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ARTICLE

# Toucan Protein Is Essential for the Assembly of Syncytial Mitotic Spindles in *Drosophila Melanogaster*

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**Summary: The *toc* gene of *Drosophila melanogaster* encodes a 235-kD polypeptide with a coiled-coil domain, which is highly expressed during oogenesis (Grammont *et al.,* 1997, 2000). We now report the localization of the Toucan protein during early embryonic development. The Toucan protein is present only during the syncytial stages and is associated with the nuclear envelope and the cytoskeletal structures of the syncytial embryo. In anaphase A, Toucan is concentrated at the spindle poles near the minus end of microtubules. This microtubule association is very dynamic during the nuclear cell cycle. Mutant embryos lacking the Toucan protein are blocked in a metaphase-like state. They display abnormal and nonfunctional spindles, characterized by broad poles, detachment of the centrosomes, and failure of migration of the chromosomes. These results strongly suggest that Toucan represents a factor essential for the assembly and the function of the syncytial mitotic spindles. genesis 31:167–175, 2001.** **© 2001 Wiley-Liss, Inc.**

**Key words:** *Drosophila;* embryo; mitosis; microtubules; spindle assembly; mitotic poles; centrosomes; nuclear envelope; kinesin

## INTRODUCTION

Understanding mitotic spindle assembly represents a major issue of cell biology. In recent years it has become obvious that this phenomenon relies on cooperative mechanisms between microtubule-based motors and their associated proteins (Heald *et al.,* 1996, 1997; Gaglio *et al.,* 1997; Nedelec *et al.,* 1997; Walczak *et al.,* 1998; Heald, 2000; Sharp *et al.,* 2000b). The general idea (reviewed in Karsenti *et al.,* 1996) has emerged that chromatin stabilizes microtubules and that self-organization of the poles is achieved by focusing free microtubule ends with minus end motors. Moreover, proteins such as NuMA (Dionne *et al.,* 1999; Gaglio *et al.,* 1995; Merdes *et al.,* 1996) could belong to a complex involved in maintaining the organization of the minus ends of the spindle microtubules into a morphologic pole.

The establishment of spindle bipolarity requires multiple microtubule-based motor activities (Gaglio *et al.,* 1996; Walczak and Mitchison, 1996; Walczak *et al.,* 1998; Sharp *et al.,* 1999b; Sharp *et al.,* 2000a). Depending on the cell type, different combinations of motors and associated proteins are probably needed to organize a mitotic spindle.

The early embryo of *Drosophila melanogaster* represents an interesting case of mitotic spindle organization. This stage is characterized by a rapid succession of 13 metasynchronous divisions occurring in a syncytium. During these divisions, the mitotic spindles are assembled and disassembled with extraordinary speed. A major question is whether these syncytial mitotic spindles, relying only on maternal contributions, are different from the later ones of the cellularized embryo. As fast DNA synthesis at this stage requires multiple replication origins, one can imagine that the extremely rapid assembly/disassembly of the syncytial spindles needs special factors such as specialized forms of kinesins or centrosomal proteins. For example, two gamma-tubulins exist in *D. melanogaster,* and the syncytial mitoses are driven by only one isoform of maternal origin, the γTub37CD (Tavosanis *et al.,* 1997; Wilson and Borisy, 1998).

As a candidate for a factor with a specific role in mitosis during the syncytial phase, Toucan is particularly interesting. The *toucan* locus has been characterized as a recessive female sterile mutation in an enhancer trap mutagenesis. From sequence analysis, *toucan* is predicted to encode a 235-kD protein with a P-loop domain, several putative phosphorylation sites and a coiled-coil region in the C-terminal domain. *Toucan* is expressed and required in germline cells (Grammont *et al.,* 1997). Using specific antibodies against Toucan protein, Gram-

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mont *et al.* (2000) have shown that its localization in the oocyte is microtubule dependent. Moreover, embryos from homozygous *toc* mothers are arrested very early in their development (Grammont *et al.,* 1997).

We have investigated the precise localization of Toucan in early embryos and examined the consequence of Toucan absence on the syncytial mitoses. We report here that Toucan is present exclusively during the syncytial stage. Toucan is a very unstable protein, possibly degraded by the proteasome pathway. Toucan associates with the nuclear envelope and the cytoskeletal structures of the syncytial embryo, especially the mitotic apparatus. The localization of Toucan along spindle microtubules is not uniform and presents an extremely dynamic pattern during the course of mitosis. Mutant embryos lacking Toucan protein display abnormal and nonfunctional spindles, characterized by broad poles, detachment of the centrosomes, and lack of migration of the chromosomes. Altogether these results strongly suggest that Toucan represents a factor essential for the assembly and the function of the mitotic spindle during the early embryogenesis of *D. melanogaster.* We discuss the possible nature of Toucan, which could represent a kinesin-associated protein, or a NuMa-like protein. Moreover, as Toucan disappears completely after the blastoderm stage, and as homozygous *toucan* embryos from heterozygous mothers are viable, we hypothesize that early and late mitotic spindles require distinct factors and might be assembled in different ways.

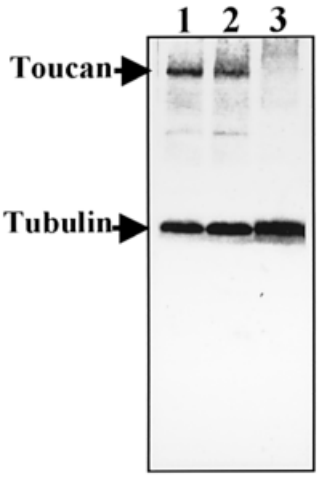


**FIG. 1.** Antibodies against the Toc protein recognize a 235-kDa protein in early embryo extracts. A 193 AA amino acid fragment (AA 749–1017) of the Toc protein was used to produce antibodies in rabbit. Western blots were probed with anti-Toc antibody and with an anti-$\alpha$-tubulin antibody. Extracts prepared from 0–1 h embryos laid by wild-type (lane 1), heterozygous (lane 2), and homozygous (lane 3) *toucan* females.

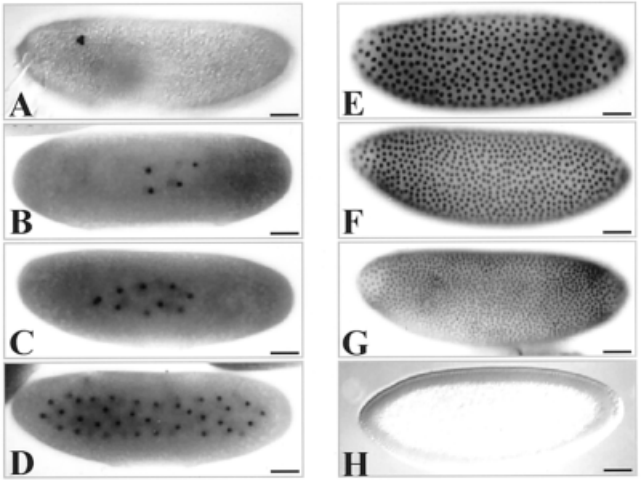


**FIG. 2.** Localization of the Toc protein in developing embryos. Nuclear cycles 2 (A), 3 (B), 4 (C), 6 (D), 9 (E), 11 (F), 13 (G), and 14 (H) are shown. Bars = 50 $\mu$m.

## RESULTS

### The Toucan Protein Is Present during All the Syncytial Divisions of the Embryo

Antibodies previously used to detect the Toucan protein during oogenesis (Grammont *et al.,* 2000) were used on early embryos. In an extract made from 0–1-h wild-type embryos, these antibodies recognize a prominent band at Mr 235 000 on a Western blot (Fig. 1). The level of this protein is reduced in embryos from heterozygous females and absent in embryos from homozygous *toucan* mutants. Using these specific anti-Toc antibodies we have studied by whole mount antibody staining and Western blotting analysis the abundance of the Toc protein during embryonic development (Figs. 2 and 3).

At the end of oogenesis, a weak accumulation of the Toc protein is detected in the oocyte nucleus (Grammont *et al.,* 1997). A similar pattern is observed in the nucleus of the zygote before and after (Fig. 2A) the first syncytial division. During the internal nuclear division stages (Fig. 2B–D), all the nuclei are strongly stained and a weak staining is detected in the cytoplasm. When the nuclei migrate to the cortex (Fig. 2E) and during the cortical nuclear division stages (Fig. 2F), the Toc protein is strongly detectable in the nuclei, whereas the cytoplasm shows a very weak staining. At the 13th division (Fig. 2G), the level of Toc protein decreases and the protein becomes undetectable by the beginning of the 14th division stage (Fig. 2H), when the cellularization process initiates.

We have also measured Toucan protein levels in precisely staged interphase embryos during all the syncytial divisions (Fig. 3). The Toc protein is detected at the expected size at Mr 235,000. The Toucan protein is present in stage 1 embryos and its level rises during the very first cycles, stays high until cycle 11, and then declines to disappear completely at interphase 14.

To summarize, we found that the Toc protein is expressed during all syncytial divisions, where it associates with the nuclei, and is degraded very quickly at the end

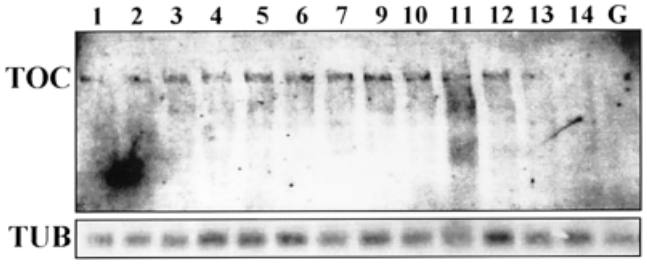


**FIG. 3.** Immunoblot detection of the Toc protein in precisely staged embryos. Proteins from three hand-selected embryos of interphase nuclear cycle 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, and gastrulating embryos (G) were analyzed. Alpha-tubulin was detected on the same blot.

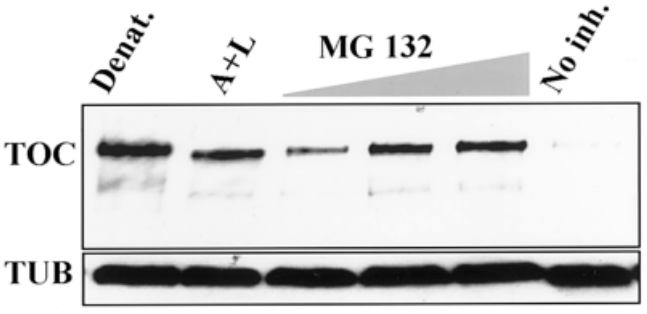


**FIG. 4.** Rapid degradation of the Toc protein in early embryos. One-hour-old wild-type embryos were dissociated in denaturing buffer (Denat.) or in a nondenaturing solubilizing buffer (Pipes 0.1 M, pH 6.6, EGTA 5 mM, MgSO4 1 mM, glycerol 6.6%, DTT 1 mM). Nondenaturing extracts were done without protease inhibitor (No inh.) or with aprotinin and leupeptin (A + L) or with increasing concentrations (20, 70, and 400 μM, respectively) of the proteosomal inhibitor MG132. In 1-h embryos the Toucan protein is resolved in one band of about 235 kDa. Alpha-tubulin was detected on the same blot.

of the syncytial divisions. These results are consistent with the previous finding of a maternal store of *toc* mRNA in the oocyte (Grammont *et al.,* 1997).

### Rapid Degradation of Toucan in vitro Is Inhibited by a Proteasome Inhibitor

We prepared soluble protein extracts from early embryos (0–1 h) under conditions that depolymerize microtubules to be able to analyze the copurification of Toucan protein with tubulin in vitro. Unfortunately, Toucan was not present in the supernatants of such extracts (data not shown), indicating that this protein is insoluble. This prevented us from testing the binding of the Toucan protein to the microtubules in vitro.

Toucan was found to be very unstable in these conditions. We observed that Toucan is completely degraded within 2 min when embryos are solubilized in a nondenaturing buffer in the absence of protease inhibitors, although the amount of tubulin is unchanged (Fig. 4). This degradation of Toucan is reduced when aprotinin (10 μg/ml) and leupeptin (10 μg/ml) are added prior to solubilization and is completely inhibited by the leupeptin analog MG132 (Affinity Research Products), a specific proteasomal inhibitor. These data indicate that Toucan is a very unstable protein.

### In the Early Embryo, Toucan Is Associated with Microtubular Structures

We have performed a detailed analysis of the localization of Toucan in *Drosophila* wild-type embryos by confocal immunofluorescence microscopy. In agreement with our previous quantitative analysis, a strong labeling was detected during the syncytial stages (nuclear divisions 1–13), but after cellularization, the embryos presented only a weak and nonspecific signal.

We found that the Toucan protein is mostly associated with microtubular structures during all phases of the mitotic cycle (Fig. 5). During interphase (Fig. 5A), the Toucan protein is associated with the nuclei. The confocal analysis clearly demonstrates that Toucan is localized to the nuclear envelope. Both centrosomes, which are attached to the nuclear envelope, are strongly labeled. At prophase (Fig. 5B), Toucan is found on the two half-spindles generated by the centrosomes. Toucan labeling follows the progressive establishment of the future spindle. During metaphase (Fig. 5C), Toucan is found on the entire spindle, which is heavily stained. Centrosomes are only weakly labeled. During anaphase, the localization of Toucan is particularly dynamic (Fig. 5D): Toucan is associated preferentially with the bundles of spindle microtubules and appears concentrated at the spindle poles close to the centrosomes. The microtubules asters were not labeled, which means that Toucan is not systematically associated with tubulin. Toucan accumulates also in the spindle midzone, between the two chromosome sets, in the region of antiparallel microtubules. During telophase (Fig. 5E), Toucan is concentrated mostly in the midbody and at the centrosomes. At this stage, microtubule asters are well developed, but they are not labeled with the anti-Toucan antibody. The localization of Toucan around the centrosomes expands toward the reforming nuclear envelope, forming a hemispheric cap. The embryo cytoplasm contains much more labeled material, in the form of diffuse material and many small granules, than during other stages.

### Toucan Is Essential for Spindle Assembly and Function

Embryos from homozygous *toucan* mothers arrest their development very early, before nuclear migration. We examined by confocal analysis such embryos after fixation and staining with antihistone and anti-CP190 (Whitfield *et al.,* 1995) antibodies. Syncytial nuclear divisions stop after only a few rounds (Fig. 6). All embryos are arrested in a metaphase-like state, with condensed chromosomes and acentrosomal spindles. On 200 mutant embryos examined, the most advanced stage contained only 16 mitotic spindles. Some embryos are arrested at the first mitosis (Fig. 6A), but most of them are stalled later, after two or three nuclear divisions. A de-

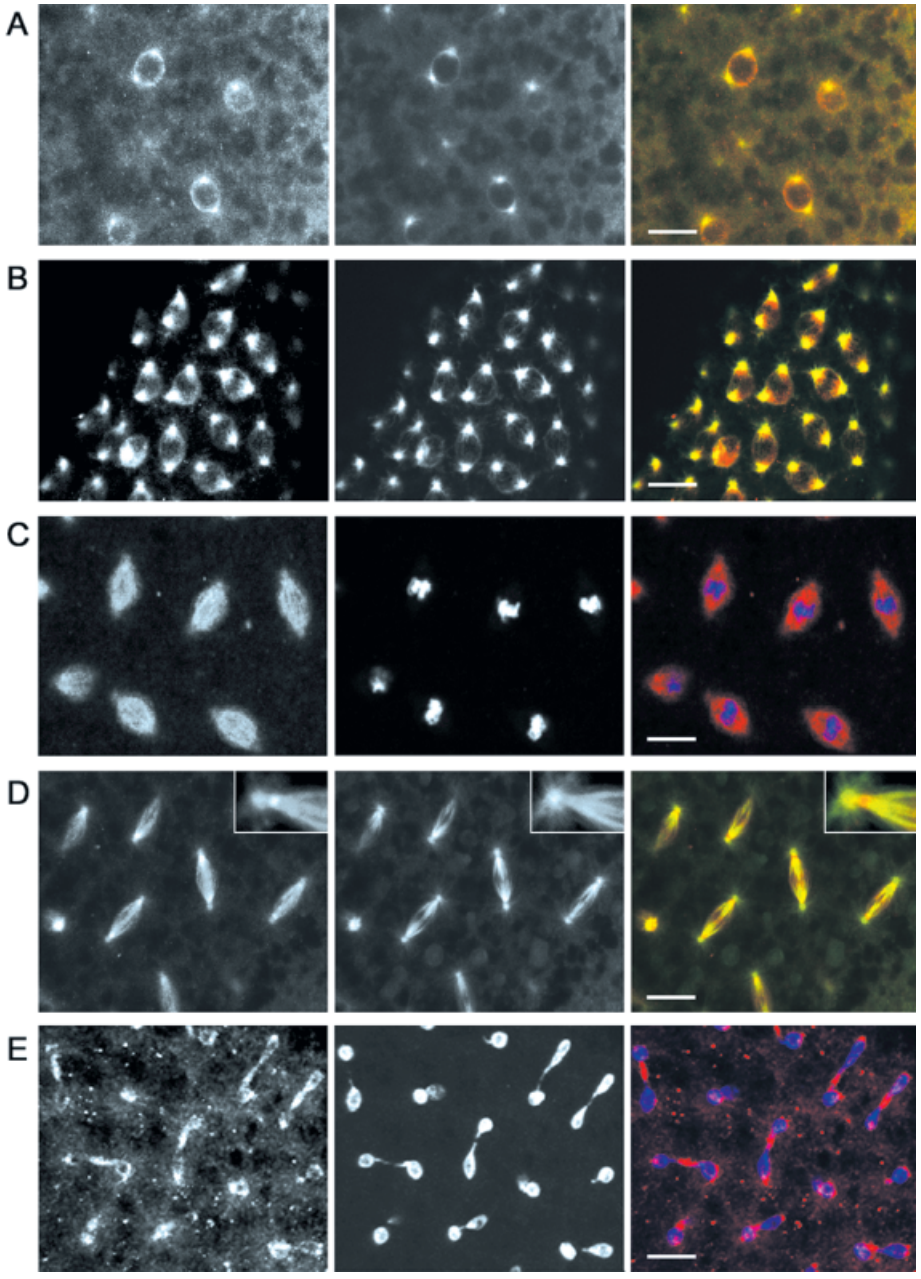


**FIG. 5.** Localization of Toucan protein during the syncytial stages in wild-type embryos. (A) Interphase. Toucan localizes to the centrosomes and the nuclear envelope (left: Toucan; middle: α tubulin; right: merge). (B) Prophase. Toucan is found on the two half-spindles (left: Toucan; middle: α tubulin; right: merge). (**C**) Metaphase. Toucan is associated with the entire spindle (left: Toucan; middle: DNA; right: merge). (**D**) Anaphase. Toucan accumulates at the spindle poles, under the centrosomes (insert: enlarged view of the localization of Toucan at the spindle pole; left: Toucan; middle: α tubulin; right: merge). (**E**) Telophase. Toucan accumulates at the midbody and in the centrosome area (left: Toucan; middle: DNA; right: merge). Bars = 10 μm.

tailed examination reveals a continuum of alterations of the mitotic spindles, which we interpret as the progressive disorganization of the mitotic apparatus after the arrest in pseudometaphase.

These spindles show broad poles, which lack a centrosome. Free centrosomes are detected in the cytoplasm at some distance of the spindles (Fig. 6B, C). In older embryos (4–5 h), centrosomes are still found in small number, suggesting an arrest or delay of free centrosomes duplication.

The spindle microtubules aspect is itself very peculiar: the tubulin staining appears very homogeneous, as if microtubule bundles no longer exist (Fig. 6C, D). The chromosomes lie in the middle of the spindle, in a metaphase plate configuration and appear to be overcondensed. No migration of chromosomes toward the poles is observed. In some cases, in which the mitotic apparatus is probably stalled for a longer time, the pseudometaphase plate collapses into a group of chromosomes with disorganized microtubule arrays.

In order to follow the altered divisions in real time, we injected fluorescently labeled tubulin into very young embryos (0–1 h collection) derived from *toc/toc* mothers. Time-lapse recordings of these embryos confirm that spindles are blocked for 15–20 min, and eventually collapse. Fig. 7 depicts a typical mutant embryo a few

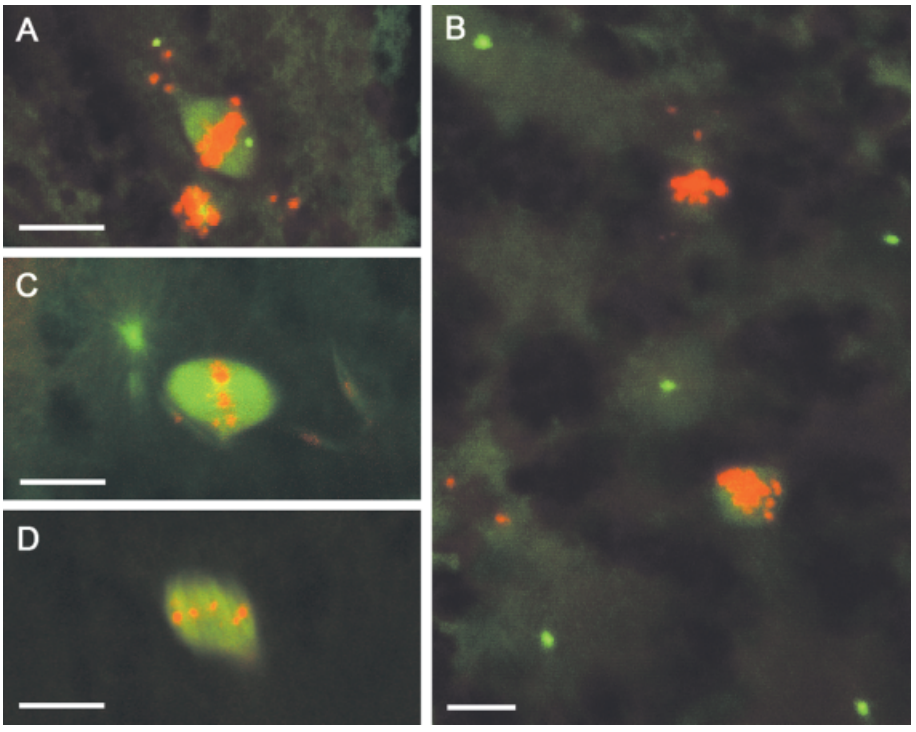


**FIG. 6.** Arrested mitosis in embryos from *toc/toc* mothers. (A, B) Chromosomes (red) are revealed with an anti-histone antibody and centrosomes (green) with the anti-CP 190 antibody Rb 188. (C, D) Double-staining of chromosomes (red) and α tubulin (green). (**A**) Block in first mitosis with the two male and female chromosome sets. (**B**) Image of two condensed metaphases taken from an embryo presenting eight metaphases. Free centrosomes are dispersed in the cytoplasm and no do not show many duplications. (**C, D**) Examples of blocked mitosis stained for α tubulin (green) and chromosomes (red). Note the large spindles poles, the overcondensed chromosomes, and the centrosome detaching from the poles. Spindle in D starts to desagregate. Bars = 10 μm.

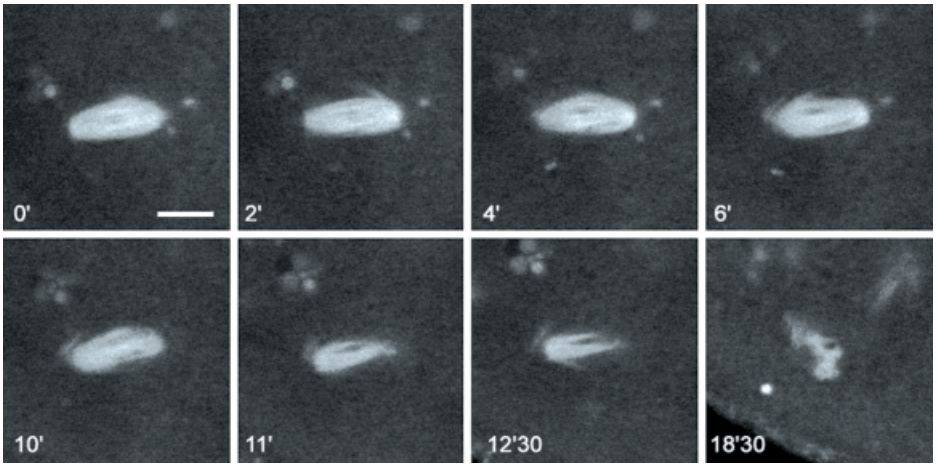


**FIG. 7.** In vivo analysis of a stalled mitosis in a toucan mutant embryo. Each picture represents selected images taken from a movie of a mitotic spindle in a toucan mutant embryo after fluorescent tubulin injection. Numbers indicate the time after the beginning of the recording. The abnormal spindle is blocked for more than 15 min and then progressively collapses and disappears. Bars = 10 μm.

minutes after injection of fluorescent tubulin. The spindle is bipolar but not biconical, and centrosomes quickly detach from the poles. The spindle is stalled in a pseudometaphase state and stays the same size. The pole-to-pole elongation typical of anaphase never occurs. After about 15 min in this state, the spindle progressively disassembles and finally disappears, leaving an unorganized mass of microtubules that disperses in the cytoplasm.

We conclude from these confocal analyses of fixed and live embryos that Toucan is essential for the assembly and the function of the syncytial mitotic spindles. Remarkably, this function is probably not required for the late divisions of the embryo.

## DISCUSSION

### Toucan Is Strictly Developmentally Regulated

Western blot analysis of staged embryos and global immunostaining reveal that Toucan is present during all of the syncytial stage. This is consistent with *toucan* mRNA accumulation in the oocyte (Grammont *et al.,* 1997). Specific anti-Toc antibodies detected one band of Mr 235,000 during all nuclear cycles. The level of Toucan protein diminishes during cycles 12–13 and falls to a very low level during interphase 14. Toucan is not detected in embryos after cellularization. This rapid disappearance of Toucan can hardly be explained by a dilu-

tion of the maternal store and likely relies on a specific degradation mechanism of Toucan. Toucan protein is unstable and is degraded very quickly in the absence of protease inhibitor. This degradation is strongly prevented by a proteasome inhibitor. The very rapid changes in Toucan subcellular localization during the different stages of the syncytial mitoses may involve a rapid degradation of the Toucan protein, triggered by the proteasome pathway. Moreover the presence of four PEST-like motifs in the N-terminal part of the Toucan protein could contribute to its instability and insure its rapid degradation. This degradation could be regulated by phosphorylation of the cdc2 phosphorylation sites that are present in Toucan, coupling its degradation with the cell cycle.

The present confocal analysis reveals that in the early embryo, Toucan is associated with the nuclei and with microtubular structures, showing a very dynamic pattern. Toucan associates with three kinds of organelles: the nuclear envelope, the centrosome, and the mitotic spindle.

### During Interphase Toucan Is Associated with the Nuclear Envelope and the Centrosomes

During telophase and all of interphase, Toucan is found associated with the cytoplasmic side of the nuclear envelope. Such localization is particularly interesting because ultrastructural observations have detected filamentous structures that link the centrosomes and the nuclei (Debec *et al.,* 1999). Through its coiled-coil domain, Toucan could associate with other proteins and form a kind of matrix in order to couple the nuclei to the cytoskeleton. Such structures could also ensure the proper migration of centrosomes along the surface of the nucleus (Robinson *et al.,* 1999).

Toucan is associated with the centrosome during most of the nuclear cycle, and particularly during all the interphase period, and until prophase. However, during metaphase and anaphase there is only a faint labeling of centrosomes. Toucan reassociates with the centrosomes during telophase. It seems therefore that Toucan is not a core component of the centrosome and is probably not involved in the microtubule nucleation function that the centrosomes play at these mitotic stages.

### Toucan Is Essential for the Assembly and Function of the Syncytial Spindles

During all phases of the mitosis, Toucan is redistributed very quickly to different parts of the spindle. In prophase, Toucan is associated with microtubules, more specifically with the + ends of the assembling spindle. During metaphase, however, Toucan is found throughout the entire spindle. At anaphase, Toucan concentrates at the poles, just under the centrosomes and also in the midzone of the spindle. During telophase, Toucan is still found in the midbody and accumulates in the centrosomal region, forming two opposite caps. Thus the relationship between Toucan and microtubules is not uniform; in particular, astral microtubules are not labeled once the spindle is fully established. All these observations demonstrate a highly dynamic redistribution of Toucan along microtubules.

All the proposed roles for Toucan based on analysis of its localization in wild-type embryos are corroborated by the mitotic defects occurring in embryos issued from homozygous *toc/toc* mothers. These embryos lack any Toucan protein, as confirmed by immunoblot analysis. They present aberrant mitotic spindles and they are all blocked in a pseudometaphase stage. This phenotype indicates that the main function of Toucan occurs during mitosis. It is clear that Toucan is essential for spindle morphogenesis and function. The defective spindle morphology in toc mutant embryos gives some clues to the possible role of Toucan. The spindle poles are very broad. There is still a bipolarization of the spindle (i.e., no multiple poles are observed), but the normal biconical shape is never attained and the spindle remains barrel shaped. The overall morphology of these spindles recalls acentrosomal spindles, observed for example in *Xenopus* egg extracts (Heald *et al.,* 1996) or in *Drosophila* cell line 1182-4 (Debec *et al.,* 1995). This strongly suggests that Toucan is involved in the organization and/or stabilization of the poles of the spindle. The centrosomes detach from the poles and are found at some distance from the spindle. Therefore Toucan may also function to tether the centrosomes to the spindle poles. Spindle microtubules appear uniform, no microtubule bundles are observed. This suggests that Toucan could cross-link microtubules and assemble them into bundles. This property of Toucan could explain the presence of Toucan in the zone of antiparallel microtubules in the midspindle during anaphase, as well as its presence in the midbody at telophase.

### What Family for Toucan?

Taken together, these observations define the main characteristics of Toucan and allow us to propose to which protein family Toucan may belong.

From sequence analysis, the *toucan* gene is predicted to encode a 235-kD protein with a coiled-coil domain in the C-terminal region between residues 1565 and 1960 (Grammont *et al.,* 1997). No homologous protein has been found in the databases. There are some significant homologies with many proteins such as pericentrin, vimentin, lamin, myosin, tropomyosin, and kinesin-like proteins. However, such homologies rely essentially on the coiled-coil domain. It is interesting to note that many of these proteins display at least one property reminiscent of Toucan, in particular either a nuclear envelope association or a spindle localization. Because of the presence of the coiled-coil domain, it is possible that Toucan is able to assemble with itself or with other proteins. Moreover, the Toucan protein contains a putative P-loop N-1 motif (GXXXXGKT/S) and a N-2 motif (NXXSSR) present in the nucleotide-binding domains of kinesins and myosins, which suggests that Toucan can bind and hydrolyse ATP or GTP, like motor proteins, and transform this chemical energy into mechanical energy.

First of all, Toucan is associated with the perinuclear matrix, and during mitosis it is tightly localized near the minus ends of microtubules at the spindle poles. In addition, embryos lacking this protein have broad spindle poles, as if microtubules were not tethered together. All these aspects are reminiscent of the NuMA protein characterized in human cells (Tousson *et al.,* 1991; Gaglio *et al.,* 1995; Merdes *et al.,* 1996; Dionne *et al.,* 1999). During interphase, NuMA is mostly a nuclear protein, but it is also found in the centrosome. NuMA redistributes during mitosis to the spindle poles where it forms two crescents (Tousson *et al.,* 1991). The role of NuMA is to form a "collar" at the poles to anchor microtubule minus ends (Dionne *et al.,* 1999). This would allow the stabilization of the poles formed through the action of minus end microtubule motors such as dynein (Karsenti *et al.,* 1996). This could correlate with a role for tethering the microtubules to the centrosomes. Unlike Toucan, however, NuMA is intranuclear and is not found in the central part of the spindle.

The other main feature of Toucan concerns its extremely dynamic redistribution along spindle microtubules and centrosomes. This strongly recalls the behavior of a kinesin-like protein (Endow *et al.,* 1994; Barton *et al.,* 1995; Williams *et al.,* 1997; Adams *et al.,* 1998; Robinson *et al.,* 1999). Because it lacks a characterized motor domain in its sequence, it is reasonable to imagine Toucan as a kinesin-associated protein. It has recently been shown that such kinesin partners not only benefit from the moving capacity of kinesins but also modulate the activity of kinesin through interaction with its cargo-binding tail domain (Coy *et al.,* 1999; Friedman and Vale, 1999; Seiler *et al.,* 2000). When not bound to a cargo, the motor protein is inhibited. In this model, Toucan would be a kinesin cargo able to move along microtubules and also able to control the motor activity of its kinesin partner. Such a KLP-Toucan complex could associate with more than one microtubule at a time, causing focusing of the poles as proposed in the model of Karsenti *et al.* (1996).

Interestingly, Toucan is also found in the midzone of the mitotic spindle during anaphase. Sharp *et al.* (1999a) have demonstrated at the ultrastructural level the presence of abundant antiparallel microtubules in the midzone of syncytial spindles of *D. melanogaster.* This suggests a role for Toucan in the elongation of the spindle, through the sliding of antiparallel arrays of microtubules (McIntosh *et al.,* 1969; Sharp *et al.,* 1999a). The redistribution of Toucan toward the + or − ends of microtubules according to the phases of mitosis could be explained by the association of Toucan with different microtubule motors that possess either a plus or a minus end directionality.

Toucan can thus be discerned as an essential protein involved in the assembly of the mitotic spindle through interactions with both microtubules and kinesins. Future work will be needed to identify proteins interacting with Toucan. Such possible partners could be Ncd (Matthies *et al.,* 1996) or KLP61F (Heck *et al.,* 1993) motor proteins.

### The Singularity of the Syncytial Spindles

Several genes have been shown to control the earliest cell cycles in the embryo, indicating that these divisions are regulated in a unique manner (Shamanski and Orr-Weaver, 1991; Lin and Wolfner, 1991; Elfring *et al.,* 1997). These genes encode maternal products required either to inhibit DNA replication in the unfertilized egg, to permit fusion of the male and female pronuclei, or to organize the several thousand mitotic apparatus required for early embryonic development. The Toucan protein is also only required for the early nuclear divisions and is not expressed zygotically in a postblastoderm embryo. This is strikingly different from cyclin B and cdc 25, which are supplied as maternal components for the 13 first divisions but are also zygotically expressed from cycle 14 onward (Edgar *et al.,* 1994). It is surprising that a protein essential for spindle assembly becomes accessory after the cellularized stage. This leads one to suppose that the syncytial spindles have unique requirements and that Toucan is part of a family of specific mitotic regulators (Inoue *et al.,* 2000) that operates only in the early embryo. Until now, the only clear characteristic of the syncytial spindles is their extreme rapidity. Comparisons of syncytial and cellularized mitotic apparatus, either at the optical or ultrastructural levels, do not reveal fundamental differences, except perhaps in the centriole, which appears to have a simpler organization during the syncytial divisions (Moritz *et al.,* 1995; Callaini *et al.,* 1997; Debec and Marcaillou, 1997; Debec *et al.,* 1999; Sharp *et al.,* 1999a). The role of Toucan could be to ensure a prompt remodeling of the microtubular structures, through the activation of kinesins. The high instability of Toucan may allow an accurate control of this process. The peculiar situation of syncytial early development in insects could therefore represent an interesting case of evolutionary selection for factors accelerating mitosis.

## MATERIAL AND METHODS

### *Drosophila* Stocks

Control embryos were obtained from the wild-type Oregon-R stock. The enhancer trap line *toucan*$^{P}$ has been previously described (Grammont *et al.,* 1997). All stocks were maintained on a standard cornmeal/molasses medium at 25°C.

### Embryo Staging, Protein Electrophoresis, and Immunoblotting

Embryos were dechorionated, fixed, and stained for DNA and cleared as described (Edgar *et al.,* 1994). Embryos were staged and selected using UV illumination on a microscope, cycle numbers were determined by the number of nuclei present and their position in the egg. Only interphase embryos were selected. Three hand-picked embryos were dissociated in 125 mM Tris-HCl,

pH 6.8, 8 M urea, 4% SDS, 10 mM DTT, 1 mM PMSF. Extracts were boiled 2 min, spun at 20,000 g for 3 min. The supernatants were electrophoresed on a 6% polyacrylamide gel. Gels were transferred to nitrocellulose membranes Optitran BA-S 85 (Schleicher and Schuell) for 1 h 30 min at 0.8 mA/cm2 in 40 mM Tris-HCl pH 7.4, 20 mM Na-acetate, 2 mM EDTA, 20% methanol, 0.05% SDS (Bolt and Mahoney, 1997).

The membrane was probed with anti-Toc antiserum at a dilution of 1:20,000. A mouse monoclonal antibody (DM1A, Sigma, T-9026) was used to detect α-tubulin at a dilution of 1:50,000. Goat anti-rabbit-HRP (horseradish peroxidase) and goat anti-mouse-HRP secondary antibodies were used at a 1:10,000 dilution. Immunoreactive protein bands were visualized using chemiluminescence ($ECL^+$, Amersham) according to the manufacturer's specifications.

### Whole-Mount Antibody Staining

Embryos were dechorionated, permeabilized 20–30 s in 100% octane and fixed 3–5 min in 37% formaldehyde according to Theurkauf (1994). The antibody staining procedure was performed as described by Sahut-Barnola *et al.* (1995). The primary antibody (Grammont *et al.,* 2000) was used at a final dilution of 1:1000.

All of the microscopy was carried out on a Zeiss Axiophot equipped with differential interference contrast optics.

### Immunofluorescence Staining

Embryos were fixed using methanol by a modification of the Mitchison and Sedat procedure (1983). This method is described in detail elsewhere (Theurkauf, 1992). In some instances the embryos were fixed using a first step of formaldehyde (37%, 5 min). Fixed embryos were labeled with primary antibodies for 1 h and extensively rinsed, then exposed to secondary antibodies for 1 h and rinsed again. All incubations were made in PBTA (PBS with 1% Bovine serum albumin, 0.05% Triton X-100). After a last rinse in PBS, embryos were mounted in a 50% glycerol, PBS solution containing 1 mg/ml *N-N*-1-4-phenylenediamine. Microtubules were detected with a monoclonal antibody against α-tubulin. The Rb 188 antibody against the centrosome-associated protein CP 190 was a gift from W. Whitfield. Chromosomes are detected either by propidium iodide or with an antihistone monoclonal antibody (MAB052, Chemicon, Temecula, CA).

Microscopy was performed using inverted confocal microscopes (Bio-Rad MRC 600 and Leica TCS-NT).

### In Vivo Fluorescence Analysis

Very young (0–1 h collection) embryos laid by *toc/toc* mothers were microinjected with rhodamine-conjugated tubulin (Molecular Probes). Microinjections were performed as described (Francis-Lang *et al.,* 1995). Observations and time-lapse recording were made on a Leica TCS-NT confocal imaging system. Time series were generated using preset programs in Leica TCS software (*xyt* mode). For these, images were acquired every 30 s.

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