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The Drosophila *embargoed* Gene Is Required for Larval Progression and Encodes the Functional Homolog of Schizosaccharomyces Crm1

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ABSTRACT

The CRM1 (Exportin 1) protein is a receptor for leucine-rich nuclear export signal sequences. We have molecularly characterized the *Drosophila melanogaster embargoed* (*emb*) gene and find that it encodes a product with 49 and 71% sequence identity to the fission yeast *Schizosaccharomyces pombe* and human CRM1 proteins, respectively. We show that expression of the *emb* cDNA is sufficient to suppress the growth phenotype of both conditional-lethal and null *S. pombe crm1*$^{-}$ mutant strains, suggesting that *emb* encodes the functional homologue of the *S. pombe* Crm1 protein. Through mutagenesis screens we have recovered a series of recessive lethal *emb* mutations. There is a substantial maternal contribution of *emb* mRNA and animals hemizygous for our *emb* alleles can develop to second instar larvae but persist at this stage and consistently fail to undergo the molt to the third instar stage. We see a nuclear accumulation of endogenous actin in the intestinal epithelial cells of the *emb* mutant larvae, consistent with a role for the *emb* gene product in nuclear export of actin protein.

THE division of eukaryotic cells into nuclear and cytoplasmic compartments has required the development of mechanisms that actively and selectively transport macromolecules through pores in the nuclear membrane. The nuclear trafficking of proteins is mediated by a group of related receptors called karyopherins that bind a peptide signal on the protein cargo and carry it through the nuclear pore (Ohno *et al.* 1998). This article describes the molecular and genetic characterization of a Drosophila homolog of the export karyopherin CRM1 (chromosome region maintenance 1), also called Exportin I. Studies have shown that nuclear CRM1 binds cooperatively to a leucine-rich nuclear export signal (NES) on its target protein and to the small GTPase Ran in its active GTP-bound form (Fornerod *et al.* 1997b). The CRM1 protein then carries its protein cargo through the nuclear pore to the cytoplasm where it is released upon the hydrolysis of RanGTP to RanGDP.

It was originally believed that one of the primary roles of the CRM1 protein is to maintain chromosome integrity, because the first cold-sensitive fission yeast (*Schizosaccharomyces pombe*) *crm1* mutants showed atypical chromatin structure when cultured at a restrictive temperature (Adachi and Yanagida 1989). *S. pombe crm1* alleles have since been recovered from a screen for caffeine resistance (Kumada *et al.* 1996) and have also been shown to confer resistance to the antibiotics spaurosine and brefeldin A (Turi *et al.* 1994). However, both the chromatin and the multidrug-resistance phenotypes are suppressed by a mutation in *pap1*, which encodes an AP-1-like transcription factor, suggesting that they are a result of deregulated *pap1* activity (Toda *et al.* 1992). It is now known that Pap1 is a substrate for CRM1-mediated nuclear export and that its nuclear accumulation in *crm1* mutants mimics the normal translocation of Pap1 to the nucleus under oxidative stress, where it regulates gene expression including the activation of drug-resistance genes (Toone *et al.* 1998). The primary role of *S. pombe* Crm1 in nuclear export is supported by the nuclear accumulation of the Dsk1 kinase in cold-sensitive *S. pombe crm1* mutant cells at a restrictive temperature (Fukuda *et al.* 1997).

A further source of *S. pombe crm1* alleles was a screen for mutations that confer resistance to leptomycin B, a Streptomyces-derived antifungal antibiotic (Nishi *et al.* 1994). Treatment of *S. pombe crm1*$^{+}$ cells with leptomycin B produces phenotypes, such as disorganized chromatin, that resemble those of *crm1* mutants. It is now known that leptomycin B inhibits Crm1 activity directly by disrupting its interaction with the NES and RanGTP (Fornerod *et al.* 1997b). This property of leptomycin B has allowed its use as a tool to study the effects of blocking CRM1-mediated nuclear export in both vertebrate

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(Ossareh-Nazari *et al.* 1997; Yang *et al.* 1998) and invertebrate (Abu-Shar *et al.* 1999; Berthelsen *et al.* 1999) cell culture systems.

Much of our understanding of the mechanisms of nuclear export has come from genetic studies in yeast and from Xenopus oocytes and mammalian cell culture systems (Ohno *et al.* 1998). In contrast, the study of nuclear trafficking in Drosophila is still in its infancy (Davis 1997). In this article we present a molecular and genetic characterization of the Drosophila *embargoed* (*emb*) gene and show that it encodes the functional homologue of *S. pombe* Crm1. We describe a series of lethal *emb* alleles recovered from mutagenesis screens and present evidence that they are associated with a defect in nuclear export.

MATERIALS AND METHODS

Molecular characterization of the *emb* gene: Restriction fragments from the genomic phage clone W5-p9 (Neumann-Silberberg and Schuepbach 1993) were used to screen 5×10^5 clones of an Oregon-R 12–24-hr embryonic plasmid cDNA library by the standard protocol (Brown and Kafatos 1988). The longest *emb* cDNA recovered (*emb*2-4) was subcloned into Bluescript SK+ (Stratagene Ltd., Cambridge, UK) and exonuclease III deletion constructs from 5′ to 3′ of the sense strand were sequenced from the M13-20 primer (ABI Prism system). Sequence data were assembled into a contig using the GCG (Genetics Computer Group, Inc.) GELASSEMBLE program. The *emb*2-4 sequence was confirmed by sequencing the opposite strand from antisense oligonucleotide primers. The same primers were used to characterize subclones of fragments from the genomic phage clone W5-p9 to establish the *emb* intron/exon structure. The best open reading frame within the *emb*2-4 cDNA sequence was identified by the positional base preference method in the standard Staden programs for nucleotide interpretation. The encoded polypeptide was compared to protein database entries using the blastp program (Altschul *et al.* 1990) and aligned to human and *S. pombe* Crm1 amino acid sequences using the ClustalW program.

Whole mount *in situ* hybridization: Digoxigenin-labeled riboprobes corresponding to the sense and antisense strands of the 3′ untranslated region of our full-length *emb* cDNA were synthesized from the T7 and T3 promoters, respectively, of the *emb* cDNA subclone 2-4XN. The 2-4XN construct consists of a 700-bp fragment of the cDNA clone *emb*2-4, extending from the *Xho*I site immediately 3′ of the *emb* coding sequence (see Figure 1B) to the downstream pNB40 vector *Not*I site, ligated into the *Xho*I/*Not*I polylinker sites of the Bluescript plasmid vector. *In situ* hybridization of the *emb* riboprobes to 0–24-hr Oregon-R embryos was undertaken essentially as described by Tautz and Pfeiffle (1989) except that prehybridization and hybridization steps were carried out at 56° rather than 45° because of the greater stability of RNA/RNA duplexes in comparison to RNA/DNA hybrids.

Construction of *emb* expression constructs: The pREPCD1*emb* and pREPCD41*emb* constructs were made in two steps. First, a restriction fragment extending from the internal *emb*2-4 cDNA *Bam*HI site (see Figure 1B) to a downstream pBluescript vector *Not*I site was ligated within the pREPCD1/41 polylinker to make a primary construct. Second, the product of a PCR amplification of the *emb*2-4 cDNA, using a primer (GTCA **GGATCC** ATG GCG ACA ATG TTG ACA TC) that introduces a *Bam*HI restriction site immediately 5′ of the putative *emb* start codon and a primer downstream of the internal *emb Bam*HI site, was *Bam*HI digested and ligated to the primary construct. The correct orientation and sequence of the cloned PCR fragment was confirmed by restriction mapping and DNA sequencing. The pREP42*emb* construct was also made in two steps. First, a PCR primer (CAAA **CAT ATG** GCG ACA ATG TTG ACA) was used to introduce a *Nsi*I recognition site overlapping the putative *emb* start codon in an amplification reaction with a second primer downstream of the internal *emb Bam*HI site. The PCR product was digested with *Nsi*I and *Bam*HI and ligated within the pREP42 polylinker to make a primary construct. The fidelity of the cloned PCR product was checked by DNA sequencing. Second, the remainder of the coding region plus the *nmt1* polyadenylation site was added by ligating the *Bam*HI/*Sst*I insert from the pREPCD1*emb* construct (see above) to the primary construct.

Suppression of fission *S. pombe crm1* mutants by *emb* expression: Standard procedures for *S. pombe* genetics were followed (Moreno *et al.* 1991). The *S. pombe* strains used in this study are shown in Table 1. For suppression of the conditional lethal mutants, *crm1-809* and *crm1-1R* strains were transformed with the plasmids pREPCD1*emb* and pREPCD41*emb* using the lithium method (Ito *et al.* 1983) and grown on plates containing 1.6% agar. For suppression of the null *crm1* mutation, diploid strains heterozygous for the $crm1^+$ gene (TP45, Table 1) were transformed with multicopy plasmids containing *emb* (pREP42-*emb*) or $crm1^+$ (pKK1, Kumada *et al.* 1996). Leu^+ transformants were allowed to sporulate on minimal plates in the absence of thiamine and remaining unsporulated diploids were killed by treatment with 0.5% glusulase. Free spores were spread in minimal plates supplemented with adenine. Leu^+ Ura^+ Ade^- colonies (*crm1*::*LEU2*-containing plasmids, TP45-1 and TP45-2, Table 1) were picked and analyzed.

Isolation and characterization of *embargoed* mutants: Flies were cultured at 25° on yeasted cornmeal agar unless stated otherwise. To screen for mutations lethal with $T(Y;2)fy^4$, X-ray- or EMS-mutagenized *b cn* males were crossed to *SM6a/Gla* females. Single *b cn/SM6a* male progeny were crossed to *C(1)M4*; $T(Y;2)fy^4/CyO$ females and single *b cn/SM6a* female progeny crossed to $T(Y;2)fy^4/CyO$ males. F_2 progeny were screened for inviability of the $T(Y;2)fy^4/b\ cn$ class. The lethal phase of hemizygous *emb* mutants was determined by collecting larval progeny of *Df(2L)N22-14/Gla Bc* males and *emb/Gla Bc* females and identifying the furthest developed Bc^+ animals. To identify the emb^1 lesion, homozygous emb^1 second instar larvae were selected from the progeny of $emb^1/Gla\ Bc$ parents on the basis of a Bc^+ phenotype. Genomic DNA was prepared from the homozygous larvae and the *emb* locus PCR amplified using gene-specific oligonucleotide primers. PCR products were directly sequenced from *emb* antisense primers (see above). The *P*-element insertion sites of *l(2)k16715* and *l(2)k06303* were identified by plasmid rescue of the *P{lacW}* element and flanking sequences using standard procedures (Hamilton *et al.* 1991). Informative restriction enzyme digests were *Pst*I for *l(2)k16715* and *Bgl*II and *Bam*HI for *l(2)k06303.* Rescued plasmids were sequenced from an oligonucleotide primer complementary to the *P*-element terminal repeat (CGACGGGACCACCTTATG).

Nuclear and actin staining of Drosophila gut epithelia: Homozygous *y w; emb* second instar larvae were selected from the progeny of *y w; emb/CyO* $[y^+]$ parents on the basis of mouth hook pigmentation. The *emb* mutant larvae and control homozygous *y w* second instar larvae were dissected and fixed in PBS with 4% paraformaldehyde. Fixed larvae were incubated with an actin antibody (Sigma A2066, raised in rabbit) at 1:200 dilution and secondary goat anti-rabbit (FITC, Jackson Immunoresearch, West Grove, PA) at 1:200 dilution following

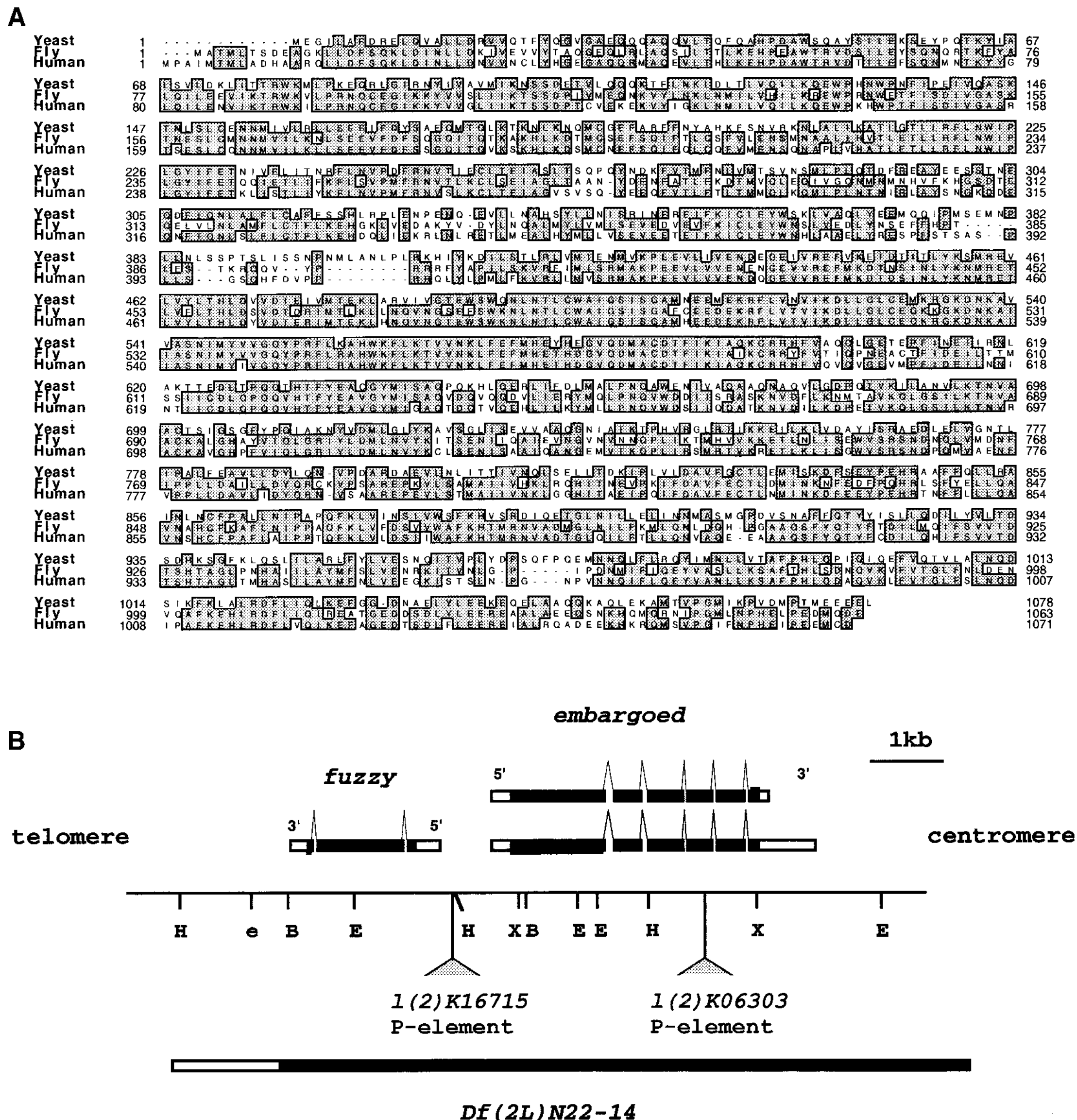

Figure 1.—(A) Alignment by homology of the predicted amino acid sequence of the *emb* gene product with the *S. pombe* and human CRM1 proteins by the ClustalW program. Identical amino acid residues are boxed together. (B) Molecular map of the *emb* locus at cytological location 29C1.2. Transcripts are indicated by boxes, the shaded portions of which represent the extent of coding sequence. The location of the *P*-element insertion sites of the *emb* mutants *l(2)k16715* and *l(2)k06303* are indicated by shaded arrowheads. The region deleted by *Df(2L)N22-14* is represented by a box, the unshaded portion of which indicates uncertainty about the distal breakpoint of the deletion. Restriction sites are B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and X, *Xho*I. The lowercase "e" is a polymorphic *Eco*RI site present on *cn bw sp* and derivative chromosomes. GenBank accession numbers are AF179360 for the *emb* cDNA and AF179361 for the *emb* genomic sequence.

standard methods for imaginal disc staining (White 1998), but including 20 μg/ml propidium iodide in the final phosphate buffered TWEEN wash to stain nuclei. Intestinal epithelia were dissected from the stained larvae and then mounted. Images were captured from a Leica confocal microscope using the TCS-NT program and were processed in Adobe Photoshop 4.

RESULTS AND DISCUSSION

Molecular characterization of the *emb* gene: In the course of screens for expressed sequences in the Drosophila chromosomal region 29C we isolated a class of cDNAs encoding a polypeptide with strong homology to the *S. pombe* Crm1 protein (Adachi and Yanagida 1989). We originally called this gene *Crm1* (Collier and Gubb 1997), but have now renamed it *embargoed* (*emb*) to reflect the nuclear export defects of the mutants (see below). We have recovered *emb* cDNA clones from 12–24-hr embryo and imaginal disc plasmid cDNA libraries (Brown and Kafatos 1988) and a 0–24-hr pupal λcDNA library. The longest of the *emb* cDNA clones is 4.2 kb and contains an open reading frame of

TABLE 1

***S. pombe* strains used in this study**

Strain	Genotype	Source
HM123	*h⁻ leu1*	Our stock
TK11R	*h⁻ leu1 crm1-1R*	Kumada *et al.* (1996)
AC1	*h⁻ leu1 crm1-809*	Adachi and Yanagida (1989)
TP45	*h⁻/h⁺ leu1/leu1 ura4/ura4 his2/+ his7/+ ade6-m210/ade6-M216 crm1::LEU2/+*	This work
TP45-*crm1*	*h⁻ leu1 ura4 ade6 crm1::LEU2* containing p(*crm1⁺*)	This work
TP45-*emb*	*h⁻ leu1 ura4 ade6 crm1::LEU2* containing pREP41*emb*	This work

1063 amino acids that is 49 and 71% identical to the *S. pombe* (Adachi and Yanagida 1989) and human (Fornerod *et al.* 1997a) CRM1 protein sequences, respectively (Figure 1A). The *emb* cDNA maps to a genomic region of just 4.5 kb (Figure 1B), with the coding sequence split by five small introns. The *emb* transcription unit lies immediately proximal on the chromosome to the tissue polarity gene *fuzzy* (*fy*, Collier and Gubb 1997), and the two genes are divergently transcribed with a little over 600 bp of genomic sequence between the 5′ ends of our longest respective cDNAs. [A recent publication (Faskin *et al.* 2000) describes an *embargoed* cDNA with an extra 131 nucleotides of 5′ sequence of which 86 nucleotides form an additional untranslated exon. This reduces the distance between the *emb* and *fy* transcripts to just 132 bp.]

***emb* gene expression:** Our longest *emb* cDNA hybridizes to two bands of ~4.2 and 3.5 kb on a Northern blot of Drosophila mRNA, the larger band having a severalfold higher intensity in all developmental stages tested (data not shown). Our *emb* cDNAs fall into two classes: a single pupal cDNA is polyadenylated just over 100 nucleotides 3′ of the termination codon and the other four cDNA clones, from embryonic and pupal libraries, are polyadenylated 660 nucleotides further downstream (Figure 1B). We conclude that the two size classes of *emb* mRNA result from differential polyadenylation and that both transcripts encode the same peptide sequence.

We have hybridized a riboprobe corresponding to an antisense strand of the 3′ untranslated region of our full-length *emb* cDNA to mixed-stage wild-type (Oregon-R) Drosophila embryos (Figure 2). We find ubiquitous expression of the *emb* transcript at all stages of embryonic development (Figure 2, A–D), as befits a gene that encodes a central component of the nuclear trafficking system. This includes a substantial maternal contribution of *emb* mRNA that is evident in very early embryos (Figure 2A). However, from the cellular blastoderm stage (Figure 2B) onward the levels of *emb* expression vary across the developing embryo. Specific tissues in which *emb* expression is relatively high are the brain, hind gut, and posterior spiracles shortly before dorsal closure (Figure 2C) and the ventral nerve cord, midgut, and somatic musculature shortly after dorsal closure (Figure 2D). In each case *emb* expression seems to increase when the tissue is well developed, suggesting that *emb* is required for the function or maintenance of these tissues rather than for their formation.

Suppression of *S. pombe* conditional-lethal *crm1* mutations by *emb* expression: The high sequence homology between the *emb* gene product and the *S. pombe* Crm1 protein prompted us to ask if the function of these proteins has also been conserved. We initially addressed this question by testing if the expression of an *emb* cDNA in conditional-lethal *S. pombe crm1* mutants could suppress the growth phenotype seen at restrictive conditions. The *emb* coding region was ligated immediately downstream of the thiamine-repressible *nmt1* promoter of the pREPCD1 expression vector and the attenuated *nmt1* promoter of pREPCD41 (Maundrell 1993). The pREPCD1*emb* and pREPCD41*emb* constructs were introduced into a cold-sensitive (*crm1-809*; Adachi and Yanagida 1989) and a temperature-sensitive (*crm1-1R*; Kumada *et al.* 1996) *S. pombe crm1* mutant. The expression of the *emb* cDNA gave analogous results for both strains (Figure 3, A and B). At the permissive temperature and in the presence of the thiamine repressor, the *emb* cDNA did not affect growth (Figure 3, Ai and Bi), but in the absence of thiamine, expression of the pREPCD1*emb* construct inhibited growth, although pREPCD41*emb* did not (Figure 3, Aii and Bii). We conclude that the levels of the *emb* gene product produced by expression from the wild-type *nmt1* promoter are toxic to the yeast cell. There are precedents for this observation since it is known that high level expression of the human Crm1 protein in *S. pombe* prevents growth (Kudo *et al.* 1997) and that overexpression of the endogenous CRM1 protein in the budding yeast *Saccharomyces cerevisiae* prevents sporulation (Toda *et al.* 1992). The toxicity of high levels of *emb* expression in *S. pombe* may result from a dominant-negative interaction, as it produces elongated cells that resemble cold-sensitive *crm1* mutant cells incubated at the restrictive temperature (Adachi and Yanagida 1989). In the presence of thiamine, the pREPCD1*emb* and pREPCD41*emb* constructs did not permit growth of the *crm1-809* and *crm1-1R* mutants at the restrictive temperature (Figure 3, Aiii and

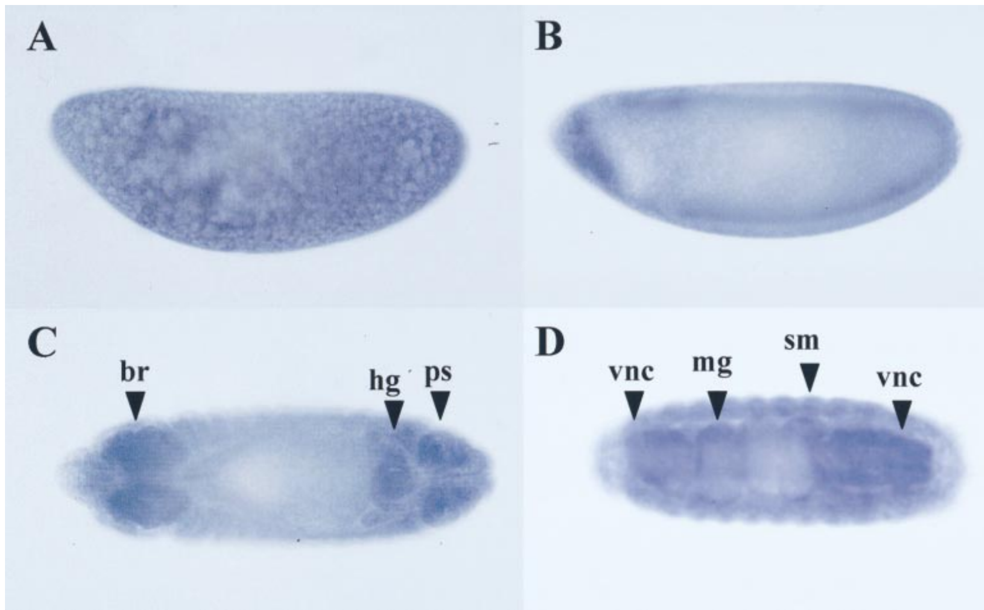

Figure 2.—*emb* expression in wild-type (Oregon-R) embryos. Anterior is left and dorsal up unless stated otherwise. (A) Lateral view of Stage 1 embryo. (B) Lateral view of Stage 5 embryo. (C) Dorsal view of Stage 13 embryo. br, brain; hg, hindgut; ps, posterior spiracles. (D) Ventral view of Stage 15 embryo. mg, midgut; sm, somatic musculature; vnc, ventral nerve cord. Photographs were taken at ×25 magnification.

Biii). In the absence of thiamine, the pREPCD1 *emb* construct also failed to support growth, presumably due to the toxicity of high levels of the *emb* gene product (Figure 3, Aiv and Biv), but the pREPCD41*emb* construct restored growth close to the level achieved by the endogenous *crm1*+ gene (Figure 3, Aiv and Biv). From these data we conclude that an appropriate level of *emb* gene expression can suppress the growth phenotype of both cold- and temperature-sensitive *S. pombe crm1* mutants.

Suppression of an *S. pombe crm1* null mutation by *emb* expression: The suppression of conditional-lethal *S. pombe crm1* mutant growth phenotypes by the expression of the *emb* cDNA suggests that the *emb* gene encodes the functional homologue of *S. pombe* Crm1. However, it remained possible that the Drosophila protein can only substitute for one activity of the yeast protein that is sensitive to the introduction of conditional-lethal mutations. We therefore attempted to suppress a null *crm1* mutant that had been made by inserting the *S. cereviseae LEU2* gene at the *crm1* locus (Adachi and Yanagida 1989), by *emb* expression. The *emb* cDNA sequence was ligated downstream of the thiamine repressible promoter of the $ura4^+$ pREP42 vector and was used to transform *S. pombe* diploid heterozygous *crm1* mutants (*crm1*::*LEU2*/+,TP45, Table 1), as haploid *crm1* null mutants are inviable (Adachi and Yanagida 1989). As a positive control the same stain was transformed with a multicopy plasmid carrying the *crm1*+ gene (pKK1; Kumada *et al.* 1996). Diploid transformants were allowed to sporulate and free spores were germinated on minimal plates supplemented with adenine. In the presence of thiamine Leu^+ Ura^+ Ade^- (*crm1*::*LEU2*) haploid cells were obtained from the diploid containing pKK1, but not from those containing pREP42*emb* (Figure 4i). However, in the absence of thiamine the diploids containing pREP42*emb* also produced Leu^+ Ura^+ Ade^- haploid cells (Figure 4ii). This result implies that the expression of the *emb* gene is sufficient to suppress the growth phenotype of a *crm1* null mutant and that the *emb* gene product is functionally homologous to the *S. pombe* Crm1 protein. This, in turn, suggests that the mechanism of CRM1-mediated nuclear export has been highly conserved through evolution and means that experiments in Drosophila can complement the ongoing studies in *S. pombe* and vertebrate cell culture systems.

Identification of *emb* mutants: The chromosome translocation $T(Y;2)fy^A$ breaks in the cytological region 29B4-C2 and was recovered from an X-ray screen for new *fy* alleles (Collier and Gubb 1997). Flies heterozygous for $T(Y;2)fy^A$ and a *fy* null allele have the typical cold-sensitive amorphic *fy* phenotype. Although the $T(Y;2)fy^A$ break places heterochromatin adjacent to the *fy* locus, the associated *fy* phenotype does not appear to be subject to position effect variegation as it is not ameliorated by introducing additional Y chromosome material (data not shown). Unlike other amorphic *fy* alleles, however, $T(Y;2)fy^A$ is lethal over the deficiency *Df(2L)N22-14* (29C1.2;30C8.9). To investigate the source of this lethality we performed F_2 lethal X-ray and EMS mutagenic screens. Four lethal alleles ($l(2)fy^{A\text{-}1}$, $l(2)fy^{A\text{-}2}$, $l(2)fy^{A\text{-}3}$, and $l(2)fy^{A\text{-}5}$) were recovered from 2496 chromosomes in EMS screens and a single lethal allele ($l(2)fy^{A\text{-}4}$) from 2076 in an X-ray screen, all five alleles belonging to a single complementation group (Table 2). The $T(Y;2)fy^A$ break must be close to the *fy* locus as it is associated with an amorphic, nonvariegating *fy* phenotype, but cannot map significantly distal to *fy* as it is also lethal with *Df(2L)N22-14* (see Figure 1B). Therefore, the best candidate for the $l(2)fy^A$ lethality was the next essential gene proximal to *fy* and most probably *emb.* To test this, we PCR-amplified and sequenced the *emb* locus from genomic DNA prepared from $l(2)fy^{A\text{-}1}$ (emb^1)

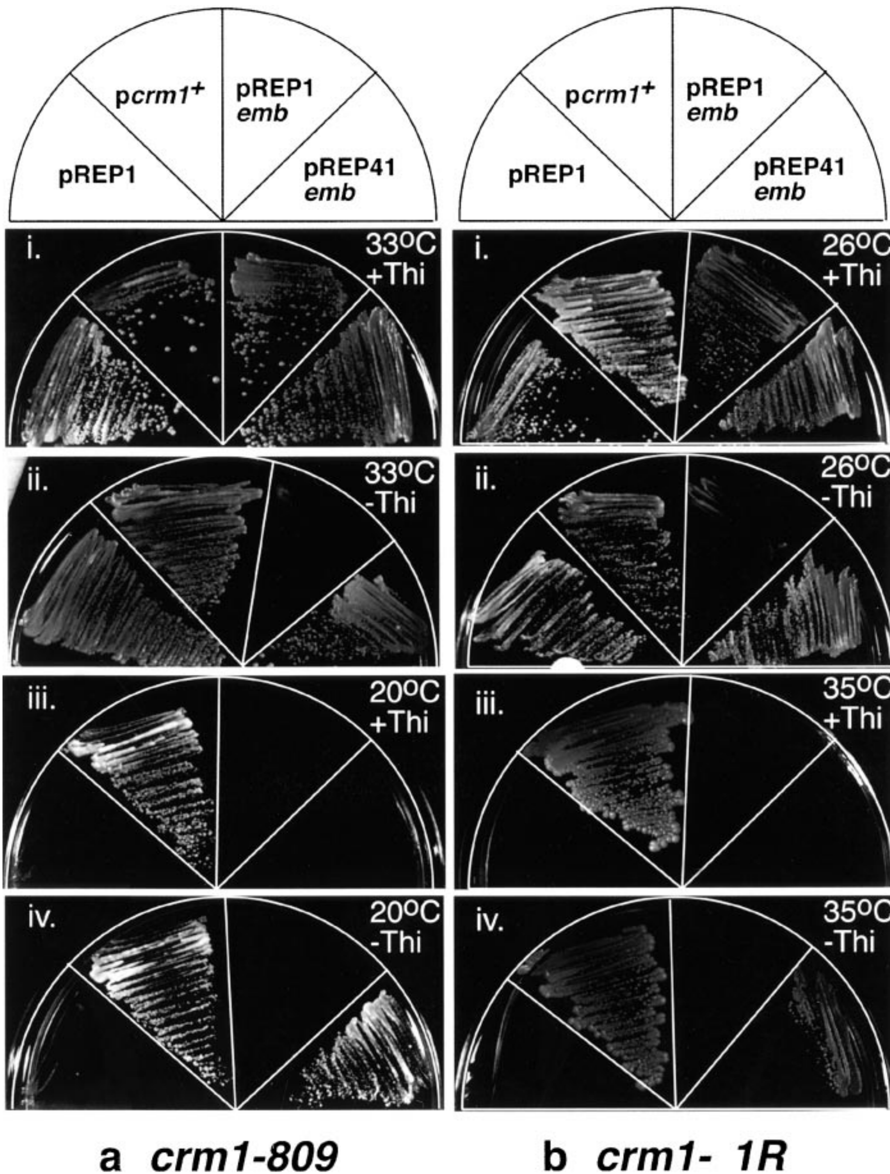

Figure 3.—Suppression of the growth phenotype of conditional-lethal *S. pombe* *crm1* alleles by expression of the *emb* cDNA. (a) Results of *emb* expression in the cold-sensitive *crm1-809* allele. (b) Results of *emb* expression in the temperature-sensitive *crm1-1R* allele. Expression constructs present in the *crm1* mutant strains are indicated on the template above. Incubation conditions are indicated adjacent to each image. Thi, thiamine.

homozygous second instar larvae and found a G-to-A transition at the 5′ donor site of intron 4. The splice site mutation was not found on the *b cn* progenitor chromosome or in other *emb* alleles from the same EMS screen. Since intron 4 is 66 nucleotides in length with no in-frame termination codons, the *emb*[1] mutant transcript is expected to encode a protein with an additional 22 residues inserted at amino acid position 872. The high degree of sequence conservation along the entire length of the *S. pombe*, Drosophila, and human CRM1 proteins (Figure 1A) suggests that this insertion will seriously compromise the function of the *emb* product and, indeed, the lethal phenotype of *emb*[1] hemizygotes is indistinguishable from that of a putative *emb* null [*l(2)k06303*, see Table 2 and below].

Our *emb* alleles fail to complement the Berkeley *Drosophila* Genome Project *P*-element insertion lines *l(2)k06303* and *l(2)k16715*, which had both been mapped to 29C3-C4 (Torok *et al.* 1993; Spradling *et al.* 1995). By plasmid rescue of the *P{lacW}* element and flanking sequences we have found that the *l(2)k06303* insert is within exon 4 and the *l(2)k16715* insert is ~400 bp upstream of the *emb* transcription start site (Figure 1B). The lethal phenotypes of *l(2)k16715* and *l(2)k06303* hemizygotes are indistinguishable (Table 2). The fact that the *l(2)k16715* insertion site is within the 5′ untranslated exon of the *emb* gene that has recently been described (Faskin *et al.* 2000) explains why its associated phenotype is as severe as *l(2)k06303*, which we believe to be an *emb* null allele. However, despite the *l(2)k16715* insertion being within 200 bp of the *fy* transcription start site, it does not significantly compromise *fy* expression, as flies heterozygous for *l(2)k16715* and an amorphic *fy* allele have no detectable tissue polarity phenotype when cultured at the restrictive temperature of 18° (Collier and Gubb 1997; data not shown).

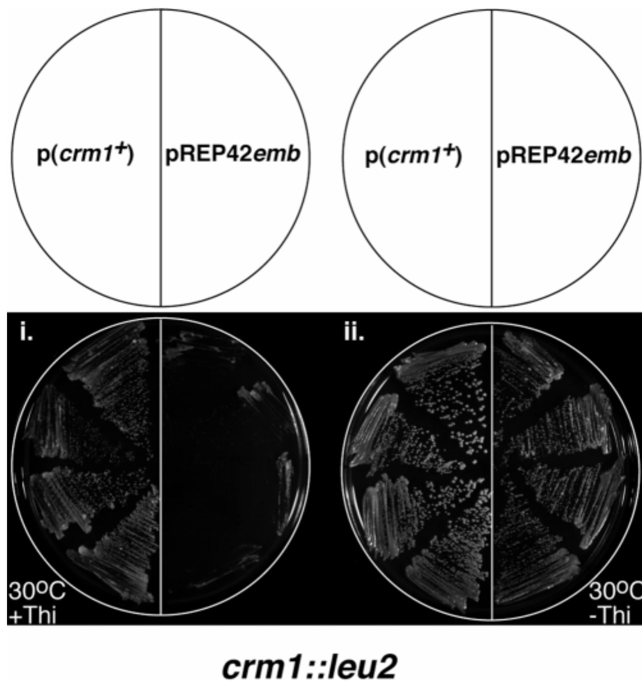

Figure 4.—Suppression of the growth phenotype of a *S. pombe crm1* null mutant by expression of *emb.* Independent *crm1*-deleted haploids were streaked on minimal plates (supplemented with adenine) and incubated at 30° for 3 days. Expression constructs present are indicated on the template above. Incubation conditions are indicated adjacent to each image. Thi, thiamine.

The similar lethal phase of the *emb* alleles suggests either that they are all genuine amorphs, perhaps reflecting the vulnerability of the highly conserved CRM1 protein to introduction of null mutations. Alternatively, the *emb* alleles are of differing strengths, but a high demand for the *emb* gene function prior to the second larval molt means that even hypomorphic *emb* alleles fail to support ecdysis (see below).

The lethal stage of *emb* mutants: We identified the lethal stage of hemizygous *emb* flies by collecting larval progeny from *emb/Gla Bc* females crossed to *Df(2L)N22-14/Gla Bc* males (see Table 2). For all *emb* alleles, *emb* hemizygous Bc^+ second instar larvae were abundant, but no hemizygous third instar larvae were seen. This was not due simply to developmental delay, as isolated second instar *emb* hemizygotes can survive for up to 7 days at 25° and still do not undergo the L2/L3 molt. This absolute block of ecdysis might mean that some component of the ecdysone signaling pathway is dependent upon *emb*-mediated nuclear export. Such consistent arrest in larval development is reminiscent of hypomorphic mutations of the *stranded at second* (*sas*) gene that encodes a cell surface receptor protein containing fibronectin type III class repeats (Schonbaum *et al.* 1992). In common with *sas* mutants, second instar *emb* mutant larvae are usually smaller than wild type and can display unusually convoluted tracheae. It is possible that *sas* activity is dependent upon nuclear export or that the *sas* receptor is a component of a signaling pathway required for *emb* expression.

The CRM1 protein is a fundamental component of the nuclear export machinery that we would expect to play a vital role in the development of the embryo to larval stages. Therefore, the survival of animals carrying *emb* null alleles to second instar larvae is probably dependent upon the maternally contributed *emb* mRNA present in early embryogenesis (Figure 2A), particularly as there is no closely related protein encoded by the Drosophila genome that might readily substitute for *emb* function.

Nuclear accumulation of actin in gut cells of *emb*

TABLE 2

***embargoed* mutant alleles**

Allele	Original name	Source	Mutagen	Molecular lesion	Lethal phase of hemizygote[a]
T(Y;2)fy^4		Collier and Gubb (1997)	X rays	Break to Y chromosome at cytological location 29B4-C2	Embryonic
emb^1	*l(2)fy*$^{4\text{-}1}$	This work	EMS	Intron 4 splice donor site mutation	2nd larval instar
emb^2	*l(2)fy*$^{4\text{-}2}$	This work	EMS	n.d.[b]	2nd larval instar
emb^3	*l(2)fy*$^{4\text{-}3}$	This work	EMS	n.d.[b]	2nd larval instar
emb^4	*l(2)fy*$^{4\text{-}4}$	This work	X rays	n.d.	2nd larval instar
l(2)k06303		Torok *et al.* (1993) Spradling *et al.* (1995)	*P* element	*P*-element insertion in exon 4	2nd larval instar
l(2)k16715		Torok *et al.* (1993) Spradling *et al.* (1995)	*P* element	*P*-element insertion 400 bp 5′ of transcriptional start site	2nd larval instar
l(2)k06318		Torok *et al.* (1993) Spradling *et al.* (1995)	*P* element	n.d.	n.d.

[a] The lethal phase is defined as the most advanced developmental stage at which hemizygous mutant animals were seen.
[b] Although the *emb*^{2} and *emb*^{3} alleles were recovered from the same EMS screen as *emb*^{1}, they are independent mutations as they do not carry the intron 4 splice site mutation. EMS, ethyl methanesulfonate; n.d., not determined.

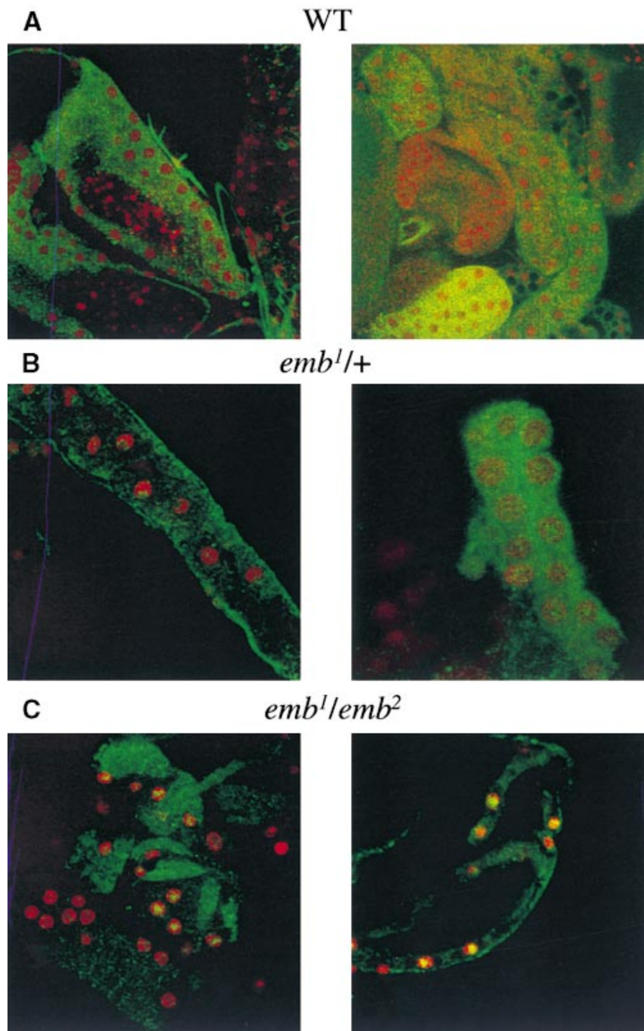

Figure 5.—Actin distribution in intestinal epithelial cells of wild-type (WT) and *emb* mutant second instar larvae. Antibody staining of actin is green and propidium iodide staining of nuclei is red; nuclear actin appears yellow. (A) *y w*, (B) *emb*^{1}/+ heteroygotes, (C) *emb*^{1}/*emb*^{2} mutant.

mutant larvae: To confirm the role of the *emb* gene product in nuclear export we looked for an abnormal subcellular distribution of actin in our *emb* mutants. Due to its low molecular weight (~42 kD), actin is believed to migrate freely through nuclear pores, but becomes localized exclusively in the cytoplasm due to CRM1-dependent nuclear export (Wada *et al.* 1998). Our analysis of the six characterized Drosophila actin proteins (actin 5C, 42A, 57B, 79B, 87C, and 88F; Flybase 1999) reveals that they are all identical in sequence to mammalian α-actin at the sites of the two internal NES sequences, suggesting that they are also substrates for Crm1-mediated nuclear export. We stained intestinal epithelia of *emb* and *y w* mutant second instar larvae with an actin antibody and with propidium iodide to highlight nuclei (Figure 5). In wild-type epithelial cells actin is exclusively cytoplasmic (Figure 5A), but in ~50% of cells from *emb*^{1}/*emb*^{2} mutant larvae actin staining is both nuclear and cytoplasmic (Figure 5C). Similar nuclear accumulation of actin occurs when rat tissue culture cells are incubated with the CRM1 inhibitor Leptomycin B (Wada *et al.* 1998), suggesting that our observations reflect a deficiency in nuclear export caused by the *emb* mutation. Surprisingly, ~50% of cells in *emb*^{1} heterozygotes also show some nuclear localization of actin (Figure 5B), although this is apparently benign, as heterozygous *emb*^{1} animals are viable and appear phenotypically wild type. This dominant *emb*^{1} phenotype may result from nuclear NES sequences being in excess of the reduced number of export receptors or, alternatively, from the *emb*^{1} gene product actively blocking export perhaps by binding to the NES sequences, but failing to mediate export. From these observations we conclude that the *emb* gene product is required to actively export endogenous actin from the nucleus in an analogous fashion to CRM1 in mammalian tissue culture cells. This result not only confirms the role of the *emb* gene product in nuclear export, but could potentially provide an *in vivo* assay for *emb*-mediated export activity in Drosophila.

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