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# Organisation of regulatory elements in two closely spaced *Drosophila* genes with common expression characteristics

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**Abstract**

Sperm tail proteins that are components of a specific structure formed late during spermatid elongation have been found to be encoded by the *Mst(3)CGP* gene family. These genes have been demonstrated to be regulated both at the transcriptional as well as at the translational level. We report here on the dissection of the regulatory regions for two members of the gene family, *Mst84Da* and *Mst84Db*. While high level transcription and negative translational control of *Mst84Da* is mediated by a short gene segment of 205 nt (–152/+53), *Mst84Db* expression is controlled by a number of distinct regulatory elements with different effects that all reside within the gene itself. We identify a transcriptional control element between +154 and +216, a translational repression element around +216 to +275 and an RNA stability element within the 3′UTR. Irrespective of the final common expression characteristics, correct regulation for any individual member of the gene family seems to be achieved by very different means. This confirms earlier observations that did not detect any other sequence elements in common apart from the TCE (translational control element). © 1997 Elsevier Science Ireland Ltd.

*Keywords: Drosophila*; Spermatogenesis; Translational control; mRNA stability; Intragenic elements

## 1. Introduction

The production of a mature sperm with its specialized function and form in *Drosophila melanogaster* is accomplished through a complex series of differentiative events driven by molecular information already laid down premeiotically in the spermatocyte stage after which essentially all transcriptional activity ceases (for a recent review see Fuller, 1993). At least part of this molecular information is represented by RNA transcripts whose translation is properly delayed in order to achieve the appropriate timing between the synthesis of specific protein products and the morphogenetic processes in which they are involved (reviewed in Schäfer et al., 1995). Due to these special characteristics, spermatogenesis in *Drosophila* can be considered an ideal model system for a comprehensive analysis of the different strategies employed by eukaryotic cells in the temporal regulation of gene expression.

One group of protein products involved in the formation of the sperm tail is synthesized by the *Mst(3)CGP* gene family. These proteins are components of the coarse fibers within the satellites found around the accessory tubules that are formed very late in spermiogenesis and that are homologous to the outer dense fibers in the mammalian sperm (Baccetti et al., 1973). One member of this gene family, *Mst87F*, has been analysed in detail (Kuhn et al., 1988b). Gene fusion studies demonstrated that it contains a key element in the 5′UTR, the translational control element (TCE), that is essential for negative translational control and at the same time can exert an effect on transcription of a heterologous gene (Schäfer et al., 1990; Kempe et al., 1993). Since all members of this gene family encode very similar gene products it has been assumed that these have similar or identical function and that the genes therefore might be regulated in similar or identical ways. Moreover, sequence analysis had demonstrated that they all share the

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TCE that is conserved not only in sequence but also in position relative to the transcription start site. Additional common regulatory aspects like secondary polyadenylation concomitant with translational activation at the end of spermiogenesis have been demonstrated to exist (Kuhn et al., 1991; Schäfer et al., 1993). We report here on the regulation of two genes within the 84D cluster. At this location four members of the gene family have been identified (Kuhn et al., 1991). The first two genes, *Mst84Da* and *Mst84Db*, are divergently transcribed and are separated by a 194 nt short intergenic region. We show that, in spite of their close spacing, their similar structural characteristics and their common expression patterns, the control of expression for these two genes is achieved by very different strategies. Moreover, we dissect the gene regulation mechanism of the *Mst84Db* gene in its components and highlight the specific sequence requirements involved in every individual expression control level.

## 2. Results

### *2.1. An enhancing element resides in the proximal 5′ flanking region of Mst84Da*

To identify sequences necessary for correct regulation of *Mst84Da* various 5′ fragments of the gene region were fused to the β-galactosidase gene (Fig. 1). RNA was isolated from transgenic flies and transcript levels were analysed in Northern experiments. Inclusion of 49 nucleotides (–30/+53 and –49/+53, Fig. 1) in front of the transcription start site was not sufficient to allow detectable RNA accumulation (Fig. 2). Addition of 103 nt (–152/+53) from the flanking region resulted in high level transcription. Inclusion of further 5′ adjacent sequences (–348/+92 and –1061/+92) was without any significant effect. The presence of additional intragenic sequences corresponding to the entire 5′UTR (untranslated region) does not result in further increase of transcript levels either. Therefore, all elements necessary for transcriptional control have to reside within the 205 nt long segment around the transcription start site, consisting of 152 nt 5′ flanking sequences and the first 53 nt of the 5′UTR.

Since the short gene fragment (–49/+53) does not promote any detectable transcriptional activity, an enhancing element has to be located in the 5′ flanking region between nucleotides –49 and –152. For quantitative regulation, further 5′ flanking sequences are not required, and even the inclusion of the entire adjacent *Mst84Db* gene has little if any effect on the expression of the *Mst84Da:lacZ* fusion genes (Fig. 2). Therefore, any regulatory elements present within this segment, that might affect *Mst84Db* expression, do not exert a drastic or exclusive effect on *Mst84Da* regulation.

### *2.2. Translational control for Mst84Da is mediated by the 5′UTR*

Since it has been demonstrated that expression of the *Mst(3)CGP* gene family is under translational control, the temporal regulation of translation from the generated fusion genes was tested. A gene fusion containing *Mst84Da* sequences from nucleotide –152 to +53 is transcribed with high efficiency (Fig. 2) and a corresponding level of β-galactosidase activity can be documented in adult testes (Fig. 3b) in which all of the different stages of spermatogenesis are represented at all times. The staining is restricted to elongated stages since the tip of the testis which contains cysts with premeiotic germ cells remains free of staining as well as part of the lumen that contains cysts with postmeiotic germ cells in different stages of elongation (Fig. 3b). In contrast, larval testes, which contain premeiotic stages of spermatogenesis only, remained essentially free of staining (Fig. 3a). Therefore, all sequences necessary for proper translational control have to be contained within the

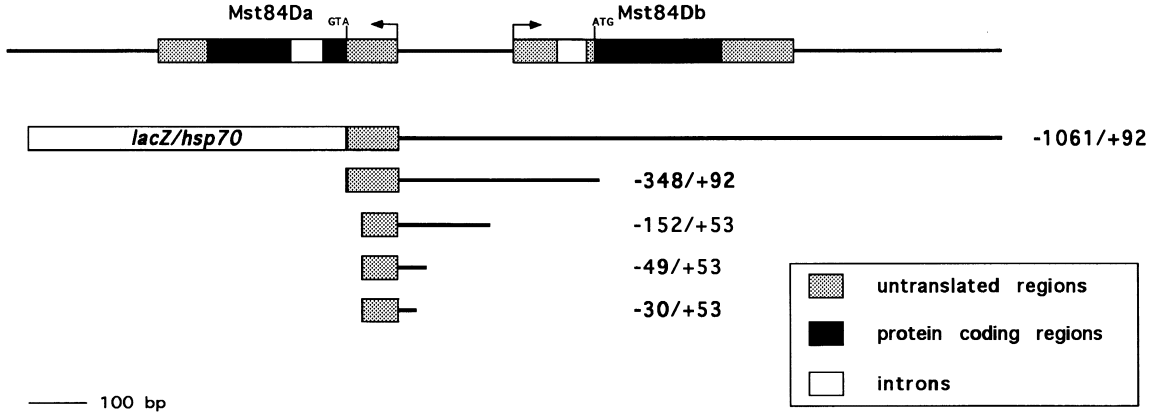


Fig. 1. Diagram of all *Mst84Da:lacZ* gene fusions. Relative to a cartoon of the genomic region containing *Mst84Da* and *Mst84Db* a summary of all *Mst84Da:lacZ* constructs is shown. Systematic names are given that indicate the extent of *Mst84Da* sequences included in the gene fusion. The shadings characteristic for the various portions in the fusion genes are explained in the inlet. Transcription start sites are indicated by an arrow pointing in the direction of transcription, and the translation start codons are shown. Flanking genomic sequences are drawn as a solid line. Only the β-galactosidase gene terminated by the *hsp70* 3′UTR and 350 nt of flanking sequences is not drawn to scale.

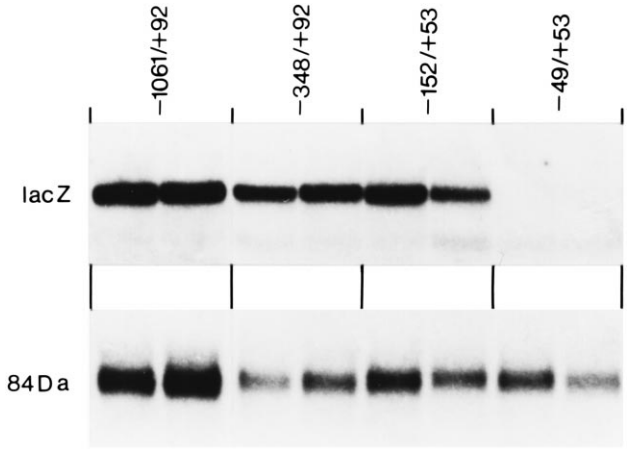


Fig. 2. Transcript levels from *Mst84Da:lacZ* gene fusions. Total RNA was isolated from transgenic adult male flies carrying the gene fusion indicated above the respective lane. The numbers indicate the extent of *Mst84Da* sequences included in those constructs with respect to the transcription start site of the gene (+1). Hybridisation levels are shown for the fusion transcripts (*lacZ*) and as a loading control for the endogenous *Mst84Da* transcripts (84Da). For each construct, two different lines chosen at random were analysed and are shown in neighboring lanes.

5′UTR. This is in accord with previous experiments in which a central importance for translational regulation has been assigned to the TCE that resides within the 5′UTR of all members of this gene family (Schäfer et al., 1990, 1993).

### *2.3. An enhancing element of Mst84Db resides within the coding region*

In order to analyse the sequence requirements for the regulation of *Mst84Db* we first focused on the hypothesis that regulatory elements could be contained within a short interval 5′ to the gene as it is known for the related genes *Mst87F* and *Mst84Da* and several other genes expressed in the male germ line. Even elements shared among *Mst84Da* and *Mst84Db* were a possible way of regulation. Surprisingly, a gene segment spanning the entire intergenic region of 194 nucleotides and the 5′UTR of *Mst84Db* (–286/+154 in Fig. 4) allows only low level expression of the fusion gene (Fig. 6). Extension of the 5′ flanking region to nucleotide –880 (–880/+157, Fig. 4) which comprises the entire *Mst84Da* gene does not increase the observed transcript level (data not shown).

Since the constructs –348/+92 *Mst84Da:lacZ* and –286/+154 *Mst84Db:lacZ* contain the identical fragment fused to the *lacZ* gene in opposite orientation, a direct comparison of the regulatory capacities of this region for both genes is possible. Transcript levels are ten times higher from the *Mst84Da* promoter ($AUG_{Da}$) compared to those from the *Mst84Db* promoter ($AUG_{Db}$; Fig. 5). Therefore, all elements present within this region operate in an orientation-dependent manner, and only in the case of *Mst84Da* are able to drive strong transcriptional activity.

To delimit the size of the regulatory region necessary for the wildtype level of *Mst84Db* transcription, the entire gene region (transcribed sequences and 375 nucleotides of 3′ flanking sequences) was tested (Fig. 4, maxi/375). To be able to monitor transcriptional activity, a fragment from the *β*-galactosidase gene was inserted within the coding region of the *Mst84Db* gene (see Section 4). Northern analyses demonstrated a drastic increase in transcriptional activity of the transgene to a degree comparable to the level of the endogenous gene (data not shown). Variation in the extent of 3′ flanking sequences did not alter the transcript level (data not shown), demonstrating that regulatory elements do not reside downstream of the gene. Conse-

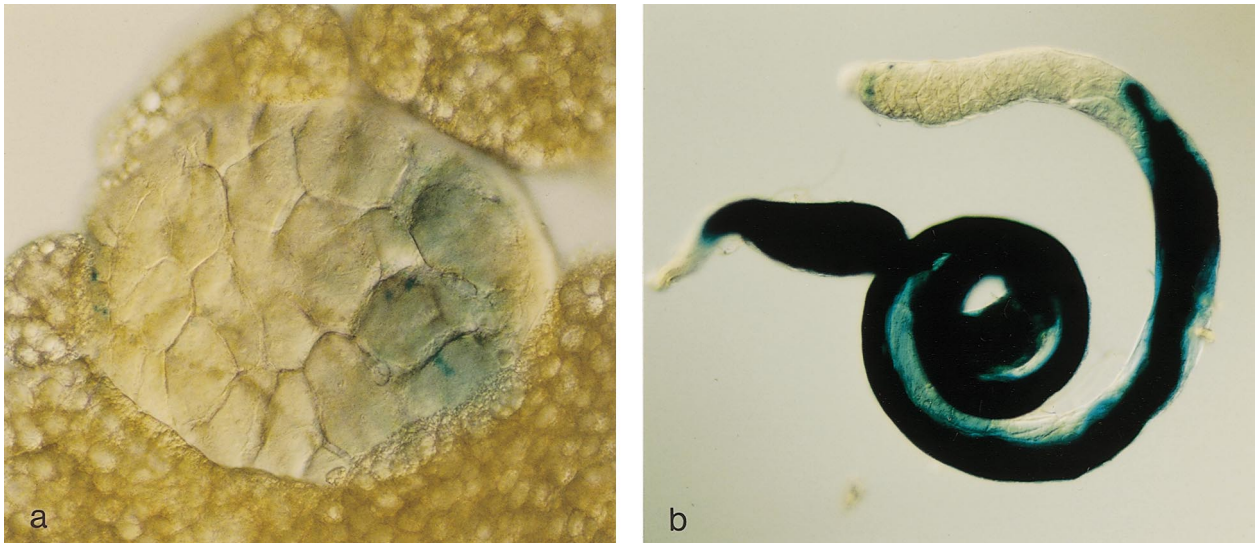


Fig. 3. Temporal profile of translational activity of *Mst84Da:lacZ* fusion genes. *β*-galactosidase activity is documented after X-gal staining overnight. Testis tissue was dissected from third larval instar and adult males of lines transformed with the –152/+53 *Mst84Da:lacZ* fusion gene. Staining was routinely monitored in comparison to tissue from $w^{1118}$ flies. The larval testis (a) remains free of staining, while the adult tissue (b) shows intense blue staining over most of the testis tube, excluding the testis tip.

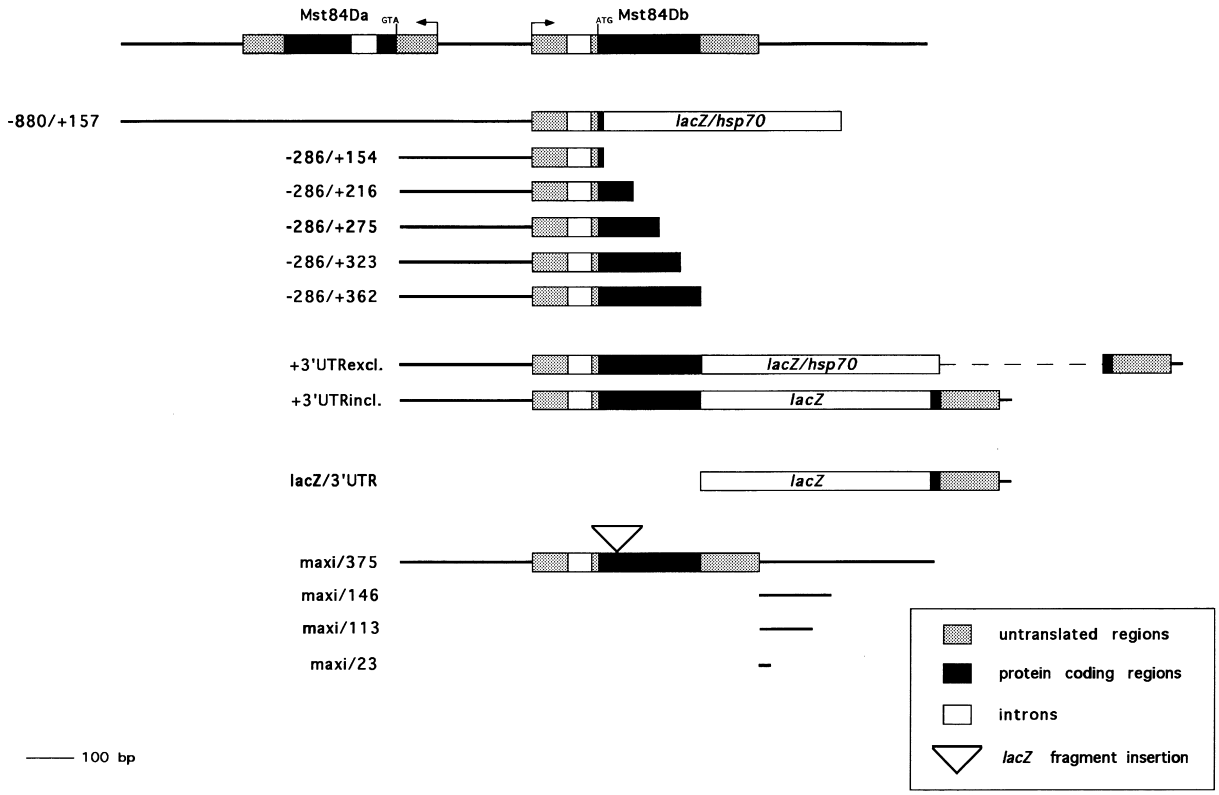


Fig. 4. Diagram of the *Mst84Db:lacZ* gene fusions. Below the cartoon of the relevant genomic region the summary of all constructs with *Mst84Db* sequences is shown. Characteristic shadings for the different portions in the fusion genes are identical to those in Fig. 1 and are again given in the inlet. Additional 350 nt of hsp70 3′ flanking sequences contained in all the fusions are indicated as a discontinuous line in front of the added *Mst84Db* 3′UTR.

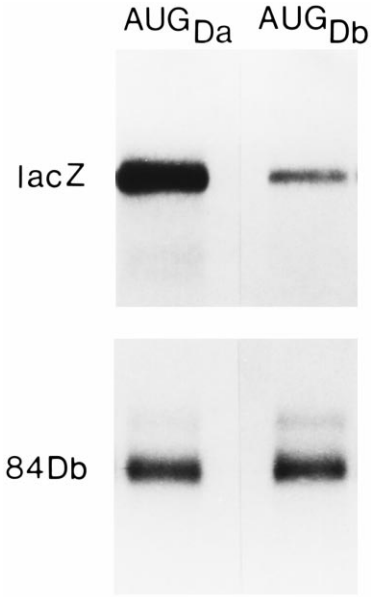


Fig. 5. Comparison of –348/+92 *Mst84Da:lacZ* ($AUG_{Da}$) and –286/+154 *Mst84Db:lacZ* ($AUG_{Db}$) transcript levels. The identical fragment of 440 nucleotides length that spans the intergenic region and includes the 5′UTRs of both genes has been fused in frame in either orientation to the $\beta$-galactosidase gene. The fusion transcript generated from the *Mst84Da* promoter is abbreviated with $AUG_{Da}$, that generated from *Mst84Db* is designated $AUG_{Db}$. Total RNA was extracted from transformed adult male flies. Control hybridisation in this case was performed with an *antisense* transcript of the 3′UTR from *Mst84Db*.

quently, the transcribed region had to be searched for control elements.

Since the 5′ flanking sequences did not contribute to transcriptional regulation of *Mst84Db* beyond a low level, the 5′ flanking region present was kept constant in all further constructs tested (286 nt), and only the extent of the transcribed portion was varied (Fig. 4). Introduction of further 62 nucleotides of the gene (+154 to +216) results in a five-fold increase in transcript level, demonstrating the presence of an enhancing element within this transcribed and translated region of *Mst84Db* (Fig. 6). Even though the signal for the endogenous *Mst84Db* transcripts is increased in the +216 sample, the hybridization intensity for the fusion transcript demonstrates a much more drastic increase allowing the conclusion drawn above — this result has been confirmed in several experiments (not shown). Additional coding sequences do not alter the observed transcript levels significantly (Fig. 6 right panel). The left and right panels in Fig. 6 result from totally independent experiments and the relative hybridization intensities from fusion transcripts to endogenous transcripts are therefore very different. To allow correct assessment of the respective signals between these panels the same RNA sample for the +216 limit has been included in both panels. Due to influences of the different genomic integration sites fusion transcript levels generally vary to a certain degree among the lines tested for any given construct; with the exception of rare cases this varia-

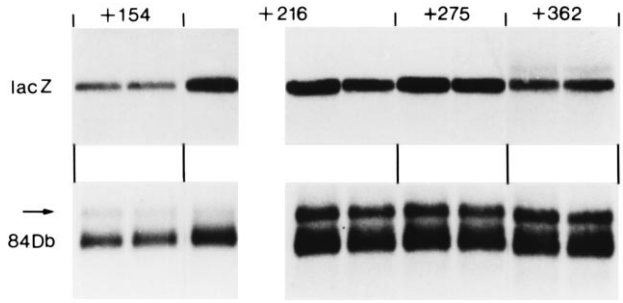


Fig. 6. Influence of the translated region of *Mst84Db* on transcriptional activity. All fusion genes contain 286 nucleotides 5′ flanking sequences (see Fig. 4). The increasing portion of the *Mst84Db* gene that has been included is indicated above the lanes. The first three lanes represent one experiment and were hybridized as a unit, while the remaining part of the figure results from a separate experiment. To allow correct comparison between both experiments, RNA of the same line containing a –286/+216 *Mst84Db:lacZ* fusion gene has been included from both experiments. The arrow in this figure (as in all other figures displaying RNA hybridizations) points to a signal that is due to cross hybridisation to a transcript most probably not residing within the 84D region. It has been observed in several completely unrelated cases by us and others and seems to occur when T7 polymerase is used to generate antisense transcripts from DNA sequences cloned in pBluescript.

tion does not exceed the factor two. The lower levels found for the +362 lines are also within that range and could therefore represent the same experimental variation. Alternatively, a regulatory element leading to a small destabilization effect could reside between +323 and +362 thus decreasing the transcript levels, however for the above mentioned reason only clear effects exceeding the experimental variations are discussed.

### *2.4. The 3′UTR of Mst84Db mediates transcript stability*

Since the fusion trancript levels discussed so far still remained below the wildtype level, the effect of the 3'UTR was tested. When the 3′UTR of *Mst84Db* was added to the gene fusion thereby forming the 3′ end of the fusion transcript, the steady state level increased by a factor of ten (Fig. 7). This additional increase in transcript accumulation has to be due to an increase in transcript stability rather than an increase in transcriptional activity for three reasons.

1. To exert its effect the postulated regulatory element has to reside within the transcript: When the same 3′UTR is included in the fusion construct but placed downstream of the *hsp70* 3′UTR plus flanking sequence and hence excluded from the transcript, the transcript level remains comparable to that observed in the absence of the *Mst84Db* 3′UTR (Fig. 7). The relative positioning within the transcript obviously does not influence its function since in the fusion transcript the 3′UTR is separated from the remaining part of the gene by the entire $\beta$-galactosidase gene.

2. Comparison of transcript levels in larval stages versus adult stages demonstrates a general increase due to the accumulation of transcripts generated in premeiotic stages and has been taken into account by loading a ten-fold lower amount of adult male RNA in Northern experiments to achieve comparable signal intensities (Fig. 8). If the fusion gene is devoid of the *Mst84Db* 3′UTR sequences, the hybridization intensity for the fusion transcript decreases in the RNA of the adult male while that of the endogenous *Mst84Db* RNA increases slightly (Fig. 8, left side). In the case of the fusion transcript containing the *Mst84Db* 3′UTR hybridization intensity clearly increases in the RNA of adult flies even though that of the endogenous *Mst84Db* RNA decreases (Fig. 8, right side). These differences can best be observed on the short exposure added on the very right side of Fig. 8, where the endogenous transcript is barely visible in the RNA of the adult fly.

3. Addition of the 3′UTR from *Mst84Db* to the 3′ end of the *lacZ* gene in the HZ50PL vector (Fig. 4) never resulted in any detectable transcriptional activity (data not shown). Therefore, the 3′UTR sequences do not contain an enhancer element operating at the transcriptional level.

### *2.5. Mst84Db transcripts are translationally repressed*

Having found that the elements of transcriptional regulation differ markedly from the other well-studied cases of the *Mst(3)CGP* genes we asked whether translational regulation was at least conserved. In contrast to our expectation, however, a gene fusion containing the segment –286/+216 from *Mst84Db* does not exhibit translational regulation although the TCE within the 5′UTR is present. The fusion transcripts

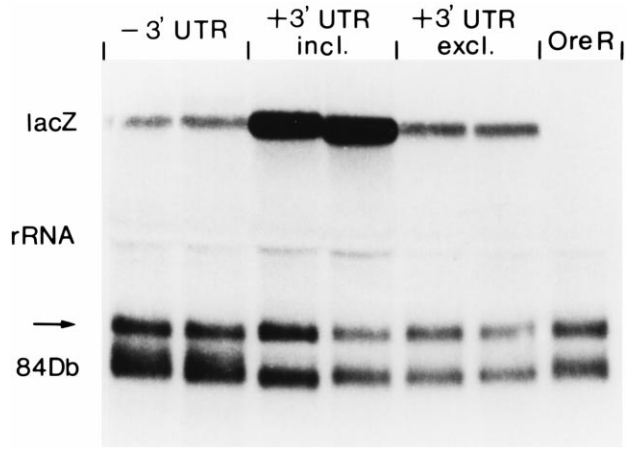


Fig. 7. Effect of the 3'UTR on mRNA levels. Transcript levels are tested in total RNA from transformed lines whose transgenes differ only with respect to the 3′UTR sequences of *Mst84Db*. The fusion gene either does not contain it ('–3′UTR' equivalent to –286/+362 *Mst84Db:lacZ*) or contains it in different locations. It is either included in the transcribed portion ('+3′UTR incl.') or excluded from it due to the presence of the *hsp70* 3′ sequences at the end of the *lacZ* gene ('+3′UTR excl.'). In this experiment again the cross hybridisation can be observed (arrow). Immediately below the rRNA (position indicated) a faint signal is observed that is due to the quenching effect of the large amount of rRNA on the filter and does not represent an additional RNA species. As a control the hybridisation pattern on wildtype OregonR RNA (OreR) is shown.

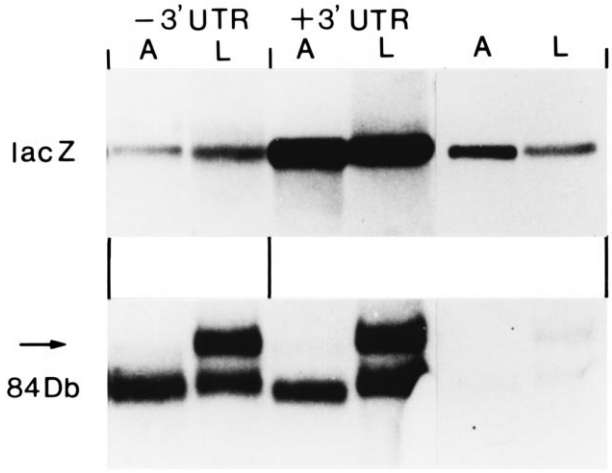


Fig. 8. Transcript accumulation dependent on 3′UTR sequences. To demonstrate a difference in transcript stability depending on the presence of the 3′UTR sequences, total RNA was prepared from male third instar larvae (L) and from adult males of the respective transformed lines (A). To obtain comparable signal intensities 4 $\mu$g of adult RNA were loaded next to 40 $\mu$g of larval RNA; this is obvious in the intensity of the cross hybridizing RNA species (arrow). Due to the drastic increase in transcript levels upon inclusion of the 3′UTR a second tenfold shorter exposure (17 vs. 170 h at −80°C) of the two relevant lanes (+3′UTR) has been added in the rightmost portion of the figure.

are translated already in premeiotic stages, resulting in high level of staining in larval testes (Fig. 9a). Thus, also translational regulation is fundamentally different from that for *Mst87F* and *Mst84Da*.

With increasing size of the included gene fragment the amount of accumulated fusion transcripts remains essentially unchanged (Fig. 6) but translational activity is drastically altered. Inclusion of gene sequences up to nucleotide +275 dramatically reduces translational activity of the fusion transcripts and only residual staining is observed in both larval and adult testes. This inhibitory effect is even a little more pronounced if the included sequences extend to nucleotide +323 or +362 (Fig. 9c,d). This demonstrates the presence of a translational repression element within the coding region of *Mst84Db*. Since the major effect is observable upon addition of nucleotides +216 to +275, the core element should reside within these 59 nucleotides and is therefore distinct from the internal coding region necessary for transcriptional regulation. Since three different gene fusions display the same effect (+275, +323 and +362) it is also apparent that the small reduction in transcript levels for +362 (see last paragraph) cannot be the cause for the drastic decrease in translational activity observed already in larval testes (Fig. 9c).

### *2.6. Stable transcripts are relieved from translational repression*

Although in larval stages RNA levels are increased by a factor of ten when the 3'UTR is included in the fusion transcript (Fig. 7), $\beta$-galactosidase staining in the larval testes remains at a comparably low residual level (Fig. 9e). This demonstrates that translational repression is not a function of RNA levels, and the mechanism efficiently represses even abundant transcripts. In adult tissue, however, a striking difference in $\beta$-galactosidase activity is apparent. Strong staining which is restricted to fully elongated spermatids is observed when the *Mst84Db* 3′UTR is contained within the fusion transcript (Fig. 9f). This demonstrates that during late spermiogenesis translational repression via the internal repressing element is relieved and thus the familiar situation known for temporal translational control is observed, i.e. no activity/staining in larval testes but strong activity in adult testes. Taken together with the observation that the 3′UTR mediates transcript stability it implies that the fusion transcripts of the constructs without *Mst84Db* 3′UTR are degraded before the late timepoint at which translational repression is relieved and, therefore, are never intensely translated.

## 3. Discussion

Divergently transcribed genes with short intergenic regions have frequently been found among members of gene families that are organized in clusters or groups, such as the salivary glue protein genes (Meyerowitz et al., 1985) and the yolk protein genes (reviewed in Bownes, 1994) which are then regulated in a very similar manner (Hofmann et al., 1991; Garabedian et al., 1985; Lossky and Wensink, 1995). Alternatively, the genes can code for products involved in the same pathway or be totally unrelated and can exhibit similar or very different expression characteristics both with respect to time and space (e.g. Gavalas and Zalkin, 1995; Driscoll and Williams, 1987; Mougneau et al., 1993; Wright et al., 1995). In any of these scenarios the organisation of regulatory elements has been found to be very different. In some cases the intergenic region is sufficient to drive correct expression from both gene promoters, and this effect is mediated by common sequence elements (e.g. Lennard and Fried, 1991; Hofmann et al., 1991). In other cases only a subset of regulatory elements operates on both genes, and they can be either exclusively located in the intergenic region or distributed in both intergenic and intragenic sequences (e.g. Heikkila et al., 1993; Punt et al., 1995). Here we have shown that in the case of the two divergently transcribed genes *Mst84Da* and *Mst84Db* the intergenic region is able to promote high *Mst84Da* transcription but confers only low transcriptional activity to the *Mst84Db* promoter. The relevant 5′ flanking element(s) required for *Mst84Da* expression are confined to a short sequence between −49 and −152, and this is in agreement with the finding that many testis specifically expressed genes contain small promoter regions. *Cis*-acting elements necessary for high level spermatocyte specific transcription have been identified very close to the transcription start site of the $\beta$2 tubulin gene (Michiels et al., 1989, 1991), the

*Mst87F* gene (Kempe et al., 1993 and unpublished data), the dihydroorotate dehydrogenase gene (Yang et al., 1995), and the janus B gene (Yanicostas and Lepesant, 1990). In contrast, in the case of *Mst84Db* even a segment of 880 nucleotides in front of the gene was not sufficient to confer high level transcription. Irrespective of the amount of flanking sequences present in the construct, fusion genes containing the first 157 nt of *Mst84Db* with the entire 5′UTR and the

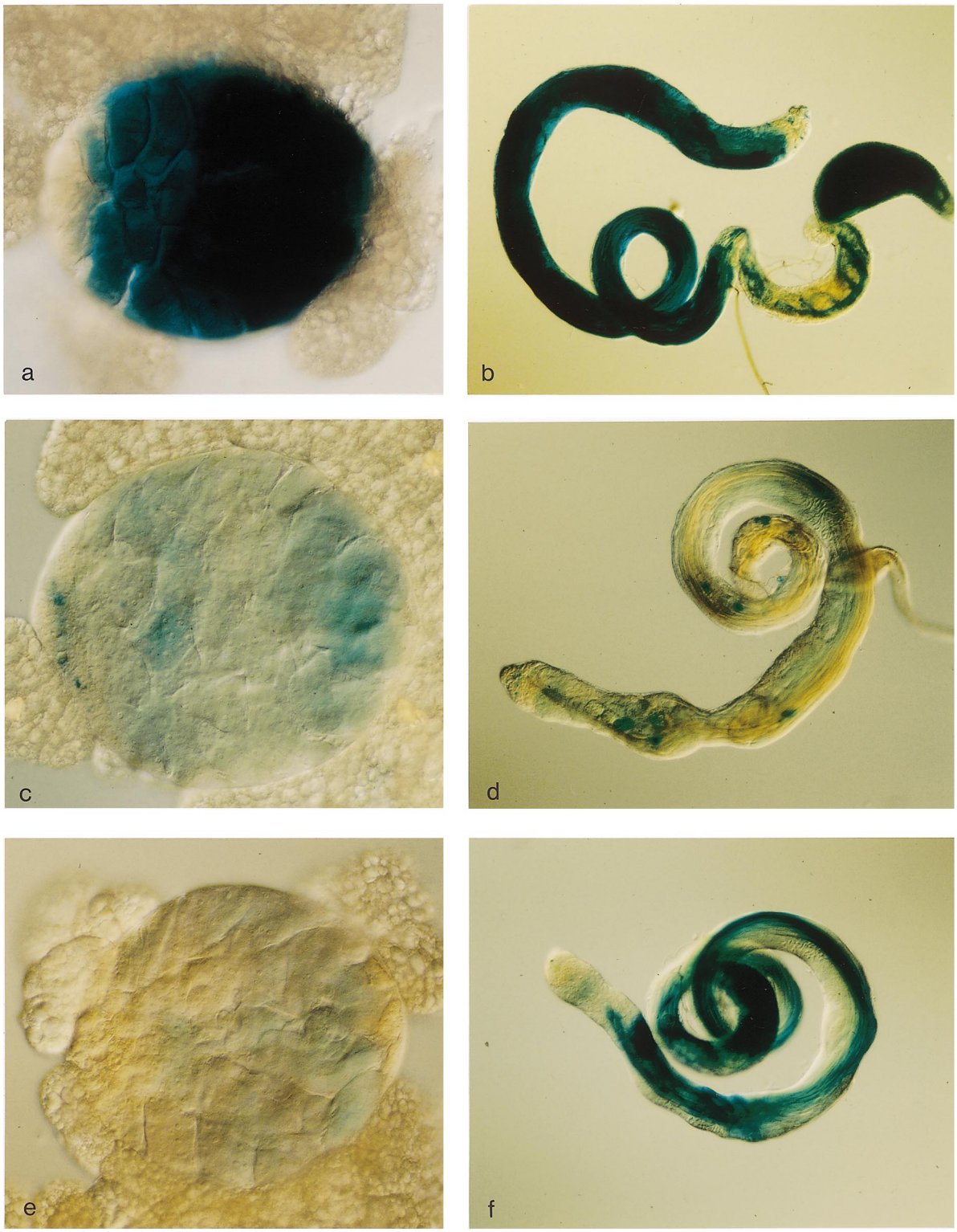


Fig. 9. Translational activity of the *Mst84Db:lacZ* fusion genes. Testes were dissected from third instar larvae and from adult males transformed with the following fusion genes (compare Fig. 4): –286/+216 *Mst84Db:lacZ* (a and b), –286/+362 *Mst84Db:lacZ* (c and d) and +3′UTRincl. (e and f). X-gal staining was again performed overnight. For corresponding transcript levels see Figs. 6 and 7.

first 12 nt of the open reading frame were only transcribed at low levels (Figs. 4, and 6). Addition of the adjacent 62 nt within the open reading frame (up to nucleotide +216) allows a five-fold enhancement of transcription. Therefore, these intragenic and even coding sequences must harbor a transcriptional enhancer element. In general, transcriptional control elements are located outside of transcribed gene regions. In the few cases in which they have been identified inside a gene, they have mostly been found within intron sequences (Banerji et al., 1983; Bornstein and McKay, 1988; Sternberg et al., 1988; Buttgereit and Renkawitz-Pohl, 1993) or within the 5′UTR (Schollen et al., 1995; Hixson et al., 1996) and in one case within the 3′UTR (Le Cam and Legraverend, 1995). In the case of the *Drosophila* white gene positive regulation of transcriptional activity has been found to require part of the coding region (Qian and Pirrotta, 1995). Dosage compensation, i.e. doubling of transcriptional activity in the male situation of this X-chromosomally located gene depends on the presence of the so-called dosage compensation determinants residing in part within the coding region. In yeast, a case has been described where a combination of activation and repression elements within the coding region govern transcription of the lipoamide dehydrogenase gene (LPD1) (Sinclair et al., 1994). Finally, also regulatory elements with negative effects on transcriptional regulation have been found within the open reading frame of a gene. In the case of a divergently transcribed *Hsp70* gene pair of the mouse a silencer element within the coding region of one gene regulates the activity of the other testis-specifically transcribed *Hsc70t* gene and possibly that of the first gene, too (Shimokawa and Fujimoto, 1996). The enhancer element of the *Mst84Db* gene represents a case in which a general positive transcription control element resides within the open reading frame of the regulated gene itself.

At the genomic region 84D the four genes of the *Mst(3)CGP* gene family are very closely spaced. In addition to the four genes identified by us (Kuhn et al., 1991), Mitcham et al. (1996) identified part of an open reading frame for an interleukin-1 receptor type protein. This open reading frame ends 45 nucleotides away from the 3′ end of the divergently transcribed *Mst84Da* gene. It is to be expected that both genes/transcripts overlap with their respective 3′ ends. In a number of densely transcribed loci at which the genes are transcribed in the same direction, for example z600 and the gonadal gene (Schulz et al., 1990) as well as the janus locus with the janA and janB gene (Yanicostas and Lepesant, 1990) the regulatory elements for one gene are found in the 3′UTR of the other gene.

An important aspect of post-transcriptional regulation is the modulation of mRNA stability which can be used to either rapidly remove specific transcripts and gene products from the cell or in the other extreme allow long-term persistence of specific mRNAs (Surdej et al., 1994). During spermatogenesis many mRNAs have to be stabilized to ensure storage through meiosis and into the transcriptionally silent postmeiotic period of spermatid differentiation (Olivieri and Olivieri, 1965; Gould-Somero and Holland, 1974). Michiels et al. (1993) have shown that an 18 nt *cis*-acting element located in the 5′ untranslated region of the *β*2 tubulin gene is able to confer mRNA stability; the 3′UTR of the exuperantia male specific mRNA fulfills a similar function (Crowley and Hazelrigg, 1995). Since the addition of *Mst84Db* 3′UTR sequences only results in high steady-state level of fusion transcripts if they are included inside the transcript, the increase also has to be due to accumulation and not to increased synthesis. Hence the 3′UTR contains a stabilizing element. Sequence comparison with the 3′UTR of exuperantia as well as the 18 bp stability element of the *β*2 tubulin gene did not show any similarities, thus excluding the possibility of a common element necessary for increased mRNA stability in spermatogenesis.

In RNA of the third larval instar the 3′UTR containing fusion transcript accumulates to a significantly higher level than the fusion transcript that does not contain the stabilizing element (Fig. 8). Therefore, much of the fusion transcript without the stabilizing element is already degraded during the transcriptionally active phase of spermatocyte growth. In adult testes the level increases in both cases due to the continuous supply of newly developing sperm and the inclusion of all further developed stages of spermatogenesis. The extent of increase, however, is more pronounced in the case of the fusion gene containing the 3′UTR due to the longer lifespan of the mRNA (Fig. 8). The increase in mRNA stability has to be high enough to ensure mRNA presence until the end of spermiogenesis i.e. 3 days after cessation of transcription since this is the time-point at which onset of translation is observed.

A further regulatory step in the control of gene expression can occur at the level of mRNA translation and is executed by preventing protein synthesis until well after transcription (Curtis et al., 1995; Schäfer et al., 1995; Seydoux, 1996). Such mechanisms are widely used in many developmental systems, and several examples have also been reported for testis specifically expressed genes in *Drosophila* (Kuhn et al., 1988b; Yanicostas and Lepesant, 1990; Schäfer et al., 1993; Yang et al., 1995; Santel et al., 1997).

The data presented here represent the first direct proof that also the *Mst84D* genes are under translational control. The regulatory mechanisms employed, however, differ drastically. In the case of *Mst84Da* a short segment at the 5′ end of the gene confers translational control. This is in agreement with earlier data in which the conserved TCE in the 5′UTR was identified as a central element for translational regulation (Schäfer et al., 1990; Kempe et al., 1993). Modification of the TCE results in premature translational activity, i.e. one element mediates both temporal translational repression as well as activation. In the case of *Mst84Db*, on the other hand, the phenomena of repression and derepression can be separated and are controlled by two different elements at two different levels of regulation. The presence of a *cis*-acting element contained in the coding

region is mandatory for translational repression. Late translational activation however is dependent on mRNA stability mediated by the 3'UTR (for a summary and comparison see Fig. 10). Whether the repressing element is sufficient to confer translational repression to a heterologous transcript has not been tested. Sequence conservation among the seven members of the *Mst(3)CGP* gene family is restricted to the coding region of the genes, and both 3′ as well as 5′UTRs have diverged so much that UTR sequences allow a gene specific hybridization on Northern blots. Within the divergent 5′UTRs mainly the block of 12 nucleotides in the TCE has been conserved. Without a conserved regulatory function of this element, the same divergence of sequences should be observed throughout the 5′UTR – the specific function of the TCE in the context of *Mst84Db* regulation however remains to be elucidated.

In a number of cases translational regulatory elements function via a specific secondary structure as for example in the case of the ferritin gene (Bettany et al., 1992; Rouault et al., 1996). A computer search of the relevant *Mst84Db* region did not reveal any prominent secondary structure that would have been unique and therefore a likely point of interaction with a regulatory protein. Alternatively, the regulatory protein could recognize a specific sequence element present at this location. The observed diversity in the organisation of the *cis*-acting regulatory elements makes it unlikely that a general mechanism operates to mask or sequester the mRNAs of the gene family. Rather at least some specific protein-RNA interactions aimed at modulating translational initiation seem to occur on every individual mRNA.

Two possible mechanisms can be invoked by which translational activity can resume late in spermiogenesis. On the one hand, a repressor molecule bound at the *cis*-acting element could dissociate due to modifications in the cellular environment and this would directly result in translational activity. On the other hand, a positive activating mechanism could be required in addition as has been found for a number of RNAs in many developmental systems. Frequently, modulation of polyA tail length has been proven to be involved in translational activation (McGrew et al., 1989; Bachvarova, 1992; Richter, 1996). Although secondary polyadenylation does occur concomitant with translational activity of the *Mst(3)CGP* gene family members, the extent of polyA elongation is markedly different from one gene to the next (Kuhn et al., 1991). *Mst84Da* transcripts undergo cytoplasmic polyadenylation to a degree comparable to that of *Mst87F* transcripts while the *Mst84Db* transcripts exhibit a size increase only in a smaller population (Schäfer et al., 1990; Kuhn et al., 1991). Taken together with the observation that in the case of *Mst84Da* the 5′UTR is sufficient to confer correct regulation it implies that the mechanism of regulation is likely to be similar for *Mst84Da* and *Mst87F*. In the case of *Mst84Db*, on the other hand, the mechanism could indeed be a default mechanism in which the persisting mRNAs can be translated as soon as the repressor has been released.

## 4. Experimental procedures

### *4.1. Fusion gene constructs of Mst84Da*

**–30/+53 *Mst84Da:lacZ*** (15 independent lines established): Plasmid clone p1172 contains a 205 bp TaqI fragment from –152 to +53 relative to the transcription start site of *Mst84Da* in pTZ 18U. An 86 bp HindIII (at position –27) /XbaI (in the multicloning site (mcs) directly behind nt +53) fragment was excised and ligated into XbaI/NotI digested pW-ATG-lac1. After ligation of the XbaI sites the incompatible NotI and HindIII ends were filled in by Klenow fragment and blunt end ligated.

**–49/+53 *Mst84Da:lacZ*** (5 lines): From the same clone p1172 (see above) a 108bp BspHI (nt -45) /XbaI fragment (see above) was isolated, filled in with Klenow fragment and ligated into NotI digested and Klenow fragment treated pW-ATG-lac1 (Kuhn et al., 1988a).

**–152/+53 *Mst84Da:lacZ*** (6 lines): During the cloning experiments two XbaI/BspHI fragments that had been generated from the two possible orientations of the aforementioned 205 bp TaqI fragment in pTZ18U were fortuitously ligated reconstituting the –152/+53 fragment flanked by XbaI sites that could then be ligated into XbaI digested

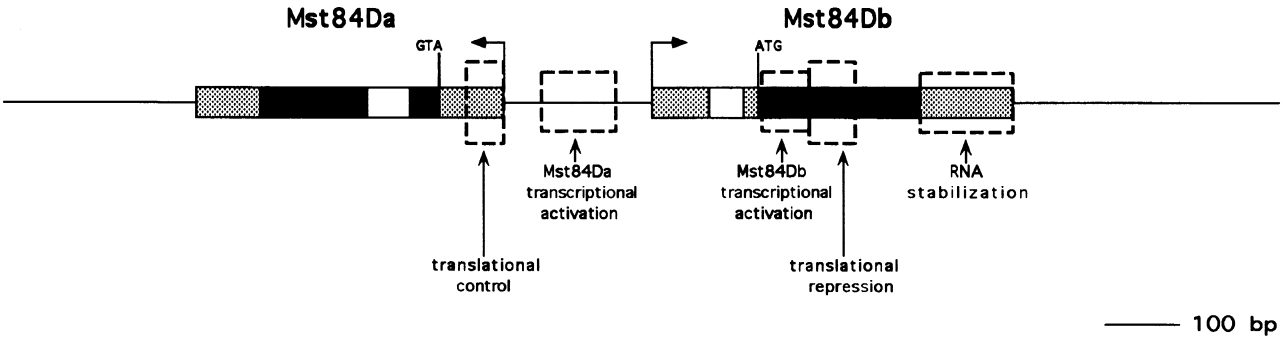


Fig. 10. Schematic representation of the regulatory regions around the two genes. Onto the cartoon of the genomic region stippled boxes have been drawn that indicate the localization of various regulatory elements. Limits are deduced from the expression analyses of the fusion genes. The respective level of regulation for each region is specified underneath. Symbols and shading of gene regions are as in Figs. 1 and 4.

pW-ATG-lac1. Correct orientation of the fragment was tested by sequencing.

**$AUG_{Da}$/$AUG_{Db}$** (6 lines/ 7 lines): Clone p949 contains an EcoRI/ApaI fragment with 1077 bp of 5′ flanking region (including the entire *Mst84Db* gene) and the first 182 bp transcribed sequences from *Mst84Da* in pBluescript KS. A 441 nt long RsaI/AvaII fragment was isolated that spans nt 593 to nt 1034 of the sequenced genomic region at 84D (Kuhn et al., 1991; accession # X67703) equivalent to nt −348/+92 with respect to the *Mst84Da* transcription start site and at the same time nt −286/+154 with respect to *Mst84Db*. Restriction sites were filled in with Klenow fragment, the orientation was verified by HindIII digestion and the correct reading frame by sequencing. This fragment was cloned into StuI digested transformation vector pWLac2 (Weinert and Schäfer, unpublished data). One orientation fused the open reading frame of the *Mst84Da* gene to *lacZ*, the other orientation fused the open reading frame of *Mst84Db* to the *lacZ* gene.

**−1061/+92 *Mst84Da:lacZ*** (17 lines): A DNA fragment spanning the gene region −1061 to +92 was generated by digestion with RsaI from clone p949 (see above) and cloned into the SmaI site of plasmid pUC18. A properly oriented insert fragment (PstI site of pUC18 at its 5′ end, KpnI site at its 3′ end) could then be cloned into the respective restriction sites of the P element transformation vector pWlac1 (Weinert and Schäfer unpublished data).

### *4.2. Fusion gene constructs of Mst84Db*

**maxi/375** (17 lines) and **shorter 3′** flanking region: The cloned *Mst84Da* segment from −1061 to +92 in pUC18 (p949, see above) spans the entire *Mst84Db* gene flanked by 286bp upstream and by 375bp downstream sequences. It was linearized by partial BglI digestion at position +188 in the *Mst84Db* sequence, the ends were filled in by Klenow fragment and ligated to a 633 bp long *lacZ* fragment (HpaI fragment from +439 to +1063 in the $\beta$-galactosidase gene cloned into SmaI digested pTZ18U, excised by BamHI/KpnI and filled in by Klenow fragment). The insert of the resulting clone, named p84Dbinslac, was excised by BamHI/KpnI in the mcs and cloned into the respective restriction sites of the modified P element transformation vector pWlac1 devoid of *lacZ* sequences.

Clone p84Dbinslac was used to generate a set of 3′ flanking region deletions by limited Bal31 digestion after linearization with PstI in the polylinker at the 3′ side of the insert. After KpnI digestion on the 5′ side of the insert a population of partially deleted fragments was isolated from a preparative agarose gel and cloned into KpnI/SmaI digested pBluescript KS. The extent of deletion was determined by DNA sequencing. Those *Mst84Db* gene fragments with the aforementioned *lacZ* fragment included at position +188 that extended to 146, 113 or 23 bp downstream of the gene were recovered by BamHI/KpnI digestion and cloned into the respective restriction sites of the pWLac1 version devoid of the *lacZ* sequences, thus generating the constructs maxi/146 (6 lines), maxi/113 (7 lines) and maxi/23 (13 lines), respectively.

**−880/+157 Mst84Db:*lacZ*** (26 lines): Clone p791 contains the entire *Mst84Db* gene together with 880bp upstream and 391bp of downstream sequences cloned into the EcoRI site of pBluescript KS. It was linearized with ClaI in the polylinker at the 3′ side of the gene, made blunt ended by Klenow fragment and digested with HindIII at position −169 relative to the *Mst84Db* transcription start site which deleted the *Mst84Db* gene and the downstream region. In addition, an AvaII fragment from the same clone p791 was isolated that contained the segment −411/+157 of *Mst84Db*. The AvaII sites were filled by the action of Klenow fragment, and the DNA fragment was subsequently digested with HindIII at position −169. The resulting segment −169 (HindIII) /+157 (AvaII blunt) was ligated to the remaining part of p791 with the −880/−169 (HindIII) gene segment. The newly combined *Mst84Db* sequences were excised by PstI (at the 5′ end) and KpnI (at the 3′ end) and cloned into the respective restriction sites of the P element transformation vector pWlac3.

**The 3′ end deleted gene fusion constructs**: The plasmid clone containing region −1061/+92 of *Mst84Da* (see above) was used to generate a set of *Mst84Db* 3'end deletions by limited Bal31 digestions starting at the PstI site in the polylinker on the 3′ side of the insert. Following EcoRI digestion on the 5′ side (which retains the KpnI site of the mcs on that side) the population of partially deleted fragments was isolated from a preparative agarose gel and cloned into the pBluescript KS vector digested with EcoRI/HincII which places a second KpnI site on the 3′ end of the insert. The extent of deletion was determined by DNA sequencing. The *Mst84Db* fragments with endpoints at +216, +275, +323 and +362 were excised as KpnI fragments and cloned into the respective site of the pWlac1 or pWlac2 (+216) P element transformation vector generating the constructs −286/+216 *Mst84Db:lacZ* (18 lines), −286/+275*Mst84Db:lacZ* (16 lines), −286/+323 *Mst84Db:lacZ* (21 lines) and −286/+362 *Mst84Db:lacZ* (20 lines). The correct insert orientation in these constructs was identified by analysing the products of restriction digestions.

**+3′UTRincl**. (17 lines). A DNA fragment containing the 3'UTR of the *Mst84Db* gene was generated from the pBluescript KS clone with the maxi/23 fragment (see above) by AvaII digestion at position +341 in the *Mst84Db* sequences and XbaI in the polylinker (including the BamHI site of the pBluescript mcs). Following Klenow enzyme filling reaction, this fragment was cloned into the HincII site of pUC19 in an orientation to be recovered by BamHI digestion and cloned into the pWlac1 P element transformation vector's BamHI site located immediately downstream of the *lacZ* sequences; the resulting recombinant that had *lacZ* and *Mst84Db* sequences in the same transcriptional orientation was identified by restriction digestion and named +3′UTRincl. Finally, the −286/+362 *Mst84Db* dele-

tion fragment cloned into pBluescript KS vector (see above) was recovered by KpnI digestion and cloned into the respective site of +3′UTRincl., identifying its correct orientation by analysing the products of restriction enzyme digestions.

**+3′UTRexcl**. (14 lines): A DNA fragment containing the *lacZ-hsp70* 3′UTR hybrid gene was generated from the pWlac1 P element transformation vector by KpnI linearization in the polylinker region at the 5′ side of the *lacZ* gene and subsequent EcoRI partial digestion in order to maintain the EcoRI site located at the 3′ side of the *hsp70* 3′UTR. The resulting DNA fragment was cloned into the KpnI/EcoRI sites of a pBluescript KS recombinant plasmid containing the 84Db3′UTR BamHI fragment (deriving from the pUC19 recombinant plasmid described above) cloned in the same transcriptional orientation into the respective restriction site. The *lacZ-hsp70/84Db3′*UTRs cassette was excised by a two-step digestion: SacII digestion in the polylinker at the 3′ side of the composite insert followed by T4 polymerase exonucleolytic treatment to create a blunt end, and KpnI digestion in the polylinker at the 5′ side. The resulting DNA fragment was cloned into the pWlac1 P element transformation vector deprived of its *lacZ-hsp70* 3′UTR hybrid gene by EcoRI digestion followed by Klenow enzyme filling reaction to create a blunt end and subsequent KpnI digestion. The obtained recombinant plasmid was named +3′UTRexcl. As before, the −286/+362 *Mst84Db* deletion fragment cloned into the pBluescript KS vector (see above) was recovered by KpnI digestion and cloned into the respective site of +3′UTRexcl., identifying its correct orientation by analysing the products of restriction digestions.

***lacZ*/3′UTR** (18 lines): The *Mst84Db*3′UTR BamHI fragment (deriving from the pUC19 recombinant plasmid described above) was made blunt ended by Klenow enzyme filling reaction and cloned into the HZ50PL P element transformation vector (Hiromi and Gehring, 1987) cut with SalI at the 3′ side of the *lacZ* gene and filled by Klenow enzyme to create blunt ends; the resulting recombinant plasmid that had the *lacZ* gene and the *Mst84Db* sequences in the same transcriptional orientation was identified by analysing the products of restriction digestions.

### *4.3. P element mediated germline transformations*

Transformations were essentially carried out as described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Injections were performed into embryos from the $w^{1118}$ strain. Plasmid DNA of the various P element constructs was purified using Quiagen-tip 500 columns, mixed with helper plasmid pUChs$\pi(\Delta)$2–3 DNA at concentrations of 500 $\mu$g/ml and 100 $\mu$g/ml, respectively, and dialyzed overnight at room temperature against transformation buffer (5 mM KCl, 0,1 mM sodium phosphate buffer, pH 6.8).

### *4.4. Northern analyses*

RNA isolations were carried out according to Chomczynski and Sacchi (1987). Electrophoresis, blotting and hybridizations were carried out as described earlier (Kuhn et al., 1991). To detect fusion transcripts *antisense* in vitro transcripts were generated from the cloned 634 nt long HpaI fragment within the $\beta$ galactosidase gene (see above) using a $^{32}$P-UTP. mRNAs from an individual gene in the cluster were detected using *antisense* in vitro transcripts from the 3′UTR of that gene (i.e. from *Mst84Da* or *Mst84Db*, as specified in Kuhn et al., 1991). For quantitation of transcript levels autoradiographs were scanned with a UMAXII scanner and intensities measured with Magic Scan (in Photoshop, Imagequant version 3.3 from Molecular Dynamics).

### *4.5. β-Galactosidase staining*

$\beta$-Galactosidase activity tests have been performed according to Glaser et al. (1986).

Larval and adult testis tissue was dissected in Ringer's solution or in PBS and stained according to standard protocols using either formaldehyde or glutaraldehyde as fixative. In every case all lines generated after injection with a gene fusion have been stained to compare expression levels. The tissue was routinely incubated overnight at RT and compared to $w^{1118}$ tissue after the same treatment. Tissue was mounted in PBS and photographed using a ZEISS Axiophot.

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