.* 1997, 1999). Here we report the cloning and phylogenetic analysis of the first complete set of importin α1, α2, and α3 genes from an invertebrate, *D. melanogaster*, thereby demonstrating that the conventional importin α gene family arose in animals prior to the split between invertebrates and vertebrates. On the basis of parsimony arguments and the fact that importin α2 and α3 genes are more similar to one another than to α1 genes (Figure 1), the phylogeny supports, but does not prove, that importin α2 and α3 genes arose from an α1 progenitor(s). Less likely is the alternative hypothesis that importin α2 and α3 progenitors were lost from fungal and plant lineages (Aravind *et al.* 2000). The *C. elegans* genome, which contains a single conventional importin α3 (IMA3) and two divergent worm-specific genes (Geles and Adam 2001), shows that the conventional importin α gene family is not strictly conserved in all animals. However, it is possible that the two nonconventional worm importin α genes (IMA1 and -2) derive from progenitor α1 and α2 genes. Because IMA1 and IMA2 are restricted to the germline, they may have evolved as rapidly as have many sex determination genes (de Bono and Hodgkin 1996; Hansen and Pilgrim 1999).

The phylogeny of the conventional importin α gene family (Figure 1) supports the adoption of a numbering scheme—importin α1, α2, and α3—to designate to which conserved clades a particular importin α gene belongs. Additional levels of gene duplication and (presumably) functional specialization have in many species given rise to additional conventional isoforms. For example, *Schizosaccharomyces pombe* contains two importin α1 isoforms, and humans contain a total of eight importin α1, α2, and α3 genes. However, because it is not always clear how, or if, isoforms within individual clades are related among species, the current phylogenetic analysis does not support a numbering system for individual isoforms (*e.g.*, importin α1-a, α1-b, α1-c, etc.).

The presence in the Drosophila genome of a single representative of each conventional clade simplifies the analysis of conventional animal importin α genes and argues that flies are the most suitable genetic system for investigating their functional relationships *in vivo*. Previous genetic studies in Drosophila addressed the *in vivo* functions of *importin α2* and *α3* genes (Küssel and Frasch 1995; Török *et al.* 1995; Máthé *et al.* 2000). The initial description of overgrowth of hematopoietic organs in homozygous *importin α2* mutant flies (Küssel and Frasch 1995; Török *et al.* 1995) proved to be incorrect (G. Adam and I. Kiss, personal communication). Similarly, we found that the mutant phenotypes ascribed to the insertion of a *P* element upstream of *importin α3* (Máthé *et al.* 2000) could be recombined away from the *P* element (A. Mason, unpublished observations).

In our study, *bona fide importin α2* null alleles provided by I. Kiss were used to investigate functional interactions among the conventional Drosophila importin α genes during gametogenesis. Interestingly, the only phenotypes associated with homozygous null *importin α2* mutations are male and female sterility. However, we cannot rule out the possibility that maternally derived Importin α2 also serves a role in early embryonic development, since oogenesis is disrupted in females lacking Importin α2. In gametogenesis, we have found that *importin α1* and *α3* transgenes can replace *importin α2* during spermatogenesis but not during oogenesis. These rescue experiments suggest that Importin α2 has distinct roles during male and female gametogenesis.

Importin α2$^{-/-}$ males exhibit defects in sperm individualization, produce very few motile sperm, and most flies are completely sterile. Sperm individualization appears to function as a quality checkpoint to weed out abnormal spermatids (Fuller 1993; Fabrizio *et al.* 1998). In fact, mature sperm bundles from wild-type testes often contain fewer than the full complement of 64 spermatids (Tokuyasu *et al.* 1972), suggesting that the removal of defective spermatids is a normal process. Also, it has been observed that spermatids with aberrant nuclear morphologies fail to individualize, presumably due to the inability of individualization complexes to assemble at the head (Tokuyasu *et al.* 1977; Fabrizio *et al.* 1998).

An intriguing example of the relationship between nuclear transport, individualization, and chromosome condensation is the segregation distorter meiotic drive system (Tokuyasu *et al.* 1977). In males, segregation distorter (*SD*) chromosomes are preferentially passed on to offspring at the expense of chromosomes that contain *SD*$^{+}$ (Sandler *et al.* 1959). Transmission electron microscopy (TEM) analysis of *SD/SD*$^{+}$ sperm bundles revealed that *SD*$^{+}$ spermatids remain in a syncytium after *SD* sperm are individualized (Tokuyasu *et al.* 1972). It has further been demonstrated that the failure of *SD*$^{+}$ spermatids to become individualized is associated with defects in chromatin condensation and nuclear morphology. Specifically, when *SD/SD*$^{+}$ postelongated sperm bundles are examined by TEM, *SD* nuclei stain much more darkly than *SD*$^{+}$ nuclei in the same bundle, presumably due to differences in chromatin condensation (Tokuyasu *et al.* 1977). The primary meiotic drive element on *SD* chromosomes is a dominant neomorphic mutation known as *Sd*. Recently, *Sd* was shown to encode a truncated form of the Ran GTPase-activating protein (RanGAP; Merrill *et al.* 1999) that is partly mislocalized to the nucleus (Kusano *et al.* 2001). Normally, RanGAP is restricted to the cytoplasm and is required to keep cytoplasmic Ran GTP levels low, while the strict nuclear localization of the Ran guanine nucleotide exchange factor (RanGEF) keeps nuclear Ran GTP levels high (Mattaj and Englmeier 1998). The resulting Ran GTP gradient provides the energy source for nuclear transport reactions. Therefore, the introduction of RanGAP activity into the nucleus in *SD* cells should affect nuclear

trafficking pathways (Mattaj and Englmeier 1998). Nuclear transport defects were, in fact, observed in the salivary glands of flies expressing the truncated RanGAP (Kusano *et al.* 2001); however, it has not been determined if a nuclear transport defect is the primary cause of the SD phenotype. Nonetheless, it is possible that the chromosome condensation defect in *SD* sperm is a consequence of defects in nuclear transport. Similarly, the effect of the *importin* $\alpha 2^{D14}$ mutation, which also likely causes a general defect in nuclear transport, on sperm individualization could be due to faulty chromosome condensation. However, TEM analysis of nuclei in $\alpha 2^{-/-}$ postelongated sperm bundles exhibited the staining intensity characteristic of normally condensed chromatin (data not shown). Therefore, the individualization defect observed in $\alpha 2^{-/-}$ flies is unlikely due to a gross defect in chromosome condensation.

Although the $\alpha 2^{-/-}$ spermatids that fail to individualize lack obvious morphological defects, they probably do have subtle defects that preclude their proper association with individualization complexes. In a percentage of mutant spermatids the defect appears to be subtle enough to escape the individualization checkpoint. Therefore, the severity of the spermatogenesis defect in $\alpha 2^{-/-}$ flies varies among spermatids within the same sperm bundle. In fact, in a small percentage of mutant flies enough motile sperm properly develop to produce small numbers of progeny.

Importin α*1*, α*2*, and α*3* transgenes all rescued the sterility defect of *importin* $\alpha 2^{-/-}$ males, demonstrating for the first time in that all three conventional importin α genes can perform a common physiologically relevant function. These results raise the question of why males missing Importin α2 are almost completely sterile if Importin α1 and α3 can perform the missing function. One possible explanation for the sterility of $\alpha 2^{-/-}$ males is that a minimum level of combined *importin* α*1*, α*2*, and α*3* expression in the testes is required for efficient spermatogenesis. The partial penetrance of $\alpha 2^{-/-}$ male sterility suggests that endogenous Importin α1 and α3 levels in the testes are sufficient in some $\alpha 2^{-/-}$ spermatids to perform the *in vivo* function(s) of the lost Importin α2. Although all three *importin* α genes are expressed in the testes, α*2* mRNA is expressed much more highly than either α*1* or α*3* mRNAs. At first glance, its loss would be expected to greatly deplete total importin α protein levels, resulting in a significant decrease in general importin α function (but see below). In mouse, representatives of each of the three conventional α1, -2, and -3 clades are all more highly expressed in testes compared to other tissues (Tsuji *et al.* 1997; Nachury *et al.* 1998), suggesting that high importin α levels may also be important during mammalian spermatogenesis. Finally, males heterozygous for both *importin* α*1* and α*2* are mostly sterile, as are males heterozygous for both the original *oho31*-containing *importin* α*2* null mutations and mutations in *Ketel*, the Drosophila importin β1 homolog (Erdélyi *et al.* 1997). These observations suggest that spermatogenesis is sensitive to a decrease in the capacity of cNLS-cargo import in the testes.

It may seem odd that a partial defect in a cellular housekeeping process like nuclear transport would cause defects only in spermatogenesis. However, *importin* α*2* is preferentially expressed in the testes compared to other adult tissues (Figure 3), so its loss would be expected to disproportionately affect spermatogenesis. Also, recessive male-sterile mutations are observed 10–15% as often as recessive lethal mutations, and the number of genes that can be mutated to cause male sterility has been estimated at between 500 and 1750 (Fuller 1993). Many of these are caused by nonnull mutations in genes required for general metabolism (Fuller 1993). Although formally null, $\alpha 2^{-/-}$ males are "functionally hypomorphic" (nonnull) for general importin α function because *importin* α*1* and α*3* are coexpressed in the testes and, presumably, can perform the same functions.

Alternatively, it might not be the combined concentration of all three importin α's that is important for spermatogenesis. Although all three importin α's are almost certainly capable of mediating general cNLS import, different paralogs are known to exhibit *in vitro* preferences for various cNLS cargoes (Miyamoto *et al.* 1997; Nadler *et al.* 1997; Prieve *et al.* 1998; Köhler *et al.* 1999). Drosophila Importin α2 could have a preference over α1 and α3 for certain cargo whose import is required for spermatogenesis. Importin α1 and α3 might still bind weakly to this cargo(s) in the absence of α2, but not strongly enough to fully supplant α2 function. In this case, when Importin α1 and α3 are expressed at high levels they can more efficiently bind the α2 specific cargo(s) and rescue sterility more consistently. The relatively low level of Importin α2 protein in testes is consistent with the hypothesis that Importin α2 serves a partially distinct specialized activity in the testes. Assuming the Western blot data (Figure 3B) reflect the true relative levels of Importin α2 and α3, the loss of α2 should not greatly reduce total importin α protein levels in testes (we are currently unable to assess Importin α1 levels). Following this line of reasoning it makes more sense that Importin α2 is required in the testes at low levels to perform an important function for which it has a higher specific activity than either α1 or α3. Finally, although the concentration of Importin α2 protein is low in adult males and in whole testes, the protein is selectively expressed in stem cells and primary and secondary spermatogonial cells (Figure 4). Therefore, the requirement for Importin α2 during spermatogenesis could derive from a necessity for a high level of cNLS transport in these specific testis cell types.

Perhaps more interesting than the redundant role(s) that the conventional Drosophila importin α genes play in spermatogenesis is the unique requirement for Importin α2 during oogenesis. *Importin* $\alpha 2^{-/-}$ females lay

severely deformed eggs and are completely sterile. Strikingly, the *importin* α*2*$^{-/-}$ female sterility phenotype was completely rescued by expression of *importin* α*2* but not α*1* or α*3*, even when the mRNA levels in the ovary from *importin* α*1* and α*3* transgenes were higher than mRNA levels from the α*2* transgene. Both Northern and Western blot analyses confirmed that all three transgenes were highly expressed in the ovary. In addition, all three transgenes are expressed by the same Gal4$^{nanos\text{-}VP16}$ driver. Therefore, it is unlikely that *importin* α*1* and α*3* transgenes are expressed in a different pattern from the rescuing *importin* α*2* transgene. We conclude that Importin α1 and α3 lack a specific biochemical activity(s) that α2 alone is capable of performing in the ovaries. It is formally possible that Importin α1 and α3 could replace Importin α2 in the female germline were they expressed at higher levels than has been achieved in these experiments. However, complete sterility is observed in females that are extensively overexpressing Importin α1 or α3, indicating that the capacity of Importin α1 or α3 to replace Importin α2 must be exceedingly low.

The unique requirement for Importin α2 during oogenesis might be due to a requirement for the nuclear transport of α2-specific NLS cargo. Thus, female infertility could be a pleiotropic consequence of the mislocalization of a nuclear protein(s) that is required for proper oogenesis. Importin α paralog-specific nuclear transport functions have been suggested by the strong preferences shown by importins α1 and α3 for certain transport cargoes, using binding and permeabilized cell import assays (Sekimoto *et al.* 1997; Köhler *et al.* 1999; Welch *et al.* 1999). Although importin α2-specific NLS cargoes have yet to be identified, not enough potential binding partners have been screened to rule out their existence.

Alternatively, Importin α2 may play an essential role in the ovary in a process(es) that is distinct from its role in nuclear transport. Indeed, there is growing experimental evidence that importin α's can perform import-independent functions. For example, mutations in one of the two *S. pombe* importin α1's, Cut15, affect mitotic chromosome condensation without reducing the efficiency of cNLS-cargo import (Matsusaka *et al.* 1998). Similarly, Srp1p, the importin α gene from *S. cerevisiae*, has been genetically linked to proteasome-mediated protein degradation (Tabb *et al.* 2000). Further experiments are needed to determine if nuclear transport-independent and/or NLS-cargo-specific transport functions explain the unique role that Importin α2 plays during oogenesis.

Importin α2 is partially phosphorylated on serine-56 in ovaries and preblastoderm embryos but not in other stages of development (Küssel and Frasch 1995; Török *et al.* 1995; Figures 3B and 5B). In contrast, Importin α3 appears not to be phosphorylated at any developmental stage or adult tissues (Máthé *et al.* 2000) (Figures 3B and 5B). Because of these observations, we tested the hypothesis that phosphorylation of Importin α2 is necessary for its unique role in oogenesis. In fact, female fertility was successfully rescued by an *importin* α*2* transgene that contained a mutation that prevented its *in vivo* phosphorylation (*importin* α*2* S56A). The function of importin α phosphorylation, which is conserved from yeast to Drosophila, and probably to mammals, remains a mystery. In Drosophila, it has been suggested that phosphorylation of Importin α2 may be related to the observation that in precellularized embryos the protein shifts between the nucleus and cytoplasm in a cell cycle-dependent fashion (Küssel and Frasch 1995; Török *et al.* 1995; Máthé *et al.* 2000). Specifically, Importin α2 protein is cytoplasmic during interphase and nuclear during mitosis (Küssel and Frasch 1995; Török *et al.* 1995). Although it is clear that phosphorylation is not essential for Importin α2 function *in vivo*, it may still play a role in regulating the protein's subcellular localization.

The fact that extant importin α2 and α3 genes occur only in the genomes of metazoan animals suggests that importin α2 and α3 genes evolved to function in cellular process(es) that are conserved among invertebrate and vertebrate lineages. Several observations suggest that multiple animal importin α genes may have arisen during the evolution of gametogenesis. Importin α2 is required predominantly for gametogenesis in Drosophila, and importin α1, -2, and -3 genes are exceptionally highly expressed in mouse testes (Tsuji *et al.* 1997; Nachury *et al.* 1998). In addition, nonconventional *C. elegans* IMA1 and IMA2 importin α genes are expressed exclusively in the gonads (Geles and Adam 2000). The sole conventional *C. elegans* importin α, IMA3, is also expressed in the gonads and is required for gametogenesis. Therefore, there is at least a consistent relationship between the expression patterns and functions of multiple importin α genes and gametogenesis in animals. If true, then the occurrence of a nonconventional importin α clade in monocotyledonous and dicotyledonous plants raises the possibility that a distinct lineage of importin α genes arose to function in plant gametogenesis.

In conclusion, these experiments lay a foundation for future studies on the roles and interplay among conventional animal importin α's. It will be interesting to identify the specific molecular function(s) of Importin α2 in oogenesis and to learn if the functions of all three conventional Drosophila importin α genes are conserved in vertebrates. Future studies on the *importin* α gene family in Drosophila will also depend on the isolation and characterization of *importin* α*1* and α*3* mutations.

We thank I. Kiss for providing the *importin* α*2*^{D14} and *importin* α*2*^{D3} strains; I. Török and E. Máthé for antibodies; M. Frasch for *importin* α*2* cDNA; P. Rørth for the UASp plasmids and the Gal4$^{nanos\text{-}VP16}$ stock; the Bloomington Stock Center for *Df(3L)kto2*, *Df(3R)by416*, Gal4^{Hsp70},

and w^{1118} stocks; K. Jensen for electron microscopy; N. Shulga for confocal microscopy; and members of the Goldfarb and Fleming labs for helpful discussions. This work was supported by grants from the National Institutes of Health (GM40362) and American Cancer Society (BE-104C) to D. S. Goldfarb and National Science Foundation (IBN-9727951) to R. Fleming.

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Communicating editor: K. V. Anderson