Related enhancers in the intron of the $\beta_1$ tubulin gene of Drosophila melanogaster are essential for maternal and CNS-specific expression during embryogenesis

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ABSTRACT

Expression of the $\beta_1$ tubulin gene of Drosophila melanogaster is under complex developmental control. For high levels of transcription in the embryonic central nervous system (CNS) different modules dispersed over 3 kb have to co-operate. Combination of a core promoter with either far upstream localized enhancer elements or, alternatively, with an enhancer from the intron results in expression limited to only a few neuronal cells. Cooperation of all three modules, however, leads to high level expression in most neuronal cells of the CNS. In the intron, we identified a 6 bp core element which is essential for transcription in the CNS, as well as an 8 bp element required for maternal expression. Interestingly, both motifs are quite similar, with CAAAAAT as the CNS core and CAAAAAT as the maternal enhancer core. Specific binding of proteins from nuclear extracts to the CNS-specific element could be demonstrated. We suggest that the $\beta_1$ tubulin gene represents an ideal marker gene to elucidate connections between pro-neural or neurogenic genes and downstream target genes throughout the CNS.

INTRODUCTION

Formation of the Drosophila central nervous system (CNS) occurs from a stereospecific set of stem cells called neuroblasts, which comprise ~20% of the neuro-ectodermal region (for reviews see 1,2). Genetic data show that these cells are singled out by a lateral inhibitory process. Their further competence to adopt a neural fate is mediated by the expression of the pro-neural genes, which include daughterless (da), the genes of the achaete–scute complex (AS-C) and the ventral nervous system defective gene (vnd) (3–7). Ongoing differentiation of the CNS is marked by the expression of a group of genes referred to as pan-neural genes (8). According to mutant phenotypes, these genes probably play a role in establishing neuronal cell type identity. This would explain why in mutants for prospero, a gene encoding a nuclear protein containing a homeodomain-like sequence, incorrectly specified progeny of certain neuroblasts are produced, which show pathfinding defects (9,10).

Concerning regulation of the transcription of neural-specific genes, little information at the molecular level is available. At least partially, activation of the proneural genes is directed by pair-rule and dorso-ventral polarity genes. Furthermore, their activity is controlled by the lateral inhibition pathway and restricted to the neural precursors. Basically, at least in the peripheral nervous system (PNS), repressing activities seem to play a major role, as in mutants for hairy, extramacrochaete and pannier ectopic expression of the AS-C genes is observed, which leads to the formation of supernumerous sensory organs (11–13). Kramatschek and Campos-Ortega (14) showed that the neurogenic genes E(spl) and HLH-m5 can be activated by the pro-neural gene products.

Few target genes of the neurogenic and pro-neural gene products expressed throughout the CNS have been identified so far. By analyzing the promoter of the neuron-specifically expressed gene elav, Yao and White (15) described a 333 bp region as sufficient for CNS-specific expression. However, no distinct elements have been identified and no transactivators for elav could be shown. Surprisingly, Ip et al. (16) recently presented data showing that expression of the snail gene in the CNS is not dependent on the pro-neural genes, demonstrating that distinct pathways must co-exist in order to define neuronal identity.

The $\beta_1$ tubulin gene of D.melanogaster is expressed maternally as well as zygotically. Transcription during embryogenesis is restricted to the CNS and PNS and to the attachment sites of the somatic muscles (17). While the $\beta_1$ protein is present at high levels in all tissues until hatching of the larvae, the maternal $\beta_1$ mRNA disappears around stage 9. Zygotic transcription in the CNS starts at stage 10, reaches its maximum level at stage 12 and decreases after stage 13 to very low levels at stage 16. In a previous report we showed that expression of the $\beta_1$ tubulin gene in the CNS is dependent on the presence of the 5′-part of the intron (17). By sequence comparison with the D.hydei $\beta_1$ tubulin gene (Buttgereit, unpublished results) we identified two conserved elements in the intron, which were termed IE1 and IE2. Deletion analysis revealed that IE1 is solely necessary for expression in the CNS during embryogenesis. By shifting its position to ~2.3 kb upstream of the start site we present evidence that this element

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acts as a classical enhancer. Based on its zygotic function, the enhancer is called the central nervous system-specific enhancer, or CNE. However, the CNE alone is not sufficient for expression in the CNS, but has to cooperate with promoterproximal and far-upstream elements in order to drive full level expression in the CNS (17). In order to identify transactivating factors, we performed in vitro assays using nuclear extracts from staged embryos. In EMSA assays we could detect specific interaction with proteins from 6–14 h old embryos, showing that factors are present which may be neural system specific. UV-crosslinking experiments revealed the specific interaction of a 71 kDa protein with the CNE in nuclear extracts. Further characterization of cis- and trans-regulatory modules will yield insight into the regulatory cascades driving development of the CNS downstream of the pro-neural and neurogenic genes.

**MATERIALS AND METHODS**

**Plasmid constructions**

The vector used for cloning was –2.2/BS, comprising β1 tubulin gene sequences from –2348 to +448 in Blueskript II KS. The clone was cut with NcoI (+160) and HindIII (+473), polylinker derived from pUC19, the 5.2 kb fragment isolated and synthetic oligonucleotides were inserted. For construct β1-1, the sequence used (MutD1, 56 bp) was 5′-CGCCAAAGTGAAGGTCCCCAATCTTTTCTCCAGATCGAGCAAAATTGACCCAGTCAATTCATGCAATTCGACAGCAGACAGTTTAAACAAATTGTGTCCACAGCAGTCGTATGGTACAGCAGTTCATGGATCCA, which mutagenizes the CNE, was inserted. To obtain construct β1-11, the oligonucleotide Mater (55 bp), 5′-GCCCAAGGTTAGTTTTCAAGTGCAGCAGAGTACAGTGCAGTTCGGCCACGACTAGTACTGGATCCA, which mutagenizes the second motif (ME), was used.

For all oligonucleotides only one strand is shown. The double-stranded oligonucleotides had at their ends the corresponding restriction half-sites as adaptors for cloning and usually an additional BamHI site for identification of positive clones. The fragments were excised from Bluescript with EcoRI and SacI and ligated to the adapter fragment Scal–XbaI from PF3 (17), containing the 3′ splice site from the β1 tubulin gene and the codons to amino acid 23 fused in-frame to the Escherichia coli lacZ gene, in the P-element transformation vector pW8 (18) cut with EcoRI and XbaI. All constructs were checked by sequencing of the intron or upstream region, respectively.

**Preparation of nuclear extracts**

Nuclear extracts were prepared according to Dignam et al. (19) with modifications. Embryos were collected on apple juice/agar plates, dechorionated with 50% Clorix, washed with 0.7% NaCl, 0.1% Triton X-100 and suspended in homogenization buffer (10 mM HEPES–KOH, pH 7.6, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.25 M sucrose, 0.5 mM DTE, 0.5 mM leupeptin). They were homogenized with a motor-driven glass/Teflon homogenizer with a tight fitting pestle at 600 r.p.m. Intact embryos and cell debris were pelleted by centrifugation for 5 min at 800 r.p.m. The supernatant was loaded onto a cushion of 30% glycerol in homogenization buffer and nuclei were collected by centrifugation at 6000 r.p.m. for 20 min. The volume of the nuclear pellet was estimated and one volume of nuclear extraction buffer was added (20 mM HEPES–KOH, pH 7.6, 420 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTE, 0.5 mM leupeptin). The suspension was stirred on ice for 30 min, the nuclei pelleted by centrifugation at 13 000 r.p.m. and the supernatant dialyzed twice against 200 volumes buffer A (20 mM HEPES–KOH, pH 7.6, 100 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTE, 2 µg/ml leupeptin). To remove precipitated material, the extract was centrifuged for 10 min at 13 000 r.p.m. and quick-frozen in small aliquots in liquid N2. Aliquots were stored at –80 °C without significant loss of activity for at least 12 months.

**Gel retardation assays**

Standard binding reactions contained up to 30 µg nuclear extract, 6 fmol 32P-labeled oligonucleotide and 200–500 ng dI dC or dA dT as non-specific competitor in 25–50 µl. Specific competition was performed in the range 5– to 500-fold excess. The gels were run in 1× TBE at 200 V for 2–3 h, dried and autoradiographed using Kodak X-Omat AR films. The oligonucleotides used for binding were ATGGCAGCAGCAGACTAGTACTGGATCCA and, as non-specific competitor, an oligonucleotide comprising a SP1 binding site, TTTGCTATGCGACAGCAGACTAGTACAGCAGTTCAGTACAGTACTGGATCCA, which mutagenizes the second motif (ME), was used.

**UV cross-linking**

Standard binding reactions were pipetted onto Parafilm™ and exposed to UV light at 254 nm for 1 min at 4000 µW/cm² using a Stratagene™ 2400 crosslinker. Samples were denatured and run
RESULTS

β1 mRNA in the CNS decays after stage 12, while protein is stable to stage 17

Previously we have shown that β1 tubulin protein is present ubiquitously in the embryo and can be detected using an anti-β1 tubulin antibody in most tissues until hatching (17). In situ hybridization to embryo sections using 3H-labeled probes indicated that the distribution of the mRNA, however, is not homogeneous, but is present at higher levels in the CNS (20). By whole mount in situ hybridization using digoxigenin-labeled probes we extended these data (Fig. 1). While maternal mRNA leads to a strong homogeneous staining of early embryos (Fig. 1A), the mRNA outside the CNS decays to undetectable levels after stage 9, while in the ventral neurogenic region three rows of cells show expression of the β1 tubulin gene at stage 10 (Fig. 1C). These cells represent neuroblasts exclusively, as double staining with an antibody against a glial-specific marker, repo (21), reveals no overlapping patterns (data not shown). In addition, β1 tubulin mRNA is detected in cells of the PNS. The mRNA levels in the CNS remain high until stage 12, after which they start to decay. Beginning at stage 13, the attachments of the somatic musculature, the apodemes, show expression of the β1 tubulin gene. At stage 15, PNS and apodemes contain high levels of β1 tubulin mRNA, while it is hardly detectable in the CNS. In contrast, the β1 tubulin protein is present at high levels in these tissues at all times (17).

Expression of the β1 tubulin gene in the CNS is dependent on the 5′-part of the intron

During the deletion analysis of the β1 tubulin gene intron we found that sequences required for high level expression in the CNS reside within the first 270 bp of the intron (17). This conclusion was drawn from the differences in the expression patterns between strains transgenic for Wβ1K and Wβ1C and the results with β1-1. Sequence comparison with the β1 tubulin gene of D.hydei revealed two blocks of homology in this region (Fig. 2; Buttgereit, unpublished results), which were named IE1 and IE2, respectively. IE1 shows 19 out of 20 bases identity, IE2 16 out of 16. The distance between the two blocks is different, with 13 bases in D.melanogaster and only four bases in D.hydei. Two closely related motifs are present in IE1: the sequence CAAAA T at +221 to +225 and the sequence A TTTTTGC at +231 to + 238. The second motif occurs a second time in IE2 on the complementary strand, from +261 to +268 (Fig. 2). The palindromic orientation of the second motif is reminescent of specific recognition elements for DNA binding proteins. To address the question whether these two homologies indeed represent cis-regulatory elements, deletions in the 5′-part of the intron were made, keeping the bases to the splice site and spacing of the elements as in the reference construct Wβ1K (17). In construct β1-1 (Fig. 3), both
elements were deleted, leaving only 49 bp of 5′ intron sequence. As in comparison with Wβ1K, which contains both IE1 and IE2, an additional 169 bp from +277 to +446 were deleted due to the cloning strategy used, a control construct, β1-2, including both boxes but deleting the same 169 bp as in β1-1, was analyzed. Both constructs were cloned in front of a lacZ gene, inserted into the P-element vector pW8 and transgenic fly strains were established. Embryos were collected and analyzed for reporter gene expression using a polyclonal anti-β-galactosidase antibody. Strains transformed with β1-2 show strong maternal expression as well as high level expression in the CNS, as does Wβ1K (Fig. 3D). This result shows that no essential elements for maternal or zygotic expression during embryogenesis are located in the 169 bp removed. Deletion of both boxes in β1-1 (Fig. 3C), however, results in a strong reduction of maternal expression and the number of reporter gene-positive cells in the CNS is greatly reduced. This expression corresponds to the pattern observed in strains transgenic for construct Wβ1C (Fig. 3B), which completely lacks the intron (17), indicating that IE1 and IE2 are the only sequences necessary for CNS and maternal expression. These data furthermore prove that the bases at the 3′-end of the intron, present in Wβ1K and β1-1, for example, are not required for CNS-specific expression. However, in contrast to Wβ1K and Wβ1C, expression of the reporter gene is observed in the PNS with both β1-1 and β1-2. Thus, putative silencing elements acting in the PNS may be present in the 169 bp removed. To narrow down the activating sequences, both boxes were tested individually for their potential neural system-specific activity. As for β1-1 and β1-2, synthetic oligonucleotides comprising either IE1 and flanking sequences (β1-3, Fig. 4A) or IE2 and flanking sequences (β1-4, Fig. 4B) were used. Analysis of the β1-3 transgenic strains show maternal as well as strong expression in the CNS. Thus, the IE1 element is capable of driving both modes of expression (Fig. 4A). Strains harboring β1-4 also show strong maternal expression, but significantly reduced expression in the CNS, as does β1-1 in which both boxes are deleted (compare Figs 4B and 3C). Thus, IE2 is not capable of enhancing

**Table 1. Expression pattern of transgenic fly strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maternal expression</th>
<th>CNS expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wβ1K (+IE1, IE2)</td>
<td>+++</td>
<td>Strong, homogeneous</td>
</tr>
<tr>
<td>Wβ1C (no intron)</td>
<td>–</td>
<td>Single cells</td>
</tr>
<tr>
<td>β1-1 (–IE1, –IE2)</td>
<td>–</td>
<td>Single cells</td>
</tr>
<tr>
<td>β1-2 (+IE1, +IE2)</td>
<td>+++</td>
<td>Strong, homogeneous</td>
</tr>
<tr>
<td>β1-3 (+IE1, -IE2)</td>
<td>++</td>
<td>Strong, nearly homogeneous</td>
</tr>
<tr>
<td>β1-4 (–IE1, +IE2)</td>
<td>++</td>
<td>Single cells</td>
</tr>
<tr>
<td>β1-7 (+IE1, +IE2)</td>
<td>++</td>
<td>Strong, nearly homogeneous</td>
</tr>
<tr>
<td>β1-8 (IE1, IE2 at 2.4 kb)</td>
<td>+++</td>
<td>Strong, homogeneous</td>
</tr>
<tr>
<td>β1-9 (IE1+2 mutagenized)</td>
<td>+/-</td>
<td>Single cells</td>
</tr>
<tr>
<td>β1-10 (CNE mutagenized)</td>
<td>+</td>
<td>Very weak, single cells</td>
</tr>
<tr>
<td>β1-11 (ME mutagenized)</td>
<td>–</td>
<td>Strong, nearly homogeneous</td>
</tr>
</tbody>
</table>

Deletion analysis reveals that only IE1 drives neurogenic expression

Table 1 shows the expression pattern of various transgenic fly strains. The strains were analyzed for maternal and CNS expression, with the following results:

- **Wβ1K (+IE1, IE2)**: Strong, homogeneous expression in both maternal and CNS.
- **Wβ1C (no intron)**: Single cells expression in maternal, no expression in CNS.
- **β1-1 (–IE1, –IE2)**: Single cells expression in maternal, no expression in CNS.
- **β1-2 (+IE1, +IE2)**: Strong, homogeneous expression in both maternal and CNS.
- **β1-3 (+IE1, -IE2)**: Strong, nearly homogeneous expression in maternal, no expression in CNS.
- **β1-4 (–IE1, +IE2)**: Single cells expression in maternal, no expression in CNS.
- **β1-7 (+IE1, +IE2)**: Strong, nearly homogeneous expression in maternal, no expression in CNS.
- **β1-8 (IE1, IE2 at 2.4 kb)**: Strong, homogeneous expression in maternal, no expression in CNS.
- **β1-9 (IE1+2 mutagenized)**: Single cells expression in maternal, no expression in CNS.
- **β1-10 (CNE mutagenized)**: Very weak, single cells expression in maternal, no expression in CNS.
- **β1-11 (ME mutagenized)**: Strong, nearly homogeneous expression in maternal, no expression in CNS.

To narrow down the activating sequences, both boxes were tested individually for their potential neural system-specific activity. As for β1-1 and β1-2, synthetic oligonucleotides comprising either IE1 and flanking sequences (β1-3, Fig. 4A) or IE2 and flanking sequences (β1-4, Fig. 4B) were used. Analysis of the β1-3 transgenic strains show maternal as well as strong expression in the CNS. Thus, the IE1 element is capable of driving both modes of expression, while IE2 is not capable of enhancing...
Figure 3. Expression pattern of transgenic fly strains. The corresponding constructs are shown schematically above the micrographs. All stainings were performed in progeny of transgenic males, removing maternal expression. (A) Ventral view of a stage 16 W\textbeta1K embryo. The CNS with the exception of the midline is strongly and homogeneously stained. (B) Stage 16 embryo transgenic for construct W\textbeta1C; only two neuronal cells per hemisegment reveal expression of the reporter gene. (C) Embryo stage 16 transgenic for construct \textbeta1-1; expression is reduced to a few cells. (D) Embryo stage 15 transgenic for construct \textbeta1-2; as in W\textbeta1K, a strong staining of the CNS with the exception of the midline is shown.

expression in the CNS, but elevates maternal transcription. As in IE1 the two motifs CAAATAT and ATTTTTGC are found, while only the second motif is repeated in IE2, the palindromic structure of the second element is not required for its function. Thus, either the first motif in IE1 alone represents the neuronal enhancer or the imperfect palindrome found in IE1 (CAAAAT---N_6---ATTTTTG) fulfills this function. No further regulatory sequences are located in these elements, as mutagenesis of both motifs in IE1 and the motif in IE2 together in construct \textbeta1-9 (Table 1) completely abolishes maternal as well as CNS expression.

Only the 5\textquotesingle-CAAAAT motif in IE1 drives expression in the CNS

To further test the regulatory potential of the motifs in IE1, both were individually mutagenized. In construct \textbeta1-10, the first motif CAAATAT was mutagenized to AGCTCA. Lines carrying this construct show intermediate levels of maternal expression, while expression in the CNS is reduced to very low levels, as in \textbeta1-1 (Fig. 4C). Conversely, in construct \textbeta1-11, the second motif ATTTTTGC was changed to ACGTCAGT. Strains transgenic for \textbeta1-11 reveal no maternal expression, while expression in the CNS is comparable to \textbeta1-3 (Fig. 4D). These results show, although both motifs are very similar, that their specificity in vivo is distinct, as the first motif exclusively drives neuronal expression, while the second motif, which is repeated in IE2, is restricted to the enhancement of maternal expression. Therefore, the first motif of the IE1 element was named CNE (central nervous system-specific enhancer) and the second motif was termed ME (maternal enhancer).

The CNE retains its function if located upstream of the \textbeta1 promoter

To check a further criterion for enhancer function of the CNE, a synthetic oligonucleotide comprising both IE1 and IE2 was cloned upstream, at \textminus2348 bp, in the construct \textbeta1-1, which lacks both IE1 and IE2 in the intron, resulting in construct \textbeta1-8 (Fig. 5). Expression monitored by anti-\textbeta-galactosidase antibody staining showed no detectable differences to \textbeta1-2, demonstrating that the CNE can also exert its enhancer activity when placed upstream of the promoter (Fig. 5). However, in contrast to classical enhancers, its activating potential is dependent on additional sequences of the \beta1 tubulin gene promoter. This was shown by combination of the intron lacking only 0.15 kb at the 3\textquotesingle-end with the heterologous hsp70 promoter (17; construct WHI), where no CNS-specific or maternal expression is observed (17). The 0.15 kb omitted in WHI themselves are not capable of driving expression in the CNS as they are present in \textbeta1-1, which definitively shows reduced expression, as does W\textbeta1C.

IE1 binds specifically to nuclear proteins in vitro

Having characterized the enhancer function of the CNE, the identification of proteins that specifically interact with this element became of interest. Nuclear extracts from embryos staged at 6–14 h were prepared in order to reduce the amount of maternally retained components and tested for specific interaction with a double-stranded oligonucleotide comprising the CNE and ME elements (Fig. 6A). Several bands were detected, but competition experiments revealed that only one specific retarded signal, complex 1, resulted from specific binding to IE1. The second complex is only competed out in the presence of both IE1
Figure 4. Embryonic expression pattern of strains β1-3, β1-4, β1-10 and β1-11. The constructs are presented above the micrographs. For β1-10 and β1-11 the sequence of IE1 is shown, the mutagenized bases are boxed. (A) Embryo transgenic for construct β1-3, stage 15/16, ventral view, without maternally derived reporter gene expression. Staining in the CNS is nearly as strong as in Wβ1K. (B) Embryo with construct β1-4, stage 15/16, ventral view, without maternal components. Staining is reduced to a few neural cells, as in β1-1, were both boxes are deleted. (C) Embryo transgenic with β1-10, stage 15/16, ventral view. Only a few cells reveal expression. (D) Embryo from β1-11 strain, stage 15/16, ventro-lateral view. Strong expression in the CNS is observed.

Figure 5. Expression pattern of the enhancer test construct β1-8. (A) Embryo stage 9, ventral view. Strong maternal expression is shown. (B) Embryo stage 15/16, ventral view, without maternal expression. Strong staining in the CNS, as in Wβ1K, is visible.

and IE2, indicating that it contains different proteins. When the CNE was mutagenized according to the strategy used for β1-7, no specific binding was observed (not shown). These results demonstrate that the core motifs identified indeed represent specific DNA binding elements. As both motifs of IE1 are very similar in their sequence, in addition to the gel retardation assays the interaction of proteins with these motifs was analyzed in UV cross-linking experiments. Standard binding reactions using the double-stranded oligonucleotide Mater (see Materials and Methods) were exposed to UV light and analyzed by 10% SDS–PAGE. A specific interaction of a 71 kDa protein was exclusively observed with the oligonucleotide Mater (Fig. 6B).
To further identify interacting proteins, we tried to purify the transactivators on different chromatographic materials, but were able to enrich the active fraction no more than 200-fold as estimated from the gel retardation assays. Further purification resulted in complete loss of activity when testing combinations of different fractions. So at the moment the identity of the transactivators is unknown.

**DISCUSSION**

The $\beta 1$ tubulin gene of *D. melanogaster* is transcribed during oogenesis as well as zygotically in the nervous system and the attachments of the somatic musculature, the apodemes. The gene is subject of a complex network of transcriptional regulation during embryogenesis. Deletion analysis revealed separate elements driving expression in the CNS, the chordotonal organs and the attachment sites of the somatic musculature (17,22). While the intron elements necessary for expression in the chordotonal organs and apodemes also act independently of the $\beta 1$ tubulin gene promoter (17,22), the situation for expression in the CNS is different. We could show that for complete expression at high levels at least three different modules have to interact: upstream sequences between –2348 and –1136, promoter-proximal elements and sequences from the 5′-part of the intron. None of these modules alone is capable of interacting with a heterologous promoter to drive expression in the CNS.

By deletion analysis we could show that the intron of the gene is essential for maternal as well as neuronal expression (17). Sequence comparison with the $\beta 1$ tubulin gene from the distantly related species *D. hydei* revealed the presence of two conserved sequence blocks in the proximal 5′-part of the intron. The elements were termed IE1 (20 bp) and IE2 (14 bp). In this report we present evidence for the neural enhancer activity of a 6 bp core element localized in IE1. Zygotically, this enhancer is specific for the neural system, because no transcription is observed in apodemes. In total, two separate motifs were detected in IE1: the 5′-located core of 6 bp, CAAAAT, which is essential for neuronal expression in the CNS, and a 3′-located 8 bp motif, ATTTTTTGC, which occurs a second time in the inverted orientation in IE2 and is required for maternal expression. Mutational analysis reveals that distortion of the 5′ core sequence reduces expression in the CNS, as does complete deletion of IE1. Therefore, this sequence was termed the central nervous system-specific enhancer or CNE. Maternal expression, in contrast, is still detectable, although at lower levels, as observed in strains harboring the complete IE1 element. This result could indicate that the CNE possibly augments the activity of the ME, by stabilizing or facilitating the interaction of transactivators with the ME. Mutagenesis of the ME completely eliminates maternal expression without reducing CNS expression, showing that the reverse interaction does not occur. These results were rather surprising, as the close relationship of the CNE and ME, CAAAAT vs CAAAAAT, indicates interaction with similar factors, although with different affinities. However, the influence of the neighboring bases of these motifs on the interaction with the regulatory proteins is not clear, although at least in vitro their mutagenesis does not alter the interaction as monitored in gel retardation assays (not shown).

Nevertheless, the CNE and ME motifs are essential for transcriptional regulation in vivo, as their mutagenesis completely knocks out the corresponding expression patterns. These results show that although the palindromic occurrence of the motifs points to an interaction with protein dimers, these motifs are able to act independently of one another, such that a synchronous binding of a single factor or the interaction of two proteins binding to these motifs is not necessary for enhancer function. As discussed above, the palindromic structure of the IE1 motif CAAAATGGCCTATTTTTG offers the possibility that homo- and heterodimers might interact with this element. However, constructs $\beta 1$-10 and $\beta 1$-11 clearly show that the motifs present in IE1, although being closely related, exert distinct functions *in vivo*: the 5′-localized CAAAAT is active only in the nervous system, while the 3′-located CATTTTTG core solely controls maternal expression. However, removal of 3′-flanking sequences in constructs $\beta 1$-1 to $\beta 1$-9 leads to an enhanced expression in the PNS which is not observed in strains transgenic for $\psi $1K. Thus, elements that negatively regulate $\beta 1$ tubulin gene expression in the PNS have been deleted in these constructs. Alternatively, elements from the 3′-part of the intron in the wild-type situation...
or in Wβ1K are positioned too far from the promoter to drive high levels of expression in the PNS. These elements are probably brought into closer proximity to the promoter in the deletion constructs, leading to the enhanced transcription in the PNS.

Having defined the CNE in vivo, we were interested in identifying transactivating proteins that might bind to sequences in IE1. In vitro binding studies using EMSA assays reveal the presence of nuclear proteins interacting with this element. A problem with these studies arises from the close relationship of the CNE and ME. As their relative affinities for interacting factors in vivo may be very similar, although in vivo sufficient for specific regulation, the identification of such factors in gel retardation assays may be quite difficult. Possible cofactors which are relevant in vivo may not function equivalently in vitro, or only with reduced efficiency. In UV cross-linking experiments, a 71 kDa protein showed a specific interaction with the CNE.

Concerning possible transactivators, only a few candidates are known. The products of the pro-neural gene class, as well as the E(spl) and HLH-5m proteins, which represent bHLH transcription factors, recognize a consensus motif CANNTG (E-box) or CACNAG (N-box), which is not found in the CNE, only the variant CAAAA TG at the 5'-end of the motif. Analysis of the β1 tubulin mRNA distribution in a mutant for a pro-neural gene belonging to the bHLH family, daughterless, revealed no significant reduction in CNS expression (Buttgereit, data not shown). Thus, the bHLH genes are unlikely to be involved in direct activation of the β1 tubulin gene expression. However, due to the high maternal mRNA level in early embryogenesis stages, which overlaps with the onset of zygotic expression, this observation remains preliminary.

For two other genes, elements for expression throughout the CNS have been mapped. In the case of the elav gene, a 333 bp promoter fragment could be identified which is capable of driving high level expression in the CNS (15). This is different from the situation in the β1 tubulin gene, where the interaction of several widely dispersed, distinct motifs are essential for activation. For the snail gene it was shown (16) that activation in the CNS, at least, is not dependent on the pathway initiated by the genes of the achaete-scute complex. In addition, elements for the CNS and PNS could be separated. Sequence comparison of the IE1 and IE2 elements revealed no homologies to either gene, indicating that the CNE represents a new DNA binding motif. Further analysis of the CNE and the specifically interacting proteins may yield insight into the regulatory cascades leading to the differentiation of the embryonic D.melanogaster CNS. So, for the first time, the differential activity of two closely related enhancer motifs active either during oogenesis or embryogenesis has been demonstrated.

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