CHARACTERIZATION OF *BRUGIA PAHANGI* ß-TUBULIN GENES AND GENE PRODUCTS.

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ABSTRACT

The ß-tubulin gene family of the parasitic nematode, *Brugia pahangi* consists of three to five ß-tubulin sequences. Two genomic clones containing ß-tubulin sequences were isolated and characterized. The ß1-tubulin gene spans 3.8 kb, is organized into 9 exons and expresses an mRNA of 1.7 kb which codes for a protein of 448 amino acids. A partial nucleotide sequence of the second clone confirmed the isolation of a distinct ß-tubulin sequence, ß2-tubulin. The ß1-tubulin transcript is found in microfilariae and adult worms, whereas the ß2-tubulin transcript is predominant in male adult worms but absent from microfilariae. Results of this study also indicate that the maturation of the ß1-tubulin message involves the acquisition of the conserved nematode 22-nucleotide splice leader sequence. Antipeptide IgGs raised against the divergent carboxy-terminal region of ß1-tubulin recognize the same ß-tubulin isoform pattern as a phylum cross-reactive monoclonal antibody. This result suggests that the ß1-tubulin is highly represented in *B. pahangi* adults and microfilariae.

ABRÉGÉ

La famille de gènes codant pour la ß-tubuline du nématode parasitaire ***Brugia pahangi*** se compose de trois à cinq séquences de ß-tubuline. Deux clones génomiques portant des séquences de ß-tubuline ont été isolés et caracterisés. Le gène codant pour la ß1-tubuline s'étend sur 3.8 kb et se divise en 9 exons. L'ARN exprimé par ce gène est de 1.7 kb et code pour une protéine de 448 acides aminés Le deuxième clone qui n'a été que partiellement caractérisé contient des séquences codant pour la ß2-tubuline. Les gènes de tubuline ß1 et ß2 se distinguent par la distribution de leurs ARN messagers. Le transcrit du gène de ß1-tubuline se retrouve chez les microfilaires et les vers adultes de ***B. pahangi***, tandis que le transcrit du gène de ß2-tubuline est exprimé surtout chez les mâles adultes et est absent chez les microfilaires. De plus, les résultats de cette étude démontrent que la maturation du transcript de ß1-tubuline entraine l'acquisition d'un "splice leader" de 22 nucléotides. Un anticorps ayant comme épitope la région carboxyl de la ß1-tubuline reconnait les mêmes isoformes qu'un anticorps monoclonal réagissant à la ß-tubuline de plusieurs organismes. Ces résultats indiquent que la ß1-tubuline est bien représentée chez les adultes et les microfilaires de ***B. pahangi***.

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Suggested short title:

ß-tubulin genes of *Brugia pahangi*

STATEMENT OF ORIGINALITY

The contributions to scientific knowledge which arise from this study are as follows:

1) This is the first report characterizing the ß-tubulin genes of a parasitic nematode. The genomic organization of the ß-tubulin genes within the *B. pahangi* genome and the analysis of their structure is significant because very little is known about the molecular genetics of filarial nematodes.

2) This study shows that members of the *B. pahangi* ß-tubulin gene family are more divergent from one another than in *C. elegans* Since *C. elegans* is the only other nematode for which ß-tubulin gene sequences have been reported the sequence analysis of the ß1-tubulin gene and the partial characterization of the ß2-tubulin gene are significant contributions to knowledge.

3) The pattern of expression demonstrated for the ß2-tubulin gene has not previously been reported for nematodes These transcripts are predominant in adult male worms which suggests that this ß-tubulin is specific to spermatozoa as found in *Drosophila*.

4) The ß1-tubulin transcripts acquire the conserved nematode 22-nucleotide splice leader sequence. This processing event has not been previously demonstrated for nematode ß-tubulin mRNAs.

5) Finally, ß-tubulin isoforms of *B. pahangi* microfilariae were characterized and compared to those of adult worms.

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To the memory of Jean Guénette

CHAPTER 1 - LITERATURE REVIEW

INTRODUCTION

The molecular genetics of filarial nematodes is an important area which has received little attention. Studies within this field will be of benefit for defining basic and molecular processes in these parasites. Tubulin is particularly suitable for initial studies of filarial nematode genes because knowledge of tubulin structure and function for other organisms is extensive. In addition, tubulin represents a major drug target for nematodes. Therefore, I have undertaken to study the tubulin gene family of *Brugia pahangi*.

A BRIEF HISTORY OF MICROTUBULE RESEARCH

Tubulin is an indispensable constituent of the cytoskeleton of all eukaryotic cells. It is a structural component of the mitotic spindle, the cytoplasmic microtubules, cilia and flagella. Factors controlling cell differentiation and progress through the cell cycle in mitosis and meiosis regulate the assembly and functional properties of these microtubule structures (Raff, 1979). Tubulin exists as a dimer (Renaud *et al.*, 1968) of two distinct polypeptide chains, α- and ß-tubulin, which are non-covalently associated. The two tubulin chains are found in equimolar amounts in cytoplasmic microtubules (Bryan and Wilson, 1971). Tubulin subunits can polymerize to form various structures *in vitro* such as sheets, rings and discs, demonstrating the intrinsic potential of tubulin subunits for a wide variety of associations. However, *in vivo* they assemble to form a hollow cylinder composed of tubulin dimer chains called protofilaments. The cytoplasmic microtubules of most organisms have 13 protofilaments, but microtubules composed of 11, 12 and 15 protofilaments have been observed in nematode cells (Chalfie and Thomson, 1982; Davis and Gull, 1983).

The notion of tubulin heterogeneity was first proposed when electron micrographs showed that microtubule containing organelles such as the mitotic spindle, the flagella and cilia behaved differently during cell division (Raff, 1979). This concept was supported by the biochemical observations that cold, pressure and antimitotic agents

could disrupt the mitotic spindle but not cilia, flagella, centrioles or the midbody (Olmstead and Borisy, 1973). Biochemical and immunological techniques were used to demonstrate the differences between cytoplasmic, ciliary and flagellar microtubules of the sea urchin, the algae ***Chlamydomonas*** and the amoeba-flagellate ***Naegleria*** (reviewed by Cleveland and Sullivan, 1985). However, it was the analysis of mammalian brain tubulin which revealed the extent of tubulin heterogeneity. Tubulin isolated from calf brain was resolved into 17 protein fractions by isoelectric focusing (George *et al.*, 1981). Subsequently, the improved resolution of two dimensional gel electrophoresis allowed the identification of 7 α and 10-14 ß isoforms of brain tubulin (Field *et al.*, 1984). This number of tubulin isoforms was shown by Gozes and Sweadner (1981) not to be due to the variety of cell types in the brain since tubulin isolated from sympathetic neurones grown in primary culture is heterogeneous. *In vitro* translation of rat brain RNA and two dimensional gel electrophoresis of the polypeptides obtained showed extensive tubulin heterogeneity indicating that the diversity of tubulin isoforms is, in part, genetically determined (Marotta *et al.*, 1979). This finding, concomitant with the development of recombinant DNA technology, has permitted the characterization of tubulin gene families from several organisms over the last decade. In invertebrates and fungi, classical and molecular genetics have also been useful for the isolation of tubulin genes. Recent studies of microtubule biology have focused on the relationship between the structure of tubulin and microtubule function. Techniques of molecular biology, biochemistry, developmental biology, cell biology and genetics have been used to examine this relationship.

TUBULIN GENES

Genomic organization

In most organisms, the α- and ß-tubulins are encoded by multiple genes (reviewed by Cleveland and Sullivan, 1985). However, in the yeasts, ***Saccharomyces cerevisiae*** (Neff *et al.*, 1983) and ***Schizosaccharomyces pombe*** (Hiraoka *et al.*, 1984) and the fungi, ***Neurospora crassa*** (Orbach *et al.*, 1986) and ***Candida albicans*** (Smith *et*

al., 1988) only a single ß-tubulin gene has been identified. In contrast, the protozoans ***Tetrahymena pyriformis*** (Barahona *et al.*, 1988), ***T. thermophila*** (Callahan *et al.*, 1984) and ***Toxoplasma gondii*** (Nagel and Boothroyd, 1988) have a single α-tubulin gene within their genomes. With the exception of the yeast ß-tubulin gene these conclusions are based on the observation that no genomic DNA fragments other than those corresponding to the cloned tubulin gene were detected on Southern blots hybridized under low stringency conditions using heterologous probes. It is therefore, possible that not all tubulin sequences were identified.

Initial Southern blot hybridization experiments suggested that the human ß-tubulin gene family contains 15 to 20 members (Cleveland *et al.*, 1980). It is now known through analysis of cDNA clones that 6 of these sequences are authentic ß-tubulin genes (Wang *et al.*, 1986; Lewis *et al.*, 1987; and Burgoyne *et al.*, 1988); the remainder are pseudogenes. Two categories of pseudogenes have been identified; the first contains intervening sequences but does not code for the complete ß-tubulin protein and the second has no intervening sequences, contains a poly-A tail and is flanked by short direct repeats (Wilde *et al.* 1982a; Wilde *et al.*, 1982b). It has been suggested that the latter category of pseudogenes was derived by integration of a cDNA copy into a staggered host chromosomal break (Wilde *et al.*, 1982a). A subgroup of the human ß-tubulin gene family consisting of four members sharing an identical ß-tubulin 3' untranslated region is composed of one gene coding for two mRNAs of 1.8 and 2.6 kb and three intronless ß-tubulin genes. Two of the intronless genes were derived by integration of the 1.8 kb mRNA into the genome and the third was derived from the 2.6 kb mRNA (Lee *et al.*, 1983). Cleveland and co-workers chose to characterize the α- and ß-tubulin genes of chicken because few pseudogenes were reported within the chicken genome. There are five functional α-tubulin genes (Valenzuela *et al.*, 1981; Pratt *et al.*, 1987; Pratt and Cleveland, 1988) and seven ß-tubulin genes (Monteiro and Cleveland, 1988) in the chicken genome. Two differentially expressed ß-tubulins of chicken, cß1 and cß2, were found to be virtually identical in amino acid sequence (99.5%) and the patterns of homology suggest that these two genes are products of gene duplication resulting from gene conversion or non-reciprocal

recombination events (Sullivan *et al.*, 1985). This is also true of two mouse ß-tubulin genes (Wang *et al.*, 1986). Although it has been difficult to distinguish functional from non-functional tubulin sequences in the mammalian genome, six mouse α-tubulin (Villasante *et al.*, 1986; Lewis *et al.*, 1985a) and six ß-tubulin genes have been described by characterizing cDNA clones. Interestingly, the amino acid sequences of four human ß-tubulin gene products are identical to the corresponding mouse ß-tubulin isotypes (Wang *et al.*, 1986).

In spite of the large number of ß-tubulin sequences identified within the human genome, they are dispersed and the functional genes exist as ingle copies (Cleveland *et al.*, 1981a). Cytogenetic studies in *Drosophila* have shown that invertebrate tubulin genes also exist as scattered single copy genes (Sanchez *et al.*, 1980; Natzle and McCarthy, 1984). This is true for tubulin gene families of other organisms (Cleveland and Sullivan, 1985), with the exception of yeast, *Chlamydomonas reinhardii* and some parasitic protozoa. In species of the parasitic protozoans *Leishmania* and *Trypanosoma*, the α- and ß-tubulin genes are linked and clustered, in the former as separate tandem repeats (α)n and (ß)n (Landfear *et al.*, 1983; Spithill and Samaras, 1985, and Huang *et al.*, 1984) and in the latter as tandem repeats of α,ß-gene pairs (Thomashow *et al.*, 1983; Seebeck *et al.*, 1983; and Esquenazi *et al.*, 1989). A single tubulin cluster in *Trypanosoma brucei* comprises about ten alternating α and ß genes with the 3' end of the cluster terminating in the middle of a ß gene. The adjacent DNA fragment shows a high degree of identity to the retrotransposon-like (RTnL) element of the trypanosome genome (Affolter *et al.*, 1989). In *Leishmania major* and *Trypanosoma cruzi*, extra minor ß-tubulin DNA fragments are located at separate chromosomal loci (Spithill and Samaras, 1985; and Maingon *et al.*, 1988). Esquenazi *et al.* (1989) propose that tubulin gene organization may be a useful tool for classification of trypanosome species. Those species with a simple α,ß gene pair repeat being characteristic of the African trypanosomes. With the exception of *L. major* ß-tubulin genes it is not yet known whether these multiple tubulin gene copies code for distinct polypeptides (Spithill and Samaras, 1987).

Tubulin gene structure

With the exception of those organisms which do not contain any conventional introns, such as trypanosomes and other kinetoplastidae, (Donelson and Zeng, 1990) the majority of expressed α- and ß-tubulin genes contain intervening sequences (Little and Seehaus, 1988). The α- and ß-tubulin genes of *T. gondi* have an unusual gene structure for protozoans because they contain two and three introns, respectively (Nagel and Boothroyd, 1988). Introns are often found in the first quarter of the α- and ß-tubulin genes, and in vertebrates the tubulin intron positions are highly conserved (Little and Seehaus, 1988). However, in the fungi and the nematode ***Caenorhabditis elegans*** the ß-tubulin genes have multiple introns which are dispersed within the coding sequence at positions which are not necessarily conserved in organisms of the same taxonomic class (Driscoll *et al.*, 1989; Smith *et al.*, 1988). The vertebrate α-tubulin gene, mα4, has an intron located immediately after the ATG coding for the initiation codon (Villasante *et al.*, 1986) and in testis the mα4 transcript is missing the amino terminal methionine (Dobner *et al.*, 1987). In the ß3-tubulin gene of ***Drosophila melanogaster***, sequences within the first intron are implicated in the regulation of this gene's expression (Bruhat *et al.*, 1990; Gasch *et al.*, 1989).

THE REGULATION OF TUBULIN GENE EXPRESSION AND TUBULIN SYNTHESIS

The spatial and temporal expression of tubulin genes

Gene specific probes have been used to measure the relative levels of expression of the ß-tubulin isotypes in vertebrates as a function of development and differentiation (Sullivan, 1988). Cell type specific expression has also been addressed with the development of ß-tubulin specific antibodies (Lopata and Cleveland, 1987; Lewis and Cowan, 1988) directed against the carboxy-terminus of the various ß-tubulin isotypes. A summary of the general expression patterns of vertebrate ß-tubulin genes is shown in Table 1.1.

In embryos, the major neuronal cß2 gene is the most abundant mRNA species, whereas the cß3 gene is barely detectable. However, in all adult tissue with the exception of the brain and lung the ß2-tubulin

TABLE 1.1. PROPERTIES OF VERTEBRATE ß-TUBULIN ISOTYPE CLASSES[1]

Isotype	Gene/Protein	C-terminal isotype defining sequence	Expression
Class I	cß7 mß5 rbt.3 hß1	EEEDFGEEAEEEA	Constitutive; many tissues
Class II	cß1/cß2 mß2 rbt.1 hß2 porc. ßA bov. ß1	DEQGEFEEEGEEDEA EG	Major neuronal; many tissues
Class III	cß4 hß4 porc. ßB bov. ß2 mß6[2]	EEEGEMYEDDEEESEQGAK S P D R	Minor neuronal; neuron specific
Class IVa	mß4 rbt.2 h5ß	EEGEFEEEAEEEVA	Major neural; brain specific
Class IVb	cß3 mß3 hß2	EEEGEFEEEAEEEAE VA	Major testes; many tissues
Class V	cß5	NDGEEEAFEDDEEEINE	Minor constitutive; absent from neurons
Class VI	cß6 mß1	DVEEYEEAEASPEKET GLEDSEEDAEEAEVEAEDKDH	Major erythrocyte/ platelets; hematopoeisis specific

[1] taken from Sullivan (1988)
[2] Burgoyne *et al.* (1988)

transcript levels are lower than those of ß3-tubulin (Havercroft and Cleveland, 1984). In adult chicken, the most striking example of differential expression is in testes where it is obvious that cß3 is the dominant ß-tubulin isotype (Havercroft and Cleveland, 1984; Sullivan *et al.*, 1986a). The 4.0 kb transcript of cß1 represents one of the major ß-tubulin transcripts in skeletal muscle myotubes, but the 1.8 kb and 4.0 kb transcripts of cß1 are found in low abundance in all other tissues (Havercroft and Cleveland, 1984). cß4 and cß5, which have a complementary pattern of expression, share identity at 16 residue positions which distinguish them from cß1, cß2 and cß3 polypeptides (Sullivan *et al.*, 1986b). It is clear from these studies of ß-tubulin gene expression, that at least two and sometimes three ß-tubulins are expressed in each cell and tissue type examined. The ß-tubulin isotypic class III and VIa transcripts are the only ß-tubulin products which are cell-type specific in vertebrates (Table 1.1).

However, within a tissue there is some cell-type specific expression. For example in mouse testis, mß2 is found only in cells of the fibrous capsule surrounding each seminiferous tubule, mß3 is found only in germ line cells (developing spermatids) and mß5 is strongly expressed in Sertoli cells which surround and interdigitate between developing spermatids (Lewis and Cowan, 1988). Furthermore, ß-tubulin transcripts in rat cerebellum have specific cellular distributions and their distribution changes during development (Burgoyne *et al.*, 1988).

In contrast to the vertebrate testis ß-tubulin isotype the ß2-tubulin of *D. melanogaster* is only expressed in the testis and is specific to germ line cells (Kemphues *et al.*, 1982). Levels of an α-tubulin transcript (α85-E) fluctuate during development and may be specific to the testis in adult *Drosophila* flies (Matthews *et al.*, 1989). In the mouse, mα3 and mα7, are expressed exclusively in the testis and code for a single α-tubulin polypeptide (Villasante *et al.*, 1986). The mα3/7 transcript is co-expressed with mß3 in mouse germ cells (Lewis and Cowan, 1988). Of the remaining three ß-tubulin genes of *D. melanogaster*, the ß1 and ß4 genes are ubiquitously expressed and the ß3 gene is developmentally regulated (Natzle and McCarthy, 1984; Bialojan *et al.*, 1984). The ß3-tubulin mRNA is transiently expressed in

the mesoderm during the pupal stage of embryogenesis, a stage at which larval muscles are histolyzed to build the adult musculature (Gasch *et al.*, 1988; Kimble *et al.*, 1989). The ß3-tubulin is a component of cytoskeletal microtubules which are involved in cell shape changes and tissue organization (Hoyle and Raff, 1990).

Two transcripts of 1.8 and 2.0 kb are produced from the testis-specific ß2-tubulin gene of *D. melanogaster*. Both transcripts are present during the prepupal and pupal periods of development, but the 1.8 kb transcript is also detectable in the 0-3 hr old embryo (Natzle and McCarthy, 1984). Although the significance of two transcript sizes is unknown, it is not uncommon for α- and ß-tubulin genes to have more than one transcript. Two transcript sizes are also observed for the chicken ß1 gene (Havercroft and Cleveland, 1984), the mouse ß5 gene (Lewis *et al.*, 1985a) and the mouse α4 gene (Villasante *et al.*, 1986). The different mRNA sizes of cß1 are due to the use of alternate polyadenylation signals (Havercroft and Cleveland, 1984) The choice of a 3' untranslated region over another is particularly interesting in view of the influence this region has on translation *in vivo* (Jackson and Standart, 1990). In the case of the human Hα44 tubulin gene, two distinct promoters are used to yield different transcripts which have unique patterns of expression (Dobner *et al.*, 1987).

Results of *in situ* hybridization experiments with an anti-ß-tubulin antibody raised against the *C. elegans* mec-7 gene product indicate that this ß-tubulin is found only in microtubules of the six touch receptor neurons which contain 15 protofilament microtubules (Mitani and Chalfie, 1990). A separate report demonstrates that it is also expressed in a pair of neurons just posterior to the pharynx (Hamelin and Culotti, 1990). However, the ben-1 ß-tubulin gene product of *C. elegans* is found in several cell types (Driscoll *et al.*, 1989).

Coordinate expression of tubulin genes

The co-expression of mß3 and mα3/7 in mouse testis is not due to the coordinate induction of these two genes (Wang *et al.*, 1986). Coordinate induction of α- and ß-tubulin expression has been reported for *C. reinhardii* and *Naegleria gruberi* following deflagellation (Brunke

et al., 1982; Shea and Walsh, 1987) and for *Tetrahymena* following deciliation (Seyfert *et al.*, 1987). The induction of tubulin gene expression in *N. gruberi* is part of the transcription of several flagellar protein genes including calmodulin, a protein which regulates intracellular calcium levels (Shea and Walsh, 1987). The ß3-tubulin of *Aspergillus nidulans* is one of approximately 1,200 new mRNAs coordinately expressed with the onset of conidiation (Weatherbee *et al.*, 1985).

Autoregulation of tubulin mRNAs

The rate of gene transcription and the stability of the mature message are factors which regulate the level of expression of a number of genes (Hunt, 1988). Tubulin synthesis in Chinese hamster ovary (CHO) cells is shut off by microtubule depolymerizing drugs such as colchicine and nocodazole because of a reduction in the level of translatable tubulin mRNA in these cells (Ben Ze'ev *et al.*, 1979). This effect is also observed after microinjection of tubulin at levels 25-50% above those found in the cell (Cleveland *et al.*, 1983). However, vinblastine, another microtubule depolymerizing drug which causes the formation of tubulin paracrystals, does not inhibit tubulin production but rather produces a small increase in tubulin synthesis (Ben Ze'ev *et al.*, 1979). No effect on the overall synthesis of tubulin is observed in CHO cells treated with taxol (Cleveland *et al.*, 1981b), a drug which stabilizes preformed microtubules and induces their polymerization (Schiff *et al.*, 1979). These results indicate that elevated levels of unpolymerized tubulin in drug treated cells inhibit the formation of new tubulin RNA and induce the existing mRNA to decay rapidly. This downregulation of tubulin synthesis in response to microtubule depolymerizing drugs occurs in several species, including humans, mouse, mosquito, *Drosophila*, chicken and sea urchins (Fellous *et al.*, 1982; Cleveland *et al.*, 1981b; Lau *et al.*, 1985; Gong and Brandhorst, 1987). This decline in mRNA is not accompanied by a decrease in transcription of tubulin genes (Cleveland and Havercroft, 1983), it is due to alterations of mRNA stability and/or translatability because enucleated mouse fibroblasts retain the ability to turn off tubulin protein synthesis in response to

colcemid (Caron *et al.*, 1985). Using a transient DNA transfection system, Gay *et al.* (1987) demonstrated that the 49 coding nucleotides of the ß-tubulin exon 1 starting at the ATG contains the regulatory sequence that modulates tubulin mRNA stability. Subsequent studies indicated that regulation of tubulin mRNA stability requires the first 13 nucleotides of ß-tubulin (Yen *et al.*, 1988a), polyribosome bound ß-tubulin mRNA and translation beyond codon 41 (Pachter *et al.*, 1987). The introduction of 25 different nucleotide base substitutions into the 13 base regulatory element of human ß-tubulin permitted Yen *et al.* (1988b) to determine that autoregulation occurred only when nucleotide changes did not alter the amino acid sequence, Met-Arg-Glu-Ile, a sequence which is conserved in all ß-tubulins sequenced to date. The interaction of the regulatory factor must occur as the amino terminal tetrapeptide emerges from the ribosome because internalization of the MREI sequence abolishes tubulin mRNA regulation (Yen *et al.*, 1988b). Furthermore, protein synthesis inhibitors block tubulin mRNA degradation indicating that this destabilization requires ongoing ribosome translocation (Gay *et al.*, 1989; Gong and Brandhorst, 1988).

In the above studies, the increase in unpolymerized tubulin subunits is dramatic and may not reflect the levels of free tubulin in resting and proliferating cells. In yeast, a significant part of the steady state control of tubulin levels occurs via protein degradation (Katz *et al.*, 1990). Yeast strains carrying an TUB1 α-tubulin and a TUB2 ß-tubulin gene copy number of approximately 2.5 fold and 2.0 fold, respectively, above the haploid wild type show a 2 fold increase and a 1.5 fold increase in TUB1 and TUB2 message, respectively. Yet, the corresponding tubulin protein levels were 150% for TUB1, 115% for TUB2, and 88% for the TUB3 (α-tubulin) of the wild type protein levels (Katz *et al.*, 1990) indicating that protein degradation does play a part in regulating tubulin levels. Furthermore, the addition of a single copy of the TUB2 ß-tubulin gene to yeast led to the doubling of the chromosomal TUB1 and TUB3 α-tubulin genes in this strain by the acquisition of an additional copy of chromosome 13 (Katz *et al.*, 1990). Overproduction of ß-tubulin was achieved by using an inducible expression vector and elevation of ß-tubulin levels by 1.4 fold was

enough to lead to the loss of microtubules in half the cells. Further increases in ß-tubulin levels lead to a dramatic loss of cell viability (Weinstein and Solomon, 1990). These researchers suggest that the regulation of tubulin levels via protein degradation or tubulin mRNA destabilization occurs as a response to the toxicity of excess ß-tubulin. Modulation of tubulin mRNAs is the mechanism regulating tubulin levels during sea urchin embryo development. In the stages prior to ciliogenesis a high concentration of unpolymerized tubulin is observed resulting in unstable tubulin mRNAs. During the later stages of embryogenesis, the increase in tubulin transcript concentrations is largely the result of the stabilization of tubulin mRNA resulting from the declining pool of unpolymerized tubulin (Gong and Brandhorst, 1987).

Regulation of tubulin synthesis during cilia and flagella formation

C. reinhardii requires protein synthesis for flagellar regeneration (Rosenbaum *et al.*, 1969), whereas the sea urchin (*Lytechinus pictus*) embryos do not require additional tubulin upon deciliation because they have a large pool of ciliary protein (Weeks and Collis, 1976). Nevertheless, a rapid transient increase in tubulin gene transcription quickly follows a single round of deciliation of sea urchin embryos (Gong and Brandhorst, 1987). The enhanced transcription of tubulin is a direct response to deciliation and is independent of tubulin dimer concentrations. In *C. reinhardii*, the stability of tubulin mRNAs increases transiently after deflagellation and this increased stability is maintained until the completion of flagellar regeneration (Baker *et al.*, 1984). Protein synthesis is required for the rapid degradation of tubulin mRNAs in *C. reinhardii* (Baker *et al.*, 1986). In contrast, the tubulin mRNA accumulation following *L. pictus* deciliation can be accounted for solely by transcription and no modulation of mRNA stability is observed (Gong and Brandhorst, 1987). The importance of the elevated transcription of tubulin genes during ciliogenesis is believed to be due to the coordinate expression of tubulin mRNAs with some newly synthesized proteins required for cilia formation (Gong and Brandhorst, 1987)

Sequences regulating tubulin gene expression

Studies of tubulin gene expression have opened the door to more detailed analyses of tubulin gene promoter regions. A germ line transformation system in *Drosophila* was used to study the promoter region of the testis-specific ß2-tubulin gene (Michiels *et al* , 1989). The promoter of the *Drosophila hydei* ß2-tubulin gene can direct the testis specific expression of the *Escherichia coli* lac Z gene in *D. melanogaster*. Using a combination of deletion analysis and *in vitro* mutagenesis these researchers show that a 14 base pair motif at position -38 to -51 is the only element controlling the testis specificity of the ß2-tubulin gene. This sequence is conserved between *D. hydei* and *D. melanogaster* and acts as a position dependent promoter element A second element between -26 and -32 has a quantitative effect on the testis-specific expression. In contrast to the simplicity of the ß2-tubulin gene, the ß3-tubulin promoter region has several regulatory elements and the first intron (4.5 kb) contains hormone responsive regulatory elements (Bruhat *et al.*, 1990). The steroid hormone 20-hydroxyecdysone (20-OH-E) is of crucial importance to insect development and regulates the expression of the ß3-tubulin gene in *Drosophila* Kc cells and during development in the flies (Sobrier *et al.*, 1989, Montpied *et al.*, 1988). Constitutive expression of the bacterial chloramphenicol acetyl transferase gene (CAT) in Kc cells was obtained when the 5' flanking region of the ß3-tubulin gene was fused to CAT coding sequences (Bruhat *et al.*, 1990). This constitutive expression was repressed when the first intron of the ß3-tubulin gene was included in the chimaeric gene. Similar to yeast silencer sequences, the negative regulatory elements of the first intron can act in either orientation whether upstream or downstream of the gene. However, in the presence of 20-OH-E the expression of the construct containing the first intron is enhanced 2 fold over the constitutive level of expression. Both positive and negative regulatory elements were localized to the extreme 0.5kb of the first intron 3' side (Bruhat *et al* , 1990) In a previous study, Gasch *et al.* (1989) demonstrated that in *Drosophila* the 5' flanking sequence and the first intron contain some sequences which direct mesoderm specific expression of the ß3-tubulin gene A sequence

similar to the ß2-tubulin testis-specific motif is also found in the ß3-tubulin promoter region. Although the function of the testis-specific element is not known for ß3-tubulin, it is important to note that the ß3-tubulin is present in the two somatic cells which enclose each gonial cell during the entire course of its differentiation to ultimately form 64 spermatozoa (Kimble *et al.*, 1989) and the ß2-tubulin is found within the differentiating spermatozoa. Therefore, this shared promoter element may direct the co-expression of the ß2 and ß3-tubulin genes within distinct cells of the testis. The identification of trans-acting factors which interact with this promoter element may confirm this hypothesis.

SOME GENERAL FEATURES OF ß-TUBULIN POLYPEPTIDES

Given the degree of conservation observed between tubulin polypeptide sequences within and between organisms it is not surprising that with the biochemical techniques available 20 years ago microtubule researchers believed that tubulin was one molecule (Raff, 1984). Tubulin genes are also highly conserved, in fact, cloned chicken ß-tubulin recognizes ß-tubulin sequences from sea urchin and humans at high stringency and from yeast and trypanosomes at reduced stringency in Southern blot analyses (Cleveland *et al.*, 1980). A comparison of ß-tubulin sequences from the two extremes of the eukaryotic evolutionary scale shows that the yeast and chicken (cß2) ß-tubulins are 70% identical. Within the chicken ß-tubulin gene family itself, substitutions have been found in 17% of the amino acid positions, but this level of divergence is primarily between the haematopoietic cß6 tubulin and the other chicken ß-tubulins (Murphy *et al.*, 1987). Similarly, the haematopoietic mouse mß1 gene shares only 78% amino acid identity with the other four fully characterized mouse ß-tubulins which are 92-95% identical to each other (Wang *et al.*, 1986). Substitutions are dispersed throughout the polypeptide backbone but a pronounced sequence divergence is seen in the carboxy-terminal moiety of ß-tubulins (Monteiro and Cleveland, 1988) and between amino acids 30 to 100 in vertebrate ß-tubulins (Sullivan and Cleveland, 1986). ß-tubulins containing highly conserved carboxy-terminal regions have been

identified from rat, chicken, mouse and humans. With the exception of the haematopoietic ß-tubulins the vertebrate ß-tubulin genes have been classified into six distinct ß-tubulin isotypes with unique carboxy-terminal consensus sequences (see Table 1.1)(Sullivan and Cleveland, 1986; Monteiro and Cleveland, 1988). Rudolph *et al*. (1987) reported that the ß2-tubulin isoform of *D. melanogaster* is present in a number of *Drosophila* species, both closely and distantly related. However, not enough sequence data is available to determine whether ß-tubulin gene isotypes have been conserved in similar taxonomic groups, like the insects. Interestingly, in vertebrates this conservation is also reflected in their patterns of expression (Table 1.1).

THE RELATIONSHIP BETWEEN TUBULIN STRUCTURE AND MICROTUBULE FUNCTION

In the last decade, microtubule research has focused on the relationship between tubulin structure and function. Two hypotheses have been proposed to account for the maintenance of these gene families in the presence of the selective pressures of evolution. The first proposed by Fulton and Simpson (1976) established a direct relationship between the differences in primary structure of tubulins and the function of each protein. The implications of such a model are that specific tubulin polypeptides would be required for particular microtubule structures. The second hypothesis, proposed by Raff (1984) postulates that tubulin gene families exist to allow differential regulation of tubulin gene expression, thus providing control over, the time, place or amount of tubulin synthesized. Evidence supporting both models has been gathered from a variety of systems.

The localization of specific tubulin polypeptides to different microtubule arrays in the sea urchin (Stephens, 1978) fuelled belief in the proposal of Fulton and Simpson that tubulin proteins were restricted by their primary sequence to a subset of microtubule structures. In 1984, Swan and Solomon demonstrated that calf brain tubulin could be used to reconstitute the chicken erythrocyte marginal band *in vitro*. In erythrocytes, all microtubules are located at the periphery of the cell in a single plane. These results suggested that, at least for erythrocytes the primary sequence of erythrocyte tubulin was not

essential for the localization and the shape of the marginal band. Subsequently, several laboratories have made use of an experimental approach developed by Bond *et al.* (1986) in which gene transfection was used as a means of assessing the role of tubulin regions in microtubule structure and function. Bond *et al.* found that introducing a chimeric ß-tubulin which contained the first 344 amino acids of the chicken ß2-tubulin and the last 113 amino acids from the *S. cerevisiae* ß-tubulin into NIH-3T3 fibroblast cells did not alter cell viability or microtubule function. The yeast ß-tubulin is 12 amino acids longer than the ß-tubulins found in mouse fibroblast cells (Neff *et al.*, 1983; Lopata and Cleveland, 1987). The chimeric ß-tubulin representing at least 10% of the total tubulin was incorporated efficiently in the fibroblast spindle (Bond *et al.*, 1986) indicating that divergent carboxy-terminal regions can be interchanged without affecting microtubule function. Using a similar approach Joshi *et al.* (1987) introduced the chicken ß-tubulin, cß6, which is normally expressed only in erythrocytes and thrombocytes into COS cells. Double-label immunofluorescence experiments with an anti-cß6 antibody and an anti-ß-tubulin monoclonal antibody demonstrated that cß6 was found in most cytoplasmic and mitotic microtubules. Immunofluorescence studies using antibodies specific for the different ß-tubulin isotypes also support the conclusions from gene transfection studies which indicate that microtubule structures are copolymers of available ß-tubulin polypeptides in cultured cells and mouse testis (Lewis *et al.*, 1987; Lopata and Cleveland, 1987; Sawada and Cabral, 1989). In Chinese hamster ovary cells, attempts to isolate more stable classes of microtubules by pretreating cells with the microtubule depolymerizing drug colcemid or by lysing cells in the presence of calcium failed to detect any preferential retention of ß-tubulin isotypes in the residual microtubule fraction (Sawada and Cabral, 1989). No isotype specific functions have been detected to date in undifferentiated cultured cells. Similar questions were addressed in organisms amenable to genetic approaches. The study of *D. melanogaster* mutants has shown that the major ß-tubulin of developing spermatocytes is present as a component of meiotic, cytoplasmic and axonemal (flagellar) microtubules (Kemphues *et*

al., 1982). In *A. nidulans*, the benA gene codes for ß1 and ß2-tubulin polypeptides which are expressed in the vegetative phase of the life cycle and tubC encodes the ß3-tubulin which is found only upon formation of conidia, the asexual spores (Sheir-Neiss *et al.*, 1978; Weatherbee and Morris, 1984). However, analysis of mutants lacking the ß3-tubulin showed that the benA gene products could support conidiation (Weatherbee *et al.*, 1985). These studies show that ß-tubulins intermingle to function in several microtubule arrays and support the hypothesis that distinct ß-tubulin genes exist to allow differential regulation of expression.

However, recent studies of *D. melanogaster*, *C. elegans* and PC12 cell ß-tubulins provide examples of ß-tubulins which are essential for distinct microtubule arrays. The ß2 and ß3-tubulins of *D. melanogaster* are considerably diverged from one another (Rudolph *et al.*, 1987). Both of these genes are expressed in testis, but the ß3-tubulin is not expressed in the male germline, it is found in the two somatic cells surrounding the differentiating spermatids (Kimble *et al.*, 1989). The introduction of a chimeric gene consisting of the 5′ untranslated region and the first 19 codons of ß2-tubulin joined to ß3-tubulin starting at codon 20 and ending in the 3′ untranslated region into a ß2 null background via P-element transformation produces male sterility. Microscopy was used to demonstrate that the ß3-tubulin can only support cytoplasmic microtubule function. In fact, when ß3-tubulin is present as more than 20% of the testis ß-tubulin pool it disrupts normal axonemal assembly (Hoyle and Raff, 1990). In *C. elegans*, mutations which render these animals touch insensitive are found in the mec-7 ß-tubulin gene. This ß-tubulin gene product is an essential structural component for the formation of 15 protofilament microtubules in the *C. elegans* touch receptor neurons (Savage *et al.*, 1989). Immunofluorescence microscopy and immunogold electron microscopy of differentiating PC12 cells reveals the preferential use of certain ß-tubulin isotypes in specialized microtubules (Asai and Remolona, 1989; Joshi and Cleveland, 1989). PC12 cells grow mitotically as round chromaffin like cells but differentiate into neuron-like cells in response to nerve growth factor (Greene and Tischler, 1976). Different α-tubulin isotypes also occur in

distinct regions within the same neuron (Hajos and Gallatz, 1985).

There is growing evidence that specific ß-tubulin polypeptides have unique assembly properties and can influence microtubule stability. The chicken haematopoietic cß6 tubulin is more hydrophobic and less acidic by 0.3 pH units than cß2 the major neuronal ß-tubulin (Murphy *et al.*, 1987). When compared to brain tubulin, erythrocyte tubulin which is 95% cß6 and 5% cß3 (Joshi *et al.*, 1987) exhibits different assembly properties *in vitro* (Rothwell *et al.*, 1986). In a recent study, Baker *et al.* (1990) showed that when samples of pre-assembled brain and erythrocyte tubulin are mixed, the incorporation of brain and erythrocyte tubulin occurs at different rates. The rate of polymerization of erythrocyte tubulin into microtubules is influenced by the slow dissociation of erythrocyte oligomers and the slow exchange of this tubulin with brain tubulin subunits. Immunofluorescence staining of the marginal band with an anti-cß3 antibody shows that staining varies in mature erythrocytes. Since this is not observed in immature cells and the number of microtubules in erythrocytes is known to decline with age (Barrett and Dawson, 1974) Joshi *et al.* (1987) propose that the differences in cß3 levels may be related to the age of the cell.

Isolation of polymerized and unpolymerized tubulin from a mouse T lymphoma and two IL-2 dependent mouse T-cell clones shows that a higher amount of T-cell clone tubulin is found in the polymerized state. The T-cell lymphoma tubulin is composed of two major isoforms mß1 (60% of total tubulin) and mß2 (40% of total tubulin) whereas the two mouse T-cell clones express only mß1 (this nomenclature differs from Table 1.1). The mß2 isoform which is found in higher proportions in unpolymerized tubulin in the T lymphoma cells and the unique properties of mß2 are confirmed by its reduced recovery following several cycles of polymerization/depolymerization of the lymphoma tubulin with brain tubulin (Harada *et al.*, 1986). The authors suggest that the presence of higher levels of unpolymerized tubulin in the lymphoma cells may confer a growth advantage to these cells. This hypothesis was derived from the observed induction of DNA synthesis and enhanced stimulation by fibroblast growth factors (FGF) and epidermal growth factors (EGF) following treatment of quiescent cells with the microtubule

depolymerizing drug colchicine (Crossin and Carney, 1981). During neurite outgrowth of PC12 cells there is preferential accumulation of type II and III ß-tubulin polypeptides and the type I and II ß-tubulin polypeptides are incorporated into microtubules in preference to the type III polypeptides. ßIII tubulin is the only isotype which becomes phosphorylated at a serine residue near the carboxy terminus upon differentiation of neuroblastoma cells (Gard and Kirschner, 1985; Ludueña *et al.*, 1988). The ßIII isotype of tubulin constitutes 25% of bovine cerebral ß-tubulin (Banerjee *et al.*, 1988). Using an antibody specific to the ßIII isotype Banerjee *et al.* (1990) have depleted ßIII containing dimers from bovine brain tubulin by immunoaffinity chromatography. The rates and extent of assembly of the ßIII depleted tubulin was enhanced as compared to the starting material, ßI-depleted and ßII-depleted tubulin. Similar results were obtained when assembly was performed in the presence of the microtubule associated proteins MAP2 and tau (Banerjee *et al.*, 1990). Therefore, isotypic composition of tubulin influences the stability and assembly properties of microtubules.

POSTTRANSLATIONAL MODIFICATIONS OF TUBULIN

Tubulin heterogeneity is in part defined by posttranslational modifications of α- and ß-tubulin nascent polypeptides. In fact, it was the detection of posttranslationally modified α-tubulin in flagella which suggested that tubulin within a particular cell is not composed of a single α- and ß-tubulin polypeptide. Three types of post-translational modifications of tubulin occur, tyrosination/ detyrosination of α-tubulin, acetylation of α- and ß-tubulin and phosphorylation of α- and ß-tubulin (reviewed by MacRae and Langdon, 1989; Joseph *et al.*, 1982; Eddé *et al.*, 1989). The Σ amino group of α-tubulin Lys 40 undergoes acetylation (LeDizet and Piperno, 1987), phosphorylation of ßIII-tubulin occurs in the carboxy-terminus at Ser residues 444 or 446 (Ludueña *et al.*, 1988) and the carboxy-terminal tyrosine of α-tubulin is involved in the tyrosination/detyrosination reactions (Arce *et al.*, 1978). In a recent study, Eddé *et al.* (1989) reported the incorporation of radiolabelled acetate into α- and ß-

tubulin isoforms of mouse neural and glial cells, but the site of modification on ß-tubulin was not identified. Detyrosination occurs primarily on tubulin dimers (Raybin and Flavin, 1977), whereas tyrosination favors polymeric tubulin as a substrate (Kumar and Flavin, 1981). It is interesting to note that the enzyme involved in tyrosination of α-tubulin anchors itself to ß-tubulin (Wehland and Weber, 1987b). The substrate specificities of enzymes involved in acetylation and phosphorylation of tubulin have not been determined but studies of developmental processes suggest that acetylation/deacetylation of α-tubulin and phosphorylation of ß-tubulin occur on tubulin dimers (L'Hernault and Rosenbaum, 1983; L'Hernault and Rosenbaum, 1985; Gard and Kirschner, 1985). A casein kinase II activity in brain may be involved in the phosphorylation of ß-tubulin *in vivo* (Serrano *et al.*, 1987). The impact of any of these post-translational modifications on microtubule function *in vivo* is unknown. However, results of several studies indicate that a link exists between tubulin modification, microtubule assembly dynamics and morphogenesis (Schulze *et al.*, 1987).

Detyrosinated microtubules are enriched in stable microtubule arrays such as cilia, basal bodies, centrioles, flagella, sperm axonemes and neurites (Kreis, 1987; Gundersen and Bulinski, 1986). Acetylated tubulin occurs in mitotic spindles, primary cilia, cytoplasmic microtubules, centrioles, and midbodies of HeLa and 3T3 cells in culture (Piperno *et al.*, 1987). Both acetylated and detyrosinated microtubules occur in the same stable microtubules but their rates and pattern of appearance differ (Schulze *et al.*, 1987; Bulinski *et al.*, 1988). In some cell lines a small subpopulation of cytoplasmic microtubules rich in detyrosinated α-tubulin (Webster *et al.*, 1987a; Kreis, 1987) and in acetylated α-tubulin (Webster and Borisy, 1989) have been shown to be extremely stable. Although this implies that detyrosination and acetylation are involved in stabilizing microtubules, studies measuring the turnover rates of detyrosinated and acetylated α-tubulin *in vivo* indicate that this is not so, rather these modifications are a consequence of microtubule stabilization (Webster *et al.*, 1987b; Webster *et al.*, 1990; Schulze *et al.*, 1987). A coincident increase in

detyrosinated α-tubulin has been noted in N115 neuroblastoma cells undergoing differentiation (Wehland and Weber, 1987a), in NIH-3T3 cells in contact with wounded cells (Gundersen and Bulinski, 1988), and in CHO cells induced to undergo differentiation to a fibroblast-like morphology (Wehland and Weber, 1987a). Extensive tyrosinolation is detected upon chemotactic stimulation of macrophage (Nath *et al.*, 1981) or O_2 stress (Nath and Gallin, 1984). Furthermore, in *T. brucei* a cell cycle modulation of detyrosination occurs with detection of a discrete gradient of tyrosinated tubulin in newly synthesized microtubules (Sherwin and Gull, 1989). These modifications occur in tubulin as a result of external stimuli which direct differentiation or morphological alterations and regulate the cell cycle.

In a mouse neuroblastoma cell line a fourfold increase in ß-tubulin phosphorylation is observed upon differentiation (Gard and Kirschner, 1985; Eddé *et al.*, 1981). A single ß-tubulin isoform similar to the one identified in differentiated neuroblastoma cells is phosphorylated in mouse primary neurons (Eddé *et al.*, 1989). The phosphorylated brain ß-tubulin has unique assembly properties, but the significance of phosphorylation to assembly dynamics *in vivo* is not known (Banerjee *et al.*, 1990). Phosphorylation of purified tubulin *in vitro* with calcium and calmodulin dependent protein kinase allows this tubulin to interact with phospholipid vesicles (Hargreaves *et al.*, 1986) probably through exposure of previously hidden hydrophobic groups. Finally, the phosphorylation of uterine smooth muscle tubulin switches from the ß to the α polypeptide under the influence of estrogen, the significance of this switch remains unclear (Joseph *et al.*, 1982). It is currently believed that posttranslational modifications are particularly important during specific developmental stages, or in response to specific extracellular signals which would cause rearrangements of the microtubular arrays.

STRUCTURAL AND FUNCTIONAL DOMAINS OF TUBULIN

A variety of biochemical and genetic approaches have been utilized to assign structural or assembly functions to different tubulin domains. These studies have provided information on the sequences of α and ß-

tubulin involved in the maintenance of assembly competent dimer structures, on the interaction of tubulin with microtubule associated proteins (MAPs), GTP, divalent cations and modifying enzymes and on the lateral interactions occurring between protofilaments within individual microtubules. A number of factors affect microtubule polymerization *in vitro*, these include the tubulin dimer concentration, nucleation sites, MAPs, pH, GTP/GDP concentrations, ionic strength and calcium ion concentrations.

GTP and microtubule dynamics

The tubulin dimer contains one GTP exchangeable site located on ß-tubulin and one non-exchangeable site which is believed to be located on α-tubulin (Sternlicht *et al.*, 1987). The GTP which is not hydrolysed (non-exchangeable for GDP) is not involved in microtubule assembly (Kobayashi, 1975). GTP binding is essential for microtubule assembly and GTP hydrolysis accompanying polymerization plays a crucial role in microtubule dynamics (Kirschner and Mitchison, 1986 ; Carlier, 1989). Mitchison and Kirschner (1984 a,b) demonstrated that rapid shrinking and growing of polymers *in vitro* and *in vivo* can occur at tubulin dimer concentrations below the critical tubulin concentrations which lead to steady growth. This phenomenon is called dynamic instability. The relationship between the molecular mechanism of GTP hydrolysis and the dynamic transitions of growing and shrinking polymers is controversial (Bayley, 1990). GTP binding to tubulin induces a small conformational change upon binding which appears to be necessary for tubulin polymerization (Kirshner and Mandelkow, 1985). Furthermore, P_i release upon GTP hydrolysis produces a slow conformational change in tubulin and some researchers believe that the release of P_i is coupled to the destabilization of microtubule polymers (Carlier, 1989). In direct conflict with Carlier's proposal, recent studies have shown that GTP hydrolysis is kinetically coupled to the addition of tubulin subunits to the ends of microtubules (Stewart *et al.*, 1990; Caplow and Shanks, 1990).

The GTP binding site of ß-tubulin is located in the amino-terminal region between amino acids 1 and 281 (Kirshner and Mandelkow, 1985; Nath

and Himes, 1986). This area of the molecule is believed to form a nucleotide binding pocket. Linse and Mandelkow (1988) show that the purine moiety of GTP binds between amino acids 63 and 77 by sequencing isolated tubulin proteolytic fragments UV cross-linked to radiolabelled GTP. The binding site for the purine moiety was confirmed by Kim *et al.* (1987) using a different cross-linking reagent. Furthermore, GTP incorporation and microtubule assembly is blocked by an antibody raised against a synthetic peptide encoding ß155-174 (Hesse *et al.*, 1987). Two models of tubulin sequences involved in GTP binding have been proposed which suggest that GTP interacts with four distinct regions of α and ß-tubulin (Sternlicht *et al.*, 1987; Mandelkow *et al.*, 1985). The Sternlicht model incorporates the interaction of one magnesium ion which is bound either as a nucleotide-metal ion complex or directly to tubulin residues (Huang *et al.*, 1985; Correia *et al.*, 1987). These models are limited to comparisons of other nucleotide binding proteins because tubulin crystals suitable for high resolution X-ray crystallography have not been isolated.

Regions of tubulin involved in dimerization and polymerization

There is a tight association between α- and ß-tubulin within the tubulin dimer. Proteolytic cleavage with trypsin which cleaves α-tubulin after a basic residue at position 339 or with chymotrypsin which cleaves ß-tubulin at Tyr 281 does not separate the α from the ß chain (Mandelkow *et al.*, 1985). In fact, these nicked dimers can polymerize in the presence of GTP, albeit to form abnormal microtubules (Mandelkow *et al.*, 1985). Protein domains responsible for the dimerization and polymerization of tubulin have been determined using chemical cross-linking and limited proteolysis (Kirshner and Mandelkow, 1985). In a microtubule polymer there are inter-dimer associations within one protofilament and intra-dimer associations between α and ß-tubulin polypeptides composing the dimer. Kirchner and Mandelkow's experiments indicate that the intra dimer bond is formed between the N-terminal domain of α-tubulin and the C-terminal domain of ß-tubulin, whereas the C-terminal domain of α-tubulin interacts with the N-terminal domain of ß-tubulin from another dimer within the longitudinal axis of the

protofilament. Furthermore, assembly of tubulin into microtubules protects the tryptic and chymotryptic sites of α and ß-tubulin, while leaving the carboxy-terminal region exposed to proteolytic degradation with subtilisin (Sackett and Wolff, 1986; Arévalo *et al.*, 1990). Two recent studies performed in Andreu's laboratory (de la Viña *et al.*, 1988; Arévalo *et al.*, 1990) utilize antibodies to synthetic peptides and limited proteolysis to map tubulin structure and regions important for polymerization. The analysis of 38 proteolytic fragments by alignment on the basis of their reactivities to six different antibodies revealed that proteolytic recognition sites are clustered in three regions within the dimer corresponding to amino acid positions 115 to 165, 260 to 300 and the C-terminus of both chains. Since proteolytic enzymes preferentially cleave exposed protein segments the zones cleaved should represent loops or hinges connecting or hanging from compact rigid segments. X-ray diffraction analysis of hydrated crystals suggests a tubulin structure of three domains connected by thinner zones (Beese *et al.*, 1987). Using antibodies against α and ß-tubulin regions which are exposed in the dimer Arévalo *et al.* (1990) identified two zones of contact between protofilaments within microtubule arrays. Antibodies to α(214-226) and ß(241-256) were unable to bind to the antigen in microtubules, sheets or rings induced *in vitro* or in isolated cytoskeletons of PTK2 cells. This suggests that these regions are involved in the longitudinal association of protofilaments. However, assembly-linked conformational changes which mask the epitope cannot be ruled out.

Chimeras of the chicken cß2 ß-tubulin containing variable substitutions of the corresponding yeast sequence at the 3' end were expressed in NIH-3T3 cells to determine which domains of ß-tubulin are essential for assembly. Replacement of the chicken ß-tubulin sequences distal to amino acid 344 with yeast ß-tubulin sequences had little or no effect on assembly (Fridovich-Keil *et al.*, 1987; Bond *et al.*, 1986). However, a moderate and severe decrease in the amount of ß-tubulin assembled was noted when the yeast substitutions extended to amino acid 282 and 201, respectively. In addition, the half life of the proteins was significantly lower than endogenous ß-tubulin and the chimeric ß-

tubulin with yeast sequences extending from amino acid 344. Assembly defective ß-tubulin mutations have also been identified in CHO cells (Boggs and Cabral, 1987; Boggs *et al.*, 1988). Disruptions in microtubule assembly in CHO cells are caused by deletions between amino acids 250 and 350 and dimers containing the mutant ß-tubulin appear to be degraded quickly (Boggs *et al.*, 1988).

A mutation of *D. melanogaster* testis ß2-tubulin which substitutes a lysine residue at position 288 for the highly conserved glutamic acid residue defines a single amino acid required for normal microtubule assembly (Rudolph *et al.*, 1987). This substitution leads to the formation of aberrant microtubule structures similar to those seen when chymotrypsin cleaved tubulin is polymerized *in vitro* (Mandelkow *et al.*, 1985) and causes male sterility in males homozygous for the mutant allele. Interestingly, this amino acid substitution falls within one of the tubulin dimer hinge regions described by de la Viña *et al.* (1988). The ß2t6 mutation of *D. melanogaster* ß2-tubulin affects a specific subset of microtubules (Fuller *et al.*, 1988). However, the nature of the amino acid substitution causing a defect in the outer doublet of *D. melanogaster* flagellar microtubules in ß2t6 mutants has not been reported (Fuller *et al.*, 1988).

The importance of the carboxy-terminal region of tubulin for microtubule assembly

Tubulin lacking the carboxy-terminus of α and ß-tubulin following subtilisin cleavage which removes approximately 40 amino acids from the carboxy-terminal can polymerize into microtubules *in vitro* but the structures formed have increased lateral interactions between polymers (Serrano *et al.*, 1984b; Sackett *et al.*, 1985). Removal of the C-termini of tubulin leads to a conformational change in the tubulin dimer (Mukhopadhyay *et al.*, 1990). Peptides corresponding to α-tubulin (430-441) and ß-tubulin (422-434) amino acid residues within the carboxy-terminal region bind to MAP2 and tau (Maccioni *et al.*, 1988). These and other microtubule associated proteins are believed to modulate microtubule dynamics and play a role in specifying microtubule structure (Kirschner and Mitchison, 1986). Rabbit antisera raised against these

peptides contain antibodies to tubulin and anti-idiotypic antibodies which react to MAP-1, MAP-2 and tau, confirming that these regions are involved in MAPs binding (Rivas *et al.*, 1988). However, the microtubule translocators kinesin and dynein bind and promote the movement of microtubules which have been cleaved with subtilisin indicating that the binding sites of the translocators do not overlap with MAPs and are not found in the carboxy-terminal 4 kDa region of α- or ß-tubulin (Rodionov *et al.*, 1990). The observed increase in lateral interactions in subtilisin cleaved tubulin is in part regulated by the properties of the last six to eight amino acids of α and ß-tubulin (Vera *et al.*, 1989). Furthermore, calcium ions do not affect polymerization nor do they depolymerize microtubules containing dimers from which the last six to eight amino acids have been removed. These results suggest that these carboxy-terminal amino acids which are not directly involved in MAPs binding are essential for the conformation of native tubulin and its sensitivity to calcium inhibition (Vera *et al.*, 1989). The possibility of isotype specific interaction with calcium was not determined in this experiment. Interestingly, the removal of the 12 extreme carboxy-terminal amino acids from yeast ß-tubulin (which is 12 amino acids longer than mammalian ß-tubulin) produces microtubules which function as well as the wild type tubulin in mitosis, meiosis and mating (Katz and Solomon, 1988). However, removal of 27 amino acids from the carboxy-terminus of ß-tubulin produced a yeast haploid strain capable of growing normally in optimal conditions (30°C), but not in suboptimal conditions (37°C) (Matsuzaki *et al.*, 1988).

THE MICROTUBULE DEPOLYMERIZING DRUGS AND THEIR TARGET

The benzimidazole drugs are a class of broad spectrum anthelmintic which were introduced in 1961 with the development of thiabendazole (Lacey, 1988). The benzimidazoles (BZs) bind to tubulin and shift the equilibrium between the tubulin subunit and microtubules resulting in the net depolymerization of microtubules (Hoebeke *et al.*, 1976; reviewed by Lacey, 1988). The BZs depolymerized intestinal cell microtubules of the parasitic intestinal nematode ***Ascaris suum*** (Borgers and De Nollin,

1975), thereby blocking the transport of secretory granules and the movement of the subcellular organelles in these cells (Borgers *et al.*, 1975). In a recent study, Eilers *et al.* (1989) showed that treatment of polarized human intestinal adenocarcinoma cells (Caco-2) with the BZ, nocodazole, blocks the transport of newly synthesized proteins to the apical cell surface, while delivery to the basolateral surface is not affected. Other effects of the BZ drugs on nematodes include alterations in the carbohydrate energy metabolism such as decreases in glucose uptake and decreases in fumarate reductase activity (Sangster and Prichard, 1985) and secretory functions (Rapson *et al* , 1981). Nevertheless, it is believed that microtubules are the primary target of BZs because colchicine and podophyllotoxin, well known microtubule depolymerizing drugs, are also inhibitors of fumarate reductase and glucose uptake and because these effects are dependent on microtubule function (Lacey, 1988). Interestingly, all microtubule depolymerizing drugs have an effect on GTP hydrolysis, they either enhance (nocodazole and colchicine) or inhibit (podophyllotoxin and vinblastine) this reaction (Lin and Hamel, 1981; Lacey, 1988). These effects may be significant considering the proposed role of GTP hydrolysis in the assembly dynamics of microtubules.

The colchicine binding site

It was the binding of the antimitotic drug, colchicine, to tubulin which permitted the identification of tubulin as the major component of microtubules (Raff, 1984). Colchicine, podophyllotoxin and BZs are believed to share the same binding site on the tubulin dimer because they competitively inhibit each others binding to nematode tubulin (Russell and Lacey, 1989) and mammalian brain tubulin (Ireland *et al.*, 1979; Hoebeke *et al.*, 1976; Laclette *et al.*, 1980). Furthermore, the binding of the BZ drugs is similar to that of colchicine because it forms a tight, pseudo-irreversible interaction which is not a covalent bond (Lacey, 1988). The observed competitive binding of these drugs to tubulin may be misleading because conformational changes in tubulin (Garland *et al.*, 1978) and in colchicine (Detrich *et al* , 1981) result from their interaction with one another. Results of drug binding

assays, electron paramagnetic resonance studies and other techniques indicate that the colchicine site is not proximal to the GTP exchangeable site, Mg^{++}, Ca^{++}, MAPs or vinblastine binding sites (Lacey, 1988). The colchicine binding site of tubulin has been localized to α-tubulin by photoaffinity labelling (Schmitt and Atlas, 1976) and by binding of radiolabelled colchicine to the 16 kDa carboxy-terminal trypsin proteolytic fragment (Serrano *et al.*, 1984a). The biochemical studies of Avila *et al.* (1987) using subtilisin digested brain tubulin show that colchicine interaction with S-tubulin and native tubulin is different. The affinity of colchicine for S-tubulin is lowered but the number of binding sites remains the same compared to native tubulin dimers. Mukhopadhyay *et al.* (1990) demonstrated through partial subtilisin cleavage of brain tubulin that the α-tubulin C-terminus (15 residues) regulates the colchicine-tubulin interaction. These studies conflict with the studies of colchicine and BZ resistant mutants which implicate ß-tubulin in drug binding. In support of the hypothesized interaction of these drugs with ß-tubulin is the observation that the cross-linking reaction of the ß-tubulin cysteine residues 239 and 354 is inhibited by colchicine and its A ring analogues (Roach *et al.*, 1985). To reconcile these two findings Sheir-Neiss *et al.* (1978) proposed that the binding site for these drugs is located in a pocket between α and ß-tubulin. Lacey (1988) has proposed a model for the colchicine site which includes three or four cysteinyl residues of α- and ß-tubulin in close proximity. The information gathered to date on the colchicine/BZ binding has not permitted the identification of the BZ binding site on tubulin.

The efficacy of BZ drugs for the treatment of filarial nematode infections

Two species of the *Brugia* genus cause lymphatic disease in humans, *B. malayi* and *B. timori* (Mak, 1987). The infective larvae of these parasites are transmitted by mosquitoes. The larvae migrate into the lymphatic vessels of the host where they mature into adult worms. It is the presence of these worms in the lymphatic vessels which causes inflammation and blockage of lymphatic circulation. The microfilariae

find their way from the lymphatic circulation into the bloodstream where they are transmitted to the vector when it takes a bloodmeal. Diethylcarbamazine citrate (DEC) is the drug currently used for the treatment of lymphatic disease. However, chemotherapy with DEC can be accompanied by severe side effects in ***Brugia*** infected patients (Subrahmanyam, 1987). ***B. pahangi*** is found in wild and domestic animals of South East Asia and natural human infections have been reported in Indonesia (Mak, 1987).

In general, the amount of BZ bound to tubulin is a good reflection of the *in vivo* toxicity of these drugs for gastrointestinal parasites and host species (Lacey, 1988; Lubega and Prichard, 1990). However, this rule does not apply to filarial nematodes, where the effectiveness of the drug is low at doses which should be active based on the tubulin/BZ binding data. The binding component of a tubulin containing extract consists of high and low affinity binding sites and the proportions of these binding sites within an extract depend on its source, therefore comparisons of BZ binding between different species or strains should be done using high affinity binding data. Although high affinity mebendazole (MBZ) binding to ***Brugia malayi*** and *B. pahangi* tubulin is 2.5 fold less than it is to the intestinal parasite ***Nippostrongylus brasiliensis*** (Tang, 1988), subcutaneous injections of MBZ and flubendazole (FBZ) in jirds (***Meriones unguiculatus***) and cats significantly reduces ***B. pahangi*** adult worm viability (Denham *et al.*, 1978; Denham ***et al.***, 1979). In a recent study, Franz *et al.* (1990) showed that intestinal microtubules of ***B. malayi*** disappeared six hours after treatment by subcutaneous injection of FBZ in infected jirds. Furthermore, marked alterations of the oogonia and embryonic cells of ***B. malayi*** females were noted 24 hours after treatment. These findings suggest that the inefficacy of BZs for treatment of the human ***Brugia*** infections via the oral route is due either to the amount of time an effective drug concentration is at the target site or the inability of the host to expel the parasite once it has been temporarily immobilized. The latter hypothesis is supported by the observations of Rahman *et al.* (1977) who found that intestinal nematodes can be expelled from the host and be recovered viable up to 24 hours post-treatment. Paralysis is one

of the effects MBZ has on nematodes (Woods *et al.* 1989). A recent study demonstrates that diethylcarbamazine (DEC) the drug currently used to treat *Brugia* infections causes abnormal polymerization of microtubules into ribbon-like structures in a rhesus monkey kidney cell line. The concentration of DEC causing a 50% decrease in kidney microtubule assembly is 2-500 fold higher than that of various BZs to brain tubulin (Fujimaki *et al.*, 1990). However, these values may not be directly comparable and this assay has not been performed on nematode microtubules.

Resistance to microtubule depolymerizing drugs

The mutations conferring BZ resistance to strains of fungi and helminths provide an important perspective for the understanding of the molecular interaction of BZ with tubulin. In BZ resistant strains of gastrointestinal nematodes the binding of BZs to parasite tubulin is greatly reduced in resistant compared to susceptible strains (Sangster *et al.*, 1985; Lacey and Prichard, 1986). In BZ resistant *H. contortus*, this is due to a decrease in the number of high affinity binding sites for the drug on tubulin (Lubega and Prichard, 1990). Furthermore, a molecular analysis of the basis for BZ resistance in *H. contortus* indicates a selection for a pre-existing worm population which contains a BZ-resistant ß-tubulin complement (Roos *et al.*, 1990). A decrease in the binding of BZs is also observed in BZ resistant fungi (Davidse and Flach, 1977). Interestingly, one ß-tubulin mutant (benA16) resistant to thiabendazole is supersensitive to carbendazim, the active component of the prodrug benomyl (a BZ) and this is reflected in their binding profiles. This and the observed conformational changes which occur upon BZ binding cautions us against assuming that all microtubule depolymerizing drug binding interactions with tubulin are identical. A list of amino acid substitutions responsible for BZ resistance are shown in Table 1.2. With the exception of a few uncharacterized α-tubulin mutants in CHO cells (Cabral *et al.*, 1986) and in *Physarum* (benC mutant)(Schedl *et al.*, 1984) and one or two uncharacterized loci which do not represent ß-tubulin in *A. nidulans* (Sheir-Ness *et al.*, 1978) and *S. pombe* (Yamamoto, 1980) the majority of BZ resistant mutations map to

TABLE 1.2. MUTATIONS CONFERRING BENZIMIDAZOLE RESISTANCE.

Organism	Amino Acid Position	Mutation	References
S. cerevisiae	318	Arg to Trp	Huffaker *et al.* 1988
"	241	Arg to His	Thomas *et al.* 1985
N. crassa	167	Phe to Tyr	Orbach *et al.* 1987
A. nidulans	6	–	Jung *et al.* 1988
"	165	–	Jung and Oakley 1990
"	198	–	Jung *et al.* 1988
"	50	–	
		Heat sensitive	"
"	134	–	
		Heat sensitive	"
"	257	–	
		Heat sensitive	"

ß-tubulin. It has been suggested by Oakley (1985) that amino acid substitutions in ß-tubulin which cause a secondary phenotype (cold sensitivity or heat sensitivity) cause a conformational change and allosteric alterations of the BZ binding site. They suggest that the region containing the three amino acid changes which do not confer conditional lethality in BZ resistant ***A. nidulans*** strains (Table 1.2) may interact to form the BZ binding site (Jung ***et al.***, 1988). Furthermore, they state that amino acid 165 may be specifically involved in the binding of the R_2 group of BZ drugs (Jung ***et al.***, 1988; Jung and Oakley, 1990). This is the chemical group which differentiates the benzimidazole carbamates from thiabendazole and cambendazole. Regardless of these speculations and those derived from biochemical studies the exact BZ binding site on the tubulin dimer remains elusive (Lacey, 1988).

BZ resistance does not only result from a decrease in the affinity of the drug for tubulin. The ben A33 mutant of ***A. nidulans*** is resistant to BZs and is blocked in mitosis at higher temperatures. At these temperatures it requires BZs for growth because it contains hyperstable spindle microtubules (Oakley and Morris, 1981). In this case it is the hyperstability of microtubules rather than decreased affinity of BZs for tubulin which causes the resistant phenotype. It is interesting to note that the isolation of suppressor mutants of the benA33 phenotype revealed a third member of the tubulin superfamily, *r*-tubulin (Oakley and Oakley, 1989). cDNAs coding for *r*-tubulin have been isolated from ***D. melanogaster*** and ***Homo sapiens*** indicating that it is an evolutionarily conserved protein (Oakley ***et al.***, 1990).

Dependence on a microtubule depolymerizing drug (colcemid) for growth has also been observed in CHO cells transformed with a CHO cell colcemid resistant ß-tubulin gene and has been associated with an increased ratio of mutant to wild type ß-tubulin (Whitfield ***et al.***, 1986). Colcemid resistant CHO cells require the assembly of the mutant ß-tubulin for the resistance phenotype to provide increased stability against the microtubule depolymerizing drug (Boggs and Cabral, 1987). This is because all three ß-tubulin isotypes within CHO cells are similar in their responses to cold, colcemid, and calcium-induced

depolymerization (Sawada and Cabral, 1989). Each of the ß-tubulin products of the two *A. nidulans* ß-tubulin genes are also benomyl-sensitive (Weatherbee *et al.*, 1985). However, this does not appear to be true of *C. elegans* ß-tubulins since the deletion of a single ß-tubulin allele confers BZ resistance indicating that remaining ß-tubulins are not susceptible to the effects of the drugs (Driscoll *et al.*, 1989). These BZ-resistant worms are lacking a single ß-tubulin isoform (bt-2) and a concomitant increase in the level of another ß tubulin (bt-3) isoform is observed (Woods *et al.*, 1989) Resistance to colchicine can be produced by microinjection of a threshold level of *Physarum* tubulin into PTK2 cells. Microtubules of *Physarum* are naturally insensitive to the action of colchicine (Prescott *et al* , 1989), but are sensitive to the action of the BZ drugs.

CHO mutants which exhibit cross-resistance to several microtubule inhibitors including the microtubule depolymerizing drugs, nocodazole, colchicine, podophyllotoxin and the microtubule stabilizing drug, taxol, has permitted the identification of proteins other than tubulin essential for microtubule function (Jindal *et al.* (1989), Ahmad *et al* , 1990). These proteins are the mammalian homolog of the highly conserved chaperonin family which play an essential role in the post-translational folding and assembly of proteins and protein complexes and in the transport of protein (hsp60) (Jindal *et al.*, 1989) and the constitutive hsp70 heat shock protein (Ahmad *et al.*, 1990) which in yeast is involved in the maintenance of protein in an unfolded conformation for transport into organelles and perhaps also for assembly into oligomeric protein complexes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988).

Hypersensitivity to BZ drugs is conferred in *A. nidulans* and *S pombe* by mutations in α-tubulin (Gambino *et al* , 1984; Umesono *et al* , 1983). Truncation of the 27 amino acids from the carboxy-terminal region of the ß-tubulin (TUB2) gene and integration of this truncated gene into the TUB2 locus of *S. cerevisiae* also produces a strain which is hypersensitive to the antimitotic drugs thiabendazole or methyl-2-benzimidazole carbamate (Matsuzaki *et al.*, 1988).

EVOLUTIONARY RELATIONSHIPS AND GENE REGULATION IN HELMINTHS

Although the helminth group diverged after the plant/animal divergence it is an evolutionarily ancient group (Tait, 1990). In fact phylogenetic studies of helminth species reveal that the evolutionary distance between the filarial nematode *B. pahangi* and the free living nematode *C. elegans* (0.172 K_{nuc}) is approximately the same as the evolutionary distance between *B. pahangi* and the mouse (0.191 K_{nuc}). Despite this evolutionary distance, the genome sizes of *B. pahangi* (1×10^{8}) and *C. elegans* (8×10^{7}) are nearly identical (Rajan, 1990; Emmons, 1988). However, the GC content of *C. elegans* is of 36% whereas it is 27.6% for *B. pahangi* (Rothstein *et al.*, 1988). In addition, histone, myosin and actin probes from *C. elegans* do not recognize these sequences from filarial nematodes on Southern blots probably because of a codon bias that is skewed towards the use of A or T in the third position of the codons of *B. pahangi* (Rothstein *et al.*, 1988). *C. elegans* introns are infrequent and in general are quite small (Emmons, 1988) but, from the single genomic sequence of a *Brugia* gene, it seems that the *Brugia* genome has numerous introns with a great deal of variation in size (Perrine *et al.*, 1988).

The trans-splicing reaction

Nevertheless, *Brugia* does acquire a conserved 22 nucleotide splice leader sequence which is involved in trans-splicing reactions in approximately 15% of *C. elegans* mRNAs (Takacs *et al.*, 1988; Bektesh *et al.*, 1988). Trans-splicing, the joining of two distinct RNAs to form a single mature message, occurs in nematodes (Nilsen, 1989, Bektesh and Hirsh, 1988), the trematode *Schistosoma mansoni* (Rajkovic *et al.*, 1990), the Trypanosomatidae (Agabian, 1990), tobacco chloroplasts (Koller *et al.*, 1987), animal viruses with RNA genomes (Makino *et al.*, 1986) and *C. reinhardtii* chloroplasts (Goldschmidt-Clermont *et al.*, 1990). A computer search of the EMBL nucleotide gene bank using known trans-splicing RNA structures predicts that trans-splicing occurs in a broad spectrum of organisms including vertebrates (Dandekar and Sibbald, 1990). With the exception of the tobacco chloroplast S12 ribosomal gene and the *C. reinhardtii* psaA gene (Goldschmidt-Clermont *et al.*, 1990),

trans-splicing results in the attachment of a 5' noncoding leader region to an mRNA precursor. In the case of the S12 trans-splicing reaction, the two copies of the first exon of the gene are located 90 kb distant on the opposite strand and 126 kb distant on the same strand from exon 2 and 3 of this gene (Koller *et al.*, 1987).

There are two *C. elegans* splice leader sequences (Huang and Hirsh, 1989), one of which (SL1) is conserved in *Onchocerca volvulus* (Zeng *et al.*, 1990), *B. malayi* (Takacs *et al.*, 1988), *Ascaris lumbricoides* (Hannon *et al.*, 1990), *Haemonchus contortus*, *Panagrellus redivivus*, and *Anasakis* species (Bektesh *et al.*, 1998). The SL1 and SL2 sequences share 17 out of 22 identical nucleotides (Huang and Hirsh, 1989). The SL1 genes are associated with the 5S rRNA locus in *C. elegans* (Krause and Hirsh, 1987) but the SL2 genes are dispersed within the *C elegans* genome (Huang and Hirsh, 1989). In *B. malayi* and *O. volvulus* the SL1 genes are linked to the repetitive 5S rRNA genes but there are other genomic locations some of which are transcribed in *O volvulus* (Takacs *et al.*, 1988; Zeng *et al.*, 1990). The majority of trans-spliced mRNAs are members of gene families (Bektesh *et al.*, 1988; Hannon *et al.*, 1990). The gene families of *C. elegans* coding for actin (Krause and Hirsh, 1987), glyceraldehyde-3-phosphate dehydrogenase (Huang and Hirsh, 1989), collagen (Park and Kramer, 1990), ubiquitin (Graham *et al.*, 1988), the heat shock protein hsp70a (Bektesh *et al.*, 1988), two ribosomal proteins (Bektesh *et al.*, 1988), the regulatory myosin light chain proteins (Cummins and Anderson, 1988) and the catalytic subunit of cAMP dependent protein kinase (Gross *et al.*, 1990) acquire the SL1 sequence. One of four glyceraldehyde-3-phosphate dehydrogenase genes acquires the SL2 sequence (Huang and Hirsh, 1989)

The mechanism of trans-splicing has been extensively studied in trypanosomes and *C. elegans* and they share some similarities Consensus sequences for 5' and 3' splice sites are found downstream of the splice acceptor and upstream of the acceptor mRNA coding region Y branched molecules consisting of the SL intron joined to the precursor RNA have been identified in trypanosomes and nematodes (Murphy *et al*, 1986; Sutton and Boothroyd, 1986; Laird *et al.*, 1987; Bektesh and Hirsh, 1988). The Y branched molecules are similar to the cis-spliced lariat

and consist of the SL RNA covalently linked to the target mRNA via a 2'-5' phosphodiester bond. The SL RNAs contain putative binding sites for the Sm antigen, which is the antigen associated with mammalian small nuclear ribonucleoprotein particles (snRNPs) (Bruzik *et al.*, 1988; Thomas *et al.*, 1988; Van Doren and Hirsh, 1988) involved in the cis splicing reaction. Bruzik *et al.* (1988) proposed that the SL RNA might autonomously activate its own 5' splice site and thereby eliminate the need for a U1 snRNP like activity. In a recent report, Tschudi and Ullu (1990) show that selective cleavage of U2, U4, and U6 snRNAs using RNase H and complementary deoxynucleotides in permeabilized trypanosome cells abolishes the appearance of trans-spliced products and intermediates. The α-tubulin transcripts produced in this system are unstable suggesting that the addition of the SL sequence to pre-mRNAs stabilizes them against degradation. In *Trypanosoma equiperdum*, the SL RNA appears to interact directly with the pre-mRNA and selects the 3' splice acceptor sites immediately 3' of the pre-mRNA/SL RNA complementary sequence (Layden and Eisen, 1988).

Despite the similarity of the trans splicing reaction of nematodes and trypanosomes some differences have been noted (reviewed by Donelson and Zeng, 1990). The most striking difference is that all trypanosomal RNAs acquire the trypanosome splice leader (Walder *et al.*, 1986), whereas only a subset of nematode RNAs undergo this processing event. Furthermore, the splice leader mRNAs of *C. elegans* and trypanosomes have different cap structures (Donelson and Zeng, 1990). The splice leader sequence and its length differ between nematodes and trypanosomes, but in trypanosomes the 39 nucleotide splice leader sequence varies in different species and genera (Donelson and Zeng, 1990). Although an A residue functions as a branch acceptor for both trypanosomes and *C. elegans*, the branch point regions differ (Patzelt *et al.*, 1989; Hannon *et al.*, 1990). In trypanosome α and ß-tubulin pre mRNAs, the branch point maps to three A residues for α-tubulin and two A residues for ß-tubulin, which are located 42 to 58 nucleotides upstream of the 3' splice site (Patzelt *et al.*, 1989). The α and ß-tubulin genes of *T. brucei*, unlike many other *T. brucei* genes has a single site for SL addition (Sather and Agabian, 1985), but there are 15 copies of these

genes in the genome and the separate branch points detected may represent differences between the tubulin gene copies. In the *T. equiperdum* variable surface glycoprotein genes, VSG-1 and VSG-28, which have multiple 3' splice acceptor sites, those sites most frequently used contain the highest pyrimidine content in the adjacent upstream sequences (Layden and Eisen, 1988). It is possible that the pyrimidine tracts, which are also seen in the α and ß-tubulin genes of *T. brucei*, contributes to the efficiency of processing, as it does in lariat formation in vertebrate cis-splicing (Reed and Maniatis, 1985). Introns of a range of non-vertebrate organisms including *C. elegans* do not contain a polypyrimidine tract (Csank *et al.*, 1990). In an *A. suum* cell free extract, either of two A residues located 18 or 19 nucleotides upstream of the 3' splice site serve equally as the branch site (Hannon *et al.*, 1990). The nucleotides adjacent to the A residue located 18 nucleotides upstream of the 3' splice site conform in 5 out of 7 positions to the weakly conserved vertebrate branch point consensus sequence (PyXPyUPuAPy) (Reed and Maniatis, 1985). However, the sequence surrounding the branch point A at -19 contains only one meaningful match. Therefore, the importance of the consensus branch point sequence in this *A. suum* RNA is uncertain. In mammalian cis introns, the first AG located 3' of the branch point/polypyrimidine tract serves as the splice acceptor. Insertion of AG dinucleotides between the branch point and the 3' splice site causes the exclusive use of the inserted AG as the 3' splice site (Smith *et al.*, 1989).

In trypanosomes the addition of the mini-exon sequence is a mechanism for processing multicistronic pre-mRNAs (Imboden *et al*, 1987; Soares *et al.*, 1989; Munich and Boothroyd, 1988; Tschudi and Ullu, 1988). It has also been suggested that the splice leader in trypanosomes serves a role as a cap donor structure which stabilizes the mRNAs (Lenardo *et al.*, 1985; Tschudi and Ullu, 1990) However, the role of trans-splicing in nematodes is still unknown.

CHAPTER 2. CHARACTERIZATION OF THE ß1-TUBULIN GENE AND THE ß-TUBULIN GENE PRODUCTS OF *BRUGIA PAHANGI*.

INTRODUCTION

Microtubules are essential for many cell processes (Dustin, 1984). Comparisons of α- and ß-tubulin sequences from a wide variety of organisms have shown that these major microtubule components are well conserved in all eukaryotic cells (Little and Seehaus, 1988). In general, α- and ß-tubulin gene families are composed of several single copy genes dispersed throughout the genome. These together with post-translational modifications are proposed to account for the large number of tubulin isoforms observed in vertebrates (Cleveland and Sullivan, 1985).

Tubulin is of particular importance in parasitic nematodes since it is the target of benzimidazole carbamates, a class of microtubule depolymerizing drugs (Lacey, 1988). These anthelmintics are used effectively for the treatment of both medical and veterinary intestinal nematode infections. Although *Brugia* infections are not effectively controlled by benzimidazole treatment (WHO, 1985), the benzimidazole carbamates are potent filaricides for *B. pahangi* (Denham *et al.*, 1978). High affinity benzimidazole binding sites are found in tubulin-enriched extracts of *B. pahangi* and *B. malayi* adult nematodes (Tang, 1988). The ß-tubulin chains are of special interest because benzimidazole resistant strains of *Physarum polycephalum* and *Caenorhabditis elegans* have altered ß-tubulins. In a parbendazole resistant strain of *P. polycephalum* an extra ß-tubulin isoform has been identified (Foster *et al.*, 1987), whereas deletion mutations which confer benomyl resistance in *C. elegans* map to the ben-1 ß-tubulin locus (Driscoll *et al.*, 1989).

A monoclonal antibody recognizing a conserved ß-tubulin epitope has previously been used to characterize ß-tubulin isoforms of *B. pahangi* adult worms by Western blot analyses of two-dimensional gels. Four to five ß-tubulin isoforms with isoelectric points ranging between pH 5.2 and 5.3 have been identified in tubulin-enriched extracts of *B. pahangi* adult worms (Tang and Prichard, 1989). In addition, immunogold

electron microscopy of *Brugia* adult worms and microfilariae using a monoclonal antibody has localized ß-tubulin to the myofibril structures of microfilariae, to muscle blocks, and the intestinal brush border of adult worms as well as the embryonic intrauterine microfilariae (Helm *et al.*, 1989).

The characterization of the ß-tubulin gene(s) and gene products of *B. pahangi* is necessary for an understanding of the interaction of benzimidazole drugs with nematode microtubules. In this chapter, I report the structure and sequence of a ß-tubulin gene of *B. pahangi* and show the isoform pattern obtained in microfilariae and adult worms using a nematode specific antipeptide antibody raised against the extreme carboxy-terminal region of this gene product.

EXPERIMENTAL PROTOCOL

Isolation of *B. pahangi* nematodes

Jirds (*Meriones unguiculatus*) infected with *B. pahangi* were obtained from J. W. McCall (Department of Parasitology, University of Georgia, USA). Adult nematodes and microfilariae were collected from the body cavities of infected jirds by dissection post-mortem. Microfilariae were separated from contaminating jird macrophages as described by Kaushal *et al.* (1982). Once isolated parasite material was either stored in liquid nitrogen or used immediately for DNA, RNA or protein extractions.

Nucleic acid isolation and hybridization

Genomic DNA was isolated from *B. pahangi* adult nematodes by the proteinase K method of Emmons *et al.* (1979). For Southern hybridization analyses, approximately 10 µg of each *B. pahangi* DNA sample cleaved with the appropriate restriction endonuclease was fractionated through a 1% agarose gel by electrophoresis and transferred to Hybond-N (Amersham) nylon membranes by capillary blotting (Southern, 1975). Hybridizations were performed with 50% or 35% formamide and 5x SSPE (1x SSPE is 0 15 M NaCl/ 0.2 M $NaH_2PO_4.H_2O$/ 1 mM EDTA) at 42°C and filters were washed with 0.5x SSC (1x SSC is 0.15 M NaCl/ 0.015 M sodium citrate) containing 0.1%

SDS at 65°C or 2x SSC, 0.1% SDS at 50°C depending on the stringency desired. Filters to be re-probed were washed in 0.4 M NaOH for 30 mins. at 45°C and transferred to 0.1x SSC, 0.1% SDS and 0.2 M Tris-Hcl pH 7.5 for 30 mins. at 45°C.

Total RNA was extracted from adult *B. pahangi* as described by Chomczynski and Sacchi (1987) using RNAzol (Cinna/Biotecx International Laboratories). For Northern hybridization analyses, 20 μg of total RNA was denatured in 1.0 M glyoxal and fractionated on a 1% agarose gel (10 mM sodium phosphate buffer pH 7.0) as described previously by Thomas (1980) and transferred to Hybond-N nylon membranes. Hybridizations were carried out as described above and the filters washed with 0.5x SSC, 0.1% SDS at 50°C.

DNA restriction fragments used as probes were isolated from the plasmid vector by fractionation through a 1% agarose gel and purified by silica gel elution using a Geneclean kit (Bio 101 Inc.) following the protocol provided by the manufacturer. Isolated DNA restriction fragments were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; ICN) using a nick-translation kit (Amersham) or an oligonucleotide labelling kit (Pharmacia) by following the manufacturer's instructions. Probes were labelled to a specific activity of about $1x10^8$ cpm/μg. Filters of Northern and Southern hybridization analyses were exposed to Kodak XAR-5 x-ray film using intensifying screens for autoradiography at -70°C.

Construction and screening of size-selected *B. pahangi* genomic DNA libraries

B. pahangi DNA (20 μg) digested with *Eco*RI or double digested with *Xba*I/*Xho*I was size-fractionated through a 1% agarose gel. Size-selected genomic DNA libraries were constructed from pooled, Geneclean purified, *Eco*RI DNA fragments ranging from 3.0 to 4.6 kb or *Xba*I/*Xho*I fragments ranging from 4.4 to 6.3 kb in size. The *Eco*RI DNA fragments were ligated with T4 DNA ligase (Pharmacia) into the alkaline phosphatase (Boehringer Mannheim) treated *Eco*RI site of the bacterial vector, pUC18. This DNA was used to transform *Escherichia coli* strain JM83 resulting in $5x10^3$ transformants. DNA fragments of the *Xba*I/*Xho*I double digest were

ligated into the *Xba*I/*Xho*I cleaved pIBI30 vector (IBI) and used to transform *E. coli* strain DH1 resulting in $3x10^3$ transformants. The libraries were screened by colony hybridization (Hanahan and Meselson, 1980) using an [α-^{32}P]dCTP labelled probe. The *Eco*RI library was screened with a *B. pahangi* ß-tubulin cDNA fragment of 125 bp from clone Bpa7 corresponding to the last 40 amino acid residues of ß-tubulin (Helm *et al.*, 1989). The *Xba*I/*Xho*I size-selected genomic library was screened with a [α-^{32}P]-labelled 0.6 kb *Xho*I/*Eco*RI genomic DNA fragment recovered from the *Eco*RI size-selected genomic DNA library. Colonies identified as positives were purified with two successive rounds of hybridization and the plasmid DNA isolated from these clones using standard techniques were mapped with restriction enzymes as described by Maniatis *et al.* (1982).

Subcloning and DNA Sequencing

The 1.8 kb *Xba*I/*Pst*I, 1.0 kb *Pst*I/*Eco*RI, 1.3 kb *Pst*I/*Sac*I and 1.8 kb *Eco*RI DNA fragments were subcloned from pBTX (see Fig. 2.3A) into pIBI30 and/or pIBI31 depending on the orientation desired. A series of deletions of these subclones were created with Exonuclease III (Boehringer Mannheim) digestion as described by Henikoff (1987).[1] Additional subclones were constructed to obtain DNA sequences overlapping the parent subclones and to obtain the sequence of the 0.4 kb *Eco*RI DNA fragment and the coding region of the 0.6 kb *Eco*RI/*Xho*I fragments of pBTX (Fig. 2.3A). The nucleotide sequence was determined from single-stranded templates prepared with the helper phage M13K07 as described by the manufacturer (IBI) using the dideoxy chain termination method of Sanger *et al.* (1977). Sequencing reactions were performed with either the Klenow enzyme kit (Pharmacia) or a Sequenase kit (United States Biochemicals Corp.) with [α-^{35}S]dATP (600 Ci/mmol; Amersham) or [α-^{32}P]dATP (3000 Ci/mmol; Amersham) using the modifications described by Klein *et al.* (1989). Two oligonucleotide primers were used to

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[1] An illustration of the products of an Exonuclease III deletion experiment is found in Appendix A.

sequence the original clones across the junctions between the *Xba*I/*Pst*I and the *Pst*I/*Eco*RI subclones, 5' ATTTTTAACTTCAGTAATCCCCTACAT 3' (Upjohn Co.) and between the 0.4 kb *Eco*RI and the 0.6 kb *Eco*RI/*Xho*I subclones, 5' GCGTTCTATGGAGGTTAT 3'. The sequencing reactions were electrophoresed through 6% acrylamide sequencing gels. The gels with sequencing reactions containing [α-^{35}S]dATP were fixed in a solution of 10% acetic acid and 10% methanol for 20 minutes before exposure. All gels were dried and exposed to Kodak XAR-5 x-ray film. Analysis of nucleotide sequences was performed using a DNA program package available from the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984) and a VAX computer..

Production of antipeptide antibodies specific to *Brugia* ß-tubulin

The peptide shown in Fig. 2.8A was synthesized using an Applied Biosystems peptide synthesizer, HPLC purified, sequenced, and coupled to the carrier protein, keyhole limpet hemocyanin (KLH), by the Alberta Peptide Institute. Cross-linked peptide/KLH with an average conjugation ratio of 3:1 was partially solubilized in phosphate-buffered saline, pH 7.2. Approximately 100 μg of peptide/KLH conjugate was emulsified with Freund's complete adjuvant and injected intramuscularly in New Zealand white rabbits. The injections were repeated twice at 21 day intervals using Freund's incomplete adjuvant. Rabbits were ear bled and tested for antibody production by Western blot analysis against total *Brugia* adult protein. The blood was collected by cardiac puncture, allowed to clot at room temperature and centrifuged. The serum recovered was stored at -20°C. Rabbit IgG was separated from other immunoglobulins by Protein A Sepharose chromatography (Pharmacia Fine Chemicals).

Preparation of *B. pahangi* soluble protein

Parasite material was washed with Hank's buffered salt solution (Gibco/BRL) or with normal saline (0.7% NaCl) and homogenized in 0.025M 2(N-morpholino)-ethane sulfonic acid (MES) buffer containing 1mM EGTA, 0.5 mM $MgSO_4$ and the following protease inhibitors, 80 μg/ml pepstatin, 10 μg/ml TLCK and 1mM PMSF. The homogenates were centrifuged at 27,000

x g for 15 mins and total protein was estimated using the Bradford dye binding assay (BioRad) as described by the manufacturer.

Protein Electrophoresis and Western blot analyses

Analysis of protein samples using polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) on 10% acrylamide gels. Two dimensional gel electrophoresis was performed as described by O'Farrell (1975) using ampholines pH 5.0–5.5 (4%) and pH 3.5–10.0 (1%) and a 10% acrylamide slab gel for the second dimension. Proteins were transferred to nitrocellulose (Trans Blot, Bio-Rad Laboratories) in the presence of 20% (v/v) methanol, 25 mM Tris, pH 8.2, 190 mM glycine at 30 V for 12–16 h (Towbin *et al.*, 1979). Filters were incubated in 10 mM Tris, pH 7.5, 300 mM glycine, 150 mM NaCl, 10% calf serum for 2 h at 37°C, then incubated for 2 h at room temperature either with antipeptide IgG diluted 1:50 or the anti-chicken ß-tubulin monoclonal antibody 357 (Amersham) diluted 1:5000. All antibody dilutions were prepared with 0.05 M Tris-HCl pH 7.5, 0.2 M NaCl containing 10% calf serum. The filters were then incubated with the second antibody, biotinylated goat anti-rabbit IgG (1:500, Bethesda Research Laboratories) and subsequently with horseradish-peroxidase-streptavidin conjugate (1:400, Amersham) for 1 h at room temperature. Antibody-protein complexes were detected with 4-chloro-1-naphtol (Bethesda Research Laboratories) as described by the manufacturer.

RESULTS

Cloning and mapping of the *B. pahangi* ß-tubulin gene

Initially, a 3.8 kb *Eco*RI ß-tubulin DNA fragment was identified by Southern hybridization analysis as the only band hybridizing to the homologous 125 bp ß-tubulin cDNA fragment isolated from clone Bpa7 (Fig. 2.1A). Therefore, a size-selected genomic library enriched for a 3.8 kb *Eco*RI ß-tubulin DNA fragment was constructed. Using the 125 bp cDNA fragment as a probe for colony hybridization (Fig. 2.1B), clone pBT41 was isolated from the *Eco*RI genomic library. Mapping of the ß-tubulin homologous sequences indicated that the 3' end of the ß-tubulin gene was

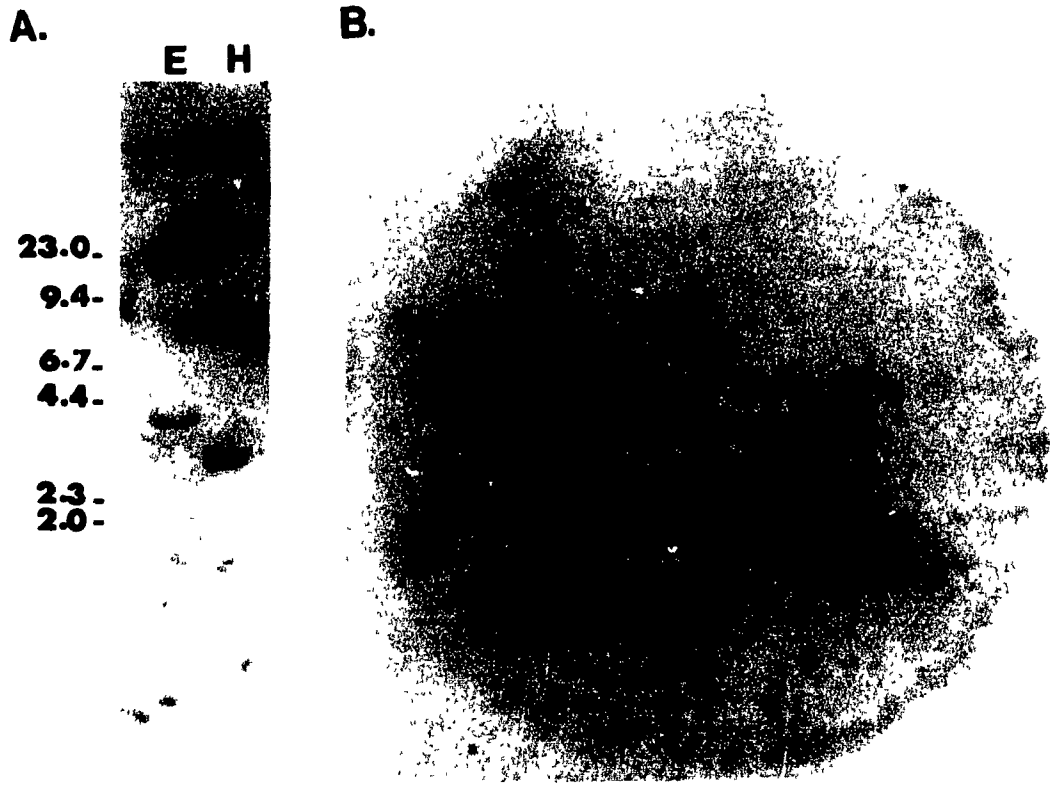

Fig. 2.1 Identification of a *B. pahangi* ß-tubulin sequence and isolation of clone pBT41. In A., *B. pahangi* geromic DNA was digested with *Eco*RI (E) and *Hind*III (H). The restriction fragments were electrophoresed through a 1% agarose gel and transferred to Hybond-N. Hybridization to an oligolabelled *B. pahangi* 125 bp ß-tubulin cDNA fragment isolated from clone Bpa7 was performed in the presence of 50% formamide at 42°C. The filter was washed in 0.5x SSC, 0.1% SDS at 65°C. B. Autoradiogram of a filter following colony hybridization to the probe described in A. Two positive colonies are visible as dark spots.

located within an *Eco*RI/*Xho*I 0.6 kb fragment. The partial nucleotide sequence of clone pBT41 revealed an open reading frame coding for 40 amino acid residues which were 70% identical to the carboxy terminus of chicken cß2 ß-tubulin (Valenzuela *et al.*, 1981). The 5' end of the gene was not present within this clone. A second genomic library was constructed using DNA fragments generated by double digestion with *Xho*I and *Xba*I. DNA fragments of 5.6 kb containing ß-tubulin sequences were identified by Southern hybridization analysis of the double digested genomic DNA using the 0.6kb *Eco*RI/*Xho*I fragment of clone pBT41 as a probe (Fig. 2.2A). Several clones were isolated from the *Xba*I/*Xho*I library and one of these clones, pBTX, containing a 5.6 kb ß-tubulin sequence was selected for further characterization. Southern hybridization analysis revealed that fragments of clone pBTX in addition to the 0.6 kb *Eco*RI/*Xho*I DNA fragment initially isolated in clone pBT41 cross-hybridize to the chicken ß2 tubulin gene indicating that additional ß1-tubulin sequences had been isolated (Fig. 2.2B). Southern hybridization analyses and nucleotide sequencing showed that pBTX contained the entire ß-tubulin gene, which will be referred to as ß1-tubulin (Fig. 2.3A).

Nucleotide and predicted amino acid sequences of ß-tubulin clones

A combination of Exonuclease III-deleted subclones and subcloned restriction enzyme fragments of clone pBTX shown in Figure 2.3A were prepared for DNA sequence analysis. DNA sequence data were obtained from both strands (with the exception of three non-coding regions from -397 to -157, 873 to 1263 and 4033 to 4215) using the sequencing strategy illustrated in Figure 2.3B. The DNA corresponding to the protein coding region spans 3.8 kb (Fig. 2.4). The predicted ß-tubulin has 448 amino acids and is organized into nine exons ranging from 57 to 227 nucleotides in length. Exon/intron boundaries of the ß-tubulin gene were identified by open reading frame homology with sequenced ß-tubulins and the presence of conserved intron/exon junction sequences GT..AG (Mount, 1982). The introns contain multiple stop codons in all three reading frames, range in size from 82 bases to 1.1 kilobases and are distributed throughout the gene (Fig. 2.3A), at predicted amino acid

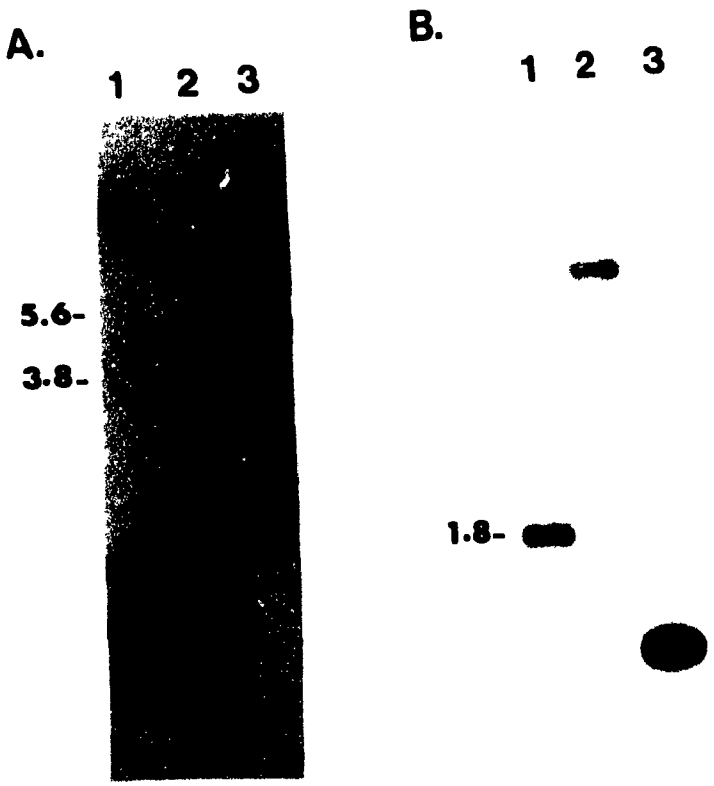

Fig. 2.2 Identification and isolation of *B. pahangi* ß-tubulin DNA fragments containing additional ß1-tubulin sequences. In A., *B. pahangi* genomic DNA was digested with *Xho*I (lane 1), *Pst*I/*Xho*I (lane 2) and *Xba*I/*Xho*I (lane 3) size-fractionated through a 1% agarose gel, transferred to Hybond-N and hybridized to the 600 bp *Eco*RI/*Xho*I DNA fragment of clone pBT41 which codes for the last 40 amino acids of ß1-tubulin. The hybridization was performed in the presence of 50% formamide at 42°C and the filter was washed in 0.2X SSC, 0.1% SDS at 65°C. B. pBTX DNA was digested with *Eco*RI (lane 1) and *Pst*I/*Kpn*I (lane 2). The probe DNA, a 1.2 kb *Pst*I fragment coding for the chicken ß2-tubulin (pT2, Valenzuela *et al.*, 1981) was fractionated (lane 3) along with the pBTX DNA fragments through a 1% agarose gel to serve as a positive control. Hybridization was performed in the presence of 35% formamide at 42°C and the filter was washed in 2X SSC, 0.1% SDS at 50°C.

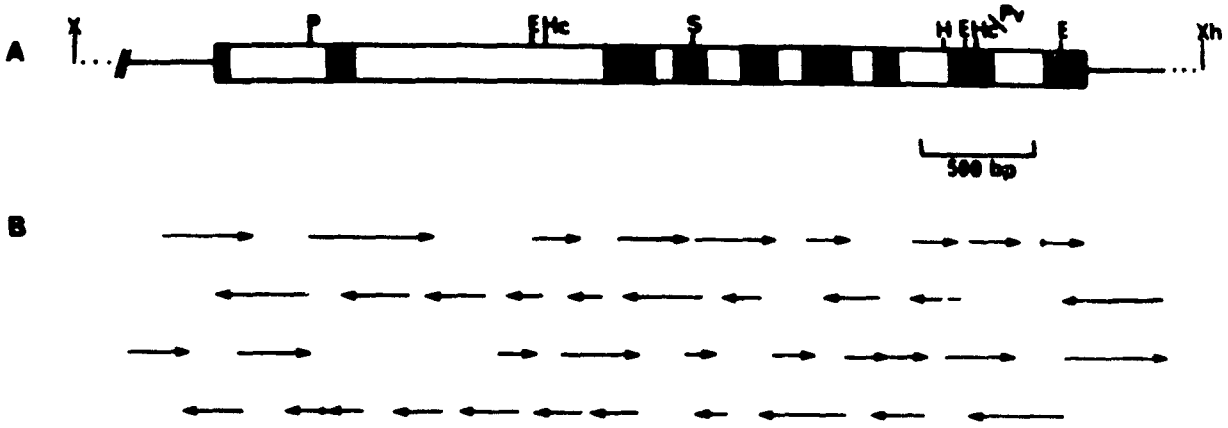

Fig. 2.3. Restriction map and sequencing strategy of the *B. pahangi* ß1-tubulin gene. A. Open boxes represent introns, dark boxes represent exons. B. Exonuclease III deletion clones of *Xba*I/*Pst*I, *Pst*I/*Eco*RI, *Pst*I/*Hinc*II and the 1.8 kb *Eco*RI DNA fragments and several additional subclones described in experimental procedures were sequenced The arrows represent sequenced DNA and their direction shows the sequence obtained relative to the vector sequencing primer. *Eco*RI (E), *Hind*III (H), *Hinc*II (Hc), *Pst*I (P), *Pvu*II (Pv), *Sac*I (S), *Xba*I (X), *Xho*I (Xh).

Fig. 2.4. Nucleotide sequence of the *B. pahangi* ß1-tubulin gene. The nucleotide sequence of the portion of clone pBTX representing the *B. pahangi* ß-tubulin open reading frames is presented in higher case letters. Presumptive intron and flanking sequences are presented in lower case letters. A potential polyadenylation site, a reverse CAAT and a TATA box are underlined. ▸

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ggtgtgcagatagcacgaggaaatgagaggagcagagaagtagggcgcagtcagacagttggatgattgcgcctcccgtgtcacttccct  -306
atatacctttgcaaggaggacatgacaaagttcgaggttatttctcgacgcgtctctctgtatactcacatgtctgtctgtctctgtt  -216
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CAG ATT GGT GCC AAG gtattgattttctgctctttctctttatcctttaggaagatgactcatcagagggtatctttccgcagaa  127

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TCA GAC CTG CAA ATT GAA CGA ATC AAC GTC TAC TAT AAT GAA GCG AAT G gttggtccattattggagttttt  628

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tttgtgttcag GG GGC AAA TAT GTG CCA CGA GCA GTC CTT GTT GAT TTG GAA CCA GGT ACC ATG GAT TCT  1778

ATT CGA GGA GGT GAG TTC GGG CAA CTA TTC CGA CCT GAC AAT TTT GTT TTT GGG CAA AGT GGA GCT GGC  1847

AAC AAC TGG GCT AAG GGA CAT TAT ACG GAA GGT GCG GAA CTA GTT GAT AAT GTG TTG GAC GTG ATA CGA  1916

AAA GAA GCT GAG GGA TGC GAT TGT CTT CAG gtacggattgccatagttttataagacattttaattgtggatgtgttttt  1996

attttaaagaaaaaattaaaaaatattttcag GGA TTT CAA CTA ACG CAT TCA CTT GGT GGT GGT ACC GGT TCC  2070

GGC ATG GGA ACA TTG CTG ATC TCG AAA ATT CGT GAG GAG TAT CCG GAT CGA ATT ATG AGC TCT TTT TCG  2139

GTT GTG CCA TCG CCC AAA gtatgtatttgaatttctgattcttatttttaacatctgttatatctattttaaacatagctccat  2223
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aacttttaagtggcggttgtctcacaaagtggtttttcggctatcatttcattttcatagttgaagaaaaagtatgctagacgtattaat 2313

gatttccag GTA TCA GAT GTT GTG TTG GAA CCC TAC AAT GCA ACA TTA TCA GTC CAC CAA CTA GTT GAA 2382

AAC ACT GAC GAA ACT TTC TGC ATT GAT AAC GAG GCT TTG TAT GAC ATC TGC TTC CGA ACG TTG AAG TTG 2451

GCA AAT CCA ACT TAC GGT GAC CTC AAC CAT TTG G gttcgtcctttaatttattcttcgaactgtccggtttaaatgca 2529
ttctatttaactatttataaaattcggatttaattgtaatttattcaactcttctaaaggttaaaatttctatttag TG TCT GTG 2614

ACA ATG TCG GGA GTA ACA ACT TGC TTA CGT TTC CCT GGA CAG TTG AAC GCC GAT CTC CGT AAA CTT GCC 2683

GTC AAT ATG GTG CCA TTC CCA CGG TTG CAT TTC TTT ATG CCA GGA TTT GCT CCT CTC TCT GCT CGT GAT 2752

GCT GCT GCT TAT CGA GCC CTC AAT GTT GCT GAA CTT ACT CAA CAG gtctctgtttatttttatggtcagggtctc 2827

ttttttgaaagtaatccatttataatcgctttaaatgtctcctgttttatgtttttaatatcttatttcttcag ATG TTT GAT 2912

GCC AAA AAT ATG ATG GCA GCA TGT GAT CCG CGT CAT GGT CGT TAC CTA ACC GTA GCT GCC ATG TTC CGA 2981

GGT AGA ATG TCT ATG CGG gtaagtgaattttgataatctccatgttaaactcagccgctgaagcatgaagattttaatccatgt 3065
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ttcttttgtggaagcttgtttttgatgtagggtttttttcttcatcgctgtttcaaagtttttaataagtaggtcttag GAA GTA GAC 3243

GAG CAA ATG ATG CAA GTA CAG AAT AAG AAT TCA TCG TAT TTC GTT GAA TGG ATT CCA AAT AAC GTA AAG 3312

ACA GCT GTT TGC GAC ATT CCA CCA CGT GGA TTA AAG ATG AGC GCA ACA TTT ATT GGA AAT ACA ACA GCT 3381

ATA CAA GAA CTT TTC AAG CGA ATT TCC GAA CAG TTT ACT G gtgaacttattaattccatctcatttgagcttagtt 3457

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tttatgcaaaaggtgaacatttttaatatgtttgttccgaatcgtctgaacttgcgttctatggaggttatacttaagtcgatccgattt 3637

tttaatgatttcag CC ATG TTC CGA CGT AAA GCA TTC TTG CAT TGG TAT ACT GGC GAA GGT ATG GAT GAA 3707

ATG GAA TTC ACG GAA GCG GAG AGT AAT ATG AAT GAC TTG GTG TCC GAA TAT CAA CAA TAT CAG GAT GCG 3776

ACG GCT GAT GAA GAA GGT GAT CTT CAG GAA GGT GAA TCG GAA TAC ATT GAA CAG GAA GAG TGA gcacaa 3845

agtgtcagctttgtgaaaaaggaacttttacagtattttatttatcgtcttttccatgtttttatgttgttacattttatttttgttactc 3935
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tatgtgggcagtggacggtattccgtgacagaagatgctacagaagattgcagcagttttt 4176

positions, 19, 56, 131, 174, 229, 293, 324 and 387. The average A+T content of introns is 70%. However, smaller introns have an A+T content of 73-78% which is similar to introns of *C. elegans* genes. The average A+T content of exons is 55% and codons having A or T in the third position are utilized 1.6 times more frequently than codons ending in C or G. A putative polyadenylation site, AATAAA is found 161 nucleotides downstream of the predicted stop codon (Fig. 2.4). In the 5' flanking region, sequences corresponding to a reverse CAAT and weak TATA box were found within the 400 nucleotides upstream of the translation start site at -327 and -301 (Fig. 2.4).

A comparison of the predicted amino acid sequence of the *B. pahangi* ß-tubulin to the *C. elegans* ben-1, tub-1, mec-7, chicken ß2, and *Haemonchus contortus* 12-16 and 8-9 ß-tubulin amino acid sequences is presented in Fig. 2.5. The percentage amino acid identity between the *B. pahangi* ß-tubulin and several other ß-tubulins are shown in Table 2.1. These results indicate that the *B. pahangi* ß1-tubulin is well conserved and shares a high degree of identity with the *C. elegans* ben-1 ß-tubulin. Five amino acid substitutions are unique to *B. pahangi* ß-tubulin: 80(Glu), 178 (Val), 277 (Asp), 279 (Ala), and 285 (Asn) and one of the amino acid substitutions, valine 178, is invariant in other ß-tubulins.

Identification of ß-tubulin sequences in the *B. pahangi* genome

We were initially interested to determine if there was more than one ß-tubulin gene within the genome which was homologous to clone pBTX. To address this issue, Southern hybridization analysis of *B. pahangi* genomic DNA was performed with restriction enzymes which cut once (*Hind*III, *Sac*I, *Pst*I, *Pvu*II) or twice (*Hinc*II) within the gene using a 5.7 kb *Xba*I/*Xho*I fragment of clone pBTX as a probe. This DNA fragment contains the entire ß-tubulin gene. Enzymes which cut once within the ß-tubulin gene would therefore be expected to yield only two bands on Southern hybridization analysis whereas enzymes cutting twice should yield three bands if there were no other closely matched sequences within the genome. As shown in Figure 2.6A, the enzymes cutting only

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                10        20        30        40        50        60
Bpß1    MREIVHVQAGQCGNQIGAKFWEVISDEHGVQPDGTYKGDSDLQIERINVYYNEANGGKYV
ben-1                                I         E    L
tub-1                    S           I     F  ET   L   D       N
mec-7        I           S           ID S Q V      L           GSN
12-16                                I    S   E    L
8-9                      S           I         E    L          H
cß2          I                       ID T S H      L          T N

                70        80        90       100       110       120
Bpß1    PRAVLVDLEPGTMDSIRGGEFGQLFRPDNFVFGQSGAGNNWAKGHYTEGAELVDNVLDVI
ben-1                   V S P                                       V
tub-1                   V S P
mec-7                   V S P          Y                            V
12-16                   V S P  A                                S    V
8-9                     F S PY         Y                            V
cß2        I            V S P    I                              S    V

               130       140       150       160       170       180
Bpß1    RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMSSFSVVPSPKVSDVVL
ben-1                                                           T V
tub-1                                                           T V
mec-7        ST                                    NT           T V
12-16                                    A                      T V
8-9                                                 A           T V
cß2        S S                                      NT    M      T V

               190       200       210       220       230       240
Bpß1    EPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLANPTYGDLNHLVSVTMSGVTTCL
ben-1                                            S
tub-1                     Y           Y          T            L
mec-7                    S                       TT           A
12-16                                            T
8-9                                              T
cß2                       Y                      TT           A

               250       260       270       280       290       300
Bpß1    RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSARDAAAYRALNVAELTQQMFDAKNMM
ben-1                                        KG Q      T
tub-1                                        KGTQ      T
mec-7                                      TS SNQQ    IT P      C
12-16                                        KG Q      T S          N
8-9                                          KG Q     ST
cß2                                        TS GSQQ     T P         S
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                310        320        330        340        350        360

Bpß1    AACDPRHGRYLTVAAMFRGRMSMREVDEQMMQVQNKNSSYFVEWIPNNVKTAVCDIPPRG
ben-1                                D   N
tub-1                                   LN
mec-7                A  I       K       LNI          D
12-16                                D   S
8-9                  A               D   S
cß2                      I       K       LN


                370        380        390        400        410        420
Bpß1    LKMSATFIGNTTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS
ben-1               S
tub-1     A   V     S                                                  I
mec-7               S
12-16     A   V     S                                  Q
8-9       A         S                                                  I
cß2                 S


                430        440
Bpß1    EYQQYQDATADEEGDLQEGESEYIEQEE
ben-1         E    E D E DGTDGDAE
tub-1         E    EDDV GYAEGEAGETY SEQ
mec-7         E A    DAAEAFDGE
12-16         E     D  EMEGAVENDTYA
8-9           E     DM   DAEGG EAYP
cß2                  Q EFE EGE DEA
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Fig. 2.5. Comparison of *B. pahangi* ß1-tubulin predicted amino acid sequence with other ß-tubulins. The amino acid sequence of the *B. pahangi* ß-tubulin derived from the nucleotide sequence is compared to six ß-tubulins; the chicken cß2 (Valenzuela *et al.*, 1981); the ben-1 (Driscoll *et al.*, 1989), tub-1 (Gremke, 1987), and mec-7 (Savage *et al.*, 1989) ß-tubulins from *C. elegans*; the 12-16, and 8-9 ß-tubulins from *H. contortus* (Klein and Geary, personal communication). The complete amino acid sequence is given in the one-letter code for the *B. pahangi* ß-tubulin predicted protein (Bpß1). In the other ß-tubulins, non-conserved amino acid substitutions relative to the *B. pahangi* tubulin are indicated as letters.

TABLE 2.1. PERCENTAGE IDENTITY SHARED BETWEEN *B. PAHANGI* ß1-TUBULIN AND OTHER ß-TUBULINS.[1]

Organism		% Identity
B. pahangi	ß1	100
C. elegans	ben-1	94
	tub-1	90
	mec-7	88
H. contortus	12-16	92
	8-9	91
Chicken[2]	ß2	89
	ß3	89
Mouse[3]	ß5	89
D. melanogaster	ß2	87

[1] References are identical to those listed in the legend for Fig. 2.5.

[2] cß2, Valenzuela *et al.* (1981); cß3, Sullivan *et al.* (1986a)

[3] Lewis *et al.* (1985a,b)

once within the gene yielded two bands and *Hinc*II, which cuts twice yielded three bands, consistent with the presence of only one ß-tubulin gene. Southern hybridization analyses under reduced stringency in the presence of 35% formamide using the 1.8 kb *Eco*RI fragment of clone pBTX as a probe revealed no additional bands, but additional bands are seen when this hybridization is performed with 20% formamide.[2] These results suggest that only one copy of this ß-tubulin gene is present within the genome and if other gene(s) exist they do not share a high level of homology with the ß-tubulin gene of clone pBTX. To further investigate the possibility of additional divergent ß-tubulin genes, the same filter was reprobed under low stringency conditions (35% formamide) with an *H. contortus* full-length ß-tubulin cDNA which is 74% identical to the nucleotide sequence of the coding region of pBTX. As shown in Figure 2.6B, hybridizing sequences in addition to the cloned ß-tubulin gene were present in this Southern blot analysis. The recognition of identical DNA fragments in low stringency hybridization analyses using different ß-tubulin probes suggests that the *B. pahangi* genome contains between three and five ß-tubulin gene(s) (Fig. 2.6B and Appendix B).

Transcription unit

Total RNA isolated from *B. pahangi* adults was subjected to Northern blot analysis under high stringency conditions using the 1.8 kb *Eco*RI genomic fragment (see Fig. 2.3A) to verify that the genomic clone was expressed and to determine the size of the corresponding mRNA. Figure 2.7 demonstrates that the ß-tubulin gene which was cloned codes for mRNA of about 1.7 kb.

Isoforms recognized by a ß-tubulin antipeptide antibody

Antipeptide antisera was raised against a carboxy-terminal peptide of the ß-tubulin coding sequence in order to characterize the ß-tubulin isoforms present in *B. pahangi* adults and microfilariae (Fig. 2.8A). This region was chosen as the antigen because of its divergence from the

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[2] Southern hybridization analyses using the 1.8 kb *Eco*RI fragment of clone pBTX are found in Appendix B1.

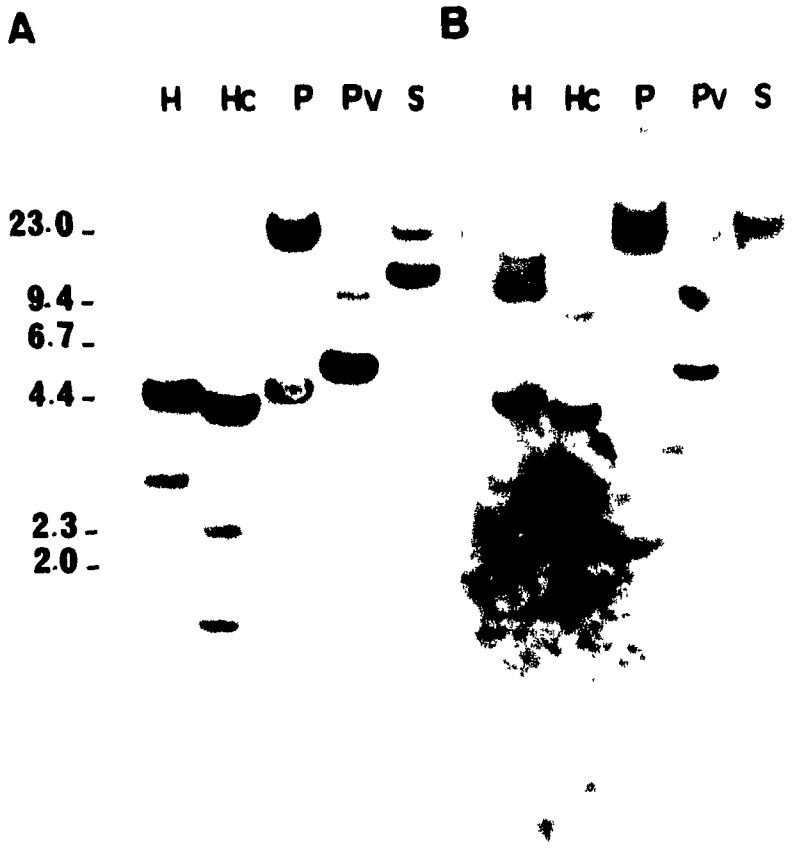

Fig. 2.6. Southern blot analyses of *B. pahangi* genomic DNA probed with nematode ß-tubulin sequences. *B. pahangi* genomic DNA was digested with *Hind*III (H), *Hinc*II (Hc), *Pst*I (P), *Pvu*II (Pv), and *Sac*I (S). The restriction fragments were size fractionated through a 1% agarose gel, transferred to Hybond-N, and hybridized in A, to the 5.7 Kb *Xba*I/*Xho*I genomic fragment of clone pBTX in the presence of 50% formamide at 42°C and washed in 0.2x SSC, 0.1% SDS at 65°C. B, the filter in A was washed and reprobed with the full-length *H. contortus* ß-tubulin cDNA insert from clone 8-9 in the presence of 35% formamide at 42°C and washed with 2x SSC, 0.1% SDS at 50°C. Molecular weight markers in kb corresponding to a *Hind*III digest of lambda DNA are shown.

Fig. 2.7. Northern blot analysis of *B. pahangi* ß-tubulin mRNA. Total RNA (20 μg) isolated from *B. pahangi* adult nematodes was denatured with 1.0 M glyoxal, electrophoresed through a 1% agarose gel, transferred to Hybond–N, and probed with the 1.8 kb *Eco*RI genomic DNA fragment of clone pBTX: lane 1, RNA from human monocyte U937 cells; lane 2, RNA from *B. pahangi* adults. Size markers in kb corresponding to mouse ribosomal RNAs are shown on the right.

carboxy termini of other ß-tubulins (see Fig. 2.5) thus, increasing the likelihood for the production of a *B. pahangi* specific anti-ß-tubulin antisera. Antibodies raised against this peptide recognize *B. pahangi* ß-tubulin but not the ß-tubulins present in mouse NIH-3T3 cells, whereas a commercially available (Amersham 357) anti-chicken ß-tubulin monoclonal antibody recognizes both the *B. pahangi* and mouse fibroblast NIH-3T3 ß- tubulins (Fig. 2.8B). The antipeptide antisera also reacted with *B. malayi* ß-tubulin but not with ß-tubulin from *Dirofilaria immitis*, also a filarial nematode.[3] These results demonstrate that the antipeptide antisera recognizes a non-conserved epitope, whereas the anti-ß-tubulin monoclonal antibody recognizes an epitope that is conserved between chicken and *B. pahangi* ß-tubulin. The epitope of the cross-reactive monoclonal antibody has been localized to a region of ß-tubulin between amino acids 339 and 417 by immunoblots of proteolytic fragments of pig brain tubulin (Serrano *et al.*, 1986). A subsequent study by de la Viña *et al.* (1988) shows that a ß-tubulin peptide 412-431 contains residues recognized by this antibody. Therefore the epitope of the cross-reactive antibody must be located between residues 412 and 417. This region is also highly conserved in nematode ß-tubulins (Fig. 2.5).

Both the antipeptide antisera and the phylum cross-reactive monoclonal antibody were used to examine ß-tubulin isoforms from adults and microfilariae by Western blot analyses of proteins separated by two-dimensional gel electrophoresis. As shown in Figure 2.9A-D, both antibodies recognize the same isoform patterns from both life cycle stages with isoelectric points ranging from 5.2 to 5.3. No isoforms containing the conserved epitope but not the parasite specific C-terminal epitope were detected by this analysis. Assuming that the phylum cross-reactive anti-tubulin monoclonal antibody recognizes the major ß-tubulin isoforms of *B. pahangi*, the results suggest that this carboxy-terminal region (Figs. 2.4 and 2.8A) is highly represented in the ß-tubulin isoforms of this nematode.

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[3] Additional Western blot analyses are found in Appendix C1.

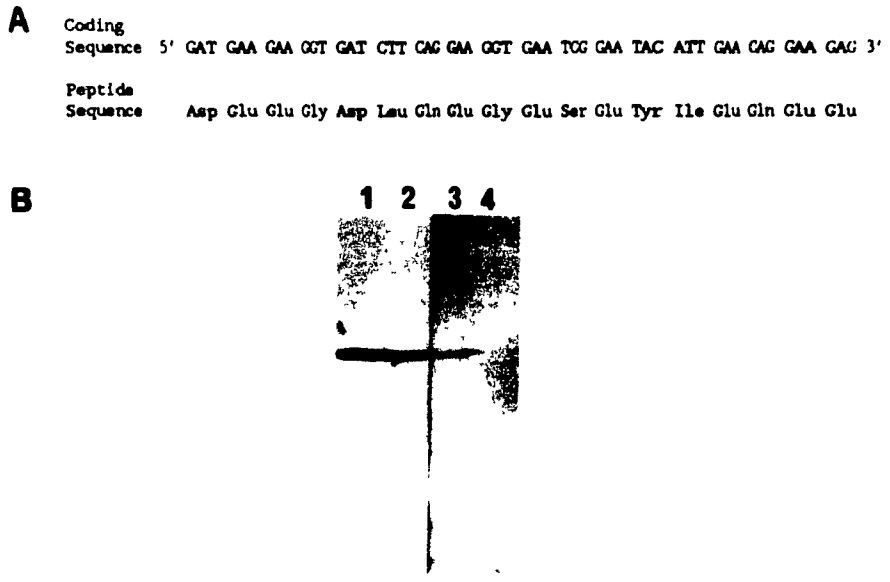


Fig. 2.8. Specificity of *B. pahangi* ß-tubulin antipeptide antibodies. A. The synthetic peptide corresponding to the carboxy-terminal sequence of *B. pahangi* ß-tubulin which was linked to KLH, then used to raise anti-peptide antibodies. B. Western blot analyses of total protein extracted from mouse fibroblast NIH-3T3 cells (lanes 2 and 4) and *B. pahangi* adult nematodes (lanes 1 and 3) using the anti-peptide antisera (lanes 3 and 4) and cross-reactive anti-ß-tubulin monoclonal antibody (lanes 1 and 2).

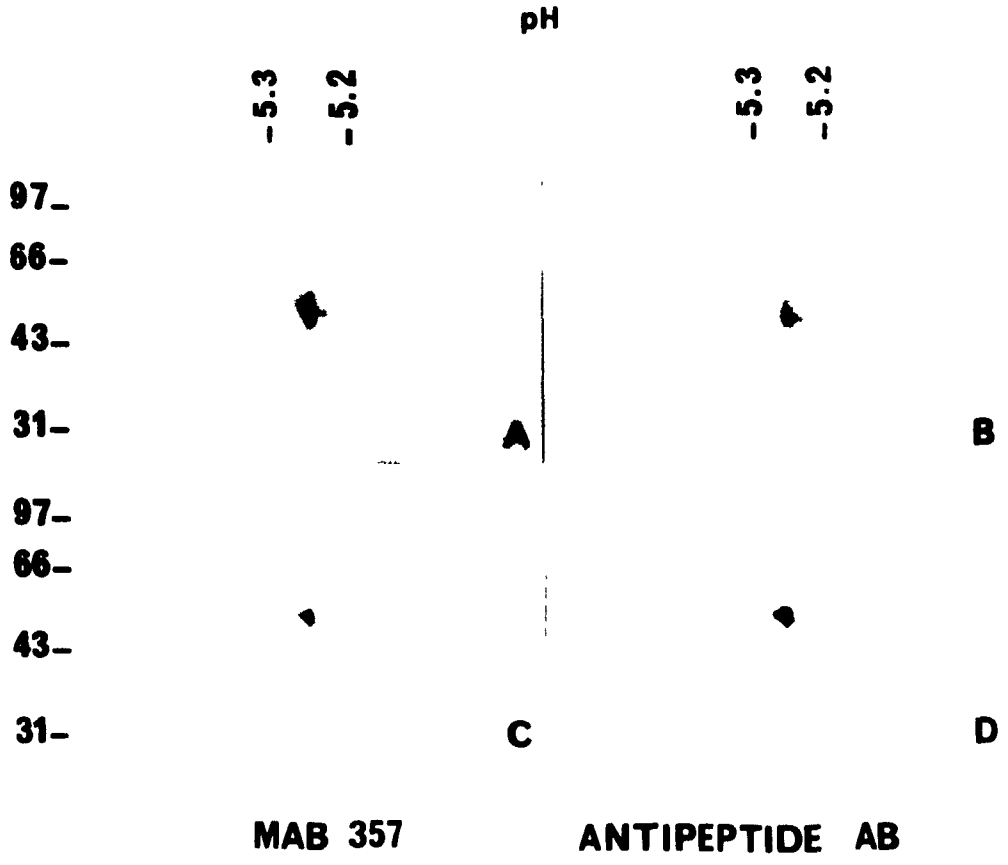


Fig. 2.9. Western blot analyses of *B. pahangi* ß-tubulin. Total protein of *B. pahangi* adult worms (A and B) and microfilariae (C and D) were separated by two dimensional gel electrophoresis and transferred to nitrocellulose. The anti-peptide IgGs were used for immunoblots in A and C and the cross-reactive monoclonal antibody was used for immunoblots in B and D. See Appendix C.2 for the detection of total protein from *B. pahangi* adult and microfilaria separated by two dimensional gel electrophoresis.

DISCUSSION

The isolation and characterization of a genomic clone representing a *B. pahangi* ß-tubulin and the characterization of the ß-tubulin isoforms of adult nematodes and microfilariae are described in this chapter. This study serves as a starting point for future studies on the molecular characterization of benzimidazole drug interaction with nematode tubulin.

The structural organization of the 5' region of the gene resembles that of vertebrate ß-tubulin genes since it contains introns at positions 19 and 56 , highly conserved vertebrate intron positions (Little and Seehaus, 1988). These data are consistent with the suggestion of Driscoll *et al.* (1989) that ancestral ß-tubulin genes had introns at these positions. The six additional introns are dispersed throughout the remaining coding sequence. Four of the introns at positions 174, 229, 293, and 387 have positions identical to those of *H. contortus* (M. Roos, personal communication) and/or *C. elegans* ß-tubulin genes (Driscoll *et al.*, 1989; Gremke, 1987). The intron located at amino acid position 131 is also conserved in the *Drosophila melanogaster* ß2-tubulin gene (Rudolph *et al.*, 1987). However, the intron located at position 324 appears to be unique to the *B. pahangi* ß-tubulin gene. The size range and number of introns observed in the ß-tubulin gene may be a general feature of *Brugia* species since multiple introns (10) ranging in size from 76 to 487 bases have been reported for a protective antigen (Perrine *et al.*, 1988). The higher A+T content of the shorter introns and the preference for codons ending in A or T may reflect the overall high A+T content of *B. pahangi* which has been reported to have an AT-rich genome (72%) (Rothstein *et al.*, 1988).

The 5' flanking region of the *B. pahangi* ß-tubulin gene has three potential splice acceptor sites (TTTAAG/CT) 3, (ATTAAG/TG) 11, and (TTGCAG/GT) 42 bases upstream of the translation initiation codon. Analysis of the RNA leader of the *B. malayi* 63 kDa protective antigen shows that a 22 nucleotide splice leader (SL), identical to the trans-SL sequence of *C. elegans* is joined to the coding RNA at a splice acceptor site located 26 nucleotides upstream of the ATG codon (Takacs *et al.*, 1988). A study of the *B. pahangi* ß-tubulin leader sequence may prove

interesting for two reasons: only a subset of nematode mRNAs studied contain the 22 nucleotide SL (Nilsen, 1989) and recently, Huang and Hirsh (1989) have identified in *C. elegans* a second 22 nucleotide SL, SL2, which is homologous to SL1 at 17 out of 22 positions.

ß-tubulin genes are members of small multigene families in many organisms including the free-living nematode *C. elegans* (Driscoll *et al.*, 1989) and the parasitic nematode *H. contortus* (Klein and Geary, personal communication). Southern hybridization analyses of *B. pahangi* DNA using a homologous probe under stringent conditions reveal banding patterns consistent with the presence of a single ß-tubulin gene and also suggest that this gene exists as a single copy in the *B. pahangi* genome. The absence of additional hybridizing bands with a homologous probe under lower stringency conditions (35% formamide, 42°C) suggests that any additional ß-tubulin sequences in the *B. pahangi* genome would have to be significantly divergent from the ß-tubulin gene we have cloned. Additional hybridizing bands can be identified by lower stringency hybridization using an *H contortus* ß-tubulin cDNA probe Whether these sequences represent novel ß-tubulin gene(s) remains to be evaluated. Although the three *C. elegans* ß-tubulin genes, mec-7, tub-1 and ben-1 cross-hybridize in the presence of 50% formamide (Gremke, 1987) there is precedent for highly divergent ß-tubulin genes within the gene families of mouse, chicken and *Drosophila*. The bone marrow ß-tubulins of mouse (Mß1) and chicken (cß6) are highly divergent ß-tubulin sequences which show 21% and 17% amino acid divergence when compared with other ß-tubulins within their respective gene families (Wang *et al.*, 1986; Murphy *et al.*, 1987). Furthermore, the *D. melanogaster* ß3 tubulin, a developmentally regulated ß-tubulin, exhibits 13% amino acid divergence when compared to the *D. melanogaster* ß2 tubulin (Rudolph *et al.*, 1987).

The functional importance of certain structural regions of the ß-tubulin gene are known. The amino terminal sequence met-arg-glu-ile, demonstrated to be required for the autoregulation of polysome bound ß-tubulin mRNAs (Yen *et al.*, 1988b), is present in the *B. pahangi* predicted ß-tubulin. The presence of this conserved amino-terminal tetrapeptide facilitated the assignment of the initiation codon for the

B. pahangi ß-tubulin.

Three of the five non-conserved amino acid changes in the *B. pahangi* ß-tubulin are clustered in the hinge region at positions 277, 279 and 285. The functional importance of the hinge region located between amino acids 260 and 300 (de la Viña *et al.*, 1988) for microtubule assembly is suggested by two observations: firstly, a *D. melanogaster* mutant (B2T8) with Lys at position 288 instead of Glu produces an assembly defective phenotype (Rudolph *et al.*, 1987) and secondly, tubulin dimers nicked by chymotrypsin at Tyr 281 retain their dimer structure but polymerize *in vitro* into aberrant structures similar to those of the *D. melanogaster* B2T8 mutant (Kirchner and Mandelkow, 1985). One or all of the amino acid substitutions occurring in the hinge region may contribute to the assembly properties of *B. pahangi* microtubules.

Most of the differences in an otherwise relatively conserved protein are found in the carboxy terminus of ß-tubulin (Sullivan and Cleveland, 1986). As with other ß-tubulins the carboxy-terminal region of ß-tubulin is highly charged and served as a good immunogen. The antipeptide antisera raised against the extreme carboxy terminus of *B. pahangi* ß-tubulin does not cross-react with mammalian ß-tubulin. As demonstrated by Western blot analyses, ß-tubulin isoforms which contained this C-terminal nematode-specific epitope also contained the conserved epitope recognized by the highly cross-reactive monoclonal antibody. These data suggest that this C-terminal region is highly represented in the ß-tubulin isoforms of adult and microfilariae. The isoform pattern of microfilarial ß-tubulins was previously unknown. It is noteworthy, in view of the identical isoform patterns of microfilarial and adult ß-tubulin that female adult worms of more than 55 days old contain microfilariae (Schacher, 1962). Therefore, the microfilarial ß-tubulin isoforms may contribute in part to the isoform pattern observed in adult nematodes.

Recently, several ß-tubulin genes of the free-living nematode *C. elegans* have been characterized. The mec-7 ß-tubulin is necessary for the formation of the 15-protofilament microtubules of touch receptor neurons (Chalfie and Thomson, 1982) whereas, the ben-1 and tub-1 ß-

tubulins are believed to contribute to 11-protofilament microtubules (Driscoll *et al.*, 1989). With the exception of the touch receptor neurons of *C. elegans* and those of *Trichostrongylus colubriformis* which have 14 protofilaments (Davis and Gull, 1983) most nematode cells have microtubule structures of 11 protofilaments (Chalfie and Thomson, 1982; Davis and Gull, 1983). The *B. pahangi* ß-tubulin shares 13 out of 15 amino acid residues common to the 11-protofilament ß-tubulins of *C. elegans* suggesting that it is a component of 11-protofilament microtubules in *B. pahangi*. This is consistent with the observation that the antipeptide antibody recognizes the major ß-tubulin isoforms of *B. pahangi* since 11-protofilament microtubules are predominant in nematode cells.

Brugia nematodes express ß-tubulin molecules which contain binding sites for the anthelmintic microtubule depolymerizing drugs, the benzimidazole carbamates (Tang and Prichard, 1989). These drugs act by disrupting the assembly of tubulin dimers into microtubules (Lacey and Prichard, 1986). Driscoll *et al.* (1989) have identified a benzimidazole-sensitive ß-tubulin called ben-1, by characterizing benomyl resistant *C. elegans* mutants. In *H. contortus* benzimidazole resistance is associated with a decrease in the binding of benzimidazoles to tubulin (Lacey and Prichard, 1986). Six of the amino acid residues unique to the ben-1 protein may correlate to benzimidazole sensitivity. Four of these are identical in the *B. pahangi* ß-tubulin characterized in this study and may therefore be involved in the benzimidazole binding site. Future studies should be initiated to address this issue.

Driscoll *et al.* (1989) have suggested that the deletion of a benzimidazole sensitive but dispensable ß-tubulin allele is a general mechanism by which nematode populations become resistant to benzimidazoles. Since the mutations conferring benzimidazole resistance are dominant, a wild-type allele of ben-1 is still expressed indicating that a reduction of a benzimidazole sensitive ß-tubulin is sufficient to provide drug resistance. This mechanism requires the presence of other ß-tubulin genes whose elevated expression can compensate for the missing ß-tubulin. The identification of additional ß-tubulin genes in the *B.*

pahangi genome will be required before the proposed mechanism for the development of benzimidazole resistance can be assessed for this nematode. This mechanism further implies that the levels of particular ß-tubulin gene products of a tissue or cell type contribute to the sensitivity of this tissue or cell type to these drugs. This may be the case for the observed embryostatic effects of benzimidazoles in *B. pahangi* (WHO, 1985).

CHAPTER 3. TWO *BRUGIA PAHANGI* ß-TUBULIN GENES DIFFER IN THEIR PATTERNS OF EXPRESSION AND THEIR MODES OF SPLICING.

INTRODUCTION

The α- and ß-tubulins of multicellular organisms are generally encoded by small multigene families (MacRae and Langdon, 1989). It was of interest to characterize the ß-tubulin genes of *B. pahangi* for two reasons, 1) very little is known about the molecular genetics of filarial nematodes and, 2) the αß tubulin dimer is the target of the benzimidazole microtubule depolymerizing drugs, which are used to treat nematode infections (Lacey, 1988). To date the most extensively characterized nematode ß-tubulin gene family is that of the free living nematode, *Caenorhabditis elegans*, for which three ß-tubulin genes have been cloned and sequenced (Gremke, 1987; Savage *et al.*, 1989; Driscoll *et al.*, 1989). Functional members of the ß-tubulin gene families are differentially expressed. For example, the *C. elegans* ben-1 ß-tubulin gene is expressed in many cells (Driscoll *et al.*, 1989), whereas the transcripts for the mec-7 ß-tubulin gene are found only in the six touch receptor neurons (Savage *et al.*, 1989; Mitani and Chalfie, 1990) and at low levels in a pair of neurons located posterior to the pharynx (Hamelin and Culotti, 1990). The spatial and temporal nature of ß-tubulin gene expression for the parasitic nematodes is unknown In fact, the sheep intestinal nematode *H. contortus* is the only parasitic nematode for which more than one ß-tubulin gene has been characterized (Klein and Geary, personal communication) but their expression patterns have not been determined. In the previous chapter the characterization of a *B. pahangi* ß-tubulin gene (ß1) which shares 94%, and 90% identity with the ben-1 and tub-1 ß-tubulins of *C. elegans*, respectively, is described. The data also show that under low stringency hybridization conditions an *H. contortus* ß-tubulin cDNA insert recognizes several bands in Southern blot analyses, suggesting that other ß-tubulin sequences may be present in the *B. pahangi* genome. In the present chapter, the identification of a second *B. pahangi* ß-tubulin (ß2) gene and the distinct pattern of expression of the ß1- and ß2-tubulin genes

of this nematode is presented. The ß1-tubulin transcripts are found in microfilariae (larval stage) and adult nematodes, whereas the ß2-tubulin transcripts are absent from microfilariae and are expressed predominantly in adult male worms.

Trans-splicing, the splicing of exons from two distinct RNAs to form a single RNA, is an event necessary for the maturation of 10-15% of nematode mRNAs (Bektesh and Hirsh, 1988; Hannon *et al.*, 1990; Bektesh *et al.*, 1988). A conserved 22 nucleotide splice leader sequence (SL1) is added to precursor mRNAs in the free living nematodes, *C. elegans* and *Panagrellus redivivus* (Bektesh *et al.*, 1988), the intestinal parasitic nematodes, *Ascaris suum* and *H. contortus* (Bektesh *et al.*, 1988) and the filarial nematodes *Brugia malayi* (Takacs *et al.*, 1988) and *Onchocerca volvulus* (Zeng *et al.*, 1990). The splice leader, SL1, has been identified in the *C. elegans* mRNAs coding for three actin proteins (Krause and Hirsh, 1987), two ribosomal proteins (Bektesh *et al.*, 1988), three glyceraldehyde-3-phosphate dehydrogenase proteins (Huang and Hirsh, 1989), a heat shock protein (hsp70a) (Bektesh *et al.*, 1988), ubiquitin (Bektesh *et al.*, 1988; Graham *et al.*, 1988), one myosin light chain protein (Cummins and Anderson, 1988), one copy of a duplicated collagen gene (Park and Kramer, 1990) and the catalytic subunit of cAMP-dependent protein kinase (Gross *et al.*, 1990). It has also been identified in the mRNAs coding for a potentially protective *B. malayi* antigen which is found in several copies in the *B. malayi* genome (Takacs *et al.*, 1988). The presence of a splice acceptor site upstream of the open reading frame does not appear to be sufficient for the addition of a splice leader. The *C. elegans* genes, act-4 and mlc-1, have a consensus splice acceptor site upstream of the initiation codon, yet they undergo neither cis nor trans-splicing reactions (Kraus and Hirsh, 1987; Cummins and Anderson, 1988). Since the SL1 splice leader is involved in the maturation of mRNAs in the closely related nematode, *B. malayi*, and three splice acceptor sites are located upstream of the *B. pahangi* ß1-tubulin initiation codon (chapter 2) it was of interest to determine whether the ß-tubulin mRNAs of *B. pahangi* acquire the SL1 sequence. Results of this study show that the 22 nt SL1 splice leader

was detected on *B. pahangi* ß1-tubulin mRNA but not on ß2-tubulin mRNA. The SL1 sequence is added to the ß1-tubulin splice acceptor site located 42 nucleotides upstream of the initiation codon. Factors determining the choice of splice acceptor site in the maturation of the ß1-tubulin mRNAs are unknown, but there is a sequence which matches the weakly conserved eukaryotic cis-splicing branch point consensus sequence at 6 out of 7 positions located 19 nucleotides upstream of the chosen splice acceptor site (Reed and Maniatis, 1985).

EXPERIMENTAL PROTOCOL

Parasite Material

B. pahangi adults and microfilariae were isolated from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) obtained from J.W. McCall (Department of Parasitology, Georgia University, USA) by dissection post-mortem. The smaller size, the presence of a spicule and the characteristic curling of the posterior end of adult male worms were all criteria used to separate adult males from females worms The microfilariae were separated from the peritoneal macrophage as previously described (Kaushal *et al.*, 1982).

Nucleic Acid Isolation and Filter Hybridizations

Procedures for *B. pahangi* genomic DNA extraction, total RNA extraction, nucleic acid transfer to nitrocellulose and filter hybridizations using nick-translated probes are identical to those described in chapter 2. mRNA was isolated using the Fast track mRNA isolation kit (Invitrogen Corp.).

Construction and screening of a *B. pahangi* genomic DNA library

The *B. pahangi* genomic library was constructed using a new cloning strategy that relies on the specificity with which partially filled-in *Xho*I lambdaGEM-11 arms can be combined with partially filled-in *Sau*3AI digested genomic DNA (Zabarovsky and Allikmets, 1986). *B. pahangi* DNA was partially digested with Sau3AI (Maniatis *et al.*, 1982) and these fragments were filled-in using the Klenow fragment (Pharmacia Fine

Chemicals) and a fill-in buffer containing dGTP and dATP. These *Sau*3AI DNA fragments were ligated into lambdaGEM-11 half site arms (Promega) using T4 DNA ligase (Pharmacia Fine Chemicals), the products were packaged into phage heads and plated onto *E. coli* LE392. The genomic library was screened for phage that hybridized to the 1.0 kb *Eco*RI *H. contortus* ß-tubulin cDNA fragment of clone 12-16 (kindly provided by R. Klein) but which did not hybridize to the 1.8 kb *Eco*RI *B. pahangi* ß-tubulin fragmen· of clone pBTX (Chapter 2), which we will call ß1-tubulin. Plaque hybridizations and phage DNA isolation were performed using standard protocols (Maniatis *et al.*, 1982). DNA was isolated from three genomic clones (3-21, 3-22, and 1-21) carrying ß-tubulin sequences distinct from the ß1-tubulin gene.

DNA Sequence Analysis

A 2.4 kb *Eco*RI ß-tubulin fragment of clone 3-21 was subcloned into the *Eco*RI site of pIBI30 (IBI). Single stranded DNAs were prepared using the helper phage M13K07 as described by IBI. These single stranded DNAs served as template for the dideoxy chain termination sequencing reactions performed with the modified T7 DNA polymerase, Sequenase (United States Biochemicals Corp.).

Primer Extension Analysis

A 24-mer oligonucleotide complementary to amino acids 10 to 17 of the ß1-tubulin coding strand, 5' ACCAATCTGGTTGCCACATTGACC 3', was synthesized by the Calgary Oligonucleotide Synthesis Laboratory. This oligonucleotide was end labelled with [γ-^{32}P]dATP (ICN) using T4 polynucleotide kinase (Pharmacia Fine Chemicals). The oligonucleotide (10 pmoles) was hybridized to adult *B. pahangi* mRNA (2-4 μg) in the presence of 80% formamide at 48°C for 15 hours (Boorstein and Craig, 1989). The RNA-DNA hybrids were precipitated several times prior to elongation with avian myeloblastosis virus reverse transcriptase (Molecular Genetic Systems) for 75 min. at 42°C. The extension products were analyzed by electrophoresis through 8% sequencing gels. A sequencing ladder was used to accurately determine the size of the extended product.

Detection of the 22 nucleotide splice leader on ß-tubulin RNA using the Polymerase Chain Reaction (PCR)

First strand cDNAs synthesized from *B. pahangi* total RNA (0.1-5 µg) served as templates for the PCR in a one-step amplification protocol (Goblet *et al.*, 1989). However, for the reverse transcriptase reaction, incubation at 42°C was performed for 30 min. and the RNA-DNA hybrids were heated to 95°C for 5 mins. before proceeding with 30 cycles of PCR This step denatures the RNA-DNA hybrids and inactivates the reverse transcriptase. One cycle consisted of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C. The final step consisted of a 5 min. incubation at 72°C. Taq polymerase was obtained from Pharmacia Fine Chemicals or BioCan Scientific. The reaction mixture contained the following primer combinations (100 ng each): a 24-mer oligonucleotide primer complementary to ß1-tubulin RNA sequences coding for amino acids 124 to 130, 5' CTGAAGACAATCGCATCCCTCAGC 3', and a 22-mer oligonucleotide corresponding to the trans-splice leader RNA (SL1), 5' GGTTTAATTACCCAAGTTTGAG 3' (Bektesh *et al.*, 1988); a 20-mer corresponding to nucleotides -39 to -20 of the ß1-tubulin gene, 5' CTCATTTCGGTCGACAAGAT 3', and the ß1-tubulin specific oligonucleotide; an 18-mer oligonucleotide complementary to ß2-tubulin RNA se uences coding for amino acids 311 to 316, 5' CATAACCGCCACCGTAAG 3', and the trans-splice leader sequence. PCR amplified DNAs were transferred to Hybond-N and filters were either hybridized to a 1.0 kb *Pst*I/*Eco*RI ß1-tubulin fragment, or a 1.9 kb *Hind*III ß2-tubulin fragment.

RESULTS

Isolation and partial characterization of a second *B. pahangi* ß-tubulin gene (ß2)

A *B. pahangi* genomic library was constructed in the bacteriophage lambda derivative lambdaGEM-11. Approximately 90,000 recombinant phage plaques were screened for ß-tubulin sequences by using a differential screening procedure which distinguished previously cloned ß1-tubulin sequences from novel ß-tubulin sequences. The *H. contortus* ß-tubulin cDNA clones 8-9 and 12-16 were useful ß-tubulin probes because they

recognize several *Eco*RI fragments when hybridized in the presence of 35-40% formamide, including the 1.8 kb *Eco*RI ß1-tubulin sequence (Fig. 3.1 and Appendix B.2). A 2.4 kb *Eco*RI DNA fragment is detected in the presence of 50% formamide by both probes. Therefore, the library was screened by hybridization to replica filters using either the 1.8 kb *Eco*RI ß1-tubulin fragment or the 1.0 kb *Eco*RI *H. contortus* ß-tubulin fragment of clone 12-16 as probes. This approach allowed us to identify those recombinant phage containing ß-tubulin sequences which differ from ß1-tubulin. Of 20 plaques which hybridized to the *H. contortus* probe but which did not hybridize to the *B. pahangi* ß1-tubulin, three were purified through three successive rounds of replating and hybridization. The *B. pahangi* genomic DNA inserts for these three clones ranged in size from 12 to 15 kb. Southern blot analyses of *Eco*RI digests of the three clones using the *H. contortus* ß-tubulin probe showed that for all three clones, identical *Eco*RI restriction fragments hybridized to the probe. To confirm that we had in fact isolated a novel ß-tubulin sequence, the 2.4 kb *Eco*RI DNA fragment was subcloned from clone 3-21 into the *Eco*RI site of the plasmid pIBI30. A partial restriction enzyme map of this clone is shown in Fig. 3.2a.[1] A partial sequence (Fig. 3.2b) of this subclone revealed that the *B. pahangi* genomic DNA isolated codes for a ß-tubulin sequence distinct from the ß1-tubulin gene, which we have termed ß2-tubulin. Based on the previously characterized ß1-tubulin gene (chapter 2) the sequenced DNA probably codes for amino acids 311-338 and is interrupted by an intron of 141 nucleotides which is located at the predicted amino acid position, 324. This intron position was also found in the ß1-tubulin gene and is unique to *B. pahangi* ß-tubulin genes (see chapter 2). A comparison of the predicted amino acid sequence of the *B. pahangi* ß2-tubulin to the *B. pahangi* ß1-tubulin (Fig. 3.2) shows that these two ß-tubulin sequences are distinct from one another with 7 out of 28 amino acid substitutions. Results of Southern blot analyses

[1] Southern hybridization analyses of clone 3-21 identifying DNA fragments corresponding to ß-tubulin are shown in Appendix D.1.

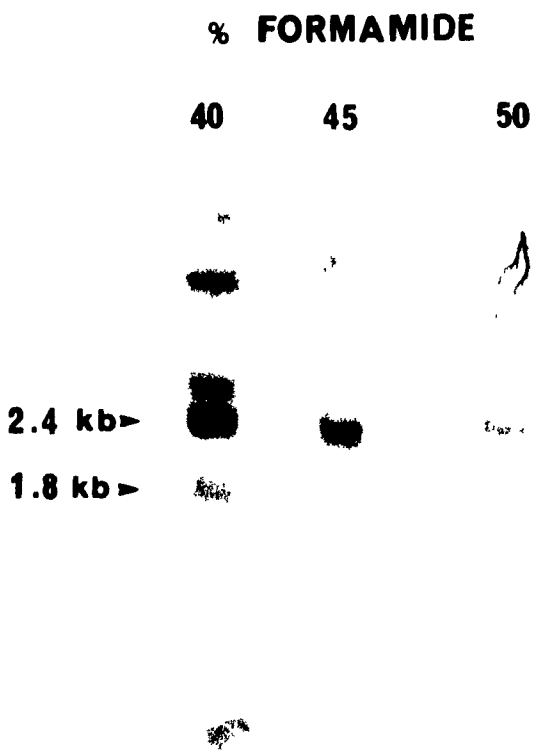
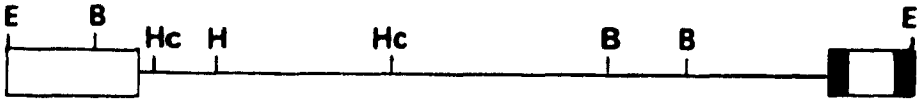


Fig. 3.1. Identification of *B. pahangi* genomic *Eco*RI fragments corresponding to ß-tubulin using an *H. contortus* ß-tubulin cDNA insert. *B. pahangi* genomic DNA digested with *Eco*RI was transferred to three separate Hybond-N strips and hybridized to the full-length ß-tubulin cDNA insert of the *H. contortus* clone 8-9 in the presence of 40%, 45% and 50% formamide, respectively. The filters were washed in 2x SSC, 0.1% SDS at 65°C. The 1.8 kb DNA fragment corresponds to ß1-tubulin and the 2.4 kb DNA fragment corresponds to ß2-tubulin.

A.



B.

```
    CTT ACG GTG GCG GTT ATG TTC CGT GGC TCG ATG TCG ATG AGC gtatgtgt  50
ß2  L   T   V   A   V   M   F   R   G   S   M   S   M   S
ß1                  A                   R               R
    ttttatagataattctgcatctttttttttaagttcagcactgttcatttgatgatgtttttt 114
    tttattttgtttgaaaacgttttcttctttttaaaattccatggattctttggatacaacatga 178
    attttag GAA GTG GAC AAT CAG ATG TTC AAT GTA CAG GAT AAG AAT TC    224
ß2          E   V   D   N   Q   M   F   N   V   Q   D   K   N   S
ß1                      E           M   Q           N
```

Fig. 3.2. Nucleotide sequence and comparison of the predicted amino acid sequence of the *B. pahangi* ß2-tubulin to the *B. pahangi* ß1-tubulin. A. Partial restriction enzyme map of the 2.4 kb *Eco*RI fragment subcloned from recombinant phage 3-21. The dark box represents the ß2-tubulin coding region identified by sequence analysis, the open box represents non-coding DNA and the line represents unsequenced DNA. *Hind*III (H), *Hinc*II (Hc), *Eco*RI (E), *Bgl*II (B). B. The nucleotide sequence of a portion of the *B. pahangi* 2.4 kb *Eco*RI genomic DNA fragment which represents the ß2-tubulin open reading frames between amino acid 311 and 338 is shown in higher case letters and the intron located at amino acid 324 is presented in lower case letters. The ß2-tubulin amino acid sequence derived from the nucleotide sequence is presented below the corresponding codons in the one-letter code. The amino acid substitutions found in the ß1-tubulin sequence are indicated by a letter.

shown in Fig. 3.3 confirm the isolation of a novel ß-tubulin sequence. In Figure 3.3A the filter was hybridized with the *B. pahangi* 2.4 kb *Eco*RI genomic DNA fragment of the ß2-tubulin gene and in Fig. 3 3B the filter was hybridized to the 1.8 kb *Eco*RI fragment of the ß1-tubulin gene. In both Figures 3.3A and 3.3B the number of hybridizing bands observed for each restriction enzyme digest is consistent with the interpretation that ß1-tubulin and ß2-tubulin sequences exist within the *B. pahangi* genome as single copy genes.[2]

Expression of the ß2-tubulin gene is developmentally regulated

Northern hybridization analyses of total RNA isolated from *B. pahangi* adult female worms, adult male worms, microfilariae and mouse macrophage were performed using the 2.4 kb *Eco*RI fragment of the ß2-tubulin gene as a probe (Fig. 3.4A). The same filter was then washed and reprobed with the 1.8 kb *Eco*RI fragment of the ß1-tubulin gene (Fig 3.4B). Mouse macrophage RNA was included as a control to ensure that the hybridization signal observed for microfilarial RNA was not due to contaminating gerbil macrophage RNA. The results shown in Figure 3 4 indicate that the ß1-tubulin gene is expressed as a 1 7 kb transcript in both female and male adult worms and in microfilariae. In contrast, ß2-tubulin transcripts are not detected in microfilariae and are predominant in male adult worms. The ß2-tubulin gene yields two transcripts of 1.7 kb and 2.4 kb in adult worms whereas the ß1-tubulin gene only yields two transcripts of 1.7 kb and 2.0 kb in adult female worms. Neither ß1-tubulin nor ß2-tubulin probes cross-hybridize with macrophage ß-tubulin RNA. These results indicate that the ß1-tubulin is constitutively expressed and the ß2-tubulin is developmentally regulated.

The maturation of ß1-tubulin mRNA requires the addition of the 22-nt SL

The 5' flanking region of the ß1-tubulin gene was previously found

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[2] Results of a Southern hybridization analysis under reduced stringency using ß2-tubulin DNA as the probe are shown in Appendix B.3.

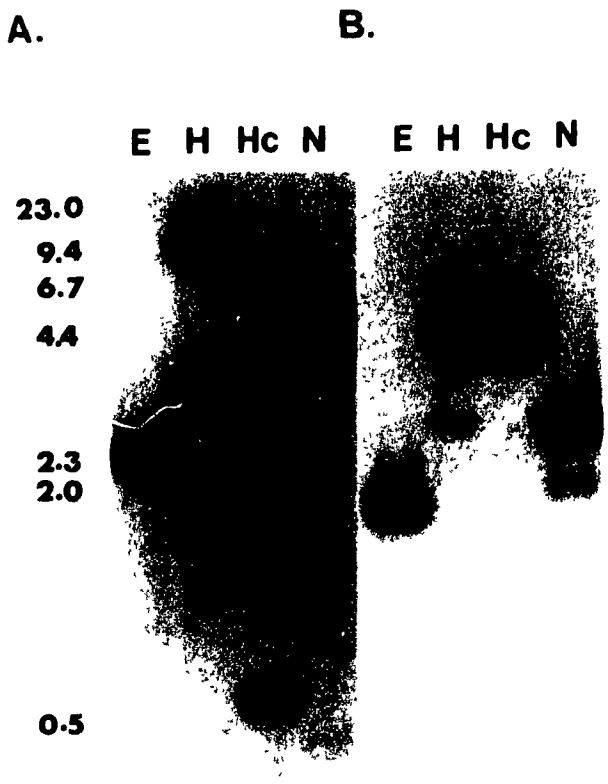

Fig. 3.3. Southern blots of *B. pahangi* genomic DNA probed with *B. pahangi* ß1- and ß2-tubulin sequences. *B. pahangi* genomic DNA was digested with *Eco*RI (E), *Hind*III (H), *Hinc*II (Hc) and *Nde*I (N). The restriction fragments obtained were size fractionated through a 1% agarose gel, transferred to Hybond-N, and hybridized in A., to the 2.4 kb *Eco*RI ß2-tubulin fragment and B., to the 1.8 kb *Eco*RI ß1-tubulin fragment. Hybridization was carried out at 42°C in the presence of 50% formamide and filters were washed in 0.1x SSC, 0.1% SDS at 65°C.

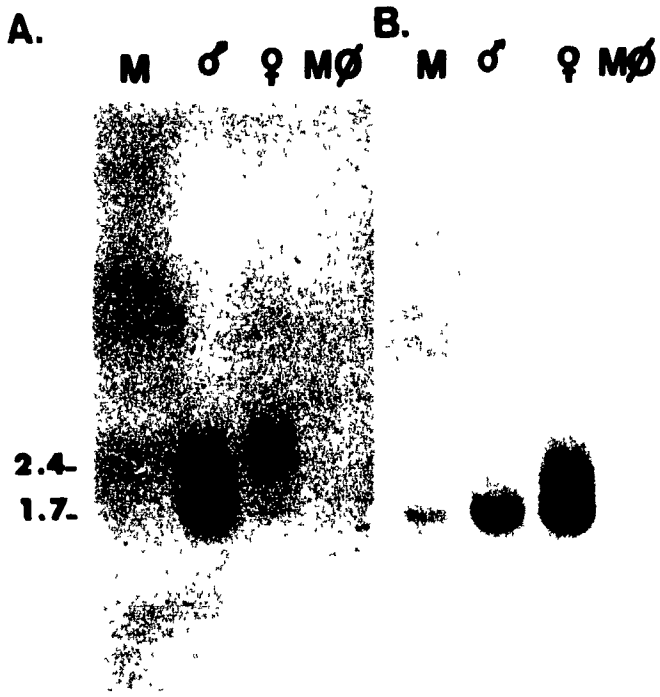

Fig. 3.4. Expression of the *B. pahangi* ß1- and ß2-tubulin genes. Total RNA isolated from *B. pahangi* microfilariae (M), adult male (♂), adult female (♀) and mouse macrophages (Mϕ) was denatured with 1.0 M glyoxal, electrophoresed through a 1% agarose gel, transferred to Hybond-N and probed with A) the 2.4 kb *Eco*R1 ß2-tubulin DNA fragment and in B) with the 1.8 kb *Eco*RI ß1-tubulin DNA fragment.

Fig. 3.5. Primer extension analysis of the ß1-tubulin mRNAs. An end-labelled oligonucleotide complementary to nucleotides 28-51 of the *B. pahangi* ß1-tubulin gene was hybridized to adult *B. pahangi* mRNA in 80% formamide for 15 hours at 48°C. The primer/RNA hybrid was extended for 45 mins. at 42°C using AMV-RT. The lane labelled (-) represents a sample containing only probe DNA. The extended product is indicated by an arrow. A sequencing reaction using the same oligonucleotide primer is shown to the left of the primer extension reaction and the DNA sequence of the 50 nucleotides located upstream of the initiation codon of the *B. pahangi* ß1-tubulin gene is found underneath the primer extension reaction. The 3' splice acceptor sites are underlined. ▸

-70 ttgatcagtaattaccggattga

-47 ttgcaggtctcatttcggtcgac

-24 aagatttcattaagtgtttaagct

to contain three possible splice acceptor sites at 3, 11 and 42 nucleotides upstream of the initiation codon (Fig. 3.5). Primer extension analysis using an end labelled synthetic oligonucleotide primer complementary to bases 28–51 of the ß1–tubulin gene shows a single primer extension product. The length of the extended product (114 nt) indicates that the 5' flanking region of the ß1–tubulin RNA is of 64 bases (Fig. 3.5). This result provides circumstantial evidence for the presence of a 22 nt splice leader on ß1–tubulin RNA, because the joining of the 22 nt splice leader to the splice acceptor site situated 42 bases upstream of the initiation codon would produce an RNA leader of exactly 64 bases in length. Primer extension analysis was not performed on ß2–tubulin RNA because of the low recovery of adult male worm tissue from infected gerbils.

In order to determine whether the acquisition of the 22 nt splice leader sequence is involved in the maturation of the *B. pahangi* ß–tubulin RNAs, the polymerase chain reaction was used to amplify ß–tubulin cDNAs carrying the 22 nt splice leader sequence The results of PCR using oligonucleotides complementary to either the coding sequence of ß1 or ß2–tubulin RNAs and an oligonucleotide corresponding to the 22 nt splice leader RNA sequence are shown in Figure 3.6 and 3.7, respectively. Of the three amplified products obtained in Figure 3.6A, a 456 bp fragment corresponding to the expected ß1–tubulin cDNA amplification product hybridized to the 1.0 kb *Pst*I/*Eco*RI ß1–tubulin probe (Fig. 3.6B). The results of Figure 3.6B show that the ß1–tubulin RNA isolated from female adult worms carries the 22 nt splice leader sequence. To verify whether splice leader addition was constitutive this experiment was repeated on RNA isolated from adult male *B. pahangi* worms and microfilariae (Fig. 3.6C). It was found that splice leader addition was not limited to a particular stage or tissue in *B. pahangi* However, using the same approach no ß2–tubulin cDNA fragment was amplified after three separate attempts suggesting that the ß2–tubulin RNA of male worms does not acquire the same 22 nt splice leader (Fig. 3.7a).

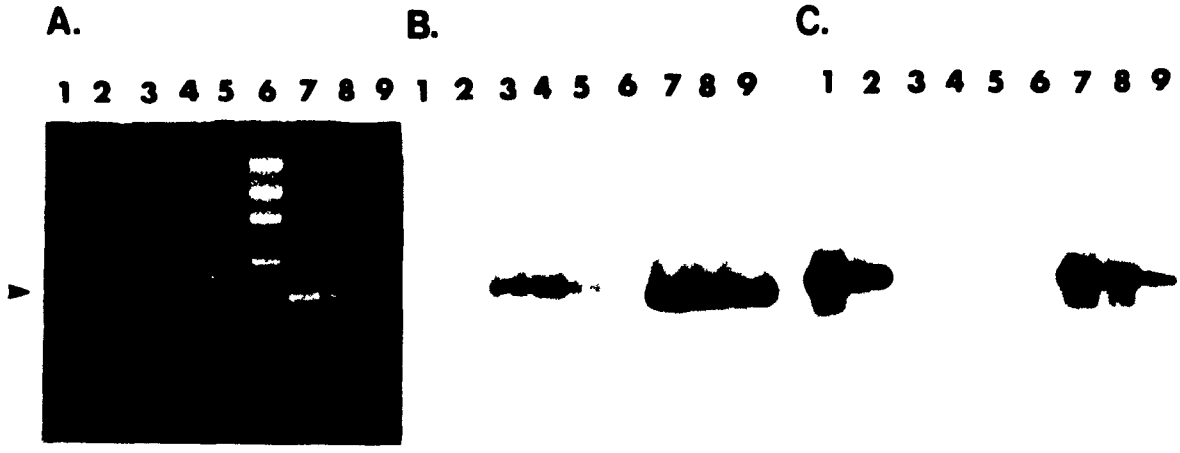

Fig. 3.6. PCR experiment using an oligonucleotide complementary to the ß1-tubulin RNA and the conserved 22 nt splice leader sequence. A. PCR amplification products stained with ethidium bromide after separation through a 1.5 % agarose gel. The first strand cDNA was synthesized from adult female *B. pahangi* total RNA, 5, 1 and 0.1 µg (lanes 3–5 and 7–9, respectively). The primers used for reactions in lanes 3–5 were the splice leader oligonucleotide and an oligonucleotide complementary to the ß1-tubulin sequence coding for amino acids 124 to 130 and for reactions in lanes 7–9 an oligonucleotide corresponding to the ß1-tubulin 5′ flanking sequences −41 to −20 and the complementary ß1-tubulin oligonucleotide. The arrow represents the expected 456 bp PCR product. B. Autoradiogram of a Southern blot in which the DNAs shown in A. were probed with a 1.0 kb *Pst*I/*Eco*RI ß1-tubulin fragment which codes for amino acids 20 to 56. C. Autoradiogram of a Southern blot of PCR amplified products probed with the 1.0 kb *Pst*I/*Eco*RI ß1-tubulin fragment. In this case the DNAs were amplified from *B. pahangi* adult male total RNA, 1 and 0.1 µg (lanes 1 and 2, respectively) and microfilarial total RNA, 5, 1, and 0.1 µg (lanes 7–9, respectively). The primers used were the splice leader oligonucleotide and the ß1-tubulin-specific oligonucleotide as in A). Lanes 1 and 2 in A. and B. and lanes 4-6 in C. represent control reactions with no RNA. Lane 6 in A. and B. and lane 3 in C. contain a *Hae*III digest of ΦX174 DNA.

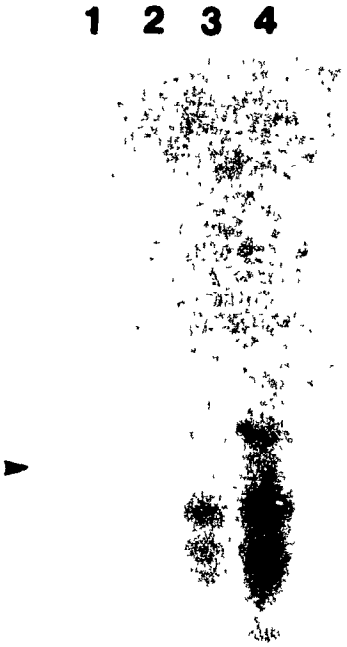

Fig. 3.7. PCR experiment using an oligonucleotide complementary to ß2-tubulin RNAs and the conserved 22 nt splice leader sequence. Southern blot analysis of PCR amplification products obtained from a reaction mixture containing total male RNA, 1 and 0.1 µg (lanes 3 and 4) and oligonucleotides complementary to ß2-tubulin sequences coding for amino acids 311 to 317 and an oligonucleotide corresponding to the conserved 22 nt nematode splice leader. Control reactions with no RNA (lanes 1 and 2). The filters were hybridized to a 1.9 kb *Hind*III fragment coding for ß2-tubulin sequences which includes the ß2-tubulin complementary oligonucleotide used as a primer. The hybridization reaction was performed in the presence of 50% formamide at 42°C and the filter was washed in 0.5x SSC at 65°C. The arrow represents the minimum size of the expected PCR product which equals 951 bp (3 x 317 codons + 5' untranslated sequences downstream of a putative splice acceptor).

DISCUSSION

There are at least two ß-tubulin genes in the filarial nematode *B. pahangi*. Both of these ß-tubulin sequences exist as a single copy within the *B. pahangi* genome and in this respect the ß-tubulin gene family of *B. pahangi* resembles that of *C. elegans* (Gremke, 1987). In contrast to the high degree of identity shared between members of the ß-tubulin gene families of *H. contortus* and *C. elegans* there are no ß-tubulin sequences within the *B. pahangi* genome which share enough identity with either ß1- or ß2-tubulin sequences to allow their detection under high stringency hybridization conditions. This allowed us to use the coding region of these genes as probes to determine their expression pattern in microfilariae and adult worms.

The ß1-tubulin gene is expressed in both developmental stages examined, this is consistent with the finding presented in chapter 2, that the ß1-tubulin C-terminal region is highly represented in the ß-tubulin isoforms of adult and microfilariae. In contrast, the expression of the ß2-tubulin gene is developmentally regulated. The transcripts are found predominantly in male adult worms. A ß-tubulin gene which is expressed at low levels in several tissues but which is dominant in the testis was identified in the mouse, human and chicken (Sullivan *et al.*, 1986a; Lewis *et al.*, 1985b; Monteiro and Cleveland, 1988). Furthermore, the invertebrate *Drosophila melanogaster* has a testis-specific ß-tubulin gene (Rudolph *et al.*, 1987). The testis specific *D. melanogaster* ß-tubulin is involved in spermatogenesis and mutants defective in this ß-tubulin have disrupted sperm structures and functions (Kemphues *et al.*, 1982). The detection of low levels of the ß2-tubulin transcripts in *B. pahangi* adult female worms does not preclude testis-specificity. A 1.8 kb transcript of the testis-specific ß2-tubulin of *D. melanogaster* is expressed in 0 to 3 hour old embryos (Natzle and McCarthy, 1984) and gravid *B. pahangi* female worms are likely to contain embryos in various stages of development (Schacher, 1962). Although there are no reports of a testis-specific ß-tubulin gene for the nematode *C. elegans*, the detection of such an expression

pattern in *C. elegans* would not be trivial since it is a hermaphrodite.

The ß2-tubulin gene of *B. pahangi* produces two transcripts of 1.7 and 2.4 kb. The production of two transcripts for one ß-tubulin gene is not uncommon. The testis-specific *D. melanogaster* ß-tubulin has transcripts of 1.8 and 2.0 kb which are detected at different stages of development (Natzle and McCarthy, 1984) and the chicken ß5-tubulin which is found at low levels in most cells has a 2.2 kb mRNA but testis RNA also contains a shorter 1.7 kb cß5 mRNA. These differences in cß5 mRNA sizes are attributed to the use of different polyadenylation processing sites (Sullivan *et al.*, 1986b).

In this chapter the presence of the conserved 22 nt splice leader on ß1-tubulin mRNA of the filarial nematode *B. pahangi* is reported. This 22 nt splice leader sequence is acquired through a trans-splicing mechanism in *C. elegans* and *A. lumbricoides* (Hannon *et al.*, 1990; Bektesh *et al.*, 1988) and has been identified in *B. malayi* (Zeng *et al.*, 1990). As demonstrated, the ß-tubulins of *B. pahangi* are encoded by a gene family and most of the previously reported mRNAs which carry a splice leader sequence are products of gene families (Bektesh *et al.*, 1988; Takacs *et al.*, 1988). It has also been shown that the *B. pahangi* ß1-tubulin mRNAs of both microfilariae and adult worms acquire the conserved 22 nt splice leader sequence. However, this splice leader sequence was not detected on the ß2-tubulin mRNAs. Detailed characterization of the 5' flanking regions of the ß2-tubulin gene is necessary to confirm the above result.

The PCR and primer extension data indicate that the 3' splice acceptor chosen for the addition of the 22 nt splice leader is located 42 bases upstream of the initiation codon. It is interesting to note that there is an adenosine residue (see Fig. 3.5B) located 19 bases away from the chosen 3' splice acceptor site and the sequence surrounding this adenosine residue conforms to the cis-splicing branch point consensus sequence (Py-X-Py-U-Pu-A-Py) in 6 out of 7 positions (Reed and Maniatis, 1985). Y intermediates characteristic of trans-splicing are formed at A residues 18 and 19 nucleotides upstream of a splice acceptor in an *A. lumbricoides* cell free extract. The sequence surrounding A(-18) conforms to the consensus branch point cis-splicing sequence at 5

out of seven positions (Hannon *et al.*, 1990). The presence of this A residue and its position relative to the 3' splice site at -42 in the *B. pahangi* ß1-tubulin 5' flanking sequence may be responsible for the selection of this 3' splice site. An A residue, surrounded by a similar consensus sequence is located 19 nucleotides upstream of the putative splice acceptor at -3, but the splice acceptor at -11 is found between these two sequences. Given the similarities observed between cis-splicing and trans-splicing (Tschudi and Ullu, 1990), the observation in cis-splicing that the AG dinucleotide closest to the branch point sequence is used exclusively as the 3' splice acceptor site and that cis-splicing is significantly reduced when the branch point distance from the 3' splice site is only 9 nucleotides (Smith *et al.*, 1989), it seems reasonable to assume that the splice acceptor at position -42 in the ß1-tubulin 5' flanking sequence is chosen because of its location at a suitable distance from a putative branch point A residue.

This is the first report of a nematode ß-tubulin (ß2) which is predominantly expressed in adult male worms and the identification of a conserved 22 nt splice leader on a nematode ß-tubulin mRNA.

CHAPTER 4 - GENERAL DISCUSSION

This study presents the isolation, characterization and distinct expression patterns of two *Brugia pahangi* ß-tubulin genes. The ß1-tubulin transcripts are found in adult worms and microfilariae and they acquire the conserved 22 nucleotide nematode spliced leader during maturation. In contrast, the ß2-tubulin is expressed primarily in adult male worms and no DNA fragments corresponding to ß-tubulin could be generated in PCR reactions containing the conserved 22 nucleotide splice leader sequence. A detailed analysis of *B. pahangi* microfilarial and adult ß-tubulin isoforms is also presented and suggests that the ß1-tubulin isoforms are highly represented in *B. pahangi*. These findings contribute to our limited knowledge of nematode tubulin gene families, their products and filarial molecular genetics.

The Southern blot hybridization analyses presented in this study indicate that *B. pahangi* ß-tubulin is the product of a gene family composed of members which are more divergent from one another than those within the *Caenorhabditis elegans* genome. These analyses also show that the *Haemonchus contortus* ß-tubulin cDNAs recognize *B. pahangi* ß-tubulin DNA fragments more readily (at higher stringency) than the homologous ß1-tubulin sequence. Southern blot hybridizations of *B. pahangi* genomic DNA digested with several restriction enzymes performed in the presence of 20-40% formamide using several probes show 3-5 hybridizing DNA fragments suggesting that this number of ß-tubulins genes may be present in the *B. pahangi* genome (Fig. 2.6 and Appendix B) This may be an overestimate since one pseudogene was found in the potentially protective 63 kDa antigen gene family of *B. malayi* (Perrine *et al.*, 1988). The two ß-tubulin genes which have been characterized in this study are found within the *B. pahangi* genome as single copy genes Thus, it appears that the organization of the *B. pahangi* ß-tubulin gene family resembles that of other invertebrates such as *C. elegans* which has 3-4 ß-tubulin genes (Gremke, 1987; Savage *et al.*, 1989; Driscoll *et al.*, 1989) and *D. melanogaster* which has 4 ß-tubulin genes (Natzle and McCarthy, 1984).

Current research efforts are directed at characterizing the ß-

tubulin gene families of the parasitic intestinal nematodes, *H. contortus* (Klein and Geary; Roos *et al.*, personal communications) and *Trichostrongylus colubriformis* (LeJambre *et al.*, personal communication), but the only complete nematode ß-tubulin gene sequences reported in the literature are those of the free-living nematode *C. elegans* (Gremke, 1987; Savage *et al.*, 1989; Driscoll *et al.*, 1989). Despite the evolutionary distance between nematode species (Qu *et al.*, 1986; Gill *et al.*, 1988) a high level of sequence identity between ß-tubulin sequences is observed. For example, the amino acid identity shared between the *B. pahangi* ß1-tubulin and the *C. elegans* ben-1, tub-1 and mec-7 ß-tubulins is of 94%, 90% and 88%, respectively. The later two comparisons are similar to the comparison of the *B. pahangi* ß1-tubulin and the chicken ß2 (89%), the chicken ß3 (89%) and the mouse ß5 (89%) tubulins (Table 2.1). This is not surprising given the conserved nature of tubulin proteins (Little and Seehaus, 1988). Little (1985) states that tubulin is not suitable as a molecular clock because of its non-linear evolution. His conclusions were derived from the observations that functionally similar tubulins from taxonomically distant species can share a higher percentage identity than functionally dissimilar tubulins from the same organism. Since mec-7 and ben-1 ß-tubulins of *C. elegans* (involved in 15-protofilament and 11-protofilament microtubules respectively) are 92% identical and ben-1 and the *B. pahangi* ß1-tubulin are 94% identical, it is likely that the ß1-tubulin of *B. pahangi* is a component of 11-protofilament microtubules. A recent re-classification of ß-tubulin amino acid sequences takes into account non-vertebrate ß-tubulin sequences (Burns and Surridge, 1990). These researchers suggest that ß-tubulins fall into three broad types and that most non-vertebrate ß-tubulins share a very high sequence identity with the vertebrate type I tubulins. This is based on the sequence identity of amino acids flanking residues 35, 55-57 and 124, which exhibit intra-species heterogeneity but inter-species conservation not unlike the isotype defining carboxy-terminal regions of ß-tubulin (Sullivan and Cleveland, 1986). The vertebrate type I ß-tubulins include the class I and IV ß-tubulins of the Sullivan classification scheme, which include mß3, mß5, cß4 and cß3 tubulins (Table 1.1). The

B. pahangi ß1-tubulin sequences for the three regions within the amino terminus resemble those of the vertebrate type I ß-tubulins, but it has a significantly divergent C-terminus compared to the type I vertebrate ß-tubulins. A similar observation was made for the ß1 and ß2-tubulins of the ciliate *Stylonichia* which was included in the analysis performed by Burns and Surridge (1990). A look at the carboxy-terminal regions of the nematode ß-tubulins presented in Fig. 2.5 shows the absence of any recognizable isotypic class for nematodes. However, the anti-peptide antibody raised against the carboxy-terminal domain of the *B. pahangi* ß1-tubulin recognizes ß-tubulin from *B. malayi*, a closely related species (see Appendix C1). Furthermore, the *B. pahangi* ß1-tubulin DNA fragment coding for the last 40 amino acids hybridizes to *B. malayi* genomic DNA fragments under high stringency conditions suggesting that a homologous ß-tubulin gene is found in *B. malayi* (Tang, 1988). These observations are analogous to those of Rudolph *et al.* (1987) who showed that the carboxy-terminal region of the ß2-tubulin of *D. melanogaster* is conserved in several *Drosophila* species. The recognition of the *A. suum* ß-tubulin(s) with the *B. pahangi* ß1-tubulin antipeptide antibody (Appendix C1) is more puzzling and would require sequence comparisons of the ß-tubulin carboxy-terminal regions of *A. suum* and *B. pahangi*, but sequences for the *A. suum* ß-tubulins have not been reported. Neither the ß-tubulins of *H. contortus* eggs nor those of adult *D. immitis* are recognized by this antibody.

An interesting proposal which arises from the analysis of tubulin genes, their patterns of expression and the microtubule arrays they take part in, is that ß-tubulins which are divergent are limited in the microtubule arrays in which they function. For example, the *P. polycephalum* multinucleate plasmodial ß2-tubulin functions solely in the mitotic spindle (Burland *et al.*, 1988). This cell type does not contain cytoskeletal, flagellar or centriolar microtubules which are found in the amoebal or flagellate cell types of *Physarum* (Havercroft and Gull, 1983). The identity of the *Physarum* ß2-tubulin to the *Physarum* amoebal ß1-tubulin is only 83% suggesting that the divergence has arisen as a result of amino acid substitutions which are tolerated in ß-tubulins functioning in few microtubule arrays (Burland *et al.*, 1988). As

mentioned previously, the ß3-tubulin of *D. melanogaster* has a limited microtubule function (Hoyle and Raff, 1990) and it diverges from ß2-tubulin by 13% (Rudolph *et al.*, 1987). If this proposal is extended to the *B. pahangi* ß1-tubulin, while taking into consideration the following observations: ß1-tubulin is highly represented in *B. pahangi* adults and microfilariae and the ß1-tubulin shares the highest degree of identity with the *C. elegans* ben-1 ß-tubulin which is found in several cell types; it is not unreasonable to postulate that the ß1-tubulin of *B. pahangi* is a component of many nematode cell types and is involved in many microtubule structures (few cells have such limited microtubule arrays). Immunocytochemistry using an antibody specific to ß1-tubulin is required to support this postulate. Helm *et al.* (1989) developed a monoclonal antibody against a *B. pahangi* fusion protein containing the 40 carboxy-terminal amino acids of ß1-tubulin. The monoclonal antibody produced (6D8) reacted with muscle blocks, the intestinal brush border and embryonic uterine microfilariae (Helm *et al.*, 1989). However, the study of Helm *et al.* (1989) does not provide any conclusive data on the cellular distribution of ß1-tubulin because 6D8 may cross-react with other *B. pahangi* ß-tubulins since it cross-reacts with chicken ß-tubulins. The transcripts of the *B. pahangi* ß2-tubulin are predominant in adult male worms and therefore, may be testis-specific like the *D. melanogaster* ß2-tubulin (Kemphues *et al.*, 1982). The *D. melanogaster* ß2-tubulin 1.8 kb transcripts are found in 0-3 hour old embryos (Natzle and McCarthy, 1984). Assuming that the *B. pahangi* ß2-tubulin is testis-specific the detection of ß2-tubulin mRNAs in mature *B. pahangi* females can be explained by the presence of embryos in various stages of development. The *D. melanogaster* testis specific ß2-tubulin, which is incorporated into all sperm microtubule structures including the flagellar microtubules, is highly homologous to the ubiquitous *D. melanogaster* ß1-tubulin and the vertebrate major neuronal isoform, cß2 (94 and 95%, respectively). Hoyle and Raff (1990) have provided direct experimental evidence that doublet microtubules of the axoneme cannot be formed by the divergent ß3-tubulin. This is consistent with the proposal of Little (1985) who suggested that it is the incorporation of ß-tubulin into the axonemal microtubules of cilia and flagella which

constrains the mutation rate of ß-tubulin. In contrast, the *B. pahangi* ß2-tubulin appears to have diverged significantly from the *B. pahangi* ß1-tubulin because hybridization of ß1 to ß2-tubulin fragments occurs only under conditions which allow highly mismatched ß-tubulin DNAs to cross-hybridize (20% formamide). Furthermore, a comparison of the 28 predicted amino acids of *B. pahangi* ß2-tubulin to ß1-tubulin shows an identity lower (75%) than the two most divergent ß-tubulins of *C. elegans*, mec-7 and ben-1, which for this region are 82% identical. Given these observations one might question the proposed sperm-specific expression of the *B. pahangi* ß2-tubulin because of its apparent divergence from the *B. pahangi* ß1-tubulin. However, the nematode spermatozoa are non-flagellated crawling cells (Anya, 1976). Therefore, the absence of flagella in nematode sperm cells may have allowed the ß2-tubulin of *B. pahangi* to diverge from ß1-tubulin. It would be interesting to determine whether the more conserved ß1-tubulin is incorporated in the axonemal microtubules of the cilia found in the sensory organs of *B. pahangi* (Laurence and Simpson, 1974).

The presence of a trans-spliced leader sequence has not previously been reported for nematode tubulin mRNAs. The tubulin transcripts of *Trypanosomes* (Imboden *et al.*, 1986; Sather and Agabian, 1985) acquire a 39-nucleotide splice leader. However, hybrid arrested translation experiments indicate that all trypanosome mRNAs acquire the SL (Walder *et al.*, 1986). Results of this study show that the ß1-tubulin transcripts of *B. pahangi* acquire the conserved 22-nucleotide nematode splice leader. Further sequence analysis of the ß2-tubulin gene and characterization of its mRNA products are required before any conclusions can be made about the steps involved in the maturation of ß2-tubulin mRNAs. Since the function of the splice leader is unknown for nematodes, it is difficult to speculate about the impact of this processing event on tubulin gene expression and tubulin synthesis. However, studies of trypanosome trans-splicing suggest that the acquisition of a trans-splice leader sequence may influence mRNA stability, increase translational initiation or direct intracellular transport and/or location (Donelson and Zeng, 1990). If the former two suggestions prove to be true this will be one more mechanism, aside from

the possible autoregulation and the choice between alternative 3' flanking sequences, through which tubulin mRNA levels and tubulin synthesis can be regulated in nematode cells.

One of our initial aims in this study was to provide information about the ß-tubulins of nematodes in order to increase our understanding of the molecular interaction of BZ drugs with nematode microtubules. The most compelling evidence for the identification of a BZ/tubulin binding region is provided by Jung and Oakley (1990), which implicates the region surrounding residue 165 in the binding of the BZ R_2 group. Their conclusions are based on the differential sensitivity of the *A. nidulans* benA16 mutant for growth on media containing either thiabendazole or benomyl. This mutant which carries a valine in the place of alanine at residue 165 is resistant to thiabendazole, but supersensitive to benomyl, yet these two drugs only differ in the R_2 group. A summary of amino acid residues at position 165 provided by these authors indicates that six fungal ß-tubulins have alanine at this residue, metazoan ß-tubulins including insects and vertebrates have asparagine at this residue, in higher plants and algae either a leucine or a methionine is found at amino acid 165 and protozoans have either aspartic acid, cysteine, glutamic acid or methionine at this position. The nematode ß-tubulins sequenced thus far (Fig 2.5), have either alanine, serine or in the case of mec-7, which is involved in benomyl resistant 15-protofilament microtubules (Chalfie and Thomson, 1982), there is an asparagine residue at position 165. It is interesting to note that the 15-protofilament microtubules of *C. elegans* are depolymerized by colchicine (Chalfie and Thomson, 1982). Since serine can be thought of as a hydroxylated version of alanine, the ß-tubulins of nematodes resemble fungal ß-tubulins at position 165 and this is reflected in the sensitivity to BZ drugs shared by these organisms Both mebendazole and benomyl resistant mutants of *C. elegans* carry deletions for the ben-1 ß-tubulin gene (Woods *et al.*, 1989; Driscoll *et al.*, 1989). The ß-tubulin product of ben-1 is believed to be the only BZ-sensitive ß-tubulin expressed in *C. elegans* (Driscoll et al., 1989) and it has a serine residue at amino acid position 165. However, so does the tub-1 gene of *C. elegans*. Since Jung and Oakley (1990) do not

suggest that amino acid 165 is the only BZ-binding site of ß-tubulin and the ben-1 and tub-1 ß-tubulins differ in six amino acid residues between 1 and 430, these two findings may eventually be reconciled when the BZ binding site of tubulin is defined. Given the complexity of the interaction between BZs and tubulin, this interaction is best studied by directly relating amino acid substitutions to *in vivo* effectiveness of the BZ drugs or to *in vitro* binding of the BZs to tubulin using drugs which differ only in one chemical group. The results presented within provide the initial information required for the pursuit of such experiments.

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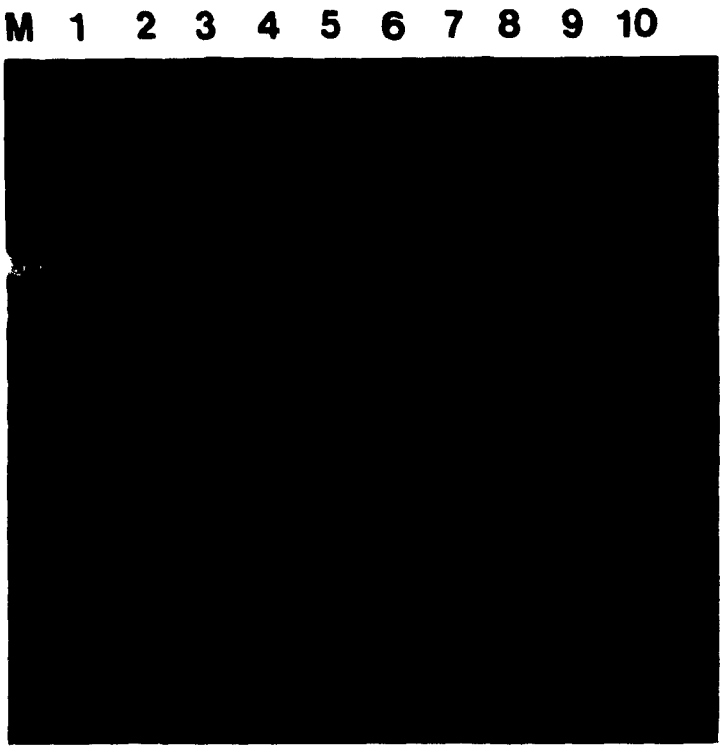
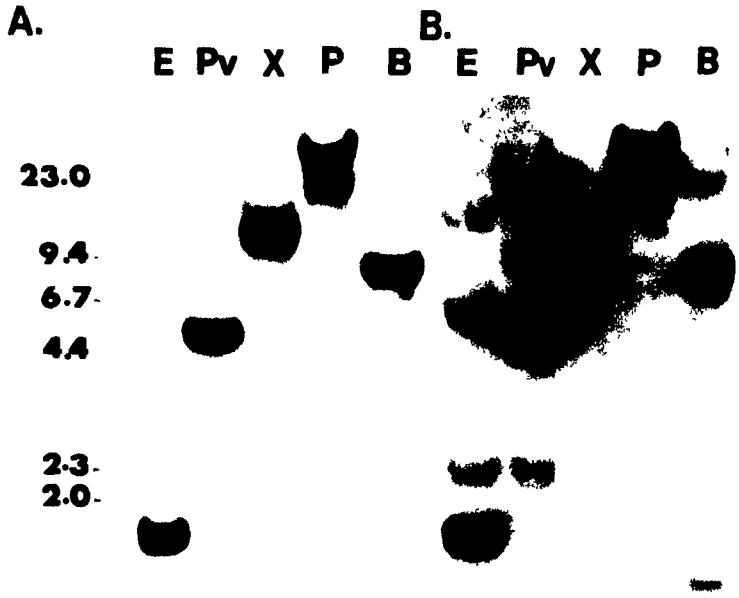
APPENDIX A. CREATION OF EXONUCLEASE III DELETIONS FOR DNA SEQUENCING

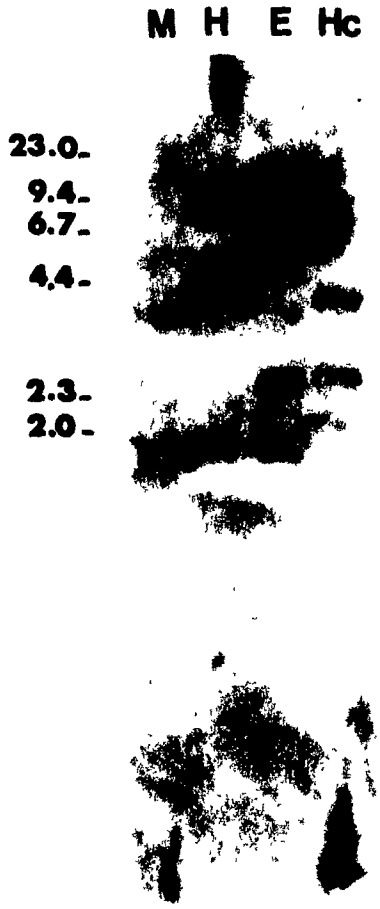


Appendix A.1 Exonuclease III digest of a pBTX subclone. The 1.8 kb *Eco*RI fragment of pBTX was subcloned into the *Eco*RI site of pIBI30 and digested with *Xba*I and *Pst*I. The linearized plasmid obtained was digested with Exonuclease III and aliquots of this digest were transferred to an S1 nuclease containing mixture at 30 s intervals. The S1 nuclease reaction was allowed to continue for 30 mins. at room temperature. After stopping the reaction aliquots at time 0 (lane 1), 30 s (lane 2) and increasing time increments of 1 min. (lanes 3-10) were electrophoresed through a 1% agarose gel containing ethidium bromide (0.5 ug/mL). A *Hind*III digest of lambda DNA was used as a size marker (lane M).

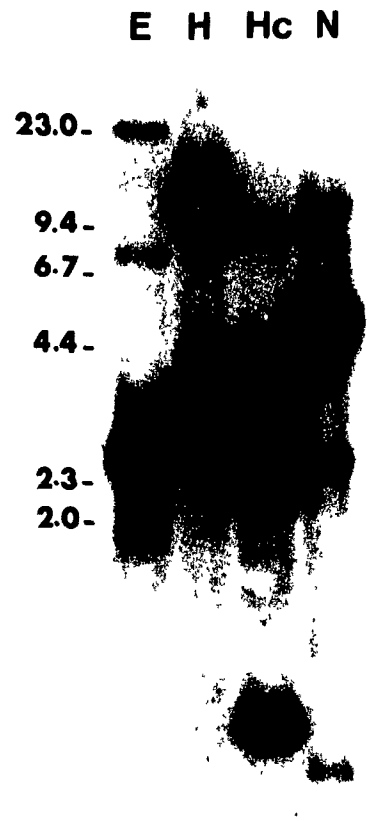
APPENDIX B. IDENTIFICATION OF THE *B. PAHANGI* ß-TUBULIN GENE FAMILY USING DIFFERENT ß-TUBULIN GENES AS PROBES



Appendix B.1. Southern blot analyses of *B. pahangi* genomic DNA probed with the 1.8 kb *Eco*RI fragment of clone pBTX. *B. pahangi* genomic DNA was digested with *Eco*RI (E), *Pvu*II (Pv), *Xba*I (X), *Pst*I (P), and *Bgl*II (B). 20 µg of each digest were loaded into two wells of the same 1% agarose gel in order to create duplicate filters after Southern transfer to Hybond-N. The filter in A. was hybridized in the presence of 50% formamide at 42°C and washed in 0.2x SSC, 0.1% SDS at 65°C. The filter in B. was hybridized in the presence of 20% formamide and washed in 2x SSC, 0.1% SDS at 50°C. Molecular weight markers in kilobases correspond to a *Hind*III digest of lambda DNA.

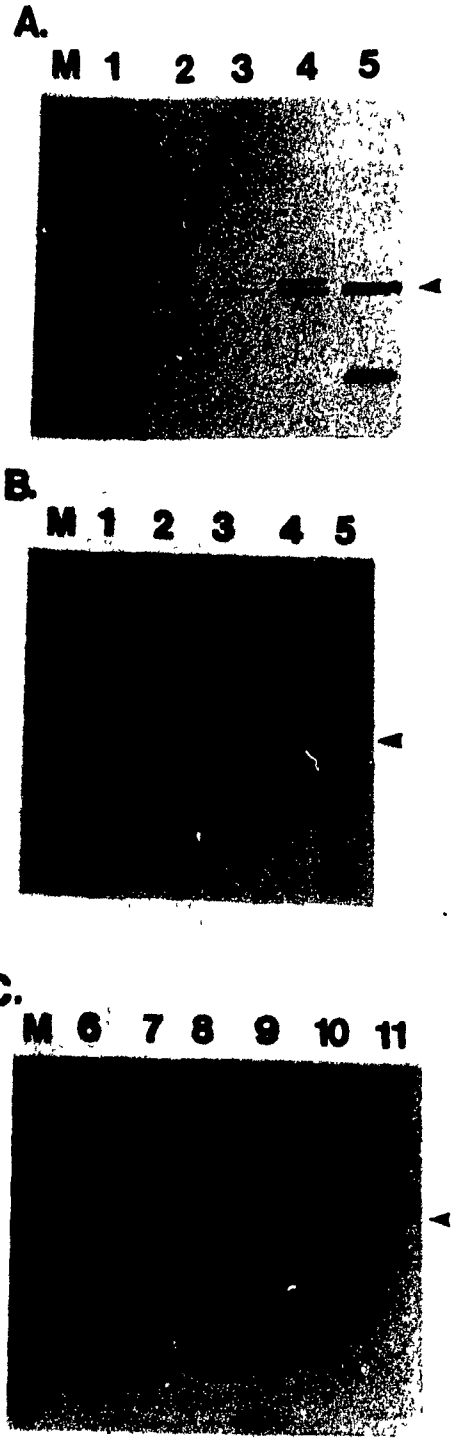


Appendix B.2. Southern blot analysis of *B. pahangi* genomic DNA probed with the 1.0 kb *Eco*RI fragment of the *H. contortus* cDNA clone 12-16. *B. pahangi* genomic DNA was digested with *Hind*III (H), *Eco*RI (E), and *Hinc*II (Hc). The DNA fragments were fractionated through a 1% agarose gel and transferred to Hybond-N. The hybridization was performed in the presence of 35% formamide at 42°C and the filter was washed in 2x SSC, 0.1% SDS at 65°C. M. represents a lane containing *Hind*III fragments of lambda DNA.

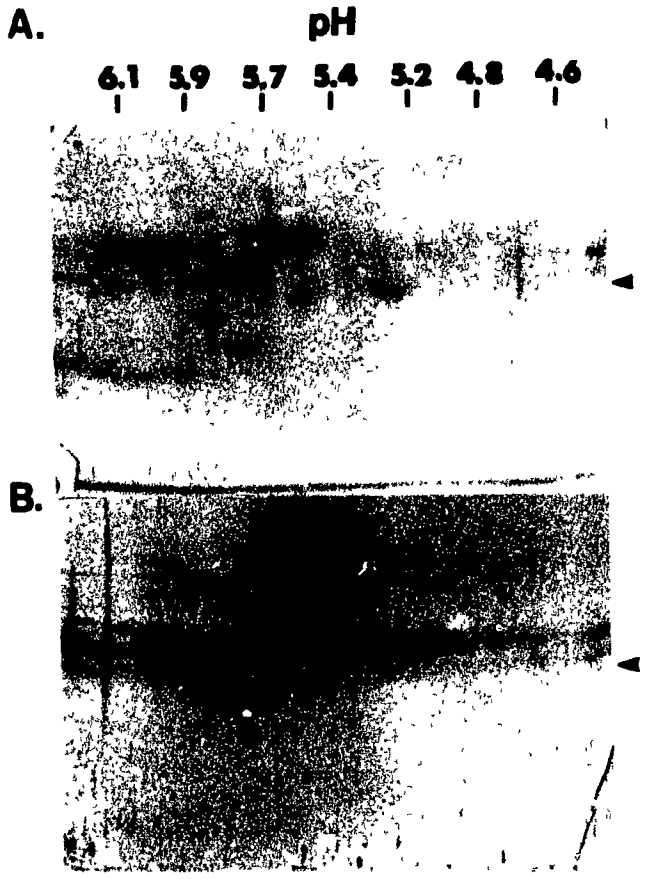


Appendix B.3. Southern blot analysis of *B. pahangi* DNA hybridized to the 2.4 kb *Eco*RI fragment of the recombinant phage 3-21 coding for ß2-tubulin. ***B. pahangi*** genomic DNA was digested with *Eco*RI (E), *Hind*III (H), *Hinc*II, (Hc) and *Nde*I. The DNA fragments were electrophoresed through a 1% agarose gel, transferred to Hybond-N and hybridized to the 2.4 kb *Eco*RI fragment of the recombinant phage 3-21 in the presence of 35% formamide at 42°C. The filter was washed in 2x SSC, 0.1% SDS at 50°C.

APPENDIX C. CROSS-REACTIVITY OF THE *B. PAHANGI* ANTIPEPTIDE ANTIBODY AND DETECTION OF TOTAL PROTEIN SEPARATED BY TWO-DIMENSIONAL GEL ELECTROPHORESIS.



See legend page 94



Appendix C.2. Detection of *B. pahangi* adult worm and microfilarial total protein separated by two dimensional gel electrophoresis. Protein of *B. pahangi* adult worms (A) and microfilariae (B) were separated by two dimensional gel electrophoresis and transferred to nitrocellulose. Proteins on the filters were detected using the BioRad Total Protein Detection kit as described by the manufacturer.

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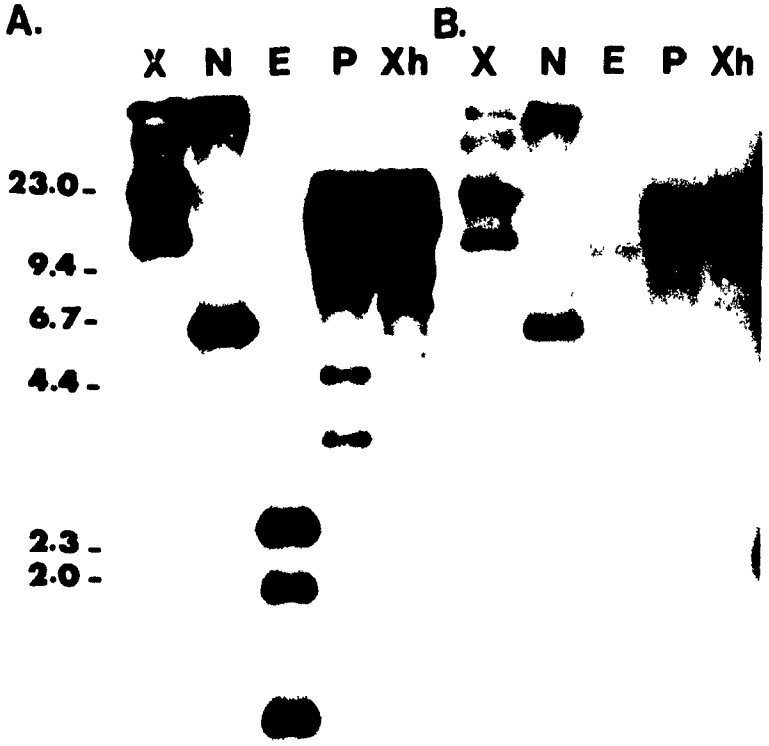
Appendix C.1. Cross-reactivity of the *B. pahangi* antipeptide antibody to ß-tubulins from various organisms. Extracts of total protein (20 μg) from various sources were electrophoresed through acrylamide gels (10% in A. and B. and 12% in C.) and transferred to nitrocellulose. The filter in A. was incubated with the phylum cross-reactive anti-ß-tubulin monoclonal antibody (1:2000 dilution) and the filters in B. and C. were incubated with the antipeptide antibody (1:50 dilution). Details of the method are found in the Experimental Protocol section of Chapter 2. The arrow indicates the tubulin band. Total protein was isolated from *B. pahangi* female worms (lane 1), *H. contortus* eggs (lane 2), *A. suum* muscle (lane 3), *A. suum* intestine (lane 4), *B. malayi* adult worms (lane 5), NIH-3T3 cells (lane 6), *B. pahangi* adult worms (lane 7), Jird peritoneal macrophage (lane 8), *B. pahangi* microfilariae (lane 9), *B. malayi* adult worms (lane 10) and *D. immitis* (lane 11).

APPENDIX D. IDENTIFICATION OF ß2-TUBULIN CODING REGIONS OF THE RECOMBINANT PHAGE 3-21.



Appendix D.1. Identification of restriction enzyme fragments of recombinant phage 3-21 coding for ß-tubulin. Phage 3-21 DNA containing *B. pahangi* genomic DNA coding for ß2-tubulin was digested with *Xba*I (X), *Nde*I (N), *Eco*RI (E), *Pst*I (P) and *Xho*I (Xh). Two identical Southern blots were obtained following transfer of size fractionated (1% agarose gel) DNA fragments to Hybond-N. The filter in A. was hybridized to the 1.0 kb *Eco*RI fragment of the *H. contortus* cDNA clone 12-16 and the filter in B. was hybridized to the 200 bp τ-labelled *Eco*RI fragment of clone 12-16 which codes for amino acids 338-407 in the presence of 50% formamide at 42°C. The filters were washed in 1x SSC at 65°C.