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Muscle development and attachment to the epidermis is accompanied by expression of ß3 and ß1 tubulin isotypes, respectively

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ABSTRACT **In *Drosophila* ß tubulins are encoded by a small gene family whose members are differentially expressed in a highly cell and tissue specific manner. Here we focus on the expression of the ß3 tubulin isotype during mesoderm differentiation and *ß1 tubulin* expression in the apodemes during embryonic development. The ß3 tubulin isotype is first detectable at the extended germband stage shortly before the separation of somatic and visceral derivatives. Comparing the distribution of the ß3 mRNA and the ß3 isotype shows that the transcription of the *ß3 tubulin* gene is cell type specifically repressed during differentiation of individual mesodermal derivatives, from which the dorsal vessel remains transcriptionally active until shortly before hatching. In contrast the ß3 tubulin protein is detectable in all mesodermal derivatives. The ß3 tubulin is an excellent marker to study mesoderm differentiation on a regulatory and cellular level using both genetics and molecular biology. In the visceral mesoderm, the expression of the *ß3 tubulin* gene is regulated by homeotic gene products, while other transactivators regulate expression in the dorsal vessel and the body wall musculature. In the somatic mesoderm, the ß3 tubulin allows to visualize myotube formation and insertion into the epidermis. This contact to the epidermal attachment sites (apodemes) induces *ß1 tubulin* expression, as can be seen in double staining experiments. We determined a 14bp cis-regulatory enhancer element guiding expression of the *ß1 tubulin* gene in these attachment sites. Using the ß1 and ß3 tubulin isotypes as markers we started to isolate mutants which are disturbed in muscle formation.**

KEY WORDS: *Drosophila embryogenesis, β tubulin, muscle development, muscle attachment, gene regulation*

The formation of the mesoderm and its derivatives is controlled by a cascade of transcriptional activators

Mesoderm formation in *Drosophila* is a well studied biological process. The dorsoventral axis is prefigured in the egg by maternally active genes culminating in the nuclear localization of the transcription factor dorsal (for review see St. Johnston and Nüsslein-Volhardt, 1992). Dorsal activates the genes *twist* and *snail* and thus leads to invagination of mesodermal cells during gastrulation (Leptin *et al.*, 1992; Reuter and Leptin, 1994). From this time point onwards the mesodermal cells express *mef2*, a gene encoding a homolog to the transcription factor Mef2 of vertebrates, which activates a number of muscle specific genes (Lilly *et al.*, 1994; Nguyen *et al.*, 1994). At the end of gastrulation the ventral region, from which the somatic mesoderm will derive, is in direct contact to the neurogenic region. Shortly after the mesoderm starts to divide into its derivatives: the somatic mesoderm gives rise to the body wall musculature, while the visceral mesoderm develops into the muscle layer surrounding the gut, the mesoderm of the gonads and the dorsal vessel, which has his origin in the most dorsally localized mesodermal cells. At least two genes are involved in the specification of the mesodermal derivatives. *bagpipe* is responsible for the development of the visceral mesoderm while *tinman* is involved in the specification of the visceral mesoderm as well as of the dorsal vessel (Bodmer *et al.*, 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). On the cytological level Bate (1990) described that first muscle founder cells are formed, which recruit some myoblasts to form muscle precursor cells, which then induce fusion of more myoblasts to form individual myotubes. Several genes have been analyzed as being expressed in such subsets of muscle precursor cells of the somatic mesoderm for example *nautilus* (Michelson *et al.*, 1990; Paterson *et al.*, 1991; Abmayr *et al.*, 1992), *S59* (Dohrmann *et al.*, 1990), *connectin* (Nose *et al.*, 1992) and *apterous* (Cohen *et al.*, 1992 and Bourgouin *et al.*, 1992). All of which might be involved in specification of the final muscle pattern. All genes involved in this genetic cascade encode DNA binding proteins. Once the myotubes are formed they are anchored in the epidermis at the muscle attachment sites, the

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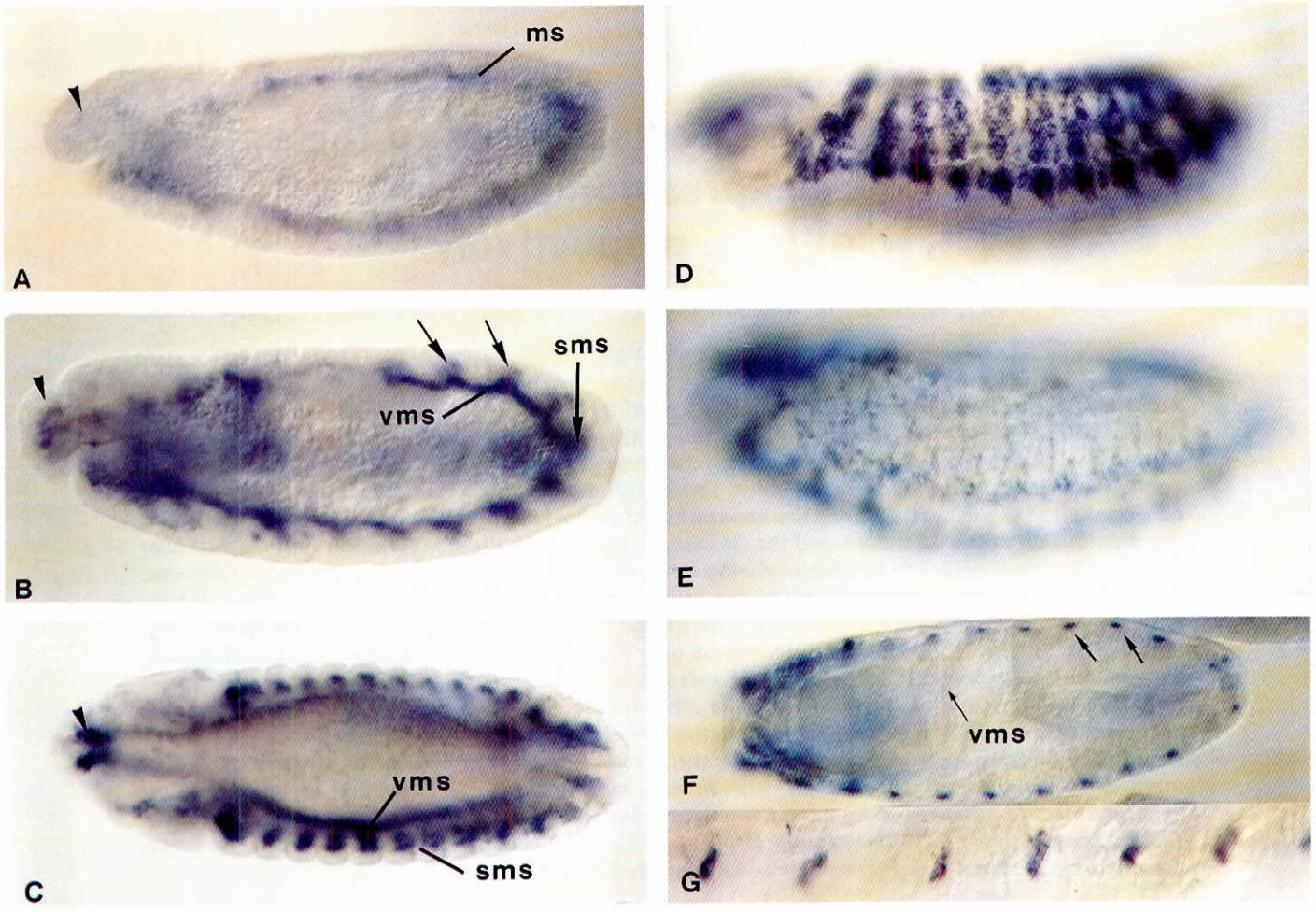

Fig. 1. β3 tubulin mRNA localization during stage 10 to 16 of embryogenesis as revealed by whole-mount *in situ* hybridization *(Tautz and Pfeiffle, 1989). Staging follows Campos-Ortega and Hartenstein (1985).* **(A)** *Lateral view of a stage 10/11 embryo. Staining is visible in the mesodermal germlayer (ms).* **(B)** *The separation of the visceral (vms) and somatic (sms) mesoderm becomes visible (late stage 11). In each segment a group of cells (arrow), precursors of the somatic muscles, appears between the visceral mesoderm and the outer ectoderm. A weak staining is visible in the pharynx muscles (arrowhead).* **(C)** *The separation of the visceral and somatic muscles is complete (stage 12).* **(D)** *At stage 14/15 β3 tubulin mRNA is detectable in all somatic muscles.* **(E)** *The β3 mRNA expression level in the somatic muscles decreases dramatically during stage 15 (with some exceptions, see text).* **(F)** *At stage 16 (dorsal view) the β3 mRNA is restricted mainly to cells of the chordotonal organs (arrows), and the dorsal vessel (Fig. 4).* **(G)** *Higher magnification of the chordotonal organs in a lateral view.*

apodemes. Electronmicroscopic analysis by Crossley (1978) revealed that numerous microtubular arrays accumulate at the muscle edge inserting into the apodemes, as well as at the side of the epidermal cells. Furthermore specific integrins are localized in the apodemes which are essential for the connection between muscles and epidermis, which is clearly evident from *myospehroid* mutants (Leptin *et al.*, 1989; Brown, 1994). Several other genes have been shown to be specifically expressed in the apodemes. Two of them encode transcription factors (*stripe*; Volk *et al.*, 1993 Lee *et al.*, 1995; *delilah;* Armand *et al.*, 1994), while others like *tiggrin* (Fogerty *et al.*, 1994) or *msp310* (Volk, 1992, Volk and VijayRaghavan, 1994) represent extracellular matrix components. For none of these genes is the expression depending on muscle insertion into the epidermis, while there is strong evidence that the *β1 tubulin* expression is indeed induced by the muscle insertion (Buttgereit, 1993).

β tubulin isotypes are cell type specifically expressed during *Drosophila* development

In *Drosophila*, α and β tubulins are encoded by a small gene family (Sullivan, 1985). Isotype specific antibodies raised against the C-terminal isotype specific regions allowed us to analyze the *in vivo* distribution of individual β tubulin isotypes (Leiss *et al.*, 1988; Buttgereit *et al.*, 1991; Kaltschmidt *et al.*, 1991). During spermatogenesis, the β1 tubulin isotype is characteristic for mitotically active germ cells while the β2 tubulin isotype characterizes all microtubular arrays from meiotic prophase onward (Kaltschmidt *et al.*, 1991). The cell type specific expression is under control of short regulatory elements in the male germ line (Michiels *et al.*, 1989, 1991, 1993; Buttgereit and Renkawitz-Pohl, 1993). During embryogenesis *β1* and *β3 tubulin* are expressed (Rudolph *et al.*, 1987; Gasch *et al.*, 1988). The *β3 tubulin* is mainly expressed during differentiation of the meso-

dermal germ layer (Leiss *et al.*, 1988; Kimble *et al.*, 1989). The *β1 tubulin* is encoded maternally and supplies the embryo with β tubulin for the early stages of development. Zygotic expression of *β1 tubulin* is limited to the central and peripheral nervous system and the apodemes (Buttgereit *et al.*, 1991). Thus, at the sites of muscle insertions in the epidermis, the β3 tubulin is in the microtubular arrays of the myotubes, while β1 tubulin is characteristic for the apodemes (see below).

Expression pattern of the *β3 tubulin* gene follows mesodermal differentiation during embryogenesis

Using whole-mount *in situ* hybridization we looked for the expression pattern of the *β3 tubulin* gene during mesoderm differentiation. The β3 tubulin mRNA amount changes in a time and tissue specific manner during embryogenesis. Some of these changes are not detectable with the β3 tubulin antibody (Leiss *et al.*, 1988) because of the high stability of the β3 tubulin protein. Here we focused on some of the differences between protein and mRNA distribution which have not been published yet.

The time course of the β3 tubulin mRNA expression is shown in Fig. 1A-G. At stage 10/11 the β3 tubulin mRNA is clearly visible in the mesodermal germlayer (Fig. 1A). At this stage the precursor cells of the pharynx musculature are stained very weakly but become clearly visible from stage 12 onward (Fig. 1A-C, arrowhead). When the mesoderm splits into a somatic and a visceral component, cells from both tissues transcribe the *β3 tubulin* gene (Fig. 1B). Our results indicate that first single cells which are in close contact to the outer side of the mesodermal layer are positive for β3 tubulin. During the next minutes the size of the cell clusters, which express *β3 tubulin*, increases (Fig. 1B+C).

In difference to some other genes like *S59* (Dohrmann *et al.*, 1990), *nautilus* (Abmayr *et al.*, 1989; Michelson *et al.*, 1990; Peterson *et al.*, 1991), *apterous* (Cohen *et al.*, 1992, Bourgouin *et al.*, 1992), which are expressed in a subset of the muscle precursors, the β3 tubulin mRNA is present in all muscle precursors and the fusing myoblasts of the somatic mesoderm (Fig. 1D). Expression of the *β3 tubulin* gene starts earlier than of the genes encoding the transcriptional activators mentioned above with the exception of Mef2, a transcription factor that is expressed early in all somatic muscle precursors well before the separation of somatic and visceral precursors (Lilly *et al.*, 1994; Nguyen *et al.*, 1994). Thus, the *mef2* gene product is a potential regulator of the *β3 tubulin* gene.

From stage 14 onward distinct differences between the β3 mRNA pattern (Fig. 1D-F) and the protein distribution (Fig. 2+3) become evident (Leiss *et al.*, 1988). Within a hemisegment the mRNA level decreases gradually from the dorsal group to the ventral group of body wall muscles (Figs. 1E and 2A), while we observe no differences along the anterior-posterior axis. In contrast, the protein is found up to stage 16/17 in all somatic and visceral derivatives, including the muscles of the body wall (Fig. 3D), the gut, heart and pharynx (Fig. 2C+D). The β3 tubulin mRNA level, however, decreases at stage 14/15 in a time and tissue specific manner. At this time the mRNA also vanishes from the gut musculature. The mRNA in the cardioblast cells of the dorsal vessel and the pharynx muscles (Fig. 2A+B), and in some cells of the chordotonal organs remains detectable during dorsal closure until shortly before hatching (Fig. 1F+G). Therefore we

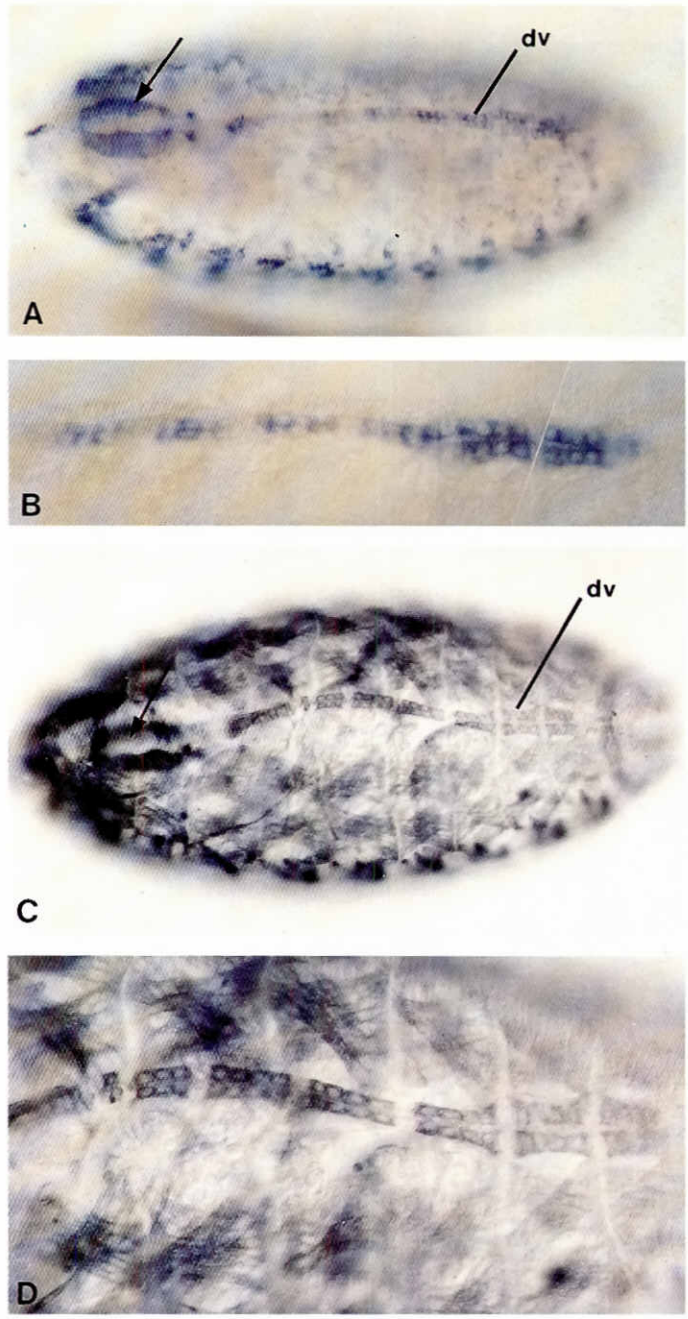

Fig. 2. Localization of the β3 tubulin mRNA becomes restricted to single tissues during late stages of embryogenesis. (A and B) *A β3 tubulin whole-mount in situ hybridization staining;* **(C and D)** *an anti β3 tubulin antibody staining.* **(A)** *Stage 15 embryo in a dorso-lateral view. The β3 tubulin mRNA level decreases in the body wall musculature, starting in the dorsal muscle group (compare with* **C***). Staining in the pharynx muscles (arrow) and the cardioblasts of the dorsal vessel (dv) is visible.* **(B)** *Expression of β3 tubulin in the dorsal vessel of a stage 15/16 embryo. The segmentally 4-cell-wide stripe expression pattern is clearly visible. Only cardioblasts, but not pericardial cells are stained.* **(C)** *Stage 15 embryo in a dorso-lateral view. The β3 tubulin protein is present in all muscles of the body wall musculature. Staining in the pharynx muscles (arrow) and the cardioblasts of the dorsal vessel (dv) is visible.* **(B)** *Higher magnification of the dorsal vessel. The segmentally 4-cell-wide stripe protein pattern is clearly visible. Only cardioblasts, but not pericardial cells are stained.*

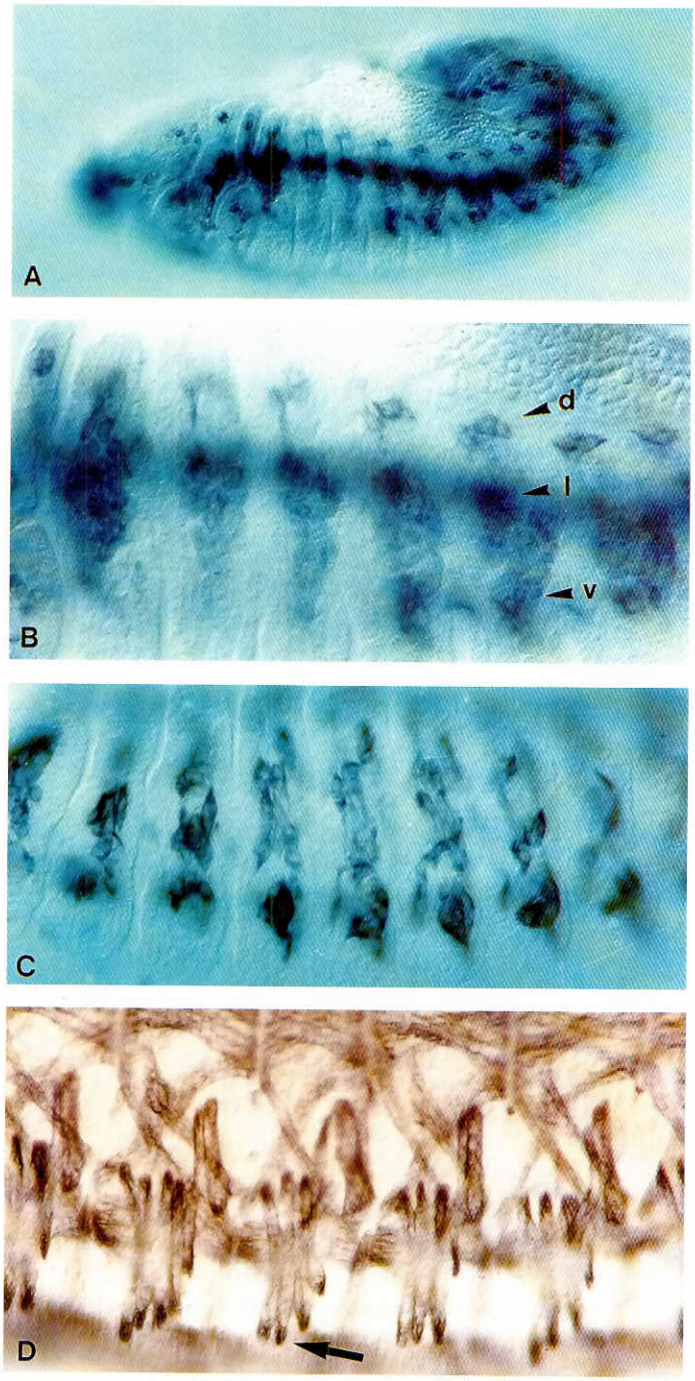

Fig. 3. β3 tubulin protein distribution in the body wall musculature. **(A)** *Embryo at the stage of germband retraction.* **(B)** *The same embryo at a higher magnification. The formation of the segmentally arranged dorsal (d), pleural (l) and ventral (v) somatic muscle group is visible.* **(C)** *Stage 13/14 embryo. The fusion of myoblasts to myotubes can be followed in each of the dorsal, lateral and ventral muscle group.* **(D)** *At stage 16 the subcellular localization of the β3 tubulin protein at the tips and the surface of single myotubes is clearly visible (arrow). At this timepoint the myotubes do not express the β3 tubulin mRNA as shown with in situ hybridization (Fig. 1), whereas in other tissues (e.g. heart) expression persists.*

postulate regulatory elements in the *β3 tubulin* gene driving the differential expression e.g. in the somatic muscles and the heart muscles during late embryogenesis (see below). Besides *mef2*, which is also expressed in the dorsal vessel, the *tinman* gene product is a potential regulator for the heart specific expression of *β3 tubulin*. This transcription factor is expressed in the same pattern of segmentally repeated 4-cell-wide stripes as has been shown for the *β3 tubulin* gene (Azpiazu *et al.*, 1993; Bodmer 1993). The four cells extend from the anterior to the posterior end of a segment, whereas the *β3 tubulin* non-expressing cells mark the border between two segments (Fig. 2A-D).

At stage 13 the fusions of somatic myoblasts to syncitial myotubes is visible by following the β3 tubulin antibody staining (Fig. 3C). Each somatic muscle precursor group at stage 12 contains approximately 4-8 spindle-shaped cells, some of them are arranged in close contact, which might be a starting position before the onset of fusions. After reaching the syncitial stage, the cytoplasmic β3 tubulin is localized mainly at the tips of the myotubes inserting into the epidermis, which can be seen very clearly during late stages (Fig. 3C).

In conclusion, the *β3 tubulin* gene is an excellent tool to analyze embryonic muscles and allows to follow the fusion process of embryonic myoblasts to myotubes (Leiss *et al.*, 1988; Bate, 1990). We have used this tool to find new mutants with defects in the muscle differentiation program. The first results of this screen indicate that the *not enough muscles (nem)* gene is essential for the development of the complete pattern of the body wall musculature (Burchard *et al.*, 1995). The *rolling stone* gene (*rost)* is necessary for the fusion of myoblasts to myotubes (Paululat *et al.*, 1995).

Different regulatory elements guide *β3 tubulin* expression in individual mesodermal derivatives

The mesodermal cells express numerous DNA-binding proteins in a spatially regulated manner, for example Mef2, Nautilus (MyoD homolog), Apterous and S59 in the somatic mesoderm and developing myotubes (Abmayr *et al.*, 1989; Dohrmann *et al.*, 1990; Michelson *et al.*, 1990; Peterson *et al.*, 1991; Bourgouin *et al.*, 1992; Cohen *et al.*, 1992; Lilly *et al.*, 1994; Nguyen *et al.*, 1994). *tinman* and *mef2* are expressed in the cardioblasts of the dorsal vessel (Azpiazu *et al.*, 1993; Bodmer, 1993). The *tinman* gene is furthermore expressed in the visceral mesoderm, as are *bagpipe* and *H2.0*, which code for homeobox containing proteins (Barad *et al.*, 1988; Azpiazu *et al.*, 1993; Bodmer, 1993). Further transcription factors encoded by homeotic genes like *sex comb reduced*, *antennapedia*, *ultrabithorax* and *abdominal A* are expressed in the visceral mesoderm in a temporally and spatially regulated manner. This expression pattern is essential for gut morphogenesis (for review see Bienz, 1994).

We aimed to clarify whether these transcription factors play a role in the regulation of the *β3 tubulin* gene. Therefore we performed an extensive deletion analysis of the *β3 tubulin* gene, the results of which are summarized in Figure 4. The *β3 tubulin* gene is first transcribed in the mesoderm, shortly before the separation of the visceral and somatic precursors is visible (Leiss *et al.*, 1988). The separation of mesodermal derivatives is reflected in the regulatory capacity of the *β3 tubulin* gene. Thus, the upstream region between -1,2 and -6,0 contains separable reg-

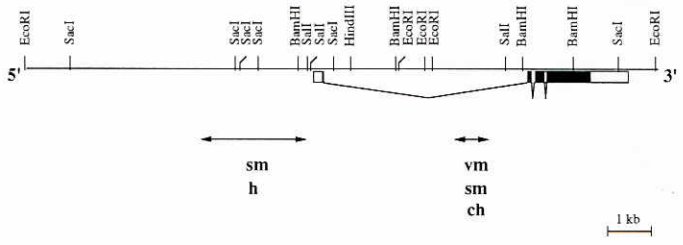

Fig. 4. Structure and regulatory units of the *β3 tubulin* gene. *The gene is interrupted by one large and two small introns. Expression in the dorsal vessel (h, heart) and in the somatic mesoderm (sm) is regulated by upstream sequences. Enhancers guiding transcription in the visceral muscles (vm) and the chordotonal organs (ch) are localized in the large intron, where also regulatory sequences for expression in the somatic musculature are found.*

ulatory units for the expression in the dorsal vessel and the somatic musculature (Gasch *et al.*, 1989; Wolk, 1994; Wolk *et al.*, in preparation). The *β3 tubulin* expression in the visceral mesoderm is guided by enhancer elements in the first 4.6 kb long intron (Gasch *et al.*, 1989). An extensive deletion analysis of this intron showed that the *β3 tubulin* gene is regulated along the anterior posterior axis in the visceral mesoderm (Hinz *et al.*, 1992). This expression pattern suggested that homeotic genes regulate the *β3 tubulin* gene expression as an effector gene, which was first shown for *Ultrabithorax* (Hinz *et al.*, 1992). The homeotic genes fulfil their regulatory function in combination with tissue specific regulators, for which Tinman is a good candidate. Currently, other transactivators are under investigation for their ability to bind otherwise identified regulatory motives. A summary of the hitherto characterized regulatory units of the *β3 tubulin* gene is presented in Figure 4.

Accumulation of β1 tubulin mRNA in apodemes starts after muscle insertion

During early embryogenesis the origin of β1 tubulin mRNA is maternal, while zygotic expression is observed in the nervous system from gastrulation onward (Gasch *et al.*, 1988; Buttgereit *et al.*, 1991). Whole-mount *in situ* hybridizations of late embryonic stages allow to visualize a further distinct mode of expression in parts of the chordotonal organs and, as mentioned above, the *β1 tubulin* is expressed in the apodemes, the muscle attachment sites (Fig. 5). Using this method we looked in detail at the time course of *β1 tubulin* expression in the apodemes. The first epidermal cells that show zygotic expression of the gene occur around stage 13, when the elongating muscles have already contacted their attachment sites (Fig. 5A). At this stage, only intersegmental attachment sites show expression of the *β1 tubulin* gene. Following embryogenesis, the pattern of *β1 tubulin* expression in individual apodemes is enhanced according to the further progress of muscle formation. At stage 16, when the larval muscle pattern is established finally, all apodemes show a high level of *β1 tubulin* mRNA (Fig. 5B-D). Four different sets of attachment sites can be distinguished: One intersegmental (iapo) as well as the two intrasegmental (ina) apodemes form rows of single cells, while the attachment sites of the lateral and dorsal transversal muscles exist as single cells.

Every somatic muscle inserts into a single defined attachment site

To describe the apodeme/muscle relation precisely, double-stainings for the β1 tubulin mRNA and the β3 tubulin protein were performed. Figure 6A shows an overview of the dorsal muscle and apodeme pattern. Figure 6B presents an embryo at late stage 13. Looking at the abdominal part, β1 tubulin positive cells already in contact to muscles are detectable, while other apodemes devoid of muscle contact reveal no expression. At stage 16 (Fig. 6C) every muscle can be attributed to a distinct β1 tubulin positive tendon cell in the epidermis. According to the muscle nomenclature developed by Bate (1990), we would propose a similar scheme for the attachments simply by adding the prefix aa- for anterior, pa- for posterior, da- for dorsal and va- for ventral attachment. So for the lateral and dorsal transverse mus-

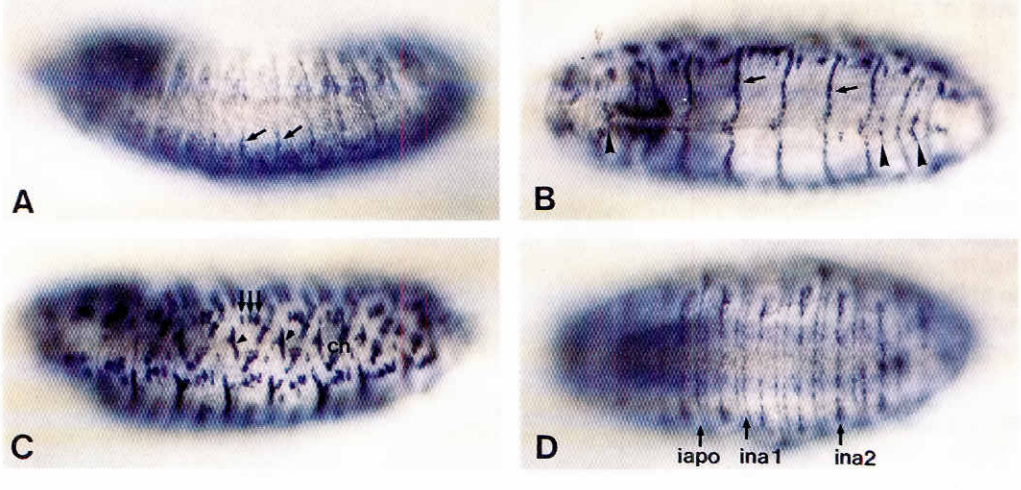

Fig 5. Localization of the β1 tubulin mRNA. (A) *Embryo stage 13, lateral view; expression of the β1 tubulin is detectable in the segmental furrows, where the intersegmental attachments (iapo's) form (arrows).* **(B)** *Embryo stage 15, dorsal view; the iapo's present a high level of mRNA (arrows). In addition, in the posterior abdominal as well in the anterior thoracic parts patches of 3-6 cells localized most dorsally are stained (arrowheads).* **(C)** *Embryo stage 16, lateral view; marked by arrows are the attachments of the pleural external muscles (pet's); most lateral, strings of cells represent the final appearance of the iapo's in that domain (arrowheads). In addition, the chordotonal organs (ch) show a high level of β1 mRNA.* **(D)** *Embryo stage 16, ventral view; the iapo's as well as ina1 extend over the complete surface, while the ina2 attachments are restricted to the most ventral parts.*

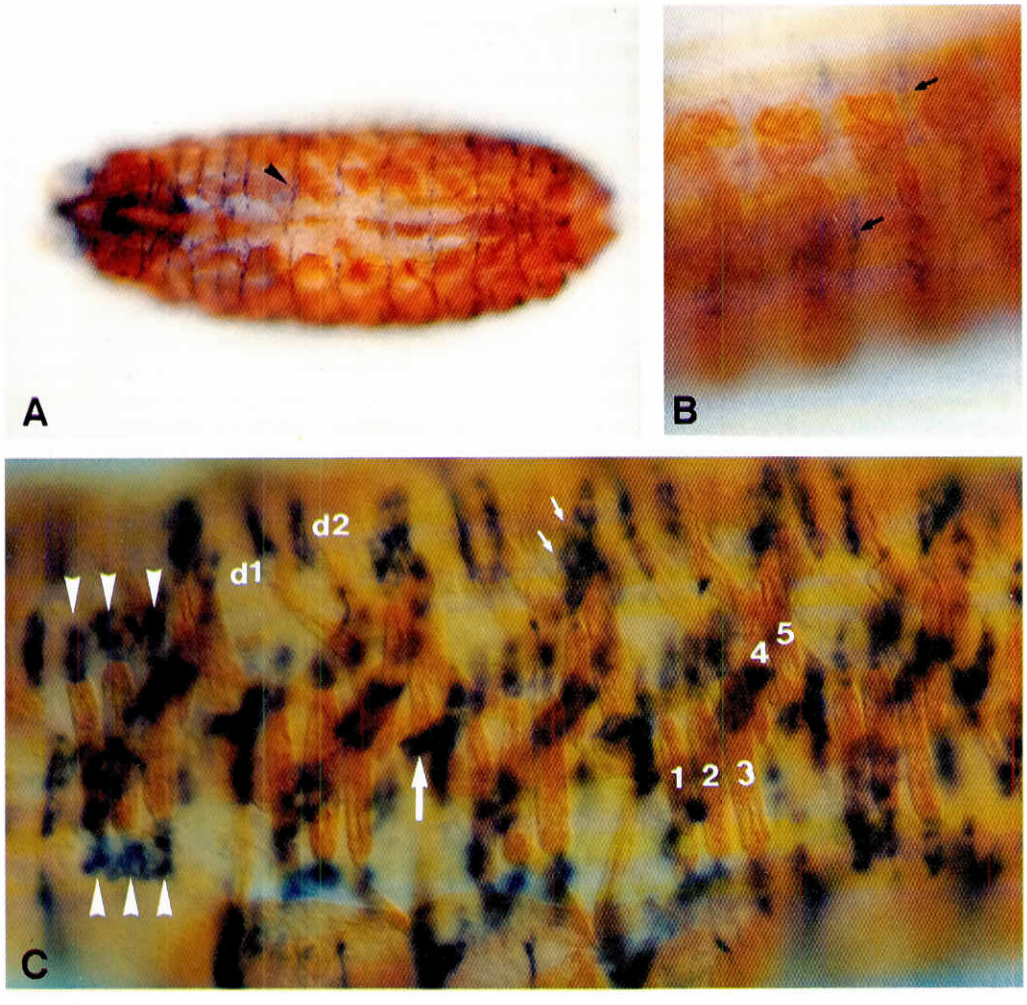

Fig 6. Localization of β1 mRNA and β3 tubulin protein. (A) *Embryo stage 15, dorsal view; the attachment sites appear in blue, the muscles in brown.* **(B)** *Embryo stage 13, lateral view; only attachment sites that are already in contact to muscles show β1 transcripts (arrows).* **(C)** *Sector magnification of the abdominal part from a stage 16 embryo; focus is on the pet muscles, so the attachments are slightly out of plane. While the LT 1-3 muscles (1,2,3) contact single cell attachments dorsally and ventrally (arrowheads), LT4 and DT1 insert dorsally into in to three-cell-cluster (small arrows), LT4 inserts ventrally into a single cell, while the ventral attachment of DT1 is in contact to the iapo (large arrow). In addition, the dorsal external oblique muscles 1 (deo 1) inserting into iapo (d1) and deo 2 inserting into ina1 are marked (d2).*

cles the according tendon cells would be designated da-LT/DT and va-LT/DT, respectively.

Expression of the *β1 tubulin* gene in the attachment sites is controlled by three copies of a 14bp element in the first intron

Extensive deletion analysis of the *β1 tubulin* gene revealed that the sequences necessary for expression in the attachment sites are localized in the first intron and can act as enhancers also on the hsp70 basal promoter. Figure 7A shows an stage 13 embryo transformed with the construct (Wβ1HI) (Buttgereit, 1993) comprising the intron of the *β1 tubulin* gene cloned into the vector pWHL in front of a truncated hsp70 promoter. As for the *β1 tubulin* mRNA (Fig. 5B), expression of the lacZ reporter gene is first detectable in the intersegmental apodemes just after the insertion of muscles (Fig. 7A). At stage 16 the expression pattern

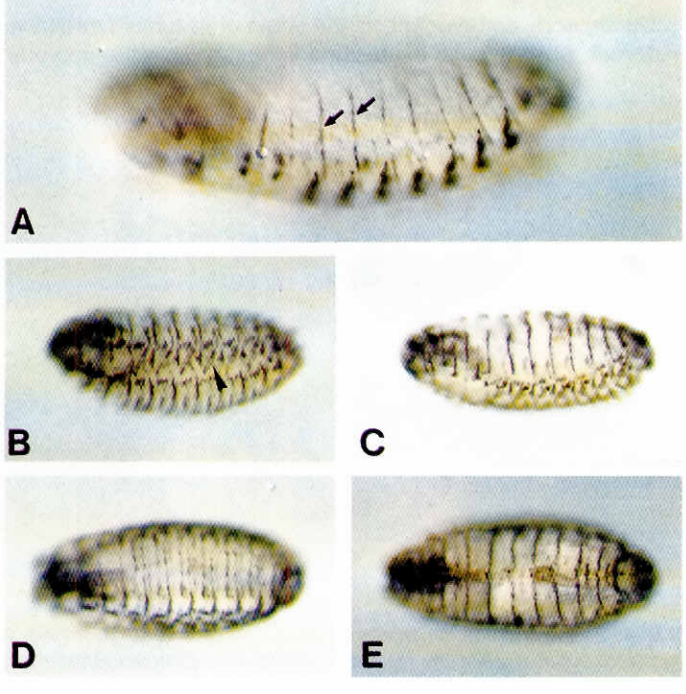

Fig 7. Anti-β-gal antibody staining of Wβ1HI transformed embryos. (A) *Stage 13 embryo, lateral view; as for the β1 tubulin, the iapo's are the first attachments presenting reporter gene expression (arrows).* **(B-E)** *As for the β1 mRNA, all muscle attachments are stained and identical to the β1 distribution. In addition, also the chordotonal organs show reporter gene expression (arrowhead).*

in the attachment sites is indistinguishable from the *β1 tubulin* mRNA distribution and the complete set of muscle attachment sites has formed (Fig. 7 B-E).

The intron furthermore contains enhancers driving *β1 tubulin* expression in cells of the chordotonal organs. A detailed deletion analysis showed that enhancers for expression in chordotonal organs are separable from those responsible for *β1 tubulin* expression in apodemes. Narrowing down the essential sequences for apodeme expression of the intron resulted in the identification of an 14bp element present in three repeats, the action of which is very likely to be additive, rather than synergistic.

Taken together, the data suggest that the activation of *β1 tubulin* expression in the apodemes is initiated in response to the insertion of the somatic muscles, and represents a very interesting model system for induction processes mediated by cell-cell interaction.

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