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The *Ketel* Gene Encodes a Drosophila Homologue of Importin-β

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ABSTRACT

The *Drosophila melanogaster Ketel* gene was identified via the *Ketel*^{D} dominant female sterile mutations and their *ketel*^{r} revertant alleles that are recessive zygotic lethals. The maternally acting *Ketel*^{D} mutations inhibit cleavage nuclei formation. We cloned the *Ketel* gene on the basis of a common breakpoint in 38E1.2-3 in four *ketel*^{r} alleles. The *Ketel*$^{+}$ transgenes rescue *ketel*^{r}-associated zygotic lethality and slightly reduce *Ketel*^{D}-associated dominant female sterility. *Ketel* is a single copy gene. It is transcribed to a single 3.6-kb mRNA, predicted to encode the 97-kD Ketel protein. The 884-amino-acid sequence of Ketel is 60% identical and 78% similar to that of human importin-β, the nuclear import receptor for proteins with a classical NLS. Indeed, Ketel supports import of appropriately designed substrates into nuclei of digitonin-permeabilized HeLa cells. As shown by a polyclonal anti-Ketel antibody, nurse cells synthesize and transfer Ketel protein into the oocyte cytoplasm from stage 11 of oogenesis. In cleavage embryos the Ketel protein is cytoplasmic. The *Ketel* gene appears to be ubiquitously expressed in embryonic cells. Western blot analysis revealed that the *Ketel* gene is not expressed in several larval cell types of late third instar larvae.

ALONG a genetic dissection of maternal effects in Drosophila, we isolated 75 dominant female sterile (*Fs*) mutations (Erdélyi and Szabad 1989; Szabad *et al.* 1989). In 32 of the *Fs* mutations the *Fs*/+ females deposit normal-looking eggs, and although the eggs are fertilized embryogenesis does not commence or ceases after a few abnormal cleavage divisions. The 32 *Fs* mutations identify 21 genes, suggesting that products of several genes are required for commencement and the initial steps of embryogenesis. This conclusion is supported by the fact that very few, if any, of the zygotic genes are expressed during early embryogenesis and evidently the initial steps of embryogenesis are under maternal control (Wieschaus 1996).

The *Ketel* gene, which was identified by four *Fs(2)Ketel* (= *Ketel*^{D}) mutations, is one of the 21 genes mentioned above (Szabad *et al.* 1989; Erdélyi *et al.* 1997). As described in the accompanying article (Tirián *et al.* 2000), embryogenesis is terminated in *Ketel*^{D} eggs, which are deposited by the *Ketel*^{D}/+ females, soon after fertilization due to the failure of cleavage nuclei formation. When injected into wild-type cleavage embryos, the *Ketel*^{D} egg cytoplasm is toxic: it hinders formation of cleavage nuclei following mitosis most likely through the prevention of nuclear envelope (NE) assembly and/or function. The mutant phenotype suggests involvement of the *Ketel* gene in a NE-related function and motivated cloning of the gene.

As described in this article, the *Ketel* gene encodes for the Drosophila homologue of importin-β, a key player in nuclear protein import. (For recent reviews on nuclear protein import see Corbett and Silver 1997; Görlich and Mattaj 1997; Mattaj and Englmeier 1998; Melchior and Gerace 1998; Pemberton *et al.* 1998; Weis 1998; Wozniak *et al.* 1998; Görlich and Kutay 1999). Briefly, importin-β, the founding member of the importin-β superfamily, was originally described to participate in import of proteins that carry a classical nuclear localization signal (cNLS) into the nucleus. The C-terminal section of importin-β associates with importin-α, an adapter molecule, that binds to the cNLS-containing import substrate. Importin-β forms 19 HEAT- and armadillo-resembling repeats and wraps around the importin-β-binding (IBB) domain of importin-α (Cingolani *et al.* 1999). The substrate-importin-α-importin-β complex docks, in an energy-independent manner, on the cytoplasmic side of the nuclear pore complexes (NPCs). During translocation through the NPCs, importin-β interacts with a number of nucleoporins with its NPC binding domains located toward the N terminus (Kutay *et al.* 1997; Wozniak *et al.* 1998). Import of the cNLS-containing nuclear protein is completed on the nuclear surface of the NPCs, where following interaction of the transport complex with Ran-GTP, the substrate-importin-α-importin-β complex disassembles. (Ran is a

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Ras-related G protein without a membrane anchoring site. For a recent review see Azuma and Dasso 2000.) The import substrate stays in the nucleus, while importin-α and importin-β are recycled to initiate a new import cycle: importin-β returns to the cytoplasm in a complex with Ran-GTP. In the cytoplasm Ran-GTP dissociates from importin-β and is converted to Ran-GDP by RanGTP-ase activating protein (RanGAP) and the Ran binding protein 1 (RanBP1); thus importin-β can participate in a new transport cycle (Bischoff and Görlich 1997; Azuma and Dasso 2000).

This article describes a combined genetic, molecular, and cell biological approach and reveals novel features of importin-β. The Ketel protein shows characteristic features of importin-β: (i) it supports import of a cNLS-containing substrate into nuclei of digitonin-permeabilized HeLa cells and (ii) it is largely cytoplasmic with pronounced accumulation in the NE. Surprisingly, the highly "toxic" *Ketel*^{D} egg cytoplasm, which prevents NE assembly following mitosis, does not prevent nuclear protein import. Unexpectedly, as revealed by Western blot analysis, the *Ketel* gene is not expressed in most cells of the larvae and adults, raising questions about cellular functions of importin-β and nuclear import of the cNLS-containing proteins.

MATERIALS AND METHODS

The *Ketel* mutant alleles: The EMS-induced *Ketel*^{D} alleles were isolated following EMS mutagenesis in a screen for dominant female-sterile mutations (Szabad *et al.* 1989). The 27 recessive *ketel*^{r} alleles were generated through second mutagenesis of the *Ketel*^{D} alleles (Erdélyi *et al.* 1997; Szabad *et al.* 1989). The *ketel*^{r}/− and the *Ketel*^{D}/− hemizygotes were produced by crossing *y/y; ketel*^{rX32}/y^+ *CyO* females with *y/Y; ketel*^{r}/y^+ *CyO* and *y/Y; Ketel*^{D}/y^+ *CyO* males, respectively. [The *ketel*^{rX32} allele, abbreviated as −, is a small deficiency that removes the *Ketel* and a few adjacent loci (Erdélyi *et al.* 1997). The y^+ *CyO* balancer chromosome carries a y^+ transgene (Timmons *et al.* 1993).] Head skeleton and ventral setae of the descending *ketel*^{r}/− and the *Ketel*^{D}/− larvae are yellow and allow their separation from the heterozygous nonyellow (y^+ *CyO*) siblings that have dark chitinous structures. For an explanation of the genetic symbols see Lindsley and Zimm (1992) and the FlyBase website (http://flybase.bio.indiana.edu). All experiments were carried out at 25°.

Molecular cloning of the *Ketel* gene: DNA manipulations, plasmid constructions, restriction mapping, Southern and Northern hybridizations, and Western blotting were done according to standard procedures. For identification of the breakpoints in the four *ketel*^{rX}-associated rearrangements, we isolated DNA from *ketel*^{rX}/+ adult flies. The DNA was digested with *Eco*RV and hybridized on Southern blots. The ^{32}P-labeled probes for Southern hybridizations were generated by random primer labeling of restriction fragments that had been isolated from a λEMBL4 library and from *CoSpeR* clones identified in a chromosomal walk. The chromosomal walk initiated from a clone that hybridized to the 38E1.2-3 cytological region and was kindly provided by Dr. P. Maróy. Cloning the *Ketel* gene was also confirmed by *in situ* hybridizations on salivary gland chromosomes of the *ketel*^{rX}/+ larvae. A detailed restriction map of the *Ketel* region was constructed and the subfragments were cloned into pBluescriptII KS+ vector. We used the subclones to precisely map the *ketel*^{rX}-associated breakpoints for sequencing and screening cDNA libraries.

The *Ketel* cDNA clones were isolated from a λgt10 cDNA library constructed from mRNAs of 0- to 4-hr-old Drosophila embryos. (The cDNA library was a kind gift from Dr. J. Tamkun.) The screening of $\sim 1.5 \times 10^5$ independent plaques resulted in 17 cDNA clones that hybridized with at least one of the subclones covering part of the *Ketel* gene. The overlapping cDNA clones were identified and subcloned into the pBluescriptII KS+ vector. Sequencing of genomic and cDNA clones were done by the dideoxy method in an IBI automated sequenator on both strands. The 5′ end of the mRNA was determined by primer extension. The primer extension was done by using total mRNA isolated from adult females and the synthetic olygonucleotide 5′GCTCTTTGCTCCTATATGAT TTCTAC3′, which hybridized close to the 5′ end of the isolated cDNA. The 3′ end was present in some of the isolated cDNAs as revealed by the poly(A) tail. The intron-exon composition of the region that encodes the 3.6-kb *Ketel* mRNA was determined by sequencing and analyzing a 7870-bp genomic fragment. For developmental Northern analysis poly(A) mRNAs were purified, blotted, and probed with the cDNA that corresponds to the 3.6-kb *Ketel* mRNA. Digoxigenin (DIG)-labeled *Ketel* cDNA was used for the detection of *Ketel* mRNA during oogenesis and embryogenesis, according to standard procedures.

Homology search and putative function of the *Ketel* gene: Having the above-mentioned sequences and to establish possible function of the Ketel protein, we screened databases with the BLAST service of the National Center for Biotechnology Information for identifying sequences displaying homology with the *Ketel* cDNA. Protein alignments were done using the MaxHom EMBL multiple sequence alignment program.

The *Ketel*$^+$ transgenes: We constructed three different types of *Ketel*$^+$ (K^+) transgenes. The first type included the entire 22-kb fragment shown in Figure 1A. The second type covered a 13.8-kb *Xba* genomic fragment (Figure 1B). In the third type a 4.0-kb *Xba-Bam*HI genomic fragment—including the *Ketel* promoter and the 5′ segment of the *Ketel* coding region—was combined with a 2.3-kb cDNA fragment that corresponded to the rest of the transcribed part of the *Ketel* gene (see Figure 1B). The above sequences were cloned into the *CaSpeR* vector with the *mini-white* marker gene and germline transformants were generated by standard procedures. The K^+ transgene-carrying flies have light to orange-yellowish eyes on the *white* genetic background. The K^+ transgenes were used for the construction of K^+; *ketel*^{r}/− and K^+; *Ketel*^{D}/− as well as K^+; *Ketel*^{D}/+ and K^+/K^+; *Ketel*^{D}/+ zygotes. Their viability and the fertility of the females were tested.

Production of the Ketel protein in bacteria and the generation of anti-Ketel polyclonal antibodies: A pGEX-Ketel plasmid was constructed first by the insertion of the *Bam*HI-*Eco*RI fragment of the *Ketel* cDNA (Figure 1A) into the corresponding sites of a pGEX4T-1 vector and glutathione-S-transferase (GST)-Ketel fusion protein was produced in *Escherichia coli*. The fusion protein consisted of the GST moiety fused in frame with the 147–884 amino-acid encoding segment of the Ketel protein. The GST-Ketel fusion protein was purified by affinity chromatography on a glutathion-agarose column and used for immunization of rabbits for the production of anti-Ketel polyclonal antibodies following standard protocols. After several boosts, the crude sera were analyzed for the presence of anti-Ketel antibody by Western blots. Two rabbits produced good titers of anti-Ketel sera by virtue of their ability to recognize the Ketel protein in *E. coli* extracts from strains with pGEX-Ketel but not in the control bacterial extracts.

For production of a nearly full-length Ketel protein, we

made use of the pET-His3A expression system (Chen and Hai 1994). The His-tagged Ketel protein, with amino acids 4–884, was purified by a Ni-chelating column and used for preparation of a Ketel protein affinity column.

The anti-Ketel antibody was purified in two steps: first on a protein-A and afterward on a Ketel protein affinity column. The affinity-purified anti-Ketel antibody was used both in Western blots and in confocal microscopy for the detection of Ketel protein. For Western blots protein extracts were prepared from embryos, larvae, and adults as well as from different organs of late third instar larvae. For laser scanning microscopy ovaries were dissected, fixed, and treated with antibodies. The Ketel protein was detected by the affinity-purified polyclonal anti-Ketel rabbit antibody that was made visible by a goat anti-rabbit rhodamin-labeled secondary antibody (Jackson Laboratories, West Grove, PA). The NE was made visible with a primary monoclonal anti-lamin mouse antibody (Harel *et al.* 1989; Paddy *et al.* 1996) and a fluorescein-labeled anti-mouse secondary antibody (Jackson Laboratories). Optical sections were generated in a Zeiss (Thornwood, NY) LSM 410 confocal microscope.

The *in vitro* nuclear protein import assay: Drosophila importin-β cDNA was cloned into the *Sph*I-*Xma*I sites of pQE30 (QIAGEN, Valencia, CA), expressed with an NH_2-terminal His tag and purified, on nickel-NTA agarose, followed by chromatography on a Superdex 200 gel filtration column.

The nuclear protein import assay was conducted as follows. Permeabilized HeLa cells were prepared by a modification of a published protocol (Adam *et al.* 1990). Briefly, HeLa cells were grown on coverslips to 50–80% confluence, washed in ice-cold permeabilization buffer (20 mM HEPES/KOH pH 7.5, 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA) and permeabilized for 15 min in the same buffer containing 60 μg/ml digitonin. The coverslips were washed three times in permeabilization buffer without digitonin. Coverslips were incubated as indicated with each 20 μl of import reaction. The import buffer contained 2 mg/ml nucleoplasmin core (to block nonspecific binding), 20 mM HEPES/KOH pH 7.5, 140 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA. Where indicated, reactions were supplemented with an energy-regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase) and Ran mix (3 μM Ran-GDP, 150 nM Rna1p, 300 nM NTF2, 150 nM RanBP1). Nuclear import of a fluorescent substrate was monitored in optical sections. The substrate was the pentamer of a fusion protein in which the nucleoplasmin core domain was combined with the importin-β-binding domain from importin-α (IBB core pentamer). Import reaction samples contained 0.24 μM fluorescein-labeled IBB core pentamer. In the indicated reactions 1.2 μM Drosophila importin-β, Ran, and an energy-regenerating system were added. Reactions were stopped after 5 min by fixation in 3% paraformaldehyde (w/v) in PBS, washed in PBS and water, and mounted with 2 μl of vectorshield mounting medium (Vector, Burlingame, CA).

The digitonin-permeabilized HeLa cell system was also used to follow nuclear import of the cNLS-phycoerythrin (cNLS-PE; Cserpán and Udvardy 1995) substrate in presence of cytosol samples prepared from ovaries of wild-type and $Ketel^D$/+ females.

RESULTS

Molecular cloning of the *Ketel* gene of Drosophila: Four of the X-ray-induced $ketel^{rX}$ revertant alleles have a common breakpoint in the 38E1.2-3 cytological region (Erdélyi *et al.* 1997). The common breakpoint both delineated the *Ketel* locus and allowed molecular cloning of the *Ketel* gene. To identify the breakpoints in the four $ketel^{rX}$ alleles, we initiated a genomic walk from a nearby genomic fragment. The genomic walk covered ∼60 kb and resulted in a 5.5-kb genomic *Bgl*II-*Bam*HI fragment that included all four of the $ketel^{rX}$-associated breakpoints (Figure 1A). The corresponding region of the Drosophila genome is included in a cosmid clone that we isolated from a *CoSpeR Not-Bam-Not* cosmid library (Figure 1A). On Northern blots, the *Bgl*II-*Bam*HI fragment strongly hybridized to a 3.6-kb mRNA specimen that most likely represents the *Ketel* gene. The 3.6-kb mRNA is abundant in females and young embryos and appears to be present, although in much reduced concentrations, throughout development (data not shown).

To isolate cDNA clones that correspond to the 3.6-kb *Ketel* mRNA, overlapping genomic fragments covering the *Xba*-*Eco*RV 10.7-kb region (Figure 1A) were used for the screening of a cDNA library prepared from 0- to 4-hr-old embryos. The longest isolated cDNA was 2858 bp long. However, it did not contain poly(A) tail. Overlaps of the 2858-bp cDNA clone with poly(A)-containing cDNAs allowed the reconstruction of a 3378-bp-long cDNA. *In vitro* extension was done on the basis of the mRNAs isolated from adult females and of a primer complementary to the 5′ end of the 2858-bp-long cDNA. The primer extension indicated a major transcription initiation site 478 bp upstream from the 5′ end of the 2858-bp cDNA. We concluded, after finding out about the missing 3′ and 5′ ends of the 2858-bp cDNA, that the encoded *Ketel* mRNA is 3656 nucleotides long and corresponds to the 3.6-kb mRNA detected in Northern analysis.

To determine molecular organization of the *Ketel* locus, we sequenced a 7870-bp long genomic DNA region that corresponds to the encoded cDNA and the surrounding sequences (Figure 1A). The nucleotide sequence is available in the EMBL nucleotide sequence database under the accession no. AJ002729. Comparison of the cDNA and the genomic sequences revealed that the *Ketel* gene contains 5 introns. The *Ketel* mRNA is composed from a 444-bp leader sequence, a 2652-bp open reading frame (ORF), and a 560-nucleotide-long trailer sequence (Figure 1A). To decide whether *Ketel* is a single copy gene or is present in multiple copies, we digested genomic DNA with three different restriction enzymes (*Eco*RI, *Xba*, and *Bgl*II) and carried out Southern analyses with ^{32}P-labeled cDNA fragments. In every case, the labeled cDNA fragments hybridized with a single band, making it very likely that *Ketel* is a single copy gene (data not shown). The finding that under high stringency conditions the Ketel cDNA hybridized exclusively to the 38E1.2-3 region on salivary gland chromosomes supports the above conclusion.

The $Ketel^+$ transgenes: To prove that the cloned gene is indeed *Ketel*, we generated three different types of

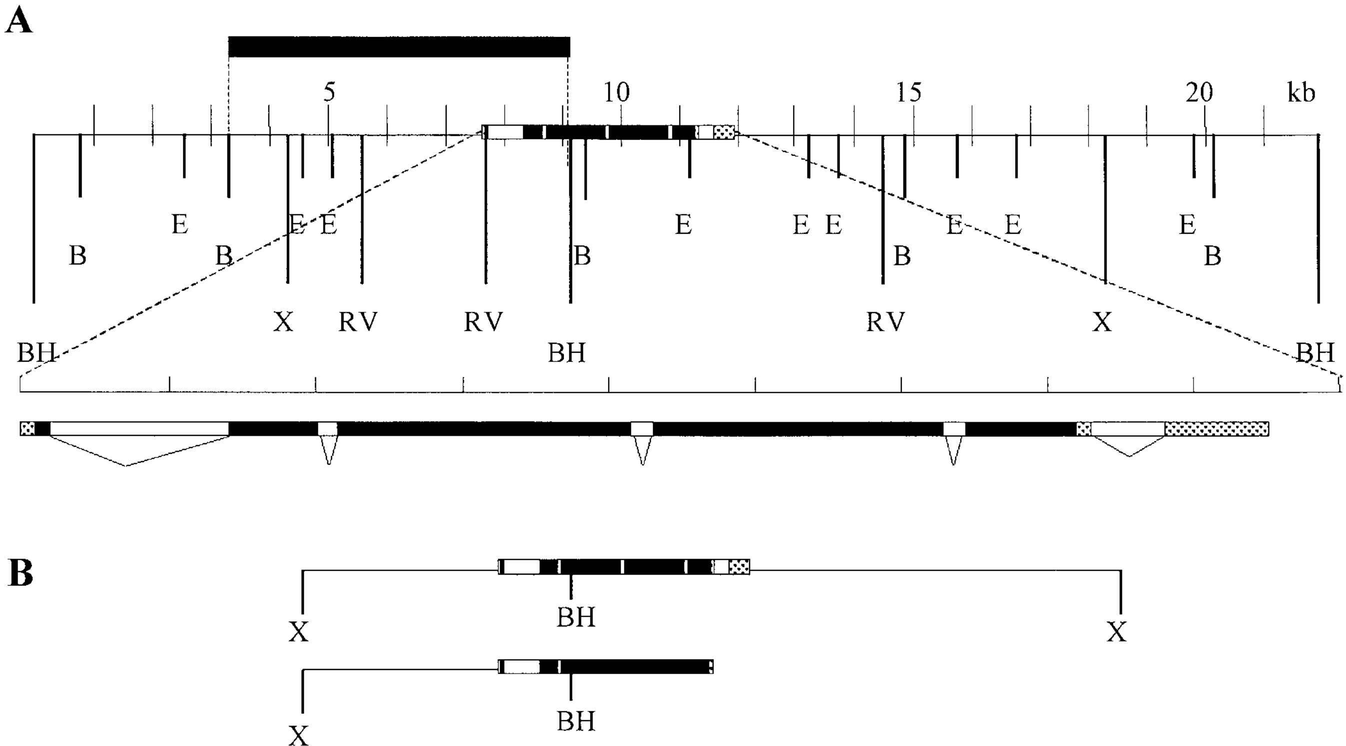

FIGURE 1.—Restriction map of a 22-kb genomic segment from the 38E1.2-3 cytological region comprising the *Ketel* gene. (A) One breakpoint of each of the four *ketel*^{rX} revertant alleles with visible chromosome rearrangements fall within the 5.5-kb *Bgl*II-*Bam*HI genomic fragment delineated by the top thick line. The box on the line below represents the *Ketel* mRNA encoding region. Detailed genomic map of the *Ketel* mRNA encoding region is shown below. Exons, introns, and the untranslated 5′ and 3′ regions are indicated by solid, open, and dotted boxes, respectively. (B). Structure of two of the three K^+ transgenes. Type I comprises the entire 22-kb genomic fragment shown in A. Type II is the 13.8-kb *Xba* genomic fragment. Type III contains the *Xba-Bam*HI 4.0-kb genomic fragment combined in frame with a 2.3-kb *Bam*HI–*Eco*RI segment of the cDNA. The indicated restriction sites are as follows: B, *Bgl*II; BH, *Bam*HI; E, *Eco*RI; RV, *Eco*RV; X, *Xba*.

altogether 21 *Ketel*$^+$ (K^+) transgenes (Figure 1B and Table 1) and analyzed their effects on both *ketel*^{r} and *Ketel*^{D} mutations. Nine of the 12 tested K^+ transgenes brought about full rescue of lethality associated with the loss-of-function *ketel*^{rX13}/− genotype: the K^+; *ketel*^{rX13}/− flies developed with the expected frequencies and were fully fertile, showing that the cloned gene is *Ketel* (Table 1; *ketel*^{rX13} is a null allele).

Type I and each of the three tested type III K^+ transgenes brought about slight rescue of the *Ketel*^{D2}-associated dominant female sterility. In ~1% of the eggs deposited by the K^+; *Ketel*^{D2}/+ females embryogenesis progressed to the stage of embryonic cuticle formation and even a few offspring developed from the K^+; *Ketel*^{D2}/+ females. However, the rate of offspring production was as low as 2–4 $\times$ 10^{-3} offspring/(female $\times$ day), as compared to the ~50 offspring/(female $\times$ day) control value. (It should be noted that cuticle and offspring did not develop from tens of thousands of eggs deposited by *Ketel*^{D2}/+ females.) One copy of the K^+ transgenes had no effects on the other three *Ketel*^{D} mutations. However, two copies of the K^+ transgenes brought

TABLE 1

The K^+ transgenes and their rescue effects on *ketel*^{r}/−-associated zygotic lethality

	Chromosome linkage				
Type and composition of the K^+ transgenes[a]	X	2nd	3rd	4th	Total
I: 22-kb genomic segment	1 (0/1)	0	0	0	1
II: 13.8-kb genomic segment	0	3	5 (4/5)	0	8
III: 4.0-kb genomic combined with a 2.3-kb cDNA segment	1 (1/1)	4	5 (3/4)	2 (1/1)	12

In parentheses, number of rescuing/tested K^+ transgenes.
[a] See Figure 1 for composition of the K^+ transgenes.

about slight reduction of female sterility when sperm with two normal Ketel gene copies fertilized the eggs (Tirián *et al.* 2000). The slight rescue of the K^+ transgenes on *Ketel*^{D}-associated dominant female sterility clearly shows that (i) the cloned gene is *Ketel* and (ii) the normal and the *Ketel*^{D}-encoded gene products participate in the same process and hence the *Ketel*^{D} alleles are strong dominant-negative mutations.

The *Ketel* gene encodes the Drosophila homologue of importin-β: The *Ketel* cDNA contains a 2652-nucleotide-long ORF encoding for a protein of 884 amino acids with a molecular mass of 97 kD. Comparison of the Ketel protein amino acid sequence with known sequences revealed a 60.3% amino acid identity and a 78.2% similarity with human importin-β, a known component of nuclear protein import (Figure 2; Adam and Adam 1994; Chi *et al.* 1995; Görlich *et al.* 1995a,b; Imamoto *et al.* 1995; Iovine *et al.* 1995; Radu *et al.* 1995). The high level of homology suggests that *Ketel* encodes the Drosophila homologue of importin-β, the founding member of the importin-β superfamily (Wozniak *et al.* 1998, Görlich and Kutay 1999).

The Ketel protein supports nuclear protein import: To decide whether the Ketel protein does indeed function as importin-β we monitored (i) the docking on the cytoplasmic surface of the NE and (ii) import into nuclei of digitonin-permeabilized HeLa cells of a fluorescent-labeled nuclear substrate in the presence of the Ketel protein and other components of the nuclear protein import apparatus (see materials and methods). As shown on Figure 3B, the substrate docked on the NE in presence of the Ketel protein, and when Ran and an energy source were added the substrate was imported into the nuclei (Figure 3, B and D). The permeabilized HeLa cell experiments clearly showed that the Ketel protein molecules function as importin-β: they can assist docking and import of nuclear proteins into the nuclei.

To understand effects of the *Ketel*^{D} mutations on nuclear protein import, we prepared cytosol from ovaries of both *Ketel*^{D}/+ and wild-type females. All the four *Ketel*^{D} mutations were included in this study. The cytosol preparations were used in the permeabilized HeLa cell assay and import of the cNLS-PE substrate was monitored (see materials and methods). In presence of the wild-type ovary cytosol the cNLS-PE substrate entered the nuclei of HeLa cells within a few minutes (Figure 4B). Surprisingly, the *Ketel*^{D}/+-derived cytosol preparations just as efficiently supported nuclear import of the cNLS-PE substrate as the wild-type ovary cytosol, showing that the *Ketel*^{D}-encoded mutant molecules do not interfere with import of the cNLS-PE substrate. Note that when injected into wild-type cleavage embryos, traces of the *Ketel*^{D} egg cytoplasm prevent the formation of cleavage nuclei at the end of mitosis (Tirián *et al.* 2000). However, nuclei of the digitonin-permeabilized HeLa cells remained intact for at least 4 hr in presence of the *Ketel*^{D}/+-derived ovary cytosol.

Expression pattern of the *Ketel* gene: To study the expression pattern of the *Ketel* gene, we detected both *Ketel* mRNA and Ketel protein during oogenesis and embryogenesis. Some *Ketel* mRNA, as detected with the DIG-labeled *Ketel* cDNA, is present in nurse cells of the stage 9 egg primordia. The concentration of *Ketel* mRNA becomes rather high by stage 10 (Figure 5A) when dumping of the *Ketel* mRNA into the oocyte cytoplasm begins. Beyond stage 11 the *Ketel* mRNA is homogeneously distributed in the oocyte cytoplasm (Figure 5B). The *Ketel* gene appears to be ubiquitously expressed in every blastoderm cell (Figure 5C) and, as far as it can be deduced from the staining patterns, also during later stages of embryogenesis (Figure 5, D–F). The *Ketel* gene seems to be intensively expressed in the central nervous system and in the larval gonads (Figure 5, E and F). The larval gonads include both ovaries and testes since the gonads possess intensive staining in each of the embryos.

We also followed *Ketel* gene expression through the detected Ketel protein with the affinity-purified polyclonal anti-Ketel antibody. The anti-Ketel antibody detected a single 97-kD protein band on Western blots with extracts prepared from different developmental stages (Figure 6). The Ketel protein is abundant in the ovaries and in the newly deposited eggs throughout embryogenesis and is present throughout all stages of development. However, when compared, *e.g.*, to ovaries, the relative Ketel protein concentration was rather low in larvae and adult females from which the ovaries were removed. To clarify the low Ketel protein content we dissected different organs from late third instar larvae and subjected them to Western blot analysis. As shown in Figure 6, while, *e.g.*, the imaginal discs contained significant amounts of the Ketel protein, there were no detectable amounts of Ketel protein present in a number of larval tissues including the salivary glands, gut, Malpighian tubules, or the larval epidermis with the overlying larval musculature.

Formation and localization of the Ketel protein was also followed in the course of oogenesis and embryogenesis by immunocytology and confocal microscopy. The Ketel protein is first detectable in nurse cells during stage 8 of oogenesis (not shown). By stage 10 the nurse cells contain large quantities of the Ketel protein. The protein is cytoplasmic with pronounced accumulation in the NEs (Figure 7, A and C). Nurse cells dump their Ketel protein contents into the oocyte cytoplasm from stage 11 of oogenesis. The follicle cells also contain Ketel protein (Figure 7A). Cytoplasm of a newly deposited egg contains stockpiles of the Ketel protein. During cleavage divisions the Ketel protein is present throughout the cleavage cycles. It is cytoplasmic and shows accumulation in the NE (Figure 7, D and F).

Immunoreactive features of the *Ketel*^{D}-encoded protein molecules are not different from wild type: amount and size of the immunoreactive components in wild-type

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              1                                                                     50
KETEL         MTSDIAMQLI AILEKTVSPD KNELLSAKNF LEQAAASNLP EFLKALSEIL VNTANSAVAR MAAGLQLKNH LTRKDEKVSQ
HUMIMP 90A    ......MELI TILEKTVSPD RLELEAAQKF LERAAVENLP TFLVELSRVL ANPGNSQVAR VAAGLQIKNS LTSKDPDIKA
IMB RAT       ......MELI TILEKTVSPD RLELEAAQKF LERAAVENLP TFLVELSRVL ANPGNSQVAR VAAGLQIR.L LTSKDPDIKA
C.ELEGANS     .......... .......... .......... .......... ......LSMIL RTQQCQSFVR QAAGLQLKNV LCAKETETKN
KAP95 YEAST   .......... .LENSILSPD QNIRLTSETQ LKKLSNDNFL QFAGLSSQVL IEGESDDVNR ATAGMLLKNS MLGGNNLIKS

                                    100                                                        150
KETEL         QYQDRWHQFP SEIRELIKNN ILAALGTENT RPSCAAQCVA YVAVIELPIN RRPMLIQTLV NKVVSEGSSE MHRESALEAI
HUMIMP 90A    QYQQRWLAID ANARREVKNY VLQTLGTETY RPSSASQCVA GIACAEIPVN QWPELIPQLV ANVTNPNSTE HMKESTLEAI
IMB RAT       QYQQRWLAID ANARREVKNY VLQTLGTETY RPSSASQCVA GIACAEIPVS QWPELIPQLV ANVTNPNSTE HMKESTLEAI
C.ELEGANS     VYLQRWLQLT AEVREQVKQN VTGTLGTEPS RPSIAAQCVA AIACAELPQN LWPNVINLLK SNVTESQSGE MLKESSLETL
KAP95 YEAST   QFAQRWIQVS PEAKNQIKTN ALTALVSIEP RANAAAQLIA AIADIELPHG AWPELMKIMV DNTGA.EQPE NVKRASLLAL

                                                        200
KETEL         GYICQDIRFGV MENQSNDVLT AITHGMRKVE PSNHVRLAAT TALHNSLEFT KSNFEKDMER NFIMEVVCEA TQCQDSQISV
HUMIMP 90A    GY.CQDIDPEQ LQDKSNEILT AIIQGMRKEE PSNNVKLAAT NALLNSLEFT KANFDKESER HFIMQVVCEA TQCPDTRVRV
IMB RAT       GY.CQDIDPEQ LQDKSNEILT AIIQGMRKEE PSNNVKLAAT NALLNSLEFT KANFDKESER HFIMQVVCEA TQCPDTRVRV
C.ELEGANS     GY.CQDIDPRV LETKANDVLT AIIHGMRPEE SSANVRFAAT NALLNSLEFT NTNFSNEAER NIIMQVVCES TSSSDQRVKV
KAP95 YEAST   GY.SADPQSQA LVSSSNNILI AIVQGAQSTE TSKAVRLAAL NALADSLIFI KNNMEREGER NYLMQVVCEA TQAEDIEVQA

                   250                                                              300
KETEL         AALQCLVKIM TLYYQYMEPY MAQALFPITL AAMKSDNDAV ALQGIEFWSN VCDEEIDLAI ESQEATDQGR APQRVSKHYA
HUMIMP 90A    AALQNLVKIM SLYYQYMETY MGPALFAITI EAMKSDIDEV ALQGIEFWSN VCDEEMDLAI EASEAAEQGR PPEHTSKFYA
IMB RAT       AALQNLVKIM SLYYQYMETY MGPALFAITI EAMKSDIDEV ALQGIEFWSN VCDEEMDLAI EASEAAEQGR PPEHTSKFYA
C.ELEGANS     AALQCLVRIM QLYYEHMLSY MGSALFQITL SAMKSQEPEV AMQGMEFWST VAEEEFDLYM TYEDEVERGA PPKCASLRFM
KAP95 YEAST   AAFGCLCKIM SLYYTFMKPY MEQALYALTI ATMKSPNDKV ASMTVEFWST ICEEEIDIAY E....LAQFP QSPLQSYNFA

                                          350                                                        400
KETEL         RGALQFLTPV LVEKLTKQDE CDDEDTWSPA KAASVCLMVL ATCCEDEIVP HVLPFIKENI ESPNWRFRDA AVMTFGSVLN
HUMIMP 90A    KGALQYLVPI LTQTLTKQDE NDDDDDWNPC KAAGVCLMLL ATCCEDDIVP HVLPFIKEHI KNPDWRYRDA AVMAFGCILE
IMB RAT       KGALQYLVPI LTQTLTKQDE NDDDDDWNPC KAAGVCLMLL STCCEDDIVP HVLPFIKEHI KNPDWRYRDA AVMAFGSILE
C.ELEGANS     EQAASHVCPV LLEAMAHHDD GDDDDDWTPA KAAGVCLML. AQCVRDDIVN HVIPFFKH.F QNPDWKYKEA AIMAFGSILD
KAP95 YEAST   LSSIKDVVPN LLNLLTRQNE DPEDDDWNVS MSAGACLQLF AQNCGNHILE PVLEFVEQNI TADNWRNREA AVMAFGSIMD

                                                                         450
KETEL         GLETNTLKPL VEQAMPTLIR LMYDSSVIVR DTIAWTFGRI CDIIPEAAIN ETYLQTLLEC FVKSLKSEPR VAANVCWAFI
HUMIMP 90A    GPEPSQLKPL VIQAMPTLIE LMKDPSVVVR DTAAWTVGRI CELLPEAAIN DVYLAPLLQC LIEGLSAEPR VASNVCWAFS
IMB RAT       GPEPNQLKPL VIQAMPTLIE LMKDPSVVVR DTTAWTVGRI CELLPEAAIN DVYLAPLLQC LIEGLSAEPR VASNVCWAFS
C.ELEGANS     GPDPKKLLPM AQEALPAIVA AMCDKNVNVR DTAAWSLGRV IDTCSELANN ELLQSVLPVL LSNGLHQEPR VANNVCWALV
KAP95 YEAST   GPDKVQRTYY VHQALPSILN LMNDQSLQVK ETTAWCIGRI ADSVAESIDP QQHLPGVVQA CLIGLQDHPK VATNCSWTII

                                  500                                                   550
KETEL         GLSDAAWEAA VTNDGETPET YALSPYF.EYI ITQLLETTDR SDGAQA.NLRC AAYQALMDMI KNSPLDCYLV VQRTTLVILE
HUMIMP 90A    SLAEAAYEAA DADDQEEPAT YCLSSSF.ELI VQKLLETTDR PDGHQN.NLRS SAYESLMEIV KNSAKDCYPA VQKTTLVIME
IMB RAT       SLAEAAYEAA DADDQEEPAT YCLSSSF.ELI VQKLLETTDR PDGHQN.NLRS SAYESLMEIV KNSAKDCYPA VQKTTLVIME
C.ELEGANS     SLVKACYESA VTDGSGQPDT FALSSVF.DPM VGELIKITDR VDGNQS.NLRI TAYEALMELI KHSPKDCYSA VRNTTVVILK
KAP95 YEAST   NLVEQLAEA. ......TP.. ...SPIYNFYP ALVDGLIGAA NRIDNEFNARA SAFSALTTMV EYATDTVAET SASISTFVMD

                                                          600
KETEL         RLNQVMQMET QINNHSDRHQ FNDLQSLLCA TLQSVLRQVH EQDAPQISDA IMTALLTMFN SSAGKSGVVQ EEAFLAVSTL
HUMIMP 90A    RLQQVLQMES HIQSTSDRIQ FNDLQSLLCA TLQNVLRKVQ HQDALQISDV VMASLLRMFQ STAG.SGGVQ EDALMAVSTL
IMB RAT       RLQQVLQMES HIQSTSDRIQ FNDLQSLLCA TLQNVLWKVQ HQDALQISDV VMASLLRMFQ STAG.SGGVQ EDALMAVSTL
C.ELEGANS     KLESLLQMES QATSEADKAQ VRDLQAMLCA TLQSVTRKMQ PADIPAVGEH IMNGLYQIMN RAATRSNAVM EEALLAVACL
KAP95 YEAST   KLGQTMSVDE NQLTLEDAQS LQELQSNILT VLAAVIRKSP .SSVEPVADM LMGLFFRLLE KKD..SAFIE DDVFYAISAL

                   650                                                              700
KETEL         VELLGAQFAK YMPAFKDFLV MGLKNFQEYQ VCCATVGLTG DIFRALKDLM VPYSNEIMTV LINNLTEPTI HRTVKPQVLS
HUMIMP 90A    VEVLGGEFLK YMEAFKPFLG IGLKNYAEYQ VCLAAVGLVG DLCRALQSNI IPFCDEVMQL LLENLGNENV HRSVKPQILS
IMB RAT       VEVLGGEFLK YMEAFKPFLG IGLKNYAECQ VCLAAVGLVG DLCRALQSNI LPFCDEVMQL LLENLGNENV HRSVKPQILS
C.ELEGANS     AE.LGKGFLS YMNVLKPYLL EGLSNTDETQ VCAAAVGLVT DLSRALEAEI MPFMDELIQK LILCLQVPRL DRNVKVVIIG
KAP95 YEAST   AASLGKGFEK YLETFSPYLL KALNQ.VDSP VSITAVGFIA DISNSLEEDF RRYSDAMMNV LAQMISNPNA RRELKPAVLS

                                      750                                                        800
KETEL         AFGDIALSIG NHFLPYLSMV LDMLAVASNL QTDANNFDMN EYINELRESI LEAYTGIIQG LKGVDQTAHT DVMHMEPHLM
HUMIMP 90A    VFGDIALAIG GEFKKYLEVV LNTLQQASQA QVDKSDYDMV DYLNELRESC LEAYTGIVQG LKGDQENVHP DVMLVQPRVE
IMB RAT       VFGDITLAIG GEFKKYLEVV LNTLQQASQA QVDKSDFDMV DYLNELRESC LEAYTGIVQG LKGDQENVHP DVMLVQPRVE
C.ELEGANS     TFADIAMAIE AHFERYVGSV VPILNNAQNA AVN..DDDQV DYVDRLREAC LNSYTGIIQG LKAIPDTTAA RNM.INVFVE
KAP95 YEAST   VFGDIASNIG ADFIPYLNDI MALCVAAQNT KPENGTLEAL DYQIKVLEAV LDAYVGIVAG LHDKPEA... ....LFPYVG

                                                          850
KETEL         HIISFIKRIA QEGDVSDSML ASAAGFIGDL LHFVWSAAIP LLDDAIITQF LAEGKRSKAQ RTKMLCTWAV KEIKKINTQV ITQ
HUMIMP 90A    FILSFIDHIA GDEDHTDGVV ACAAGLIGDL CTAFGKDVLK LVEAPMIHEL LTEGRRSKTN KAKTLATWAT KELRKLKNQ. ...
IMB RAT       FILSFIDHIA GDEDHTDGVV ACAAGLIGDL CTAFGKDVLK LVEAPMIHEL LTEGRRSKTN KAKTLATWAT KELRKLKNQ. ...
C.ELEGANS     PIVQLITRIS SMEPVSEALI ATTAGIIGDL VQLYEGDIIR FFLTDQVTQM LQKGRKSKVS KTKSMANWAT KEIKKVTLK. ...
KAP95 YEAST   TIFQFIAQVA EDPQLEDATS RAAVGLIGDI AAMFPDGSIK QFYGQDWVID YIKRTRSGQQ ATKDTARWAR EQQKR..... ...
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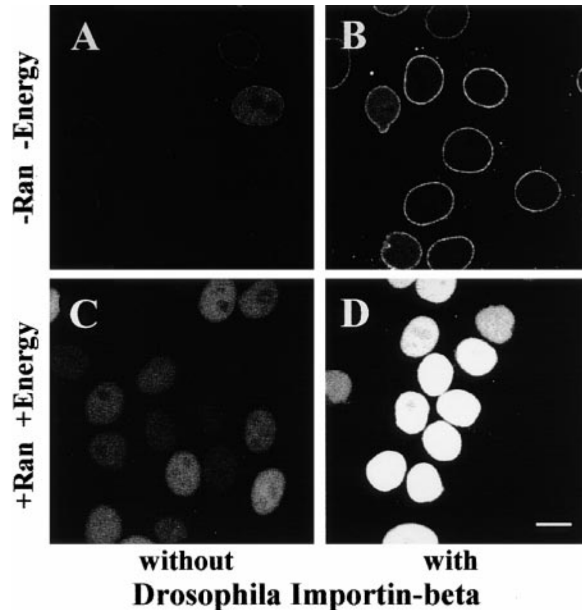


Figure 3.—Drosophila Ketel protein functions as importin-β since it promotes the docking on the NE and nuclear import of a fluorescent IBB-nucleoplasmin fusion protein as detected in optical sections. Without addition of Ran and energy supply (A) or with Ran and energy supply (C) only background signals appear due to residual components in the digitonin-permeabilized HeLa cells. When importin-β is added and no energy is supplied the substrate docks on the cytoplasmic surface of the NE (B). When, however, Ran, energy supply, and importin-β are added the substrate is imported into the nuclei (D). Bar, 10 μm.

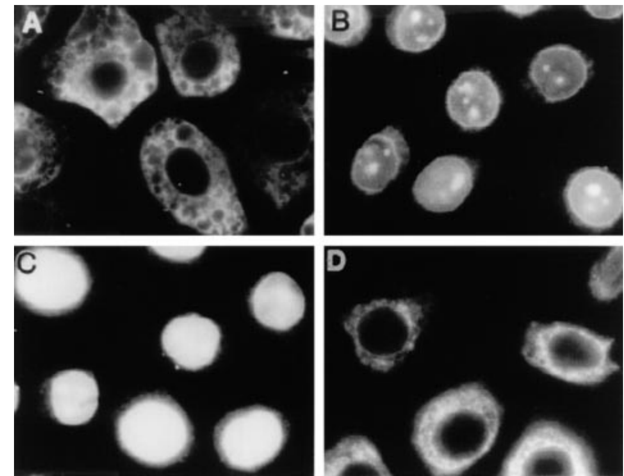


Figure 4.—Nuclear import of the cNLS-PE substrate into nuclei of digitonin-permeabilized HeLa cells in the presence of cytosol prepared from Drosophila ovaries. (A) At 0° and in the absence of ATP the cNLS-PE substrate molecules are not imported into the nuclei and do not even accumulate around the NE. (B) At 30°, the cNLS-PE substrate molecules are imported into the nuclei even in the absence of ATP. (C) Nuclear import of the cNLS-PE substrate is very effective on 30° when extraneous ATP is added. The nuclear import patterns shown on B and C are identical for wild-type and $Ketel^D$/+-derived ovary cytosol preparations. (D) Wheat germ agglutinin (200 μg/ml) effectively blocks nuclear protein import on 30° even in the presence of ATP.

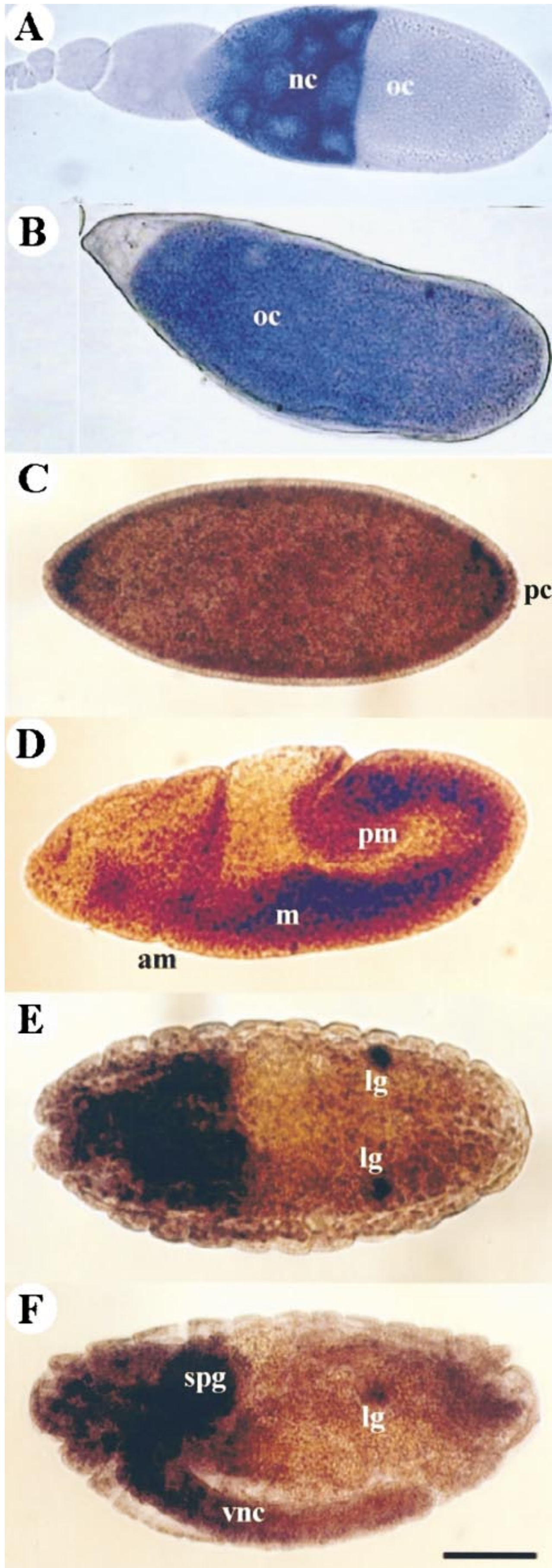
and $Ketel^D$/+ ovary and egg extracts appear identical on Western blots (data not shown). These observations suggest that the EMS-induced $Ketel^D$ mutations (i) did not alter the expression pattern of the gene and (ii) did not change the size of the encoded protein molecules.

DISCUSSION

The *Ketel* gene encodes the Drosophila homologue of importin-β: The *Ketel* gene was identified by four EMS-induced *Fs(2)Ketel* (= $Ketel^D$) dominant female sterile mutations and their $ketel^r$ revertant alleles (Szabad *et al.* 1989; Erdélyi *et al.* 1997). The $Ketel^D$ alleles are gain-of-function type and bring about dominant female sterility by inhibiting the commencement of embryogenesis. The $Ketel^D$-encoded gene products prevent cleavage nuclei assembly at the end of mitosis by, as it appears, disrupted NE formation/function and suggest a NE-related function of the normal *Ketel* gene product (Tirián *et al.* 2000). Most of the loss-of-function $ketel^r$ alleles are zygotic lethal mutations that cause death during second larval instar showing zygotic requirement of the *Ketel* gene (Erdélyi *et al.* 1997). To understand *Ketel* gene function, we cloned the *Ketel* gene. A common breakpoint in four of the X-ray-induced $ketel^{rX}$ alleles localized the gene to the 38E1.2-3 cytological position and allowed, as an outcome of a genomic walk, cloning of the *Ketel* gene. Genomic Southern and developmental Northern and Western analyses revealed that the single-copy *Ketel* gene encodes a single type of 3.6-kb mRNA and synthesis of the corresponding 97-kD Ketel protein.

To show that the cloned gene is indeed *Ketel*, we generated different types of K^+ transgenes. Because the transgenes bring about rescue of $ketel^r$-associated lethality, it is safe conclude that the cloned gene is *Ketel*. Furthermore, the K^+ transgenes slightly reduce $Ketel^D$-associated dominant female sterility, showing that the normal and the $Ketel^D$-encoded mutant gene products participate in the same pathway. The slight rescue of

Figure 2.—Alignment of the Ketel protein, human, rat, *Caenorhabditis elegans*, and yeast importin-β amino acid sequences. Boldface letters in boxes label amino acid identities among all sequences. Boxes alone indicate identity but one. The sources are as follows: Drosophila: this article, accession no. AJ002729; human: Görlich *et al.* (1995a,b), accession no. L38951, nucleotide identity no. (NID) G893287; rat: Radu *et al.* (1995), accession no. L38644; *C. elegans*: Wilson *et al.* (1994), accession no. AF003136, NID G2088700; *Saccharomyces cerevisiae*: KAP95 protein, Enenkel *et al.* (1995), accession no. S51350, Cosmid 8300, NID G2088700, EMBL U19028.



$Ketel^D$-associated dominant female sterility implies a dominant-negative nature of the $Ketel^D$ mutations; *i.e.*, the $Ketel^D$-encoded molecules impede function of the normal Ketel gene products (Tirián *et al.* 2000).

Comparison of nucleotide and amino acid sequences of the *Ketel* gene and the Ketel protein revealed strong homology with human importin-β, a component of nuclear protein import: in the two protein sequences 60% of the amino acids are identical and 78% are of similar nature.

Importin-β (also called karyopherin-β) is a major component of nuclear protein import and has been known from biochemical studies in which components of nuclear protein import were identified (Adam and Adam 1994; Chi *et al.* 1995; Görlich *et al.* 1995a,b; Imamoto *et al.* 1995; Radu *et al.* 1995). The *Ketel* gene does indeed encode the Drosophila importin-β since the Ketel protein possesses characteristic features of importin-β. In absence of an energy source the Ketel protein produced in bacteria supports docking of a IBB-nucleoplasmin core fusion protein on the NE of digitonin-permeabilized HeLa cells (Figure 3B). When an energy source is provided, the nuclear protein is imported into the nucleus.

Transport of macromolecules between the cytoplasm and the nucleus proceeds through the NPCs and is mediated by shuttling receptors of the importin-β superfamily (for reviews see Mattaj and Englmeier 1998; Pemberton *et al.* 1998; Weis 1998; Wozniak *et al.* 1998; Görlich and Kutay 1999). The importins bind their cargo, directly or through adapter molecules like importin-α, in the cytoplasm and release them in the nucleus. A RanGTP gradient provides the driving force for transport: importins bind their cargo in the cytoplasm where RanGTP levels are low and release it upon encountering high RanGTP concentration in the nucleus. The conversion of RanGTP to RanGDP in the cytoplasm is catalyzed by RanGAP1 and is further stimulated by RanBPs. RanGTP is generated from RanGDP in the nucleus by RCC1 (regulator of chromatin condensation), a chromatin-associated nucleotide exchange factor. Importin-β is engaged in nuclear import of proteins with cNLS through importin-α, an adapter molecule. Importin-β can also operate as an autonomous receptor independently of importin-α. A number of types of pro-

Figure 5.—*In situ* hybridizations for the detection of Ketel mRNA during oogenesis (A and B) and different stages of embryogenesis: cellular blastoderm, stage 5 (C), stage 8 (D), stage 14 (E), and stage 17 (F) embryos. Lateral (C, D, and F; anterior left and dorsal up) and dorsal (E) views. nc, nurse cells; oc, oocyte; pc, pole cells; am, anterior midgut primordium; pm, posterior midgut primordium; m, mesoderm; lg, larval gonad; spg, supra oesophageal ganglion; vnc, ventral nerve cord (embryos were staged as described in Campos-Ortega and Hartenstein 1997). Bar, 50 μm.

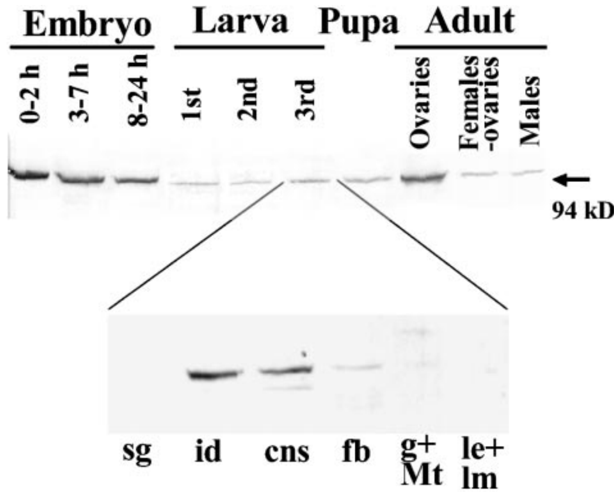


Figure 6.—Western blot analysis to detect Ketel protein with a polyclonal anti-Ketel antibody. Equal amounts of protein samples (as measured by photometry and confirmed with Ponceau-stained control gels) were loaded in the different slots. With respect to third instar larval organs, the central nervous system (cns) did not include the ring gland and the larval gonads were removed from the fat body (fb) sample. sg, salivary glands; id, imaginal discs; g + Mt, gut and Malpighian tubules; le + lm, larval epidermis with the overlaying larval musculature.

teins have been identified that can directly bind to importin-β and are imported into the nucleus, *e.g.*, some ribosomal proteins and the HIV Rev protein (Henderson and Percipalle 1997; Jäkel and Görlich 1998; Truant and Cullen 1999). Importin-β can also form a complex with importin-7 and mediate histone H1 import (Görlich *et al.* 1997; Jäkel *et al.* 1999). In addition, apart from importin-α, importin-β also uses other adapter molecules: snurportin1 for the import of m3G-capped UsnRNPs (Huber *et al.* 1998) and XRIPa for the import of the Xenopus replication protein A (Jullien *et al.* 1999).

Several members of the importin-β superfamily have been identified mainly in yeast and vertebrates (Wozniak *et al.* 1998; Görlich and Kutay 1999). A search in the Drosophila genome (at http://flybase.bio.indiana.edu) for homologues of vertebrate importin-β superfamily members identified 10 genes (Table 2). It appears that most members of the human importin-β family are also present in Drosophila (Table 2). However, there is no apparent homologue of exportin-t that is engaged in export of tRNAs from the nuclei to the cytoplasm. The closely related human importin-5 and RanBP6 (Görlich and Kutay 1999) have a single corresponding Drosophila homologue (Karybeta3). Similarly, human importin-7 and RanBP8 have a single Drosophila relative called dim-7. As in humans, there are two transportin genes in Drosophila (Table 2). Of the Drosophila importin-β family members, functions have been assigned thus far to transportin (Siomi *et al.* 1998) and to a homologue of human CRM1 *embargoed* (Fasken *et al.* 2000) and, as described in this article, to Ketel.

The Ketel protein is cytoplasmic and is not present in every cell type: As other members of the importin-β family, the Ketel protein is largely cytoplasmic (Görlich *et al.* 1995a) with pronounced accumulation in the NE (Figure 7). As predicted by features of the *Ketel*^{D} and *ketel*^{r} mutant phenotypes (Tirián *et al.* 2000), the Ketel protein is produced and is dumped into the oocyte cytoplasm during oogenesis and cleavage embryos make use of the Ketel maternal dowry. Surprisingly, however, the *Ketel* gene does not appear to be expressed in the

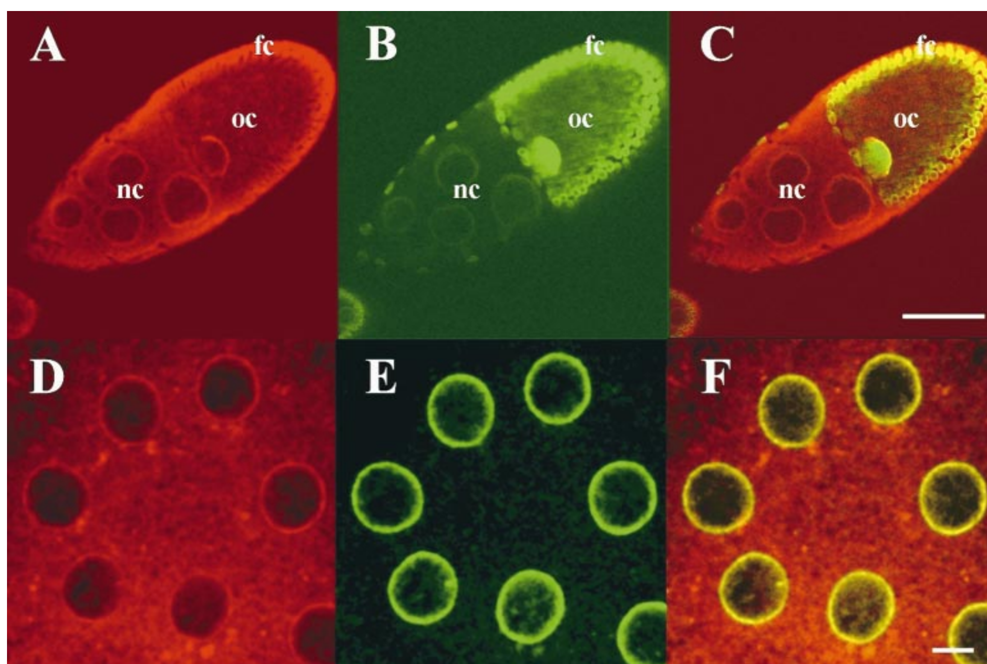


Figure 7.—Distribution of the Ketel protein, as detected in optical sections, in a stage 10 egg primordium and in an interphase cleavage embryo. The Ketel protein is shown in red (A and D), the nuclear lamina appears in green (B and E). Merged signals are shown on C and F where yellow coloration results from superimposition of green and red signals. nc, nurse cells; oc, oocyte; fc, follicle cells. Bar, 50 μm for A–C and 5 μm for D–F. As shown by the lamin signal in C and D the oocyte nucleus contains uniformly distributed lamin molecules inside (Ashery-Padan *et al.* 1997).

TABLE 2

Members of the Drosophila importin-β family and their closest human homologues on the basis of amino acid sequence identities

Drosophila protein[a]	Cytological localization	Closest human homologue(s)	Accession nos. of the human gene	Identity level (%)	References in FlyBase
Ketel (CG2637)	38 E1.2-3	Importin-β	I52907	60	This article; Szabad *et al.* (1989); Erdélyi *et al.* (1997); Kozlova *et al.* (1998)
Transportin (CG7398)	65 A6	Transportin-1	Q92973	71	Siomi *et al.* (1998); Norvell *et al.* (1999)
		Transportin-2	AF019039	68	
CG 8219	65 A6	Transportin-1	Q92973	58	
		Transportin-2	AF019039	56	
CAS (CG13281)	36 B1-2	CAS	P55060	51	Spradling *et al.* (1999)
Karybeta 3 (CG1059)	82 D1	Importin-5	Y08890	51	Colas *et al.* (1999); Spradling *et al.* (1999)
Dim-7 (CG7935)	66 B7-10	Importin-7	AF098799	52	
CG 8212	52 C1-2	RanBP11	AK001696	35	
CG2848	23 B1	Mtr10a	AJ133749	41	
CG12234	18 D10-11		KIAA1291	34	
Embargoed (CG13387)	29 C1	CRM1	Y08614	69	Fasken *et al.* (2000)

[a] Code as available from FlyBase (http://flybase.bio.indiana.edu).

fully differentiated larval cells. Apparently the Ketel protein appears to be present largely in mitotically active cells. Genetic requirement of the *Ketel* gene is discussed in the accompanying article by Tirián *et al.* (2000).

The possible mode of action of the *Ketel^D*-encoded proteins: When injected into wild-type cleavage embryos, traces of the *Ketel^D* egg cytoplasm exert deleterious effects through the prevention of cleavage nuclei formation (Tirián *et al.* 2000). Toxic effects of the *Ketel^D*-encoded molecules are perhaps an outcome of arrested nuclear protein import. To elaborate this possibility, we prepared cytosol from ovaries of the *Ketel^D*/+ females and studied their effects on nuclear protein import. Unexpectedly, the *Ketel^D* cytosol preparations did not prevent nuclear import of the cNLS-PE substrate (Figure 4). In fact, the cNLS-PE molecules were equally efficiently imported into the nuclei in the presence of the *Ketel^D* or wild-type ovary cytosol. Consistent with this observation, the *Ketel^D* egg cytoplasm did not prevent import of the cNLS-PE molecules into interphase nuclei of wild-type cleavage embryos (Tirián *et al.* 2000). Knowing that the *Ketel^D* alleles are strong dominant-negative mutations, the above results may be surprising. A number of possibilities may come to light to explain the former observation. It is very unlikely that all four of the EMS-induced *Ketel^D* alleles altered expression of the *Ketel* gene such that the cytosol preparations did not contain *Ketel^D*-encoded molecules. Although the *Ketel^D*-encoded molecules block function of the normal ones, perhaps the cNSL-PE substrate is imported into the nuclei via another nuclear protein import route powered by unidentified components of the ovary cytosol. The existence of parallel import pathways is well established. For example, the human ribosomal protein L25 is imported through at least four pathways (Jäkel and Görlich 1998). The *Ketel^D*-encoded molecules well may support nuclear protein import, a feature not known at present. It is also possible that although the *Ketel^D*-encoded molecules do not participate in nuclear protein import, they do not interfere with import function of the normal Ketel molecules, and their toxic effects become apparent when the importin-β molecules perform a function other than nuclear protein import.

Indeed, the deleterious effects of the *Ketel^D* mutations become apparent at the end of cleavage mitosis when the NE reassemble and daughter nuclei form. Remarkably, the *Ketel^D* cytosol did not disrupt HeLa cell nuclei and, along with this observation, Drosophila wild-type interphase cleavage nuclei remained intact in presence of the *Ketel^D* egg cytoplasm. Because the digitonin-permeabilized HeLa cells do not divide, they are inadequate to detect defects associated with NE assembly. It appears as if the *Ketel^D* mutations identify a novel function of importin-β required during reassembly of the NE at the end of mitosis. Perhaps importin-β is not only engaged in nuclear protein import but is also a structural component of the NPCs, as Corbett and Silver (1997) proposed, and the *Ketel^D* mutations identify the nucleoporin function of the gene.

NE assembly is a stepwise process (Marshall and Wilson 1997; Gant *et al.* 1998; Sutovsky *et al.* 1998; Zhang and Clarke 2000). First, every chromosome as-

sociates with Ran-GDP (Zhang *et al.* 1999). The chromatin-associated Ran-GDP promotes binding to chromatin of membrane vesicles and recruits RCC1, the guanine nucleotide exchange factor for Ran, and promotes the association of nucleoporins (Goldberg *et al.* 1997; Gant *et al.* 1998). RCC1 generates Ran-GTP from Ran-GDP, and Ran-GTP causes fusion of the vesicles and formation of double nuclear membrane (Gant *et al.* 1998; Zhang and Clarke 2000). Formation of the NE with NPCs establishes a condition for resumed nuclear protein import and the formation of functional nuclei. The process takes place *in vitro* where NE forms from egg cytoplasm extract components over the demembranated sperm chromatin (Burke and Gerace 1986) in a process that is similar to NE assembly around the sperm chromatin during male pronucleus formation following fertilization (Sutovsky *et al.* 1998). As described recently by Zhang and Clarke (2000), functional NEs form over Sepharose beads loaded with Ran-GDP in Xenopus egg extract in the absence of DNA or chromatin. However, the role of importin-β in NE/NPC assembly waits to be elucidated.

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