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A 14 bp promoter element directs the testis specificity of the *Drosophila* β2 tubulin gene

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To analyze the regulation of gene expression during male germ cell development, we investigated the testis-specific expression of the *Drosophila* β2 tubulin gene. Germ line transformation experiments with the upstream region of the *D.melanogaster* β2 tubulin gene fused to the *Escherichia coli lacZ* gene resulted in the correct tissue specific expression of the reporter gene. Furthermore, we showed that the upstream sequences of the β2 tubulin gene of the distantly related species *D.hydei* can drive the expression of the *lacZ* gene testis specifically in *D.melanogaster* flies. A detailed deletion analysis showed that 53 bp of upstream and 23 bp (*D.melanogaster*) or 29 bp (*D.hydei*) of leader sequences are sufficient to confer tissue specificity. The short promoter regions contain a 14 bp motif at identical positions in both species, which acts as a position-dependent promoter element. *In vitro* mutagenesis and subsequent germline transformation experiments revealed that this sequence is the only element necessary for the testis-specific transcription of the β2 tubulin gene in *Drosophila*.

Key words: β2 tubulin gene/*Drosophila*/gene regulation/spermatogenesis/tissue specificity

Introduction

Tissue-specific gene transcription is accepted to be the result of interactions between general and specific *trans*-acting factors and *cis*-acting elements, the *cis*-acting elements concerned being either part of the promoter, or of an enhancer element, which acts independently of position and orientation (for review see Maniatis *et al.*, 1987). Most of the genes studied to date are expressed in somatic tissues. In contrast, the knowledge about transcriptional regulation in germ cells is rather limited. Though a number of genes are known to be expressed during spermatogenesis, e.g. heat shock genes (Krawczyk *et al.*, 1987; Allen *et al.*, 1988), protamine genes (Peschon *et al.*, 1987 and references therein), proto-oncogenes (reviewed in Propst *et al.*, 1988), tubulin genes (reviewed in Cleveland and Sullivan, 1985) and histone genes (Busslinger *et al.*, 1985; Cole *et al.*, 1986), the lack of cultured cells has hampered the analysis of their regulation. The recent development of germ line transformation systems for mice and *Drosophila*, however, has enabled the extension of studies of gene regulation to those tissues that are not accessible by other methods.

As a model system for germ-cell-specific regulation we chose the β2 tubulin gene of *Drosophila*. This gene is expressed only in the testes and encodes an abundant product essential for spermatid differentiation (Kemphues *et al.*, 1979, 1982; Fuller *et al.*, 1987). Other members of the small β tubulin gene family are expressed with different developmental and tissue specificities (Bialojan *et al.*, 1984; Natzle and McCarthy, 1984; Gasch *et al.*, 1988; Leiss *et al.*, 1988). Most, if not all, of the transcriptional activity in differentiating *Drosophila* sperm cells ceases before the first meiotic division (Hennig, 1967; Lindsley and Tokuyasa, 1980). Thus transcriptional activity is mostly restricted to spermatocytes and can be considered to be cell type specific (for reviews of *Drosophila* spermatogenesis see Lindsley and Tokuyasu, 1980; Hackstein, 1987; Lifschytz, 1987).

As a first step towards understanding the mechanism of gene regulation during the spermatocyte stage of germ cell differentiation, we compared the regulatory regions of the testis specifically expressed β2 tubulin genes of the distantly related species *D.melanogaster* and *D.hydei*. We found that the promoter of the *D.hydei* gene is able to direct testis-specific expression of the *Escherichia coli lacZ* gene in *D.melanogaster*. We show by deletion analysis that 53 bp of upstream sequences together with the first 23 bp of transcribed sequences are sufficient for correct regulation of transcription in the spermatocyte stage. *In vitro* mutagenesis indicated that a 14 bp motif, which is conserved between *D.melanogaster* and *D.hydei*, is the only element controlling the testis specificity of the β2 tubulin gene promoter.

Results

Analogous sequences of the D.melanogaster and the D.hydei β2 tubulin genes confer testis-specific expression in D.melanogaster

We have previously determined that the β2 tubulin genes of *D.melanogaster* and *D.hydei* encode identical proteins (Michiels *et al.*, 1987). In contrast, leader and trailer sequences diverged strongly, emphasizing the evolutionary distance between these species. Northern blot experiments and sequence analysis revealed the presence of a conserved open reading frame (ORF), which is situated 5′ to the β2 tubulin genes of both species and is transcribed from the same strand. The stop codon is located at nucleotide −527 (*D.melanogaster*) and at −284 (*D.hydei*) relative to the transcription initiation sites of the β2 tubulin genes. In the intergenic regions only a few 8 to 14 bp long sequence motifs are conserved at identical or similar positions relative to the transcription initiation sites of the β2 tubulin genes. Based on this knowledge of the gene arrangements we have addressed two questions: firstly, whether the region between the β2 tubulin gene and the 5′ ORF contains all sequences necessary for testis specific expression and, secondly,

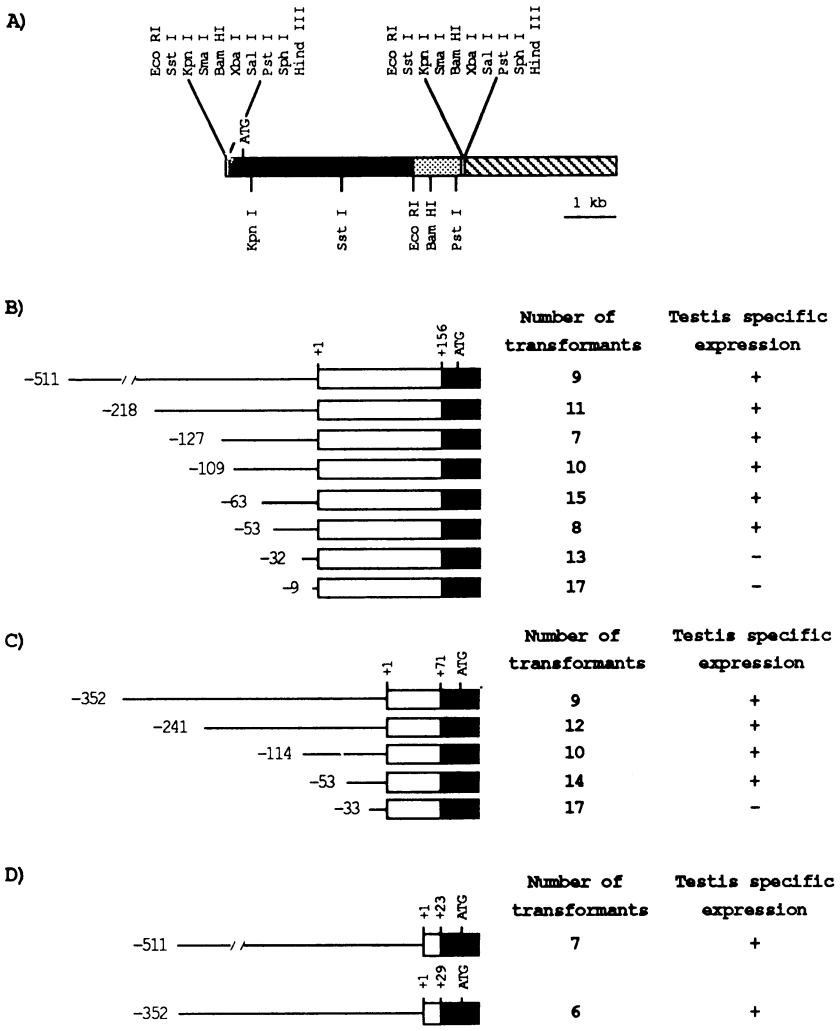

Fig. 1. Constructs used in P-element-mediated transformations. (**A**) For all *β2−lacZ* leader fusions we used the construct pUClac20, which contains a polylinker 5′ to the *E.coli lacZ* gene and one 3′ to the SV40 poly(A) sequences. The position of the translation start site of the *lacZ* gene is indicated (ATG). Since the *Sph*I site contains an additional ATG, all leader fusions were made via the 5′ *Hin*dIII site. (**B,C**) Deletions of the β2 tubulin gene promoters of *D.melanogaster* and *D.hydei* respectively. The positions of the 5′ end points of the constructs are indicated as their distance (in bp) from the transcription initiation site (+1). (**D**) 3′ deletion constructs from the *D.melanogaster* (top) and *D.hydei* (bottom) β2 tubulin genes. Black boxes, *E.coli lacZ* gene (including 5′ untranslated sequences); stippled boxes, SV40 poly(A) sequences; striped boxes, pUC sequences; open boxes, leader sequences of the β2 tubulin genes; thin lines, promoter region of the β2 tubulin genes.

whether any of the blocks of sequence homology in the 5′ regions of the *D.hydei* and *D.melanogaster* β2 tubulin genes represent transcriptional control elements.

In order to determine if the sequences between the transcription initiation site and the neighbouring ORF are conferring tissue specificity, we fused the *D.melanogaster* β2 tubulin sequences (−511 to +156) to the *E.coli lacZ* gene and performed P-element mediated transformations (see Figure 1 and Materials and methods for details). Male flies from independently transformed strains were stained for β-galactosidase activity. With the exception of the known background activity in the gut and occasionally in the sperm pump (Glaser *et al.*, 1986), β-galactosidase activity was observed only in the testes (Figure 2A). The somatic parts of the male reproductive system, e.g. paragonia, vas deferens and ejaculatory duct, were not stained (Figure 2A).

Within the testes, germ cells are linearly arranged according to their developmental stage. Germ cell precursors (spermatogonia) are localized at the very tip followed by cells which enter the meiotic prophase (spermatocytes). After meiosis, the sperm differentiation takes place. These post-meiotic stages occupy most of the adult testes. Staining of transformed flies revealed that the tip of the testes does not show detectable levels of β-galactosidase activity. This region contains spermatogonia and early spermatocytes which do not express β2 tubulin (Kemphues *et al.*, 1982; our own unpublished observation). Thus the DNA region between −511 and +156 of the β2 tubulin gene of *D.melanogaster* is sufficient for correct tissue- and cell-type-specific expression of the marker gene in *D.melanogaster*.

We reasoned that the *D.hydei* upstream sequences should also confer testis specificity to the marker gene after transformation into *D.melanogaster* if the essential regulatory elements are maintained between *D.melanogaster* and *D.hydei*. To test this we ligated a fragment from −352 to +71 of the *D.hydei* β2 tubulin gene to the *E.coli lacZ* gene (Figure 1C). This construct contains 68 bp of the neighbouring ORF, the complete intergenic region and 71 bp of the β2 leader region. As is the case for the *D.melanogaster* gene, this region is sufficient to confer cell-type-specific

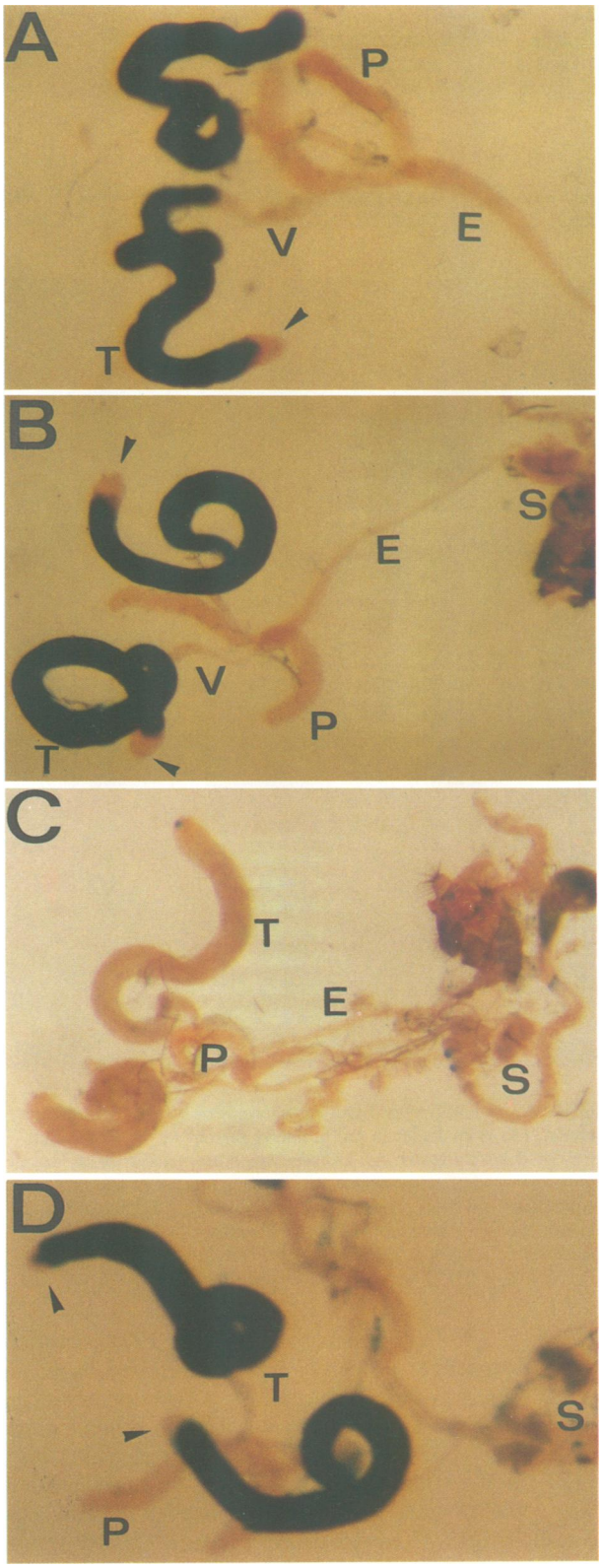

Fig. 2. Transgenic flies: testis-specific expression of β-galactosidase under the control of β2 tubulin gene sequences of *D.melanogaster* and *D.hydei*. Testes were dissected from homozygous males, transformed with the −511 (**A**), −32 (**C**) and +23 (**D**) deletion constructs of *D.melanogaster* or the −352 construct of *D.hydei* (**B**). Staining time was 4 h. Abbreviations used are: T, testis; P, paragonia; V, vas deferens; E, ejaculatory duct; S, sperm pump. The arrowheads point to the tip of the testes.

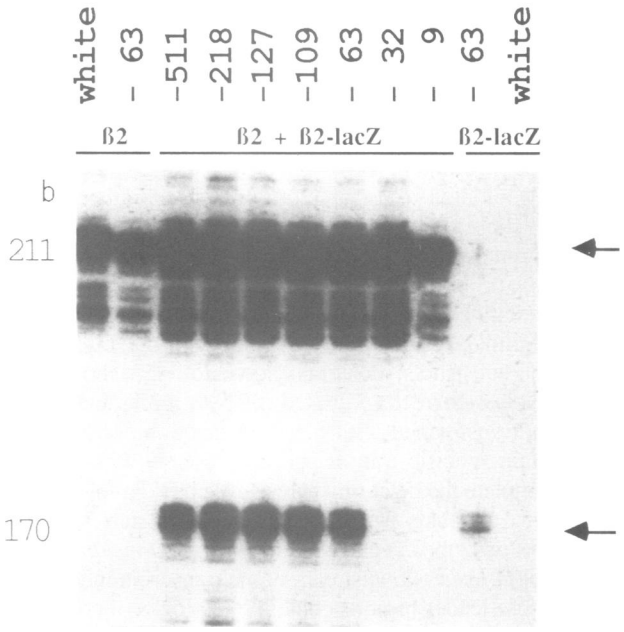

Fig. 3. Quantitative comparison of the RNA levels in the deletion mutants. For the S1 analysis total RNA was isolated from a pooled total of 30 testes dissected from males from at least seven independently transformed lines (for each construct) in order to diminish position effects. Hybridizations to the end-labeled probes were done overnight at 40°C. The endogenous β2 tubulin mRNA served as a standard. The probes used (β2 to detect transcripts of the internal β2 tubulin gene, β2−*lacZ* to probe transcripts of the transformed β2−*lacZ* gene fusions) as well as the deletion constructs used for transformation are indicated above the lanes. White indicates RNA derived from the untransformed white strain. The arrows point to the protected fragment of the internal β2 tubulin gene (211 b) and the protected fragment of the β2−*lacZ* gene fusions (170 b).

expression in the testes after transformation into *D.melanogaster* (Figure 2B). This shows that the general mechanism of β2 tubulin gene regulation during spermatogenesis is conserved between the species *D.melanogaster* and *D.hydei*, and that the conserved sequences in the upstream regions are possible candidates for regulatory elements.

53 bp of upstream sequences are sufficient to direct testis specific expression of the β2−lacZ fusion genes

Using appropriate restriction sites or exonuclease *Bal*31 digestions, we created a set of 5′ deletion mutants (see Figure 1). Deletion of sequences upstream from −53 of the *D.melanogaster* and the *D.hydei* promoters did not effect the testis-specific expression of β-galactosidase (see Figures 1 and 4C). Further deletions to −32 for the *D.melanogaster* or to −33 for the *D.hydei* upstream sequences completely abolish expression (−32 is shown as an example in Figure 2C). Thus extremely short regions are sufficient for correct tissue-specific expression. Analysis of adult flies, third instar larvae and embryos revealed that none of the deletions leads to detectable expression in other tissues, indicating that the deleted regions do not function as silencer sequences.

To clarify whether sequences upstream of −53 have an influence on the level of transcription, S1 nuclease protection experiments were performed (Figure 3). To compare mRNA levels in different deletion mutants we pooled testes from

7–10 independently transformed strains for each deletion construct, thus limiting the variation due to different chromosomal integration sites. Within the limits of detection, no *β2–lacZ* mRNA is present in testes of males transformed with the −32 and the −9 deletion mutants of the *D.melanogaster* *β2* promoter. From all other deletion constructs correctly initiated transcripts are present at rather similar amounts (Figure 3). The level of expression from the −53 deletion mutant was found to be comparable to the mutants containing 63 bp or more of upstream sequences, indicating that no additional enhancing element is present in the region tested. Similar results were obtained with the *D.hydei* *β2* promoter constructs: deletions down to −53 showed the same level of correctly initiated mRNA, while testis RNA of males transformed with the −33 deletion construct did not contain specific transcripts (data not shown).

To complete the deletion analysis we checked for possible regulatory elements 3′ of the transcription start site. The constructs described above contain 156 bp (*D.melanogaster*) or 71 bp (*D.hydei*) downstream of the transcription initiation sites. These leader regions contain two blocks of conserved sequences (see Michiels *et al.*, 1987). To eliminate these homologies we generated 3′ deletion mutants to +29 bp (*D.hydei*) and to +23 bp (*D.melanogaster*) (see Figure 1D). The +156 and the +23 constructs (*D.melanogaster*) showed the same cell type specificity and no significant difference in the level of expression (see Figure 2D). Also, no difference was observed between the *D.hydei* +71 and +29 constructs (data not shown). The remaining parts of the leader do not contain any conserved sequences (see Michiels *et al.*, 1987), making it unlikely that they are involved in the transcriptional regulation of the *β2* tubulin genes.

A conserved 14 bp sequence (β2UE1) is essential for spermatocyte-specific expression of the β2 tubulin gene

Between −53 and the transcription initiation site two sequence motifs common to both *D.melanogaster* and *D.hydei* are present at identical positions relative to the transcription initiation sites (Figure 4A). Deletions to −32 (*D.melanogaster*) and −33 (*D.hydei*) abolish expression (Figures 1 and 2C), indicating that sequences further 5′ are essential for correct expression. A 14 bp homology is present between −51 and −38 in the promoters of both *β2* tubulin genes. To test the function of this element, we mutated it in the *D.melanogaster* *β2* tubulin gene promoter by using an artificial *Bgl*II site created between −33 and −38 (see Figure 4B and Materials and methods) and assayed for testis-specific expression of the *lacZ* gene in several independently transformed strains. Construction of the *Bgl*II site (Mut 1) did not alter the testis-specific expression. In contrast, a promoter in which the 14 bp element is destroyed was not able to direct expression of the *β*-galactosidase gene in any of the nine independent strains tested (Figure 4D). Thus this element, which is hereafter referred to as *β2* upstream element 1 (*β2UE1*), or part of it, is essential for correct expression in the testes.

The β2UE1 functions in a position-dependent manner

We asked whether *β2UE1* functions as an enhancer element by testing it in connection with a heterologous promoter. The *Drosophila hsp70* promoter has been used for similar studies (Garabedian *et al.*, 1986; Hiromi and Gehring, 1987;

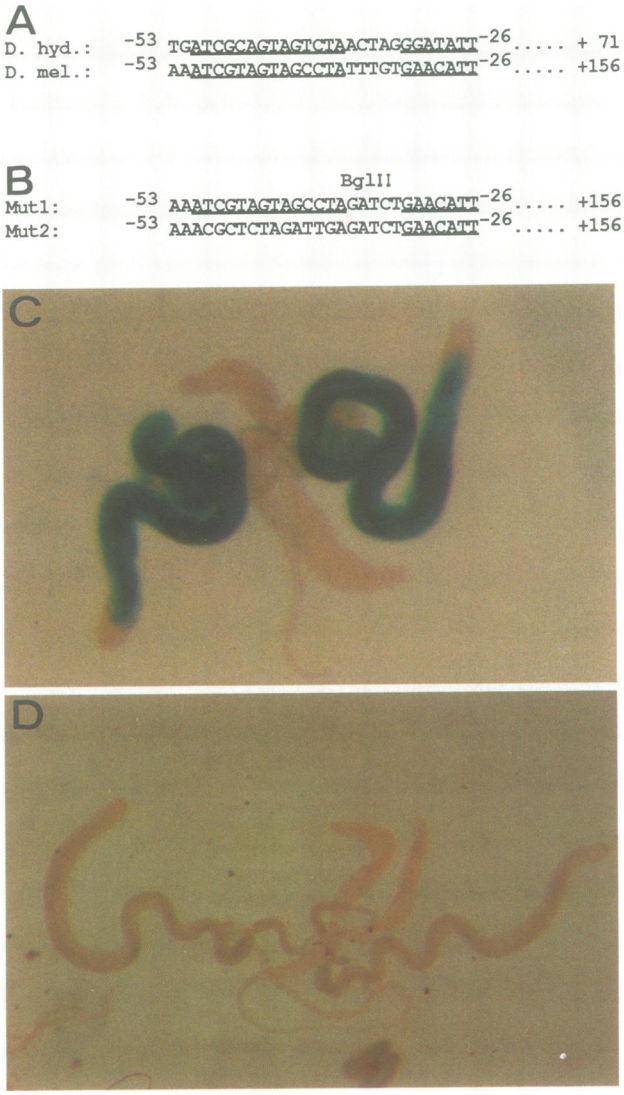

Fig. 4. Oligonucleotide-mediated mutagenesis of the 14 bp upstream element. (**A**) Homologies in the promoter regions of the *β2* tubulin genes of *D.melanogaster* and *D.hydei* which are needed for the correct expression of the marker gene. (**B**) Promoter sequences after introduction of a *Bgl*II site between the homologous regions (Mut 1), and subsequent substitution of the 14 bp element (Mut 2). (**C**, **D**) *β*-Galactosidase activity in the testes of males transformed with Mut 1 (C) and Mut 2 (D). Testes were incubated with X-gal for 4 h.

Bray *et al.*, 1988; Fischer and Maniatis, 1988). In these examples enhancer properties have been tested by fusing regulatory sequences to a truncated *hsp70* promoter lacking the heat shock responsive elements. Using synthetic oligonucleotides, we connected the 14 bp element to a *hsp70–lacZ* construct via a *Nru*I site at −50 bp relative to the *hsp70* CAP site. Analysis of transformed flies revealed no testis-specific *β*-galactosidase staining. This result was not due to a construction artifact as some of the transformed lines did show expression of the *lacZ* gene in other tissues, probably directed by sequences surrounding the integration sites. This means that the *β2UE1* does not function as a classical enhancer element.

In both the *D.melanogaster* and *D.hydei* *β2* tubulin gene promoters, *β2UE1* is located between −38 and −51 relative to the transcription initiation site. In the *β2UE1–hsp70* promoter fusions, however, the *β2UE1* was localized between

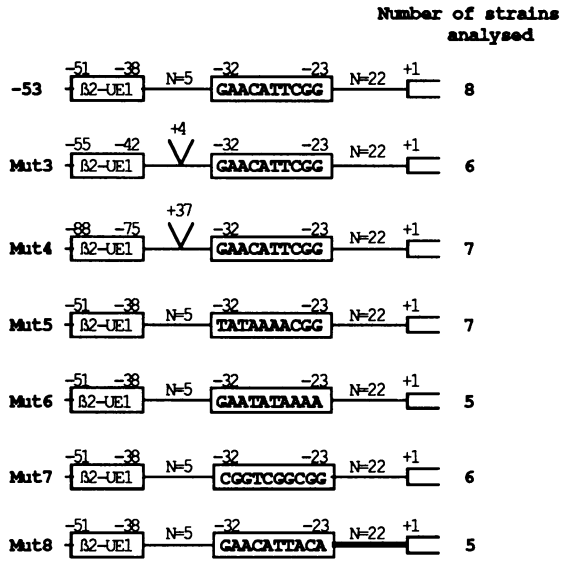

Fig. 5. Constructs used for further analysis of the β2 promoter. All constructs were fused in the leader at position +156 to the *E.coli lacZ* gene. In the wild-type construct (−53) a *Bgl*II site had been introduced between positions −33 and −38, which was used to make the other constructs. The thick line in Mut 8 indicates that all bases between −22 and −2 have been mutated. For further details, see Materials and methods.

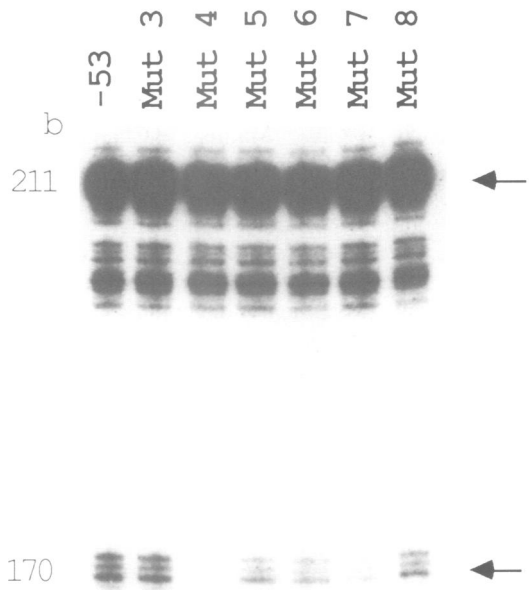

Fig. 6. Quantitative analysis of mutations within the β2 promoter. For each construct, testes from at least five independently transformed strains were collected. Strain designations correspond to the constructs presented in Figure 5. Arrows point to the protected fragments from the internal β2 tubulin gene (211 b) and from the transformed β2−*lacZ* gene fusions (170 b).

−59 and −72 relative to the *hsp70* CAP site. This prompted us to investigate whether the action of the *β2UE1* element is position dependent. We compared the expression from constructs carrying insertions of 4 and 37 bp into the artificial *Bgl*II site located between −33 and −38 (Figure 5). Insertion of 4 bp (Mut 3) does allow correct expression at a normal level (Figure 6). In contrast, analysis of transformed flies with 37 bp inserted (Mut 4) revealed that this construct is not able to direct expression (Figure 6). Thus the position of the *β2UE1* within the promoter is extremely important for the expression of the β2 tubulin gene. The *β2UE1* can therefore be considered as a classical promoter element.

β2UE1 is the only element in the upstream region required for the testis specificity

To test whether the position-dependent *β2UE1* is the only sequence required for the testis-specific expression, we checked for the presence of other testis-specific elements within the remaining β2 promoter region. Between −26 and −32, where normally a TATA element is located, the *D.melanogaster* and *D.hydei* β2 promoters contain the sequences GAACATT and GGATATT respectively. To clarify the function of this element, we mutated it to a classical TATA box consensus (TATAAAA) in Mut 5 and Mut 6 and to an unrelated sequence (CGGTCGG) in Mut 7 (see Figure 5 and Materials and methods). Staining of transformed flies revealed that the *lacZ* gene was still testis specifically expressed. S1 analysis showed that none of the mutations affected the site of transcription initiation (see Figure 6). The amount of mRNA, however, was reduced, indicating that the function of this element is quantitative.

We further asked whether sequences between this element and the transcription initiation site are relevant by mutagenizing all bases between −25 and −2 (Mut 8 in Figure 5). Flies transformed with this construct showed a slightly reduced level but correct pattern of expression (see Figure 6). Sequences between −25 and −2 are also unimportant for the specificity of the promoter.

Since the sequences between −38 and −33 were mutated in these constructs to create a *Bgl*II site at this position (see Figure 4), this means that all bases between −38 and −2 can be mutated without influence on the germ cell specificity. This makes it likely that the *β2UE1* is the only element directing the spermatocyte-specific expression of the β2 tubulin gene.

Discussion

We have begun to examine the mechanism regulating the transcription of the β2 tubulin gene during male germ cell differentiation. Our deletion analysis clearly revealed that 53 bp of immediate upstream sequences are sufficient for correct expression in the testes. Despite the evolutionary distance between *D.melanogaster* and *D.hydei*, which diverged ~60 million years ago (Beverley and Wilson, 1984), the general mechanism of transcriptional regulation of the β2 tubulin genes is conserved. Mutagenesis showed that a 14 bp element (*β2UE1*), which is conserved both in sequence and in position between these species, is the only element required for the activation of testis-specific transcription. The fact that the *β2UE1* does not direct testis-specific expression from a heterologous promoter shows that it does not function as a classical enhancer element. This was supported by our observation that the position of this element within the promoter is crucial for its functioning.

A second element, located between −26 and −32, has a quantitative effect on the testis-specific expression. In sequence and position, it resembles a TATA-like element. Mutagenesis resulted in a 2- to 3-fold depression of tran-

scription, which is less than that observed after mutation of the TATA element in other promoters (Wasylyk *et al.*, 1980; McKnight and Kingsbury, 1982; Wu *et al.*, 1987), and did not change the position of the initiation site, as is the case for the SV40 early promoter (Mathis and Chambon, 1981). It is not known whether or not there is a testis-specific factor binding to this element.

Normally, multiple *cis*-acting elements are needed to mediate tissue specific expression in eukaryotes (Serfling *et al.*, 1985; Maniatis *et al.*, 1987). For instance, the lymphocyte-specific expression of the immunoglobulin genes is regulated by at least three different sequence elements, each of which can direct tissue specificity (Grosschedl and Baltimore, 1985). Though it has recently been shown that the octamer sequence in the promoter alone, in connection with a TATA box, is sufficient for the specificity, the regulation is complicated by the fact that an enhancer sequence is needed to stimulate transcription (Wirth *et al.*, 1987). Also, the regulation of the *Drosophila* β3 tubulin gene during mesoderm development is mediated by multiple elements of which at least one acts as a tissue-specific enhancer (Gasch *et al.*, 1989). In this respect, the simplicity of the β2 promoter is remarkable in that very short promoter sequences are sufficient for regulating tissue-specific transcription.

One might expect that genes expressed in the same tissue are regulated by common *cis*-acting elements. However, the promoters of the *Drosophila* α2 tubulin gene (Kalfayan and Wensink, 1982; Theurkauf *et al.*, 1986) and *hsp26* gene (Glaser *et al.*, 1986), which are expressed in the testes, do not contain any strong sequence similarities to the *β2UE1*, indicating that their regulation might be different from the β2 tubulin gene. Also the *mst(3)gl-9* gene, which is expressed exclusively in the testes (Kuhn *et al.*, 1988), does not contain any *β2UE1*-like sequences. We do, however, find similarities to the *β2UE1* at similar though not identical positions in the promoters of the *Drosophila* β3 tubulin gene and alcohol dehydrogenase gene (*Adh*) (Michiels *et al.*, 1987; Gasch *et al.*, 1988), neither of which are expressed in spermatocytes. The *β2UE1*-like sequence in the *Adh* promoter is part of the region protected from DNase digestion by the factor Adf-1 (Heberlein *et al.*, 1985). This factor is necessary for the *in vitro* transcription of the *Adh* promoter (Heberlein and Tjian, 1988). The function of the *β2UE1*-like element within the β3 promoter is not known. We are currently testing whether *β2UE1* can be substituted by these elements.

Apart from *Drosophila*, in which the study of gene regulation *in vivo* is greatly facilitated by the availability of the P-element transformation system, testis-specific gene regulation has been studied mainly in sea urchins. Using an *in vitro* approach, Barberis *et al.* (1987) have defined sequence motifs in the sperm histone H2B-1 gene promoter which interact with protein factors. A CCAAT-displacement factor, present in tissues other than testis, may act as a repressor of transcription by preventing the interaction between the CCAAT motif and the CCAAT binding factor. A similar model could account for the specificity of the β2 promoter, although the existence of a testis-specific *β2UE1* binding factor could also explain our observations, without the need to postulate the presence of specific repressor or displacement factors in other tissues.

The identification of the *cis*-acting sequences required for the tissue-specific expression of the β2 tubulin gene will now enable us to search for *trans*-acting factors binding to these sequences. In particular, it will be interesting to see how the testis specificity of the β2 promoter is mediated, and to what extent factors binding to the *β2UE1* and *β2UE1*-like sequences are related.

Materials and methods

Construction of the P-element transposons

For all transformation experiments, fusions were performed between the leader regions of the β2 tubulin genes and the *E.coli lacZ* gene. To facilitate this, we constructed pUClac20 (see Figure 1A). From the plasmid pCH110 (Hall *et al.*, 1983) the 5.0 kb *Sca*I−*Hin*dIII fragment was isolated and ligated to the 1.8 kb *Sca*I−*Hin*dIII fragment of pUC18. The resulting construct was partially digested with *Eco*RI and the 4.5 kb fragment, containing the pUC18 polylinker, 5′ untranslated sequences, the *E.coli lacZ* gene and the SV40 poly(A) signal, was ligated into *Eco*RI-opened pUC19, giving rise to pUClac20. pUClac20 thus contains two polylinkers, one 5′ to the *lacZ* gene and one 3′ to the SV40 poly(A) sequence (Figure 1A).

For the *D.melanogaster β2−lacZ* gene fusions, a 1 kb *Hae*III fragment (−0.9 to +0.156 kb; for the determination of the transcription start site see Michiels *et al.*, 1987) was isolated and *Hin*dIII linkers (Boehringer) were added. After restriction with *Hin*dIII and *Pvu*II (−511 bp), the 0.7 kb fragment was ligated, together with an adjacent 1.1 kb *Eco*RI−*Pvu*II fragment (−1.6 to 0.511 kb), into *Eco*RI- and *Hin*dIII-cut pUC18. From this construct, pUMB2, the 0.7 kb *Pvu*II−*Hin*dIII fragment was isolated and ligated, together with a 3.8 kb *Hin*dIII−*Xba*I fragment from pUClac20 containing the *lacZ* gene, into *Hpa*I−*Xba*I-cut P-element vector pW8 (Klemenz *et al.*, 1987).

The −127 bp and −109 bp deletion constructs were generated in a similar way by cutting pUMB2 with *Sca*I−*Hin*dIII and *Hgi*AI−*Hin*dIII respectively. The *Hgi*AI site was made blunt prior to ligation into the *Hpa*I site of pW8. Further 5′ deletions from pUMB2 were made by opening the construct with *Eco*RI followed by treatment with exonuclease *Bal*31 (Boehringer). Resulting fragments, after recutting with *Hin*dIII, were ligated into *Sma*I−*Hin*dIII-cut pUC18. Deletion start sites were mapped by sequencing. Appropriate fragments were isolated from the vector by digestion with *Eco*RI and *Hin*dIII and subsequently ligated into *Eco*RI−*Xba*I-opened pW8, together with the 3.8 kb *Hin*dIII−*Xba*I fragment from pUClac20.

For the *D.hydei* β2 tubulin gene promoter fusions, a 0.4 kb *Sal*I−*Dra*I fragment (−352 bp to +71 bp; see Michiels *et al.*, 1987) was ligated into *Sal*I- and *Sph*I-opened pUC18, of which the *Sph*I site was made blunt by treatment with Exonuclease VII (BRL). From this construct the insert was mobilized by *Xba*I and *Hin*dIII and ligated into *Hpa*I−*Xba*I-opened pW8, together with a 3.8 kb *Hin*dIII−*Sma*I fragment from pUClac20. Further 5′ deletions were made in an analogous way to the *D.melanogaster* 5′ deletions.

The *D.melanogaster* +23 deletion was made by digestion of pUMB2 with *Nhe*I and filling in with the Klenow enzyme. The *D.hydei* +29 deletion mutant was created by *Bal*31 digestion of the intermediate pUC construct. All intermediate pUC constructs were sequenced over both insert junctions and all pW8 constructs were sequenced over the leader fusion site with a synthetic oligonucleotide (5′-GGGAAAATAGGTTGCGCGAG-3′) which primes in the 5′ untranslated region of the *lacZ* gene, to confirm their structure,

Oligonucleotide-mediated mutagenesis

For the oligonucleotide-mediated mutagenesis the −53 deletion mutant of pUMB2 was opened with *Eco*RI (pUC linker) and *Nhe*I, which cuts downstream of the transcription initiation site. This fragment was replaced by the following sequence (written 5′ to 3′; only one strand is shown) which has had 3 bp exchanged between −33 and −37 to create a *Bgl*II site (underlined; see also Figure 5): AATTCAAATCGTAGTAGCCT-AGATCTGAACATTCGGTGTAGTAATCCAAGCCAGGTTCAGTTC-ACCTCAGTATCAG.

The *Eco*RI−*Bgl*II fragment was subsequently replaced by the sequence AATTCAAACGCTCTAGATTGAGATC to destroy the 14 bp element within the −53 deletion mutant. To generate the position mutants, the −53 deletion mutant was linearized with *Bgl*II and either blunt ended and religated (+4 bp spacing mutant) or ligated to the sequence GATCCCGGGCTAG-CCCTAGGTCTAGAACTAGTCGAC to create the +37 bp spacing mutant. Further mutations within the −53 deletion mutant were made by replacing the *Bgl*II−*Nhe*I fragment by the sequences GATCTTATAAAA-CGGTGTAGTAATCCAAGCCAGGTTCAGTTCACCTCAGTATCAG or GATCTGAATATAAAATGTAGTAATCCAAGCCAGGTTCAGTTCA-CCTCAGTATCAG to create a TATA box consensus, by GATCT-

CGGTCGGCGGTGTAGTAATCCAAGCCAGGTTCAGTTCACCTC-AGTATCAG to destroy the TATA-like element or by GATCTGAA-CATTACAGACGTGTCGATGTCGACTAGTCAGTTCACCTCAGT-ATCAG to mutate all other 5′ sequences (mutated sequences are underlined). All sequences were confirmed by sequence analysis of both strands. Further cloning into pW8 was done as described above for the *Bal*31 deletion mutants.

P-element transformations

The recipient strain for microinjections was the *D.melanogaster* strain w^1 (Klemenz *et al.*, 1987). Microinjections were essentially performed as described by Rubin and Spradling (1982). pW8 derivatives were either purified by banding over CsCl−EtBr gradients or by treatment with RNase A and Proteinase K and elution over Elutip-D columns (Schleicher and Schüll). All injections were performed with the aid of an Eppendorf microinjector type 5242 at starting concentrations of 400 μg/ml for the pW8 constructs and 100 μg/ml for the helper plasmid pπ25.7WC (Karess and Rubin, 1984). The transformed strains used in this study were checked for having single and independent integration sites by Southern blotting experiments (Southern, 1975).

Histochemical staining

The staining of males for β-galactosidase activity was performed essentially as described by Glaser *et al.* (1986). After incubation with X-gal, the testes were rinsed three times with 0.7% NaCl, transferred to glycerol and photographed.

RNA analysis

For the S1 analysis (Gasch *et al.*, 1988), RNA was prepared according to Steller and Pirotta (1984). As probes we used a *Sca*I (−127 bp) to *Bst*EII (+211 bp) fragment for the endogenous β2 mRNA and a *Sca*I (−127 bp) to *Xho*II (+170 bp) fragment to detect mRNAs of the transformed *D.melanogaster* β2−*lacZ* gene fusions. Hybridizations were done overnight at 40°C.

Sequence analysis

Sequence reactions were performed with the dideoxy chain termination method of Sanger *et al.* (1977) on double stranded plasmids (Chen and Seeburg, 1985) with either the Klenow enzyme (Boehringer) or the Sequenase enzyme (USB). Sequence analysis was done using software from the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

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