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Review

Regulation of tubulin expression: Multiple overlapping mechanisms

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Tubulin is the main constituent of microtubules, a macromolecule participating in a variety of essential cell phenomena. Although the roles of microtubules have been extensively described, the regulation of tubulin expression remains largely unexplored. This review gives an overall view of the regulatory mechanisms of tubulin expression reported in the literature. The first model proposed to explain the regulation of tubulin expression was based on an auto-regulatory mechanism. This hypothesis suggests that soluble tubulin pools regulate the tubulin mRNA levels. This is due to the MREI sequence common to all -tubulin isotypes. Nevertheless this model does not explain variations specific for each tubulin isotype. Transcriptional regulation has been suggested in multiple models. Indeed it appears that certain isotypes are expressed in defined conditions, and that this expression depends on gene regulatory sequences. To illustrate isotype specific regulatory mechanisms, the example of 3-tubulin is presented due to its particular expression pattern as well as its importance in certain physiological phenomena and pharmacological situations.

Key words: Tubulin, expression, regulation.

INTRODUCTION

Tubulin diversity

Tubulin is a globular protein that constitutes the building block of microtubules, a major element of the cyto-skeleton. Tubulin heterodimers are composed of two major classes, -tubulin and -tubulin. Since the initial cloning of chicken and tubulin cDNA during the 1980s (Cleveland et al., 1981; 1980), tubulin genes of a wide variety of organisms have been isolated and charac-terized. Identification of functional transcripts has helped classifying and tubulin into classes named isotypes. The later has been defined according to the divergent carboxy- terminal amino acid sequences. Multiple isotypes of both and tubulin are present in vertebrates as summarized in the Table 1. Moreover, whole genome analysis has allowed identifying predicted members of the tubulin gene family. However, the major part of these sequences represents pseudogenes; therefore, functional genes need to be sorted by providing evidence of the protein functionality. For this reason, the and tubulin classification is not definitively established. In addition, the complexity of the tubulin population is amplified by various post- translational modifications (Luduena, 1998). Indeed both and tubulin possess the ability to undergo different modifications such as tyrosination/dety-rosination, acetylation/deacetylation, phosphorylation, polyglutamylation and polyglycylation. These post-trans-lational modifications allowed subdividing isotypes into several isoforms (MacRae, 1997).

Multiple roles of the tubulins

Diversity among tubulin isotypes mostly arises from the divergent C-terminal sequence permitting the classi-fication into isotypes, and the ability to undergo numerous post translational modifications. It has been shown that isotypes are differentially expressed according to the tissue and that isoforms have been associated with differ-rent functions. For example 3- tubulin is expressed in neurons and Sertoli cells of testis contrary to 4a-tubulin,

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Table 1. Vertebrate and tubulin genes. Their classification was deduced from the analysis of the carboxy-terminal sequence (Dobner et al., 1987, Khodiyar et al., 2007, Lewis and Hall, 1985 ; Sullivan and Cleveland, 1986 ; Villasante et al., 1986).

Isotype	gene name	C-terminal sequence
A1A	1A	VDSVEGEGEEEGEEY
A1B	1B	
A1C	1C	EVGADSADGEDEGEEY
A4A	4A	EVGIDSYEDEDEGEE
A3A	3A	VDSVEAEAEEGEEY
A3B	3B	
A3C	3C	
A3D	3D	
A3E	3E	
A8	8	GTDSFEEENEGEEF
AL3	-like 3	LAALLERDYEEVAQSF
I	hM40	EEEEDFGEEAEEEA
II	h 9	DEQGEFEEEEGEDEAEG
III	h 4	EEEGEMYEDDEEESESQGPK
IVa	h5	EEGEFEEEAEEEVA
IVb	h 2	
V		QEATANDGEEAFEDDEEEINE
VI	h 1	EEDEEVTEEAEMEPEDKGH

which is specifically expressed in neurons. The 5- tubulin is an abundant isotype in birds but possibly a minor constituent of most mammalian cells (Lewis, 1990). Moreover post-translational modifications occur in de-fined tissues probably in relation with specific functions. Indeed in mammals, glycylation is mainly limited to tubulin incorporated into axonemes of motile cilia and flagella, whereas glutamylation is abundant in neuronal cells, centrioles, axonemes, and the mitotic spindle. In cilia and flagella, the polyglutamylation and poly-glycylation play a role in the formation and maintenance of axonemal structures. These modifications may also influence the transport of structural and membrane com-ponents within cilia and flagella (Hammond, 2008).

These data, as well as the strong conservation of isotypes between species, support a functional role to explain the diversity of tubulin. Therefore, the multitubulin hypothesis stipulating that the isotypes are responsible for different microtubule functions has been proposed (Fulton and Simpson, 1976). However, it was shown that -tubulin expressed specially in the testis by *Drosophila melanogaster* is used as well during meiosis as in flagella (Kemphues et al., 1982). Moreover, it was found that the incorporation of chimeric isotypes does not alter icrotubule function (Bond et al., 1986).

The question of functional significance of tubulin iso-types increased in complexity after it was demonstrated that different isotypes of -tubulin display diverse stability (Schwarz, 1998). In addition, genetic studies on *D. melanogaster* have shown that the number of protofilaments incorporated into the microtubule was dependent on the -tubulin isotypes (Raff et al., 1997). Today it appears that a differential functional significance of tubulin isotypes truly exists, which is well illustrated by the ex- ample of the 3-tubulin isotype. This isotype confers a higher sensitivity to some anticancer drugs as compared to the other isotypes (Gan, 2007). More-over, recently 3-tubulin has been shown to exist in at least two iso-forms that confer different sensitivity to anti-microtubule agents (Cicchillitti et al., 2008). Even though certain physiological functions remain to be elucidated, these results confirm the specific role of some tubulin isotypes.

A key to govern tubulin diversity: the regulatory processes

Great progress in understanding of the differential expression and the significance of the post-translational modifications of tubulin isotypes has been achieved. Nevertheless, some isotypes and isoforms have not been clearly associated with a physiological function. The specific expression of a defined kind of tubulin in precise conditions, such as a specific tissue or in response to a physiological stimulus, indicates the existence of regu-latory mechanisms that are able to explain the presence of these various tubulins.

Regulation controls the quantity of the protein and its function as shown in Figure 1. The amount of protein is

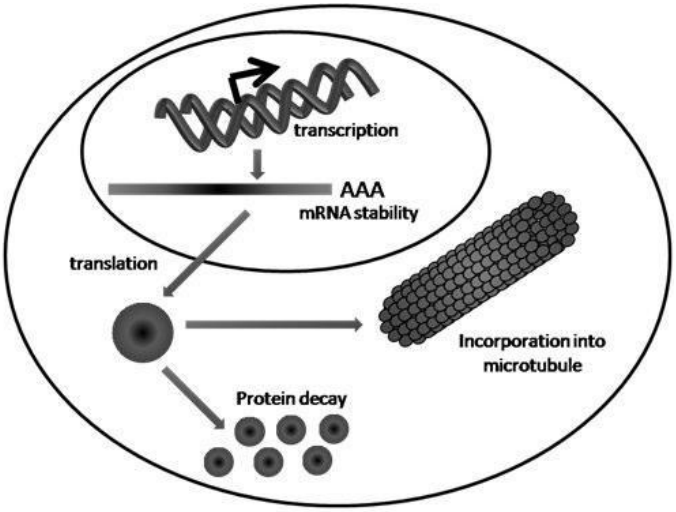

Figure 1. Tubulin regulation could occur at different levels. Firstly, transcription of various and -tubulin isotypes is controlled by promoter elements (enhancer or repressor). The translatability and stability of the produced mRNA is also controlled. The protein pool is further controlled through incorporation into microtubules or decay mechanisms.

controlled by the rate of mRNA transcription, the rate of mRNA translation and/or the mRNA half-life. Post-translational regulation modifies protein function through controlling specific folding that allows or hinders tubulin incorporation into the microtubule. This review will be focused on the regulatory processes governing tubulin expression.

Understanding regulation of the tubulin expression provides novel perspectives explaining cellular physiological as well as pharmacological activities. This review will focus firstly on the known general regulatory mechanisms, before tackling the issue of specific regulatory mechanism for 3-tubulin as an example due to its important involvement in decreased cell response to anti-microtubule drugs.

AUTOREGULATORY MECHANISM: A GENERAL PROCESS

To explain the regulation of soluble tubulin dimers, the model of an autoregulatory mechanism was proposed, based on the observed effects of colchicine and nocodazole in 3T6 mouse fibroblasts (Ben-Ze'ev et al., 1979). Both of these tubulin depolymerizing agents induced a rapid decrease in -tubulin mRNA expression with an inhibition of -tubulin synthesis. According to the autoregulatory mechanism hypothesis, the increased unpolymerized tubulin pool suppresses the formation of new mRNA and hastens the decay of existing mRNA. Moreover, microtubule destabilization without increasing the free tubulin did not induce an inhibition of tubulin synthesis. Furthermore, the assembly state of microtubule influences the tubulin synthesis since there is a correlation between amounts of tubulin mRNA and the newly synthesized protein (Cleveland et al., 1981). In fact tubulin production decreased concomitantly with the increase in tubulin monomer pool. Kinetic studies performed in mouse fibroblasts indicated that tubulin monomers may regulate the rate of mRNA transcription. Eukaryotic cells seem to exploit mRNA instability as a means to precisely control level of the monomer tubulin pool. A similar mechanism has been described in the ciliated protozoan *Tetrahymena pyriformis* by studying the control of tubulin gene expression during the cell cycle (Zimmerman et al., 1983). After having synchronized the cell culture, the mRNA tubulin level and its translatability during cell cycle were evaluated. The tubulin mRNA synthesis appeared to be periodic with the production peaking during G2 phase. Once again, the authors suggested that the tubulin soluble pool size regulated the transcription level.

The existence of the autoregulatory mechanism got a further confirmation by the microinjection of purified tubulin subunits into mammalian cells in culture. The tubulin synthesis is suppressed by the injection of an extra amount equivalent to 25 - 50% of the initial tubulin amount present in the cell (Cleveland et al., 1983). The tubulin soluble level not only modulates the rate of tubulin synthesis, but it also modulates the response to depolymerization. Using two cell lines possessing different cellular levels of soluble tubulin, fibroblasts with 55% of its tubulin in soluble form developed reduced response to depolymerizing agents in comparison to hepatocytes which contains only 15% soluble tubulin (Caron et al., 1985a).

Later studies deciphered the mechanism by which soluble -tubulin modulates mRNA level. Two groups have simultaneously established the cytoplasmic com-ponent of the regulatory mechanism (Caron et al., 1985b; Pittenger and Cleveland, 1985). They used a similar approach employing enucleated cells termed cytoplasts, produced either from mouse fibroblasts (Caron et al., 1985b) or Chinese hamster ovarian cells (Pittenger and Cleveland, 1985). These two studies reported that the regulatory process is achieved in the cytoplasm by a mechanism that controls mRNA stability and/or translatability. Therefore, the autoregulatory mechanism involves -tubulin mRNA stability.

At the end of the 1980s, it was demonstrated that the amino acid sequence MREI (methionine-arginine-glutamic acid- isoleucine) common to every -tubulin, is both sufficient and necessary to activate the cytoplasmic regulatory mechanism (Yen et al., 1988b). Experiments using a protein synthesis inhibitor in cultured animal cells, provided evidence that - tubulin mRNA encoding a truncated product containing only 26 amino acids does not constitute a substrate for the autoregulatory mechanism described previously (Pachter, 1987). This was the first

report indicating that a specific sequence may be required to trigger the regulatory process. Once the amino terminal tubulin peptide emerges from the ribosome, it is recognized to activate the mRNA degradation process (Yen et al., 1988b). To identify the minimal sequence of the nascent peptide required for autoregulation, chimeric genes containing progressively smaller -tubulin gene regions have been transfected into cultured fibroblasts (Yen et al., 1988a). This experiment showed that the autoregulatory domain contains the first 13 translated nucleotides of -tubulin mRNA encoding the first 4 translated codons MREI. The translational regulation requires the presence of a necessary co-factor which binds to the nascent -tubulin. The possibility that the tubulin monomers themselves might act as co-factor has been eliminated. Therefore, the identity of the co-factor remains unknown (Theodorakis and Cleveland, 1992).

In summary, the autoregulatory mechanism through the common MREI amino acid sequence appears to be a general regulatory mechanism for the cellular level of all tubulin that is not isotype- specific. However, tubulin isotypes possess a defined tissue-specific expression pattern, suggesting the existence of a specific regulatory mechanism that enables restricted variation of some isotype expression.

TRANSCRIPTIONAL REGULATION: A SPECIFIC SYSTEM

The development of molecular techniques has allowed investigating expression control at transcriptional level in order to explain the regulatory mechanism specific to each tubulin isotype. The unicellular green algae *Chlamydomonas reinhardtii* constitutes one of the most used models for genetic studies especially in the case of expression regulation due to the simplicity of its genome. *C. reinhardtii* possesses 4 tubulins called 1, 2, 1, and 2-tubulin, which are encoded by 4 distinct genes. In the unicellular algae, an acid shock or a mechanical stress causes rapid flagellar excision and coordinately activates transcription of a set of flagellar genes to ultimately regenerate new flagella. This property has been used to explore transcription activation of tubulin genes which belong to the induced flagellar genes. The 2-tubulin promoter was first characterized in *C. reinhardtii* after a deflagellation (Davies et al., 1992).The transcriptional activity of the promoter is increased followed the deflagellation (Davies and Grossman, 1994). The 2-tubulin promoter contains a GC-rich region between the TATA box and the transcription initiation site, and 7 copies of 10 bp sequence motifs called tub (short name of tubulin) box. These tub box motifs are involved in the induction of transcription following the deflagellation. Indeed, removing 4 or 5 tub box motifs prevents transcriptional increase by flagellar excision contrary to the change of GC-rich sequence to AT-rich region, which does not significantly affect the transcription level.

Another gene has been studied in *C. reinhardtii* after deflagellation by an acid shock to define and map the acid shock responsive element governing induction of 1-tubulin gene. It appears that this gene is not silent in non-stimulated cells but is expressed at low basal level, suggesting that one or several enhancer/silencer elements must be present to ensure a higher transcription rate. Deletions of various sequences have shown that 2 promoter regions (- 176 to -122 and -85 to -16) are especially important for regulating the 1-tubulin gene expression. Indeed, the deletion of -176 to -122 bp region resulted in an induction level of 45 - 70% of the basal expression, and the deletion of the region upstream the -56 bp resulted in a complete loss of inducibility without affecting the basal expression. Moreover, the 1-tubulin promoter region from -85 to -16 bp conferred partial acid shock inducibility to a reporter gene (Periz, 1997) . These results show that induction of 1-tubulin gene by an acid shock is a complex response involving diverse sequence elements. However, the transcription factors implicated have not been identified.

The 1-tubulin gene regulation has also been explored in the ciliated protozoan *Stylonychia lemnae*. In this case, the promoter has been characterized. The *S. lemnae* macronuclear genome consists of minichromosomes easy to study because they may encode as little as a single gene each. Moreover, the 5©-nontranscribed spacers are usually no longer than 400 bp and highly suitable for promoter characterizations. Microinjection of two artificial and differently tagged 1 tubulin minichromosomes with deletion and block substitution mutations into the macronucleus of *S. lemnae* was used as a means to characterize its promoter. The core promoter contains a critical sequence around the transcription initiation site that appears to be an initiator element, and a TATA-like element around position -25. While mutation of the TATA-like element caused aberrant transcription initiation, mutation of the sequence surrounding the transcription initiation site abolished transcription, indicating that the TATA-like element and the initiator are conserved core promoter elements of *S. lemnae* minichromosomes cooperating in the recruitment of RNA polymerase to the correct transcription initiation site. Moreover, two distinct upstream sequence elements appear to be specific for the α1 tubulin minichromosome. On the other hand, the α2 tubulin minichromosome promoter is very short, comprising the two proximal elements but not the upstream sequence elements. These structural promoter differences caused up-regulation of α2-tubulin expression in cells treated with concanavalin A lectin but not of α1 tubulin (Skovorodkin et al., 2007).

The housekeeping 2- tubulin gene of the eukaryote parasite *Giardia lamblia* appears to contain a strong promoter. Deletions of the promoter demonstrated that 2 AT- rich sequences surrounding the transcription initiation site are essential to achieve gene reporter expression. These *cis*-acting elements possess the ability to function independently of any other element to initiate transcription

Therefore, these elements were considered initiator-like elements despite the sequence differences with the eukaryotic initiator consensus sequence. Moreover, the deletion or mutation of the distal sequence containing the AT-clusters led to a strong decrease of the transcriptional activity confirming the importance of this region for promoter activity (Elmendorf and Pierce, 2001).

Regulation of tubulin expression has also been explored in vertebrate organisms. The fruit fly *D. melanogaster* possesses a tissue-specific tubulin isotype expressed exclusively during spermatogenesis. The 2-tubulin promoter sequence responsible for the tissue specific gene activation is confined in a region of 80 bp which is sufficient to drive germ -line specific expression in the testis. In addition a 14 bp activator element called 2UE1, is necessary for promoter specificity (Michiels and Renkawitz, 1989). The role of the activator element has been confirmed *in vivo* by using transgenic drosophila (Santel et al., 2000) . Besides, 1-tubulin gene is abundantly expressed in the central nervous system in the zebra fish *Danio rerio*. This neuron-specific isotype promoter possesses a 64 bp region (-469 to -406 bp) necessary to drive a reporter gene after optic nerve crush. This sequence appears not essential for promoter activation in the developing retina suggesting specificity for this region (Senut, 2004).

The vincristine-resistant mouse melanoma cell line, B16F10 is a model for an inducible tubulin gene regulation, where a large increase in 2-tubulin mRNA level was observed after overnight exposure of the parent cells to vincristine. This suggested that this variation is not the result of a resistance phenomenon but due to the exposure to the drug. The cloning of the promoter upstream to a reporter gene revealed that the promoter activity is increased only after vincristine or vinblastine exposure but not after exposure to paclitaxel indicating that the effect is perhaps specific for vinca alkaloids. The modulation of promoter activity leading to the regulation of 2-tubulin mRNA level by vinca agents was found to be mediated through the p53 protein which binds to a specific sequence located in the first intron. Vincristine appears to prevent p53 binding to its specific motif and thereby allows an increase in 2-tubulin expression. In this case the p53 specific sequence acts as a silencer element and antagonizing p53 binding triggers 2-tubulin transcription (Arai et al., 2006).

Regarding the regulation of tubulin expression, 3-tubulin is a model gene due to its specific expression pattern in physiological conditions and the clinical relevance of its expression levels in relationship to anti-microtubule drug response. Indeed in normal adult tissues, 3- tubulin is significantly expressed mostly in neuronal cells and in Sertoli cells (Easter et al., 1993), and during defined periods of development (Katsetos, 2003). This sup-ports the presence of spatial and temporal mechanisms governing its expression. In addition, increased expression of 3-tubulin has been correlated to a decreased response to anti-microtubule agents in a large variety of cancers (reviewed by (Drukman and Kavallaris, 2002). Selective expression of an isotype in the case of chemoresistance suggests once again the existence of precise regulatory mechanisms able to control expression level of a specific tubulin isotype. The subsequent section of this review will focus on such differential regulation of the 3-tubulin gene expression.

THE ISOTYPE-SPECIFIC DIFFERENTIAL REGULATION OF 3 TUBULIN EXPRESSION

A limited number of publications have focused on the regulation of 3-tubulin gene expression. The best characterized model of regulation of 3- tubulin gene expression is in *D. melanogaster*, studied towards the end of 1990s during embryo development. The 3-tubulin appears to constitute a differentiation tissue-specific factor not only during the fruit fly development but also during human development. The 3-tubulin expression occurs prominently and gradually in neuronal tissues during human fetal and postnatal development. The distinct expression pattern is exhibited following time and spatial gradients correlated with the specific development of cellular subtypes. Transcription factors involved in development have been especially identified in *Drosophila* (Katstetos et al., 2003).

During the differentiation and specification of *Drosophila* mesoderm, the ultrabithorax (Ubx) factor encoded by a homeotic gene, controls 3-tubulin gene expression. In the visceral mesoderm, 3-tubulin transcription is achieved by two separately acting enhancers with binding sites located on the first intron of the 3-tubulin gene (Hinz and Renkawitz, 1992). Contrary to the enhancing activity of the Ubx factor, the transcription factor Engrailed (En) appears to be a repressor (Serrano et al., 1997). Indeed, the 3-tubulin gene has been identified as a direct target of the nuclear regulatory protein En in *Drosophila*. Under normal conditions, 3-tubulin gene is expressed exclusively in the mesoderm. However, its expression is deregulated when En factor is abnormally expressed. Moreover, their functional binding sites have been characterized both *in vitro* and *in vivo* to be located in the first intron of the 3-tubulin gene (Serrano et al., 1997) . In addition, it has been demon-strated that 3-tubulin gene expression could be induced by steroid hormone in Drosophila. In fact, *in vitro* 3-tubulin expression is regulated by ecdysone at least in part at the transcriptional level (Bruhat et al., 1990). Furthermore, ecdysone-independent positive *cis*-acting elements are located in the 5©-flanking region of 3-tubulin gene and 3©-fragment of the first intron. Both of these sequences appear to be essential to confer an effect to ecdysone that indicates cooperation between the two regions. Deletion analysis of the 360 bp intronic region reveals that a fragment of 57 bp is crucial for the

ecdysone response of the 3-tubulin gene. This fragment contains 5©-TGA(A/C)C-3© motifs homologous to ecdysone responsive elements. Band shift assays show that this 57- bp fragment is bound by three specific complexes. One of these appears to be involved in the level of the ecdysone response (Bruhat et al., 1993).

The rat was the first vertebrate in which the promoter of 3-tubulin gene was characterized particularly during neuronal differentiation. The cloning of the rat promoter permitted the mapping of the transcriptional start site and to define the TATA box binding protein at position - 28 bp similar to other tubulins within the 30 nucleotides 5©to the established transcriptional start site. The first 131 bp are sufficient to confer a transcriptional activity to the promoter where numerous putative binding sites have been found such as Sp1, AP2, Pit1, or an Ebox (Dennis et al., 2002). The human 3-tubulin promoter has been recently described with its regulating elements involved in response to hypoxia. It does not possess a homologous structure to rat promoter. Numerous binding sites for transcription factor are also present in the human promoter but not with an analogous organization. In addition, in the case of hypoxia response in ovarian carcinoma cell line, the responding site is an HIF- 1 binding site present in the 3©-flanking region at +168 from the stop codon (Raspaglio et al., 2008).

These studies have demonstrated the presence of a regulatory mechanism at the transcription level to control the amount of mRNA. However, it appears likely that 3-tubulin is also controlled at the translational level. Indeed, an over-expression of 3-tubulin protein more than 2-3 folds has failed until now. Ranganathan and Benetatos (1998) and our group (data not published) have used a similar approach to establish stable 3-tubulin over-expressing cells with 3-tubulin cDNA cloned on an expression vec-tor. Moreover, the protein level did not correlate with the mRNA level suggesting that a mechanism interferes to prevent enhanced protein expression.

Conclusion

Tubulin mRNA is expressed during physiological defined conditions. Studies revealed a wide variety of factors influencing tubulin isotype expression level. Each isotype seems to be controlled by a particular system of regulation according to the cellular context. For example 3-tubulin mRNA is enhanced following hormonal exposition in drosophila or by hypoxia in human due to activation of specific responsive elements located on the tubulin gene promoter (Michiels and Renkawitz, 1989). Such regulatory elements were also located in the gene introns (Bruhat et al., 1990) or in the 3©-UTR regions (Raspaglio et al., 2008).The exploration of tubulin genes has thus revealed that the tubulin isotype gene expression was at least partially regulated at the transcriptional level. Nevertheless, transcriptional control cannot explain the variety of situations observed as reflected by the discordance between mRNA and protein levels. The cytoplasmic regulatory mechanism intervenes to adjust the protein expression to the adequate level necessary for the cell. This is achieved by controlling the stability of the mRNA which is governed by the soluble -tubulin pool according to the autoregulatory mechanism (Theodorakis and Cleveland, 1992).

Moreover, controlling of the protein half-life is another alternative. Such hypotheses have not been extensively explored until now. Some studies explored tubulin interactions with several cofactors acting as chaperon proteins. Indeed tubulin could be regulated not only quantitatively but also functionally. Tubulin requires to be polymerized into microtubules in order to participate in various cellular functions including mitosis or intracellular transport. The polymerization of tubulin requires interaction with numerous co-factors such as the members of the tubulin binding cofactor family which seems to influence soluble and polymerized pools (Nogales, 2000). The level of tubulin mRNA and its translation rate appear to be specifically regulated to meet the required content of this protein.

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