**Ultrabithorax is a regulator of β3 tubulin expression in the Drosophila visceral mesoderm**

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### Summary

β3 tubulin expression accompanies the specification and differentiation of the Drosophila mesoderm. The genetic programs involved in these processes are largely unknown. Our previous studies on the regulation of the β3 tubulin gene have shown that upstream sequences guide the expression in the somatic musculature, while regulatory elements in the first intron are necessary for expression in the visceral musculature. To further analyse this mode of regulation, which reflects an early embryonic specification program, we undertook a more detailed analysis of the regulatory capabilities of the intron. The results reveal not only a certain degree of redundancy in the cis-acting elements, which act at different developmental stages in the same mesodermal derivatives, but they also demonstrate in the visceral mesoderm, which forms a continuous epithelium along the body axis of the embryo, an early action of regulators guiding gene expression along the anterior-posterior axis of the embryo: an enhancer element in the intron leads to expression in a subdomain restricted along the anterior-posterior axis. This pattern is altered in mutants in the homeotic gene Ultrabithorax (Ubx), whereas ectopic Ubx expression leads to activity of the enhancer in the entire visceral mesoderm. So this element is likely to be a target of homeotic genes, which would define the β3 tubulin gene as a realisator gene under the control of selector genes.

Key words: anterior-posterior axis, β3 tubulin gene, Drosophila embryogenesis, gene regulation, mesoderm, Ultrabithorax.

### Introduction

In contrast to the well-characterised mechanisms of pattern formation in the ectoderm of Drosophila embryos (for review see Akam, 1987; Ingham, 1988), less is known about the corresponding processes in the mesoderm. The genetic program leading to mesoderm formation is relatively well understood. A cascade of maternally acting genes (reviewed by Anderson and Nüsslein-Volhard, 1986; Anderson, 1987; Govind and Steward, 1991) culminates in a gradient of nuclear localisation of the dorsal morphogen (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). On the ventral side of the embryo this gradient is interpreted by the zygotically expressed presumptive transcriptional regulators twist and snail, leading to ventral furrow invagination and mesoderm formation (Simpson, 1983; Nüsslein-Volhard et al., 1984; Boulay et al., 1987; Thissé et al., 1988; Leptin and Grunewald, 1990; Jiang et al., 1991; Pan et al., 1991; Thissé et al., 1991).

Determination of visceral versus somatic mesoderm occurs later in development (Beer et al., 1987). Genetic functions in this process have not yet been characterised. Recently, the neurogenic genes have been assigned to the mesodermal differentiation program, apparently fulfilling a role in cell fate determination (Corbin et al., 1991). Genes coding for other presumptive transcription factors, which might be involved in the processes of mesoderm specification and differentiation, have been cloned (Barad et al., 1988; Bopp et al., 1989; Bodmer et al., 1990; Dohrmann et al., 1990; Michelson et al., 1990; Paterson et al., 1991). Unfortunately, no mutations in any of these are available.

Some homeotic selector genes are expressed in the mesoderm during gastrulation (e.g. abdA (Karch et al., 1990; Macias et al., 1990), Ubx (White and Wilcox, 1985); a review concerned with the Ultrabithorax (Ubx) gene has been written by Beachy (1990)). Hooper (1986), using different alleles of the Ubx gene, has correlated the mesodermal expression domain with homeotic transformations of the larval somatic musculature. Ubx and other selector genes have been shown to have a second phase of expression in the visceral mesoderm and to have a function in gut morphogenesis (Bienz and Tremml, 1988; Tremml and Bienz, 1989; Immerglück et al., 1990; Reuter and Scott, 1990; Reuter et al., 1990).

The β3 tubulin gene (βTub60D) is a member of a small gene family in Drosophila melanogaster (reviewed by Fyrberg and Goldstein, 1990). During embryonic development, it is expressed in the differentiating mesoderm (Gasch et
al., 1988; Leiss et al., 1988; Kimble et al., 1989). As the expression starts with the first morphological signs of differentiation, the gene provides a model system to investigate gene regulation during this process. Our previous studies have already shown the gene to be independently regulated in the somatic and visceral mesoderm (Gasch et al., 1989). This supplies a tool to trace back the pathways establishing mesodermal differentiation and to identify the upstream regulatory factors involved.

In this report, we extend our analysis of the complex cis regulatory elements of the β3 tubulin gene. The first 4.6 kb intron harbours several independently acting enhancers conferring partially redundant aspects of expression. Distinct elements regulate expression in the somatic mesoderm at different steps of differentiation. Another discrete enhancer leads to expression only in the abdominal part of the visceral mesoderm, overlapping with the early mesodermal Ubx distribution. The alterations of this pattern in Ubx mutants and after ectopic Ubx expression suggest an activation of the enhancer by this homeoprotein.

Materials and methods

Construction of P-element transposons and P-element-mediated transformations

Construction of β3/Lac-0.23 was performed by ligation of a 15 kb HindIII fragment of β3/Lac-6.01 (Gasch et al., 1989), a 1.1 kb genomic BamHI-HindIII fragment spanning 0.23 kb of upstream sequences, the first exon and the 5′ part of the first intron and a 0.6 kb BamHI-HindIII fragment from pW8 (Klemenz et al., 1987).

To construct pWHL (Fig. 2A), a XbaI-PstI fragment containing 250 bp of hsp70 upstream sequences and 89 bp of leader upstream sequences and 89 bp of leader sequences from 132E3 (Karch et al., 1981) was subcloned into pUC8 (Vieira and Messing, 1982). Using the HindIII polylinker site a NruI-(−50)-HindIII fragment missing the heat shock response element was ligated to the HindIII-XbaI fragment of pUClac20 (Michiels et al., 1989) in HpaI-XbaI opened pW8 (Klemenz et al., 1987). Deletion plasmids of the β3 tubulin intron were generated using ExoIII digestion (Henikoff, 1984). The starting plasmid was prepared by ligation of a blunt-ended XmaI(+) fragment into EcoRV opened pC20H (Marsh et al., 1984). ExoIII treatment was performed after cutting with KpnI and ClaI. For 5′ deletion constructs, fragments of the resulting plasmids reaching from XbaI (polylinker) to XhoI (+4276) were subcloned into the pWHL XbaI site. 3′ deletion constructs were prepared in an analogous way, using both pC20H XbaI polylinker sites.

Fragments cloned into the pWHL SstI site were prepared as described. For pWHL/β3-2U and pWHL/β3-2UR the described (longest) XbaI fragment was blunt ended. The β3 DNA present in pWHL/β3-1 is a XbaI-(filled in)-SmaI fragment from β3/Lac-6.01 (Gasch et al., 1989). pWHL/β3-13: XbaI-(filled in, using 3′ deletion endpoint)-SnaBI fragment.

pWHL/β3-14 and pWHL/β3-16: 5′ deletion endpoints were recovered via the pC20H SphI site and after cutting with SmaI the resulting fragments cloned into SphI-SstI opened pWHL. For pWHL/β3-15, Mvnl was used instead of SnaBI.

DNA injections of w1 embryos were performed as described by Rubin and Spradling (1982) with minor modifications (Michiels et al., 1989). Immuno-staining of embryos

Preparation and staining of embryos was done essentially as described (Leiss et al., 1988), using the Vectastain Elite kit (Vector Laboratories). For double label immunostaining the procedure was modified according to Lawrence et al. (1987). Antibodies utilised were a mouse monoclonal antibody against β-galactosidase (Promega), β3-tubulin-specific antiserum from rabbit (Leiss et al., 1988) and FP3.38, a mouse monoclonal antibody against Ubx (White and Wilcox, 1984) kindly provided by R. White. After dehydration, embryos were embedded in Epon and photographed under Nomarski optics with a Leitz microscope. For sectioning embryos were embedded in Durcupan ACM (Fluka) as described by Roth et al. (1989). 10 μm sections were cut on a LKB Ultratome 8802A.

Df(2R)mod(260) (Simpson, 1983), a small deletion of the twist gene (Thiese et al., 1987) was provided by C. Nüsslein-Volhard.

Embryos heterozygous for pWHL/β3-14 and HSU and, as a control, embryos which carry only pWHL/β3-14 were collected over a period of 2 hours. They were maintained at 25°C until 4-6 hours old and were then given a 20 minute heat shock in a 37°C water-bath. Brought back to 25°C, the embryos were allowed to develop in a moist chamber until the desired stage. They were dechorionated, fixed and immunostained as described above.

Sequence analysis

Sequencing reactions were performed on both strands according to Sanger et al. (1977) and Chen and Seeburg (1985) with Sequenase enzyme (USB), using the ExonucleaseIII-deletion-plasmids described above as templates. Assembly and analysis of the sequence was done using software from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

Results

Upstream sequences drive expression in the somatic musculature, while intron sequences lead to expression in somatic and visceral musculature

We have previously shown, that 6 kb of β3 tubulin upstream sequences are capable of conferring specific expression in the somatic mesoderm to an indicator gene (Gasch et al., 1989; for the expression pattern driven by the fusion gene β3/Lac-6.0 see Fig. 5A, D). This expression starts at the extended germ band stage, in later stages expression can be observed in the somatic musculature, the pharyngeal musculature and the dorsal vessel. In contrast, regulatory elements in the first intron of the β3 tubulin gene are necessary for transcription in the visceral mesoderm (Gasch et al., 1989). This was shown using a construct that carries 6 kb of upstream sequences and the first intron of the β3 gene.

Heat shock treatment

Embryos heterozygous for pWHL/β3-14 and HSU and, as a control, embryos which carry only pWHL/β3-14 were collected over a period of 2 hours. They were maintained at 25°C until 4-6 hours old and were then given a 20 minute heat shock in a 37°C water-bath. Brought back to 25°C, the embryos were allowed to develop in a moist chamber until the desired stage. They were dechorionated, fixed and immunostained as described above.

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does a fusion gene carrying 1.2 kb of β3 upstream sequences (Gasch et al., 1989).

The β3/Lac-0.23I construct is indeed capable of driving LacZ expression in the visceral mesoderm (Fig. 1B), showing the independent action of regulatory elements in the upstream region and the first intron. To our surprise, later in development staining can also be found in the somatic mesoderm (Fig. 1C). Therefore the intron harbours a second cis-acting element driving expression in the somatic mesoderm, which works independently of the upstream element.

The enhancer elements in the intron act on the heterologous hsp70 promoter

We wondered whether the cis regulatory elements present in the first intron of the β3 tubulin gene fulfil the requirements of a classical enhancer definition (Serfling et al., 1985), which include action on a heterologous promoter. In order to test this and to facilitate subsequent characterisation of these elements, we constructed the vector pWHL, which contains the hsp70 basal promoter linked to the E. coli LacZ gene (Fig. 2A). Embryos transgenic for this vector show little or no expression of β-galactosidase.

The region of the first intron was tested in two fragments in this vector. Nucleotides 4272-4870 are present in pWHL/β3-1 shown in Fig. 2B, numbering of nucleotides is relative to the start site of transcription (Gasch et al., 1988), the sequence is shown in Fig. 3, expression patterns are summarised in Table 1. Embryos carrying this construct do not show any detectable β-galactosidase expression. This is in accordance with the negative action of this DNA region in cell transfection experiments (Bruhat et al., 1990). The edysone inductibility found to be associated with this fragment by these authors cannot be evaluated by our experiments aimed at tissue-specific expression.

In contrast, nucleotides 307-4276, present in pWHL/β3-2U, acting on the hsp promoter lead to the expression pattern shown in Fig. 4. Strong staining specific to the visceral mesoderm can be observed from the extended germ band stage onwards (stage 11 of Campos-Ortega and Hartenstein 1985; Fig. 4A, B, E). In later stages, staining also appears in the somatic mesoderm (Fig. 4C, D, F). In every aspect this expression pattern is indiscernible from that driven by β3/Lac-0.23I, as can be seen for example in the comparison of Figs 1B and 4A. We conclude, that the elements of the first intron which regulate embryonic expression are included in this region, and that they can act on the heterologous hsp70 promoter as successfully as on their own promoter.

Inversion of this DNA fragment relative to the start site of transcription (pWHL/β3-2UR) as well as cloning it downstream of the SV40 polyadenylation site present in pWHL (pWHL/β3-2D, shown in Fig. 5C, F) leads to essentially the same pattern of expression in transgenic embryos, indistinguishable from the β-galactosidase pattern driven by pWHL/β3-2U. This indicates, that these cis-acting elements present in this region act irrespectively of position and orientation, thus fulfilling all requirements for an enhancer.

The β-galactosidase pattern exhibited by embryos carrying the hsp70/LacZ fusion gene under control of the β3 intron not only allows one to follow the differentiation of the visceral mesoderm from the separation of splanchno-

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Fig. 1. The β3 tubulin intron drives expression in the visceral and somatic musculature. (A) The fusion gene β3/Lac-0.23 is shown in relation to the genomic map of the β3 tubulin gene. 0.23 kb of upstream sequences, which show no tissue-specific expression of their own, the first exon and the first intron are fused, at the codon for amino acid 25, in frame to the E. coli LacZ gene. Expression of this construct in transgenic embryos is visible after immunostaining with an antibody against β-galactosidase in whole-mount embryos. (B) shows a lateral view of an embryo at stage 11, which demonstrates staining of the visceral mesoderm. (C) A dorsal view of an embryo at stage 14 reveals expression in the visceral and somatic mesoderm. Open bars, transcribed region of the β3 tubulin gene; black bars, amino acid coding region of the β3 tubulin gene; lines, 5’ and 3’ nontranscribed sequences; stippled bars, LacZ or SV40 sequences; aa, amino acid.

In order to separate the regulatory capabilities of the 4.6 kb of intron sequences from those of elements located far upstream, we chose to similarly construct β3/Lac-0.23I (Fig. 1A). The 0.23 kb of upstream sequences should not confer any β3 tubulin gene-specific expression on their own, as a construct bearing these fused in the leader region to the LacZ gene (β3/Lac-0.23) drives no expression reminiscent of the pattern of β3 tubulin in transgenic flies, nor
Fig. 2. β3 tubulin / hsp70 / LacZ constructs used for transformation. (A) The plasmid pWHL is based on the pW8 transformation vector (Klemenz et al., 1987), which uses the Drosophila white gene (striped bar) under control of the hsp70 promoter (thick line) as a selectable marker. The E. coli LacZ gene followed by an SV40 polyadenylation site (stippled bars) is under the control of a truncated hsp70 promoter (thick line), from which the heat shock response elements have been deleted. Arrowheads represent the inverted repeats of the P-transposon. Unique restriction enzyme sites in the polylinkers upstream and downstream of this fusion gene are given in bold letters. These sites are suitable for insertion of sequences to be tested for enhancers. Embryos transgenic for this construct occasionally show expression in salivary glands, hindgut or pharyngeal musculature at very low level. (B) DNA fragments tested in pWHL in relation to a map of the first β3 tubulin intron. Flanking exon sequences are represented by black bars. Lines indicate the extent of DNA present in the construct, the exact endpoints as determined by sequencing are given, using the same numbering system as in Fig. 3. All constructs contain the β3 fragments in the same relative 5'-3' orientation to the LacZ gene as in the β3 tubulin gene, with the exception of pWHL/β3-2UR. Restriction enzyme sites given in parentheses were used for cloning (see Materials and methods), but are not unique in the region shown. Numbers of transformant lines and their expression patterns are given in Table 1.
pleura and somatopleura onwards, but also later during organogenesis, staining can be seen in the somatic mesoderm. As transcription in this mesodermal derivative is also driven by upstream sequences of the β3 tubulin gene, we compared the expression observed under the control of either element to the β3 tubulin expression pattern (Fig. 5). As can be seen in Fig. 5B, in the extended germ band (stage 11) β3 tubulin can be detected in the visceral mesoderm and in clusters of large cells in the somatic mesoderm. We have proposed (Leiss et al., 1988), that these cells are identical to muscle pioneer cells as described for the Drosophila embryo by Johansen et al. (1989) and Bate (1990). These cells can only be found stained in embryos expressing β-galactosidase under control of the β3 upstream region (Fig. 5A), not in embryos transgenic for constructs carrying only β3 tubulin intron sequences (Fig. 5C). In these embryos, expression in the somatic mesoderm begins during germ band retraction (stage 12), and can easily be observed after this process (Fig. 5F). Therefore, both intron and upstream regions harbour several elements, which lead to β3 tubulin expression in the somatic mesoderm. The difference between them is the developmental stage when they become active.

Two DNA regions lead to expression in the visceral mesoderm

To demarcate the borders of the elements responsible for the cis regulatory capacity of the intron we carried out a deletion analysis. For this purpose, progressively smaller fragments were tested in pWHL in a manner analogous to the construct pWHL/β3-2D, keeping either the 3′ or the 5′ end invariable (Fig. 2B). The β-galactosidase expression patterns in transgenic embryos were compared to those driven by pWHL/β3-2D and are listed in Table 1.

When deleting from 5′, no difference in staining patterns can be observed between embryos carrying pWHL/β3-2D or pWHL/β3-5. In the 3′ deletion series, pWHL/β3-12 still drives all tissue-specific features. This implies that the tissue-specific enhancer elements in the first intron of the β3 tubulin gene are included in the DNA region between nucleotides 3154 and 4033. No aspect of the embryonic expression pattern of β3 tubulin can be controlled by intron sequences exclusively outside this region. This is evident from embryos carrying pWHL/β3-10, which only show staining unrelated to the β3 tubulin pattern. On the 3′ side, pWHL/β3-8 does not lead to specific expression. This construct shows strong enhancer trapping qualities. Taking into account the weak staining in embryos bearing the vector pWHL, this might argue for a general enhancer element in this region.

### Table 1. Expression patterns of β3/LacZ fusion genes in transgenic flies

<table>
<thead>
<tr>
<th>Construct</th>
<th>Endpoints</th>
<th>Number of lines</th>
<th>sm early</th>
<th>sm late</th>
<th>vm (ap)</th>
<th>ch. org.</th>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>β3/Lac-0.23I</td>
<td>0.23 kb</td>
<td>4</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>a</td>
</tr>
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<td>6 kb</td>
<td>7</td>
<td>+</td>
<td>a</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>β3/Lac-6.0I</td>
<td>6 kb</td>
<td>6</td>
<td>+</td>
<td>a</td>
<td>+</td>
<td>a</td>
</tr>
<tr>
<td>pWHL (vector)</td>
<td></td>
<td>5</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pWHL/β3-1</td>
<td>4272;4870</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pWHL/β3-2U</td>
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<td>2</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>a</td>
</tr>
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<td>4276;307</td>
<td>3</td>
<td>−</td>
<td>+</td>
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<td>a</td>
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<td>−</td>
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<td>+</td>
<td>a</td>
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<td>+</td>
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<td>a</td>
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<td>−</td>
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<td>−</td>
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<td>−</td>
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<tr>
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<td>3397;3669</td>
<td>8</td>
<td>−</td>
<td>−</td>
<td>+/− 8</td>
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Expression patterns driven by the constructs β3/Lac-6.0, β3/Lac-6.0I and β3/Lac-0.23 (Gasch et al., 1989) are given for comparison. β3/Lac-6.0I contains 6 kb of upstream sequences as well as the first intron of the β3 tubulin gene fused at the identical position as in β3/Lac-0.23I in frame to the LacZ gene. β3/Lac-6.0 contains 6.0 kb, β3/Lac-0.23 0.23 kb of upstream sequences fused in the β3 leader region to the LacZ leader. ch. org., chordotonal organs; sm early, early (starting at stage 11) expression in somatic mesoderm; sm late, late (starting at stage 12) expression in somatic mesoderm; vm, expression in entire visceral mesoderm; vm (ap), expression in anterior-posterior restricted region of visceral mesoderm.

a. expression in the somatic mesoderm driven by the late element cannot be detected, if the early element is also present. Similarly, expression in the entire visceral mesoderm masks the staining in the abdominal region, although a corresponding difference in intensity seems visible in some lines.

b. all lines carrying this construct stain so weakly, that expression in chordotonal organs cannot be judged with certainty.

c. five of the eight lines show very weak staining in the visceral mesoderm of the first and second abdominal segments. Expression in the chordotonal organs can be detected in all lines.
Two non-overlapping intron fragments confer expression in the mesoderm of transformants. pWHL/β3-11 carrying embryos show weak staining in the visceral mesoderm of the first two abdominal segments. However, the DNA fragment present in pWHL/β3-7 drives expression in the embryonic mesoderm in a way similar to pWHL/β3-2D, i.e. in the somatic mesoderm and the whole visceral mesoderm. We conclude from this, that the β3 tubulin gene not only possesses separate elements leading to expression in the somatic mesoderm, but also transcription in the visceral mesoderm is regulated partially redundantly by independently acting elements.

A 500 bp fragment confers expression in a subdomain of the visceral mesoderm restricted along the anterior-posterior axis

To examine more closely the cis regulatory elements narrowed down by the deletion analysis, we decided to insert them separately, as two non-overlapping DNA fragments, into the upstream cloning site of pWHL (Fig. 2). This position resulted in stronger expression than in the downstream polylinker sites. Expression in the whole visceral mesoderm and in the somatic mesoderm is still achieved by the control elements present in pWHL/β3-13 (Fig. 6B, E, H). Considering also the 5′ breakpoint of the construct pWHL/β3-7 this implies that the regulatory elements responsible for this mode of expression are located between +3699 and +4033.

Comparison of this staining pattern and the pattern of β3 tubulin (Fig. 6C, F, I) to the β-galactosidase expression driven by pWHL/β3-14 (Fig. 6A, D, G) demonstrates that the latter one is in a striking fashion limited along the anterior-posterior axis of the embryo. Closer examination reveals that the staining intensity in this subdomain of the

Fig. 3. Sequence of the first β3 tubulin intron. Numbering is relative to the start site of transcription (Gasch et al., 1988). Flanking exon sequences are marked by underlining. These sequence data are available from EMBL/GenBank/DDBJ under accession number X68393.
Gene regulation in the Drosophila mesoderm

Visceral mesoderm is not distributed evenly, but is greatest in the first two abdominal segments. Posteriorly it declines in a shallow gradient to the seventh abdominal segment, anteriorly in a steeper one ending in the second thoracic segment. This gradient makes an exact boundary of expression difficult to determine, as that is given by the detection limit of the anti-β-galactosidase antibody and thus varies depending on staining intensity. At very low staining intensities, as found in the embryos carrying pWHL/β3-11, described in the previous section, expression is only detectable in the first and second abdominal segments. Expression driven by this fragment can furthermore be found in a small subset of somatic muscles and chordotonal organs, but this expression is also limited to the abdominal region of the embryo. We conclude, that one or several cis-acting elements present in this DNA fragment confine transcription in all these cells to the abdominal segments.

Elements leading to abdominal expression in the visceral mesoderm and in the chordotonal organs are separable

In an attempt to further delimit the cis-acting element(s) responsible for the restriction of expression to the abdominal region, we divided the DNA region between +3154 and +3669 into two partially overlapping fragments. Expression driven by both of them is limited in the described manner along the anterior-posterior axis.

Embryos that are transgenic for pWHL/β3-15 exhibit staining in the abdominal visceral mesoderm (Fig. 7A), like those carrying pWHL/β3-14. Also the specificity of the expression pattern in the somatic musculature is maintained with this smaller fragment.

In contrast, the element(s) responsible for expression in the abdominal chordotonal organs are found in pWHL/β3-16 (Fig. 7B). This nonmesodermal aspect of β3 tubulin expression is responsible for the residual staining in a segment-like spacing noted by us in dorsalisated mutants (Leiss et al., 1988) and can also be found in wild-type embryos. Fig. 7C shows pWHL/β3-16 driven β-galactosidase expression in a twist mutant embryo.

Concerning the location of the cis-acting element(s) con-
focussed on selector genes, taking the staining of the visceral mesoderm more difficult to recognize in B. In later stages, staining in the somatic mesoderm can be observed with have to be located in the overlapping DNA region between presumptive pioneer cells of the somatic musculature (B) is driven by upstream regions (A), whereas the intron mediates expression in the visceral mesoderm (C). The plane of focus is on the visceral mesoderm in C, while it is on the muscle pioneers in A and B, making the staining of the visceral mesoderm more difficult to recognize in B. In later stages, staining in the somatic mesoderm can be observed with both constructs. pWHL/β3-2D drives expression in the visceral mesoderm as well (F), which is absent in the embryo carrying β3/Lac-6.0 (D). (D) Stage 14, dorsal view; (E) stage 13, dorsal view; (F) stage 13, ventral view. Note the additivity of β3/Lac-6.0 and pWHL/β3-2D expression to give the β3 tubulin-specific pattern. mp, muscle pioneer cells; arrows, somatic mesoderm; arrowheads, visceral mesoderm.

Fig. 5. The cis-acting elements conferring expression in the somatic mesoderm become active in different developmental stages. This becomes obvious by comparing anti-β-galactosidase-stained embryos transgenic for β3/Lac-6.0 (A, D), in which β-galactosidase expression is driven by 6 kb of β3 tubulin upstream sequences (Gasch et al., 1989) to embryos, which carry pWHL/β3-2D (C, F), a construct containing the LacZ gene under control of the intron. Embryos stained using a β3 tubulin-specific antiserum (Leiss et al., 1988) are shown for comparison (B, E). Lateral views of stage-11 embryos show that by this developmental stage β3 tubulin expression in the presumptive pioneer cells of the somatic musculature (B) is driven by upstream regions (A), whereas the intron mediates expression in the visceral mesoderm (C). The plane of focus is on the visceral mesoderm in C, while it is on the muscle pioneers in A and B, making the staining of the visceral mesoderm more difficult to recognize in B. In later stages, staining in the somatic mesoderm can be observed with both constructs. pWHL/β3-2D drives expression in the visceral mesoderm as well (F), which is absent in the embryo carrying β3/Lac-6.0 (D). (D) Stage 14, dorsal view; (E) stage 13, dorsal view; (F) stage 13, ventral view. Note the additivity of β3/Lac-6.0 and pWHL/β3-2D expression to give the β3 tubulin-specific pattern. mp, muscle pioneer cells; arrows, somatic mesoderm; arrowheads, visceral mesoderm.

Expression driven by the 500 bp fragment shows Ubx dependence

As the pattern of β-galactosidase driven by pWHL/β3-14 shows a striking anterior-posterior restriction, we searched for potential regulators of this expression among the organizers of the anterior-posterior axis. Considering the developmental stage of activation of the β3 tubulin gene and the extent and heterogeneity of expression of pWHL/β3-14 we focussed on selector genes, taking the β-galactosidase staining maximum in the first two abdominal segments into account on Ubx. Ubx shows two phases of expression in the mesoderm. The first phase in the extended germ band (Fig. 8A) parallels expression in the ectoderm, but the expression domains are out of register: whereas Ubx protein can be detected in the ectoderm in parasegments 5-13, it can be found in the mesoderm in parasegments 6-12, showing a maximum in parasegments 7 and 8 (White and Wilcox, 1985). At the beginning of this expression, segregation of somatopleura and splanchnopleura has not yet occurred. Later the somatic mesoderm within this region contains Ubx product. A second phase of Ubx expression occurs in the visceral mesoderm in parasegment 7 after germ band retraction (Bienz and Tremml, 1988).

As the first phase of Ubx expression precedes and overlaps with pWHL/β3-14-driven β-galactosidase expression, we compared the spatial distribution of LacZ in embryos (Fig. 8B). The coincidence of the expression domains and maxima makes Ubx a good candidate for a regulator of the β3 tubulin gene.

To test this hypothesis we analysed pWHL/β3-14 in an Ubx mutant background. Specific staining can still be found (Fig. 8C), but the pattern is altered. As becomes evident from a comparison of Fig. 8C and 8B the maximum in the first two abdominal segments is missing in the Ubx mutants. These show instead uniform staining in the expression domain. This suggests an influence of Ubx on the expression driven by this DNA fragment, although Ubx is clearly not the only activator.

The alteration in the staining pattern might be due to a homeotic transformation of the visceral mesoderm in the relevant parasegments. To rule out this possibility we studied the β-galactosidase expression driven by pWHL/β3-14 after ectopic Ubx expression. For this purpose we made use of flies transgenic for a Ubx cDNA under control of a heat shock promoter (Gonzalez-Reyes et al., 1990). Heat shock
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Treatment leads to Ubx expression throughout these embryos (Fig. 8D) and to subsequent activation of the 500 bp fragment in the entire visceral mesoderm, also anterior of its original expression domain, even in cephalic segments. The conditions used in this experiment (see Material and methods) were relatively mild compared to those leading to transformations visible in the epidermis (Gonzalez-Reyes and Morata, 1990). Furthermore, the extension of the pWHL/β3-14 expression domain is visible in all the embryos subjected to heat shock, whereas the transformation events can only be observed in a smaller fraction (Gonzalez-Reyes and Morata, 1990). This difference in the penetrance of the observed effect argues that induction of pWHL/β3-14 occurs independently of a homeotic transformation. Therefore, the extension of the expression domain of pWHL/β3-14 is probably due to a direct or indirect action of Ubx on this enhancer of the β3 tubulin gene.

Discussion

In order to gain experimental access to the programs of gene regulation during mesoderm differentiation, a detailed study of cis-acting elements in the intron of the β3 tubulin gene has been carried out. Fig. 9 gives a simplified overview of the tissue-specific elements involved in the complex regulation of this gene during embryogenesis.

The β3 tubulin gene shows discrete regulatory mechanisms in the visceral and somatic mesoderm, not just a simple germ layer-specific regulation. This reflects programs acting after determination of mesodermal primordial cells on the visceral versus somatic fate.

For expression in the somatic mesoderm, two independently acting elements have been mapped, which become active during different developmental stages. This phenomenon, similar to the two-tiered regulation often observed with segmentation genes acting earlier in development, may be necessary due to the changing presence of transcriptional regulators. As proposed by DiNardo et al. (1988) in the case of the segmentation gene engrailed, an early transient program might be substituted by the establishment of a more stable, later program.

Also in the visceral mesoderm, transcription is achieved by separately acting enhancers. The expression driven by these elements partially overlaps in some regions, as observed by anti-β-galactosidase immunostaining. Whether this partially overlapping mode of expression reflects true functional redundancy cannot be deduced from the available data. Rescue experiments using these cis-acting elements might help to clarify this aspect. Unfortunately, a mutagenesis experiment, which has recently been carried

Fig. 6. Independently acting sequences lead to expression either throughout the visceral mesoderm or only in the abdominal visceral mesoderm. Embryos transgenic for pWHL/β3-14 (A, D, G) show β-galactosidase expression in the visceral mesoderm, but only in a restricted region. As expression shows no sharp boundaries, but a graded distribution, the limits of the stained region vary with staining intensity. A maximum of expression can be found in the first two abdominal segments. Note that in (G) staining at the body wall can be recognised. This is due to β-galactosidase expressed in chordotonal organs and a small subset of somatic muscles, where this expression is also restricted in an anterior-posterior fashion. In contrast, the non-overlapping β3 intron fragment present in pWHL/β3-13 leads to β-galactosidase expression throughout the entire visceral mesoderm (B, E, H). This fragment also contains the element(s) responsible for the late expression in the somatic musculature (E, H). The pattern of β3 tubulin, as revealed by the antibody, is shown for comparison (C, F, I). (A, B, C) Lateral views, stage 11; (D, E, F) lateral views, stage 13; (G, H, I) horizontal views, stage 15. A1, first abdominal segment; arrows, somatic mesoderm; arrowheads, visceral mesoderm.
out for the β3 tubulin gene (Kimble et al., 1990), has not led to the isolation of amorphous alleles.

It has been demonstrated in this report, that the β3 tubulin gene carries an enhancer regulating its expression along the anterior-posterior axis in a way reminiscent of the early mesodermal domains of selector genes. The majority of this pattern occurs in the first two abdominal segments and is dependent on \textit{Ubx} function. After ectopic expression of \textit{Ubx} protein, the enhancer becomes active in the entire visceral mesoderm, but not in other unrelated tissues. This suggests a combinatorial action of \textit{Ubx} or a \textit{Ubx}-dependent factor and a transcription factor specific to the visceral mesoderm on this DNA fragment.

From a teleological point of view the question remains open: is this mode of regulation due to some functional requirement, as β3 tubulin is expressed in the entire visceral mesoderm? An explanation might be that the β3 tubulin gene falls under the control of transcription factors that happen to be present at this developmental stage in this mesodermal derivative anyway. Possibly the transcriptional regulators organising and specifying the anterior-posterior axis are also used for more general purposes. However, further analysis of the element described using biochemical and genetic means will provide more insight into the processes establishing regional specification in the mesoderm.

This analysis will show whether the action of \textit{Ubx} on the β3 tubulin gene is direct or mediated by another transcriptional regulator. This should help to close the gap between selector genes and the structural genes realising the positional information. Recently, an approach to identify target genes under homeotic gene control by isolating \textit{Ubx} binding sites from embryonic chromatin has been reported (Gould et al., 1990). ‘Climbing uphill’ might complement the approach coined as ‘fishing downstream’ by Morata and Struhl (1990).

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**Fig. 8.** *Ubx* is a regulator of the expression driven by the 500 bp fragment. Shown are lateral views of stage-11 embryos. At this stage *Ubx* is expressed in a broad region (parasegments 6-12) in the mesoderm, as detected by a monoclonal antibody (A, White and Wilcox, 1984). (B) *LacZ* expression driven by pWHL/β3-14 showing the limited expression domain and the maximum in A1 and A2. (C) In *Ubx* mutants (*Ubx*6.28, Kerridge and Morata, 1982) the maximum pWHL/β3-14 expression in the first two abdominal segments is no longer detectable (compare with B). Staining intensity is uniform over the whole expression domain. (D) Ectopic *Ubx* expression after heat shock induction in embryos heterozygous for HSU (Gonzalez-Reyes et al., 1990) and pWHL/β3-14 leads to extension of *LacZ* expression to the cephalic segments, although it is still limited to the visceral mesoderm. This embryo is double-stained for *Ubx* (red) and β-galactosidase (black). Arrowheads point to the visceral mesoderm. A1, first abdominal segment; ms, mesoderm. 

*Ultrabithorax* mutations in constant and variable regions of the protein coding sequence. *Genes Dev.* **1**, 386-397.


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