**β3-tubulin** is directly repressed by the Engrailed protein in *Drosophila*

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**SUMMARY**

In *Drosophila*, Engrailed is a nuclear regulatory protein with essential roles during embryonic development. Although Engrailed is a transcription factor, little progress has been achieved in identifying its target genes. We report here the identification of an effector gene, the β3-tubulin gene, as a direct target of Engrailed. The cytological location of β3-tubulin, 60C, is a strong site of Engrailed binding on polytene chromosomes. Immunostaining analysis of a transgenic line containing a P[β3-tubulin-lacZ] construct shows an additional site of Engrailed binding at the location of the transgene. Molecular analysis allowed identification of several Engrailed binding sites, both in vitro and in vivo, within the first intron of the β3-tubulin locus. Engrailed binding sites identified in vitro are active in larvae. Furthermore, expression of β3-tubulin is repressed in the ectoderm of *engrailed* mutant embryos. Repression of β3-tubulin by Engrailed is also obtained when Engrailed is ectopically expressed in embryonic mesoderm. Finally, two different sets of Engrailed binding sites are shown to be involved in the early and late regulation of β3-tubulin by Engrailed during embryogenesis.

Key words: Engrailed, β3-tubulin, target genes, *Drosophila*

**INTRODUCTION**

Induction and maintenance of a differentiated state during embryonic development depends on expression of a large number of genes acting at multiple levels. These genes can be grouped in two classes. The first class includes genes encoding regulatory factors, named ‘selector’ genes, which are generally transcription factors. The second class corresponds to the ‘effector’ genes, which encode products directly responsible for morphogenesis. Transcriptional regulation of these two classes of genes is the fundamental mechanism responsible for the differential expression of the genetic program. Genetic and molecular studies in *Drosophila* have largely contributed to the elaboration of this conceptual framework.

During embryonic development of *Drosophila*, the anteroposterior organization is generated by the activity of several classes of maternal and zygotic genes (reviewed by Ingham, 1988; Kornberg and Tabata, 1993). Many of these genes encode transcriptional regulators which, through a regulatory cascade, sequentially subdivide the embryo into smaller domains along the anteroposterior axis (Hoch and Jäckle, 1993). The last genes in this cascade are the ‘segment polarity’ genes, which encode products necessary for the establishment of compartments within segments (Kornberg and Tabata, 1993). The *engrailed* (*en*) gene belongs to this last class of genes and plays an essential role during *Drosophila* development.

Genetic and molecular studies have shown that the Engrailed (En) protein is necessary to specify posterior compartment cell identity (Morata and Lawrence, 1975; Kornberg, 1981; Guillem et al., 1995; Tabata et al., 1995). The *en* gene also plays an essential role in the establishment of compartment boundaries within the insect body segments (Lawrence and Morata, 1976). Among other possible roles, these anteroposterior boundaries established before gastrulation serve as reference for the later and spatially restricted expression of homeotic genes of the *Antennapedia* and *Bithorax* complexes (Lawrence and Morata, 1994). This last class of genes is necessary to confer segmental identities (McGinnis and Krumlauf, 1992; Lawrence and Morata, 1994).

En is a nuclear protein of 552 amino acids, which contains a homeodomain (Fjose et al., 1985; Poole et al., 1985). En is able to bind DNA with high affinity through its homeodomain (Desplan et al., 1985). In addition, En acts as a transcription factor after transfection into cultured cells (Jaynes and O’Farrell, 1988; Ohkuma et al., 1990; Jaynes and O’Farrell, 1991). These results suggest that En could directly regulate the expression of particular genes at the transcriptional level. Thus, the identification and the analysis of the role of these target genes is an essential step in understanding *en* function during development.

The identification of target genes for transcription factors has been undertaken using different techniques, based on two main ideas. The first approach is functional and is based on the existence of genetic interactions between two genes. The second approach is based on the capacity of transcription
factors to bind specific DNA sequences. This second approach has been successful for identifying target genes for Ultrabithorax (Gould et al., 1990; Gould and White, 1992; Graba et al., 1992; Capovilla et al., 1994; Graba et al., 1995; Mastick et al., 1995; Botas and Auwers, 1996) and Antennapedia (Heuer et al., 1995).

Although En is a homeodomain-containing protein that is known to specifically bind DNA and act as a transcription factor, little progress has been made in identifying its target genes. Genetic experiments have shown that cubitus-interruptus (Eaton and Kornberg, 1990; Schwartz et al., 1995), hedgehog (Tabata et al., 1992), decapentaplegic (Raftery et al., 1991) and Ultrabithorax (Mann, 1994) could be targets of en regulation. Only cubitus-interruptus was actually shown to be a direct target of en regulation (Schwartz et al., 1995). Two other target genes have been identified so far by taking advantage of the ability of En to specifically bind DNA, poly-homoeotic (ph), a Polycomb group gene, was identified on the basis of En binding to polytene chromosomes (Serrano et al., 1995) and Msr-110 was identified as a screen for En-binding sequences (Saenz-Robles et al., 1995).

Using immunostaining of polytene chromosomes and molecular analysis, we have identified a new target of en regulation, the β3-tubulin gene. The cytological location of β3-tubulin, 60A, is a strong En binding site on polytene chromosomes. In addition, transposition of a portion of the β3-tubulin region to a new location on polytene chromosomes resulted in a new site of En binding. The expression pattern of β3-tubulin is deregulated in en mutant embryos. Moreover, when ectopically expressed in the mesoderm, Engrailed is able to repress β3-tubulin in the somatic mesoderm. Finally, En binding sites within the β3-tubulin locus were identified in vitro and in vivo, and were shown to respond to En in vivo.

MATERIALS AND METHODS

Fly strains
Canton S was used as wild type. The transgenic line hsen-3 (Poole and Kornberg, 1988) bears a P-element containing the engrailed coding sequence driven by a hsp70 promoter and is localized in 34B. For heat-shock, larvae were kept at 37°C for 30 minutes and then returned to room temperature for 15 minutes for recovery before dissection. Df(2R)en<sup>5FX31</sup> is a deficiency of the 48AB region that deletes the engrailed and invected genes, as well as several neighbouring transcription units (Kuner et al., 1985). en<sup>F14</sup> is an EMS induced allele with a translation stop at codon 422 (Gustavson et al., 1996). The strain is maintained on a Cy balancer which contains a fjt-lacZ transgene, which allows identification of homozygous en mutant embryos. en<sup>B86</sup> is an EMS induced allele, isolated by Wieschaus and Nüsslein-Volhard, with a 104 residue altered coding sequence (Gustavson et al., 1996). tkll (Xiong and Montell, 1993) is a P-element-induced mutation in the tramtrack locus. The β3-tubulin transgenic lines were from Renkawitz-Pohl’s laboratory and have already been described (Gasch et al., 1989; Hinz et al., 1992). The lines used in this study are described in Fig. 4B. UAS-en; twist-Gal4 embryos were obtained by crossing UAS-en females (T. Tabata et al., 1995) with twist-Gal4; 24B-Gal4 males (Staehr-Hampton et al., 1994).

Antibodies
Anti-Engrailed antibodies used were: (i) a goat polyclonal antibody (Gustavson et al., 1996); (ii) a monoclonal 4D9 antibody (Patel et al., 1989); (iii) a monoclonal 4F11 antibody, recognizing an epitope outside the homeodomain provided by T. Kornberg’s laboratory and described by Serrano et al. (1995); (iv) a rabbit polyclonal antibody raised against a T7-En bacterial protein (F. Fayre and A. Vincent, unpublished). The anti-β3-Tubulin is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the 15 C-terminal amino acids of the β3-Tubulin protein (Kimble et al., 1989). Monoclonal anti-β-galactosidase antibody was provided by Promega.

Immunostaining and in situ hybridization of polytene chromosomes
Immunostaining of polytene chromosomes essentially followed the procedures of Zink and Paro (1989), using modifications detailed in Serrano et al. (1995).

To localize the inserts of transgenic lines, in situ hybridizations to polytene chromosomes were carried out with a biotinylated DNA probe, using the non-radioactive gene detection system (BRL Bluegene 8279SA). As a probe, we used the β3-lacZ 0.1 DNA, corresponding to the entire 4.6 kb intron, plus 6 kb upstream region of β3-tubulin, fused to lacZ (Hinz et al., 1992).

Library of genomic targets for Engrailed protein
A library of genomic DNA fragments associated in vivo with En protein was constructed according to a procedure described by Serrano et al. (1995). Nuclei were isolated from 0-16 hours embryos and protein/DNA interactions were blocked by u.v. irradiation. Chromatin was subsequently prepared and digested with Sau3A, to generate small fragments of around 400 bp. The endogenous En protein normally present in embryos, plus associated DNA was specifically immunoprecipitated with anti-En antibody. This step is an in vivo immunopurification, since the only source of En protein is the endogenous En protein normally present in the embryos. We verified, at that step, that, whereas 1% of the chromatin was retained during the immunoprecipitation, the En protein concentration was much higher in the immunopurified chromatin (data not shown). After this in vivo immunoprecipitation, DNA fragments were ligated into a Bluescript vector. Three rounds of in vitro immunopurification of En binding sequences were then performed in the presence of protein A-Sepharose beads, 4F11 monoclonal anti-En antibody and HS-EN exogenous En protein. After each in vitro immunopurification step, the recovered fragments were PCR amplified using T3 and T7 primers, contained in the Bluescript vector. Following a last PCR amplification, the DNA was cloned into the BamHI site of a Bluescript vector. As controls, we tested for the presence of different DNA fragments corresponding to gene promoters which are known to be related or not to en regulation, either by hybridization of 5000 ordered clones or by PCR amplification, using specific sets of primers. As positive controls, we screened for the presence of the En binding fragments identified in vitro within the en gene itself (Desplan et al., 1985) and within the ph locus (Serrano et al., 1995). As negative controls, the presence of the Fb1 (Maschat et al., 1991) and of janus (Yanicosostas and Lepesant, 1990) promoter regions have been tested. Whereas all the fragments were present before the in vivo immunopurification step, only en and ph fragments which are known to be involved in en regulation were recovered in our library (data not shown). We also verified, in the case of ph, that fragments which are involved in en regulation are present just after the in vivo immunopurification step and are amplified during the in vitro steps (Serrano et al., 1995).

Fixation and staining of embryos
For antibody double stainings, embryos were fixed as described by Sullivan et al. (1993). Fixed embryos were stained with different antibodies diluted to the desired concentration. Primary antibodies were detected either by immunofluorescence (with rhodamine-conjugated or fluorescein-conjugated secondary antibodies), or by peroxidase-conjugated secondary antibody (Vector-Vectorstain) and alkaline phos-
phatase (Vector) as indicated in the text. Embryos were mounted in Citifluor AF1 for immunofluorescence and in 80% glycerol for peroxidase and alkaline phosphatase stainings. Double staining with primary antisera from rabbit and mouse was carried out for both antisera simultaneously.

For double stainings with anti-Engrailed antibody and whole-mount RNA in situ hybridization, the antibody staining was first carried out until the detection step. Embryos were then rinsed and the RNA in situ hybridization was then performed according to O’Niell and Bier (1994), using the digoxigenin-alkaline phosphatase reaction. The 3'-tubulin DNA template used for in vitro transcription corresponds to the entire genomic coding region cloned into a Bluescript vector.

Fixation and X-gal staining of larval tissues
In situ detection of the β-galactosidase activity was carried out as described by Glaser et al. (1986).

Preparation of Engrailed protein
Nuclear extracts from a Schneider 2 cell line (termed HS-EN) transformed with a gene fusion composed of the Drosophila hsp70 promoter, the coding portion of the engrailed cDNA and the engrailed polyadenylation site (Gay et al., 1988), was prepared as described by Serrano et al. (1995).

Purified 90B1 Engrailed protein was kindly provided by H. Bourbon. This protein corresponds to a truncated Engrailed protein, lacking the first 297 amino acids of the N terminus. After transcription from the T7 promoter induced by addition of IPTG, further purifications were performed on a phosphocellulose column, and then on a FPLC Mono S column (Bourbon et al., 1995).

Gel shift assays
DNA probes were synthesized by PCR amplification using specific primers. After gel purification, these fragments were end-labeled using T4 kinase. Binding assays contained approximately 10 pmol of poly(dI:dC). DNA-protein complexes were resolved on 6% native polyacrylamide minigels in 0.5x TBE (pH 8.3) buffer. Gels were prerun at 4°C for 1 hour at 100 volts and run at 4°C for 2 hours at 120 volts.

RESULTS

Immunostaining of polytene chromosomes
En binds specifically to polytene chromosomes in vivo (Serrano et al., 1995). This fact was used to identify a Polycomb group gene, polyhomeotic as a direct target of En (Serrano et al., 1995). We therefore decided to look for new En target genes by analysing strong En binding sites on polytene chromosomes.

Since en is not normally expressed in salivary glands, we characterized its distribution on polytene chromosomes in a transgenic line (hsen-3) carrying the en gene controlled by a heat-shock promoter (Poole and Kornberg, 1988). After heat-shock, En protein is highly expressed in all salivary gland nuclei in this strain (data not shown). Analysis of En distribution on polytene chromosomes showed that En binds specifically to a restricted number of sites. Region 60C is a strong site of En binding, as detected by immunostaining with a polyclonal anti-En antibody (Fig. 1). Subsequent genetic and molecular analysis showed that the 3'-tubulin gene, located in 60C, is a target of en regulation.

engrailed represses 3'-tubulin expression during embryogenesis
To verify that 3'-tubulin is regulated by En during development, expression patterns of en and of 3'-tubulin have been compared, initially during embryogenesis. If 3'-tubulin is activated by en, their patterns of expression should be coincident, while they should be complementary if 3'-tubulin is repressed by en. Confocal analysis of en and 3'-tubulin expression (Fig. 2A) in late embryos (stage 17) has shown that 3'-Tubulin (green) forms a network in the mesodermal cell layer that is segmentally reiterated. At this stage, en (red) is expressed in a single cell-wide stripe in the posterior compartment of each segment (Fig. 2A). Cells expressing en, never express 3'-tubulin (Fig. 2A), en and 3'-tubulin expressions are thus mutually exclusive.

If 3'-tubulin is indeed a target of en regulation, 3'-tubulin expression should be affected in en mutant embryos. Fig. 2C shows that the 3'-Tubulin network is disorganized in stage 17 en mutant embryos, compared to wild-type embryos (Fig. 2B). In addition, the presence of 3'-Tubulin protein in the epidermis is always associated with this abnormal Tubulin network. Since the expression of this gene is normally strictly mesodermal, except for expression in the chordotonal organs (Hinz et al., 1992), this result suggests that 3'-tubulin might be repressed by Engrailed in the ectodermal cell layer.

This repression is better shown when engrailed is ectopically expressed in the mesoderm, by using a Gal4 line, promoted by twist (Staehling-Hampton et al., 1994) after crossing with a UAS-en line. In stage 17 embryos, 3'-tubulin is normally highly expressed in the pharyngeal musculature and in the somatic musculature (Fig. 3A). In twist-Gal4/UAS-en embryos of the same age, en is expressed in both the pharyngeal and the somatic musculatures, as well as in salivary glands (not shown). In such embryos, using double staining with an anti-Engrailed antibody and a RNA-digoxigenin 3'-tubulin probe, we can no longer detect 3'-tubulin in the somatic mesoderm, while the expression is still detectable in the pharyngeal musculature (Fig. 3B). This might be due to the presence of strong activators in pharyngeal muscles that cannot be removed by En. It is apparent that both in the somatic mesoderm and in salivary glands (where 3'-tubulin is not normally expressed), no 3'-tubulin is detectable in presence of En (Fig. 3B). This suggests that 3'-tubulin is completely repressed by En in the somatic mesoderm, under these conditions.

Localization of Engrailed binding sites within the 3'-tubulin locus
In order to verify that this regulation by en was direct, we first analysed whether En protein was able to bind to the 3'-tubulin locus in vitro. To better understand how engrailed regulates 3'-tubulin, we identified in detail En binding sites within the 3'-tubulin locus.

DNA genomic fragments covering the 3'-tubulin locus (6 kb of upstream sequences and the entire coding region) were first in vitro immunoprecipitated in presence of En protein (data not shown). A 3 kb BamHI fragment located within the first intron was specifically bound by En (Fig. 4A). Interestingly, the first intron was shown to contain the promoter region of the 3'-tubulin gene (Gasch et al., 1989; Hinz et al., 1992). Using gel shift assays (Fig. 4C), En binding sites were verified to lie...
within five fragments in the first intron, named from A to E (Fig. 4A). En affinities for these different fragments were estimated by gel shift assays in which the concentration of En was varied relative to a constant amount of DNA (Fig. 4C). Binding sites were titrated either with purified 90B1 En protein (corresponding to an En protein missing the first 297 amino acids of the N terminus; data not shown) or with HS-EN protein (corresponding to a Schneider cells nuclear extract, expressing the entire En protein after heat-shock; Gay et al., 1988; Fig. 4C). The affinity for the HS-EN protein is estimated to be 10-fold higher than the affinity for the purified 90B1 En bacterial protein for the same binding site (data not shown). This is probably due to posttranslational modifications, such as phosphorylation, that occur in Drosophila cells (Gay et al., 1988). The estimated $K_D$ with the HS-EN protein for all the En binding fragments are around $10^{-9}$ M (Fig. 4C), which corresponds to the affinity of one TTAATGCAT consensus binding sequence for the En protein (data not shown). Sequence analysis of these five $\beta_3$-tubulin fragments bound by En revealed the presence of ten sites related to the En consensus binding sequence TTAATTCAT (Hinz et al., 1992; indicated as * in Fig. 4). The TTAATTCAT consensus sequence or related sequences has been shown to mediate repression by En, when placed upstream of a minimal Adh promoter fused to the CAT gene in transfected cells (data not shown; Jaynes and O’Farrell, 1988). En binding to this type of sequence within the $\beta_3$-tubulin locus is thus consistent with the apparent role of en in repressing $\beta_3$-tubulin expression in the ectoderm.

Engrailed also binds $\beta_3$-tubulin in vivo

Engrailed binding on polytene chromosomes of a $\beta_3$-tubulin transgenic line

A transgenic line, $\beta 3023I$ (Hinz et al., 1992; Fig. 4B) containing all the $\beta_3$-tubulin first intron region, as well as 0.23 kb upstream of the transcriptional start site, fused to a lacZ reporter gene, was used to show that En binds to the $\beta_3$-tubulin gene in vivo. In this strain, lacZ expression follows the endogenous $\beta_3$-tubulin expression (Hinz et al., 1992). In situ hybridization on polytene chromosomes was carried out and showed that the insert was localized at position 3C on the X chromosome (Hinz et al., 1992). To verify that the signal, detected at 60C on polytene chromosomes, results from En binding to the $\beta_3$-tubulin gene, we examined En antibody polyclonal binding sites in the $\beta 3032I$ transformed strain. We took advantage of the fact that En is expressed ectopically in salivary glands of a tramtrack (ttk1) mutant (Xiong and Montell, 1993). In this strain, the chromosome morphology is more easily seen than in the hasen-3 line under heat-shock conditions. Fig. 5B shows...
During embryogenesis, expression in the visceral and late embryogenesis
Role of the different Engrailed binding sites during
in both embryos and larvae.

Genomic DNA fragments associated with En protein
in vivo

En binding to the
the signal detected at 60°C can be attributed, at least in part, to
En binding to the β3-tubulin gene. We conclude that the β3-
tubulin gene is a good candidate to be a direct target of En in vivo.

In vivo immunopurification of Engrailed binding sites
within the β3-tubulin locus
The presence of the En binding fragments, identified in vitro
within the β3-tubulin locus, has been tested in a library of
genomic DNA fragments associated in vivo with En protein
and further purified in vitro for their capacity to bind En
(Serrano et al., 1995; detailed in Materials and Methods). By
PCR amplification, using specific sets of primers, the A to E
fragments were shown to be present in this library (Fig. 6). The
resultant PCR products hybridized specifically with a β3-tubulin
probe (data not shown). In this plasmid library, the F fragment
(Fig. 4A), which cannot bind En in vitro, is not detected (Fig.
6). This result shows that, during embryogenesis, En is able to
bind in vivo, within the β3-tubulin locus, to the five A to E
fragments identified in vitro. The in vitro and in vivo binding
analysis indicate that regulation of β3-tubulin by En might be
mediated through sequences in fragments A to E.

Analysis of the in vivo role of the different Engrailed
binding sites identified within the β3-tubulin first
intron
To determine whether the identified En binding sites are active
in vivo, we analysed the expression pattern of several strains
carrying different fragments from the β3-tubulin locus
upstream of a lacZ reporter gene (Fig. 4B; Hinz et al., 1992)
in both embryos and larvae.

Role of the different Engrailed binding sites during
embryogenesis
During embryogenesis, expression in the visceral and late
somatic musculatures is regulated by sequences contained
within the first intron of β3-tubulin, while upstream
sequences are necessary for early expression in somatic muscle,
suggesting that β3-tubulin regulation is achieved by
an early transient program until stage 12 and a more stable late
program for later stages (Gasch et al., 1989; Hinz et al., 1992).
Indeed the β3-6 line (Fig. 4B) drives expression in the
dicotonal organs and to a lesser extent in the visceral mesoderm,
but not in the somatic mesoderm in early germ-band-extended
embryos, whereas it mimics the normal β3-tubulin expression
in later germ-band-retracted embryos (Hinz et al., 1992; Fig.
7). In this line, wild-type and en mutant embryos present a
similar pattern of lacZ expression, both before and after stage
12 (Fig. 7). This suggests that the sequences within the D
fragment, present in this line (Fig. 4B), are not sufficient to
respond to En regulation during embryogenesis, at least when
isolated. In the β3-4 transgenic line (containing the A to D
fragments, but not the E fragment; Fig. 4B), lacZ expression is
only deregulated in late embryonic stages, after germ band
retraction and not in germ-band-extended en mutant embryos
(Fig. 7), suggesting that the sequences in the A to D fragments
are involved in late engrafted regulation. In the AI15.2 line,
lacZ expression is similar to endogenous β3-tubulin expression
(Gasch et al., 1989; Fig. 7). In this line, lacZ expression is
deregulated in en mutant embryos. Ectopic expression appears
in the ectodermal cell layer of these [AI15.2; en+] mutant
embryos, both at germ band extension and at germ band retraction
stages (Fig. 7). Only the E fragment is missing in the β3-
4 line, compared to the AI15.2 line, suggesting that the
sequences related to the En consensus within the E fragment
might be implicated in the early regulation of β3-tubulin
expression by En. Interestingly, in the WHL4 line which only
contains the E fragment (Fig. 4B), lacZ is not expressed during
embryogenesis and no lacZ expression could be detected in an
en mutant background (data not shown).

These results suggest that the sequences lying within the A
to D fragments are involved in late regulation by En, while
sequences in the E fragment are involved in early regulation,
preceding stage 12 embryonic development. These experiments were carried out with different en mutants, corresponding either to point mutations, interrupting the en transcription unit (like en² or enB86) or corresponding to a deletion covering the engrailed and the invected loci (enX31) (Gustavson et al., 1996). The results were similar with all these mutants (data not shown), suggesting that Engrailed rather than Invected might be responsible for the repression of β3-tubulin.

Role of engrailed during larval development
We compared lacZ expression patterns in WHL4 and AI15.2 lines (Fig. 4B). In the AI15.2 strain, which contains all the En binding sites, the lacZ expression pattern is similar to the endogenous β3-tubulin expression in larvae (data not shown). In particular, β3-tubulin is not normally expressed in the larval hindgut (Fig. 8A). In contrast, the DNA fragment inserted in the WHL4 strain only covers the E fragment. This fragment was
previously shown to contain all the sequences of the \( \beta_3 \)-tubulin locus necessary for the response to ecdysone at the end of the third larval stage, before metamorphosis (Bruhat et al., 1990). In this line, lacZ is expressed ubiquitously in all larval tissues, as shown in the hindgut (Fig. 8A). In order to analyse the repression of \( \beta_3 \)-tubulin by En, we used these two transgenic lines in the presence of hsen-3, to increase the level of En protein (Poole and Kornberg, 1988). Under heat-shock conditions, high amounts of En protein are synthesized ubiquitously in all larval tissues (data not shown). Repression of lacZ expression is observed in [WHL4; hsen-3] larvae in half of the cells longitudinally, in a pattern resembling normal en expression (Hama et al., 1990). This shows that the sequences in the E fragment are able to respond in vivo to differences in En protein concentration and might be part of the regulation of \( \beta_3 \)-tubulin expression in one half of the larval hindgut. The presence of other En binding sequences in the AII15.2 strain seems to be sufficient to get a complete repression of the \( \beta_3 \)-tubulin in presence of either normal or high levels of En protein (Fig. 8A). These results suggest that, under physiological conditions, the En binding sites present in the AII15.2 strain are involved in repression of the \( \beta_3 \)-tubulin in the hindgut. In addition, the E fragment is able to mediate repression by En in the posterior compartment only when En is overexpressed, under non physiological conditions. This response is, nevertheless, probably specific to En, since it is only observed in larval tissues normally sensitive to en expression, as imaginal discs or hindgut, but not in salivary glands for instance (data not shown).

Comparison of lacZ expression patterns in \( \beta_3 \)-6 and \( \beta_3 \)-7 lines provided direct insight into the in vivo function of the D fragment. While the \( \beta_3 \)-7 line contains no En binding site upstream of lacZ (Fig. 4B), the \( \beta_3 \)-6 line contains an additional fragment which covers the D En binding site (Fig. 4B). In the \( \beta_3 \)-7 line, lacZ shows a higher expression in the compartment expressing En (Fig. 8B). Strikingly, in the \( \beta_3 \)-6 strain, no lacZ expression can be detected in the hindgut (Fig. 8B). This result shows that the addition of the D fragment leads to a repression of lacZ expression in the hindgut. It suggests that En might be responsible for the observed repression, at least in the posterior compartment, through its binding to D. Thus, the sequences in the D fragment seem to be active in vivo during larval hindgut development under physiological conditions and could also be responsible of lacZ repression in AII15.2 strain.

**DISCUSSION**

Only a few genes have been identified as putative targets of En, based on the existence of genetic interactions. Most of these are themselves segment polarity genes or homeotic genes and show phenotypes that allow convenient analysis. Only one of them, cubitus interruptus has actually been shown to be a direct target of En (Schwartz et al., 1995). Most of structural genes belong to multigenic families, in which different members often encode redundant functions (Cleveland, 1987). Using genetic interactions might therefore not be an efficient method of identifying target genes encoding effector proteins. Our strategy takes advantage of En’s capacity to bind specific DNA sequences in vivo. Relative to the genetic approach, it is blind because it does not require a preconceived idea about the function of putative en targets. Two genes, polyhometric (Serrano et al., 1995) and Msr-110 (Saenz-Robles et al., 1995) have already been identified as En targets using this method.

In this report, we describe the identification of another direct target of En. Several lines of evidence demonstrate that \( \beta_3 \)-tubulin is an in vivo target of En. Immunostaining of polytene chromosomes with an anti-En antibody reveals a strong En binding site at 60C, the cytological location of \( \beta_3 \)-tubulin. In a transgenic line containing sequences from the \( \beta_3 \)-tubulin promoter, a new En binding site on polytene chromosomes is detected at the position where the transgene is located. In en mutant embryos, \( \beta_3 \)-tubulin expression is derepressed. This \( \beta_3 \)-tubulin isoform, which is normally strictly mesodermal except for expression in the chordotonal organs, is ectopically expressed in the ectoderm of en mutant embryos. Furthermore, when en is ectopically expressed in the mesoderm, no expression of \( \beta_3 \)-tubulin transcripts are detectable in the somatic mesoderm, showing that this repression by en, might occur at the transcriptional level.

Five En binding fragments were identified in vitro within the first intron of the \( \beta_3 \)-tubulin locus, and these fragments contain several sequences related to the En consensus binding site, TTAATTGCAT, which has been shown to be able to mediate repression by En in transfected cells (data not shown; Jaynes and O’Farrell, 1988). This agrees with the observation that En represses \( \beta_3 \)-tubulin expression in the ectodermal cell layer during embryogenesis. The different En binding fragments identified in vitro within the \( \beta_3 \)-tubulin locus are also present in a plasmid library of in vivo En target genes. Thus, En binds to these fragments in the embryo under physiological conditions.

It is of interest to note the complexity of the spatial and temporal regulation of \( \beta_3 \)-tubulin expression. The final pattern of expression is achieved through the juxtaposition of several negative and positive regulatory elements, that are dispensable by themselves. For instance, within the \( \beta_3 \)-7 line (Fig. 4B), a 500 bp fragment (which does not contain En binding sites and is localized between region D and E), was shown to be sufficient to confer, to a lacZ reporter gene, a pattern similar to the endogenous \( \beta_3 \)-tubulin expression in the visceral mesoderm and in the late somatic mesoderm (Hinz et al., 1992; data not shown). The addition of the D fragment in the \( \beta_3 \)-6 shows a particularly high level of lacZ expression in the chordotonal organs, a place of normal expression of \( \beta_3 \)-tubulin (Hinz et al., 1992; Fig 7). However, adding more regulatory sequences to this fragment deregulates this lacZ expression prior to stage 12. Indeed, in the \( \beta_3 \)-4 line, the highest lacZ expression is now detected in neuroblasts, a place where \( \beta_3 \)-tubulin is not normally expressed (Fig. 7). This indicates that this 500 bp fragment in \( \beta_3 \)-7 contains all the enhancer sequences necessary to get normal expression of the \( \beta_3 \)-tubulin in visceral mesoderm and late somatic mesoderm, while surrounding sequences contain repressor sequences, as well as enhancer sequences involved in other tissues, prior to stage 12. Interestingly, the En binding sites are localized outside of this 500 bp fragment (Fig. 4B). Only the presence of the entire 4.6 kb intron and of 0.23 kb of upstream sequences finally restores a normal expression profile. This intricate regulatory system of independently acting enhancers and silencers might confer redundant regulation of expression to prevent errors in the final expression profile of structural genes. This also indicates that before stage 12, a complicated network of regulation seems to be present in embryos.
Fig. 5. En immunodetection in the \( \beta 3023I \) transgenic line.
(A) Localization of the insert to region 3C of the X chromosome by in situ hybridization, using a biotinylated \( \beta 3 \)-tubulin probe.
(B, C) Polyten chromosomes bound by the rabbit polyclonal anti-En antibody, secondary detected by peroxidase. (B) Immunostaining of the X chromosome of the \( \beta 3023I \) transgenic line in a \( ttk1 \) background. (C) Immunostaining of the X chromosome of a \( ttk1 \) line. Common sites are indicated and the new signal detected at region 3C in the \( \beta 3023I \) transgenic line (B) is indicated by an arrow.

Fig. 6. Identification of sites bound in vivo by En protein. The presence of the En binding sites, identified in vitro within the \( \beta 3 \)-tubulin locus, was tested by PCR amplification in the in vivo En targets library. The amplification was carried out using specific sets of primers. The primers were chosen to lie within \( Sau3A \) fragments and were contained within A to F fragments. 10 ng of total library DNA was amplified. L corresponds to the amplification product from the library DNA. C is a control of PCR amplification specific for each fragment. N is a negative control, in which PCR amplification has been carried out in the same conditions as in the L and C lanes, but without any DNA matrix. One fifth of each amplification product was loaded. M is the 1 kb ladder migration control (Gibco, BRL).

Fig. 7. Roles of the different En binding sites during embryogenesis. \( lacZ \) expression is detected by a monoclonal anti-\( \beta \)-galactosidase antibody, secondary detected by the peroxidase (brown), in the \( \beta 3 \)-6; \( \beta 3 \)-4 and \( A115.2 \) transgenic lines shown in Fig. 4B. Embryos either contain two functional copies of the \( en \) gene (WT) or are homozygous for \( Df(2R)en^{SFX31} \) (\( EN^- \)). \( en \) mutant embryos are selected because they do not express En (normally detected by a rabbit polyclonal anti-En antibody, secondary detected by the alkaline phosphatase, visualized in blue in wild-type embryos). Early: embryos prior to stage 12 (germ-band extended embryos). Late: embryos after stage 12.
The different β-Tubulin isoforms could have the capacity to be expressed in different types of cells in early development as a means to adapt to particular functional or regulatory requirements. If β-Tubulins can indeed be functionally redundant, this might account for the difficulties to isolate embryonic lethal mutations for such genes (Kimble et al., 1990).

It has been shown that distinct elements regulate β3-tubulin expression in the somatic mesoderm at different stages of development (Hinz et al., 1992). Similarly, different sets of regulatory sequences are involved in the regulation by Engrailed before and after stage 12. Indeed, during embryogenesis, two different sets of En binding fragments mediate the early and the late repression by En. The E fragment is necessary to obtain repression by En during the germ band extension, while A to D fragments mediate repression after germ band retraction, even though when isolated, the D fragment is not sufficient to respond to En regulation (see β3-6 line in Fig. 7). However, D might be necessary for En response, since it is present in our embryonic En binding sites library (Fig. 6).

In vivo analysis was also carried out during larval development, using different transgenic lines. These studies showed that the E fragment is able to respond to high levels of en expression in the hindgut. The presence of the other En binding sites in the AI15.2 line might be responsible for the β3-tubulin repression in the posterior hindgut, under physiological conditions. Indeed, the presence of the 60 bp D fragment is already sufficient to drive lacZ repression in the larval hindgut, even though the hypothesis that En is involved cannot be directly tested because of the lack of appropriate en viable mutants.

β3-tubulin is the first effector gene identified as being directly regulated by en. This finding is interesting and fits with the idea that en not only is a segmentation gene, but also behaves like a selector gene, since its activity is responsible for the posterior identity cell fate (Guillen et al., 1995; Tabata et al., 1995). The fact that en can directly regulate structural genes suggests an additional role for En in directly regulating morphogenesis.

Furthermore, en is specifically expressed in the ectodermal cell layer, unlike β3-tubulin, which, apart from expression in the chordotonal organs, is exclusively mesodermal. The direct interaction between these two genes strongly supports the idea of the existence of a dialogue between different cell layers (Lawrence et al., 1994). Actually, a regulatory cascade between different cell layers has already been reported in the specification of endoderm by homeotic genes (Immerglück et al., 1990). Thus en could have a role, not only in directing posterior cell fate, but also in distinguishing between the ectodermal and mesodermal cell fates.

The way regulatory genes eventually specify morphogenesis remains a black box in Drosophila development. The downstream genes of selector genes must directly regulate processes that govern morphogenesis. Among the target genes identified are two Ultrabithorax targets scabrous, a gene encoding a secreted polypeptide with homology to fibrinogen (Graba et al., 1992) and connectin, encoding a cell surface molecule (Gould and White, 1992), as well as an Antennapedia target, centrosomin, an essential component of the centrosome (Heuer et al., 1995). Strikingly, all have been identified on the basis of the capacity of homeotic proteins to bind DNA specifically. The β3-tubulin gene is also regulated by Ultrabithorax, although the direct nature of this regulation remains to be demonstrated (Hinz et al., 1992). The identification of the β3-tubulin gene as a target of en may allow us to start making the connection between transcription factors, structural genes and morphogenesis.

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Bruhat, A., Tourmente, S., Chapel, S., Sobrier, M. L., Coudert, J. L. and Dastugue, B. (1990). Regulatory elements in the first intron contribute to β3-tubulin expression on en expression in larval hindgut. lacZ expression is detected by β-galactosidase staining in transgenic lines shown in Fig. 4B. (A) lacZ pattern is shown in the hindgut of WHL4 or AI15.2 strains in absence (WT) or in presence of hsem-3 transgene, under heat shock conditions (HS-EN). (B) Comparison of the hindgut expression profiles of β3-7 and β3-6 strains. In β3-7, lacZ expression is higher in the compartment expressing En (data not shown). The arrow indicates the position of the posterior compartment.


