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Indirect evidence for *Delta*-dependent intracellular processing of Notch in *Drosophila* embryos

Magalie Lecourtois and François Schweisguth

Cell–cell signalling mediated by the receptor Notch regulates the differentiation of a wide variety of cell types in invertebrate and vertebrate species [1], but the mechanism of signal transduction following receptor activation is unknown. A recent model proposes that ligand binding induces intracellular processing of Notch [2–4]; the processed intracellular form of Notch then translocates to the nucleus and interacts with DNA-bound Suppressor of Hairless (Su(H)), a transcription factor required for target gene expression [5–8]. As intracellular processing of endogenous Notch has so far escaped immunodetection [1], we devised a sensitive nuclear-activity assay to monitor indirectly the processing of an engineered Notch *in vivo*. First, we show that the intracellular domain of Notch, fused to the DNA-binding domain of Gal4, regulated transcription, in a *Delta*-independent manner. Second, we show that full-length Notch, containing the Gal4 DNA-binding domain inserted 27 amino acids carboxy terminal to the transmembrane domain, activated transcription in a *Delta*-dependent manner. These results provide indirect evidence for a ligand-dependent intracellular processing event *in vivo*, supporting the view that Su(H)-dependent Notch signalling involves intracellular cleavage, and transcriptional regulation by processed Notch.

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Received: **23 March 1998**
Revised: **29 April 1998**
Accepted: **11 May 1998**

Published: **8 June 1998**

Current Biology 1998, 8:771–774
http://biomednet.com/elecref/0960982200800771

© Current Biology Ltd ISSN 0960-9822

Results and discussion

During early neurogenesis in *Drosophila*, Notch signalling leads to the Su(H)-dependent transcription of several genes of the *Enhancer of split* (*E(spl)*) complex [7,8]. To determine whether the postulated intracellular form of Notch, N^{intra} (amino acids 1791–2703 of Notch), can activate the transcription of target genes of Su(H) *in vivo*, we analysed the effect of a Gal4–N^{intra} fusion protein on the expression of a *lacZ* reporter gene that was coupled to the *m5* gene of the *E(spl)* complex (*E(spl)-m5*), in which all four Su(H)-binding sites were substituted by upstream activator sequence (UAS) elements (Figure 1a,b).

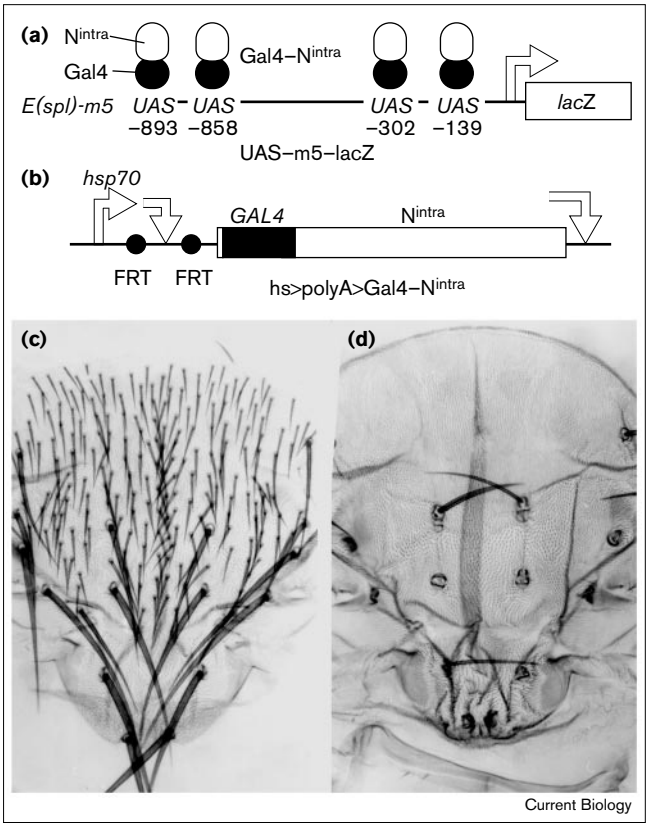
First, we established that the DNA-binding domain of Gal4 did not interfere with the function of N^{intra}. Expression of N^{intra} [4] in the developing notum has previously been shown to block neural differentiation, resulting in a bristle-loss phenotype [9]. Overexpression of Gal4–N^{intra} had a similar effect (Figure 1c,d). We conclude that Gal4–N^{intra}, like N^{intra}, behaves as a constitutively activated form of Notch.

We then examined the regulatory activity of Gal4–N^{intra}. A fragment of the promoter from the *E(spl)-m5* gene (nucleotides –897 to +20) conferred upon a *lacZ* reporter gene the same dynamic expression pattern as the endogenous gene at stages 9–10 of embryogenesis (Figure 2a). This DNA fragment contains four Su(H)-binding sites that are strictly required for Notch-dependent regulation [8]. Replacement of all Su(H)-binding sites by Gal4-binding sites in the UAS–m5–lacZ reporter construct (Figure 1a) abolished *lacZ* expression in the neuroectoderm (Figure 2b). Expression of Gal4–N^{intra} restored this expression in a subset of neuroectodermal cells. This was seen with both heat-induced and basal levels of Gal4–N^{intra} expression (Figure 2c,d). N^{intra}, in contrast to Gal4–N^{intra}, failed to induce UAS-mediated transcription, showing that transcriptional activation by Gal4–N^{intra} required DNA binding (data not shown). Although Gal4–N^{intra} was presumably expressed in all cells following heat shock, transcriptional activation was restricted to the neuroectoderm in stage 9–10 embryos. This specificity may in part be imposed by the *E(spl)-m5* regulatory sequences included in the reporter construct, as ubiquitous expression of full-length Gal4, using the daughterless–Gal4 driver line [10], also preferentially induced UAS–m5–lacZ expression in the neuroectoderm (data not shown). Thus, the observation that Gal4–N^{intra} activated transcription in *Drosophila* embryos confirms and extends previous results from transfection studies in mammals, demonstrating that the intracellular domain of Notch can activate transcription [2,11,12].

We next examined whether *Delta* (*Dl*) activity was required for regulation by Gal4–N^{intra}. Figure 2e shows that Gal4–N^{intra} activated UAS–m5–lacZ expression in a *Dl*-mutant embryo, consistent with the idea that signalling by activated Notch involves transcriptional regulation.

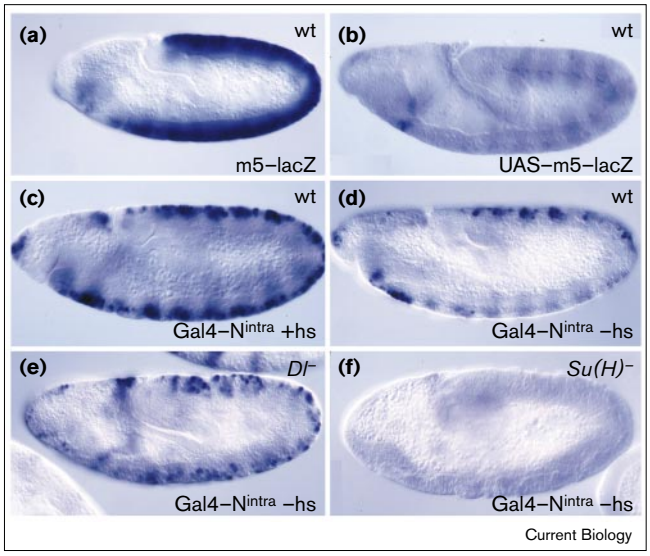
Finally, if Su(H) simply functions in tethering a processed form of Notch to DNA, then the binding of N^{intra} at the

Figure 1



Structure and signalling activity of Gal4–N^{intra}. **(a)** Structure of the UAS–m5–lacZ construct and strategy, using Gal4, to tether the intracellular domain of Notch at the positions of the Su(H)-binding sites within the regulatory sequences of the *E(spl)-m5* gene. The four Su(H)-binding sites, whose nucleotide positions are indicated, were substituted by UAS elements to which Gal4 binds. The reporter gene was *lacZ*. The position of the transcriptional start site is indicated by an arrow. **(b)** Diagram of the hs>polyA>Gal4–N^{intra} construct, where > indicates the Flp recombinase target (FRT) sequence. A DNA fragment encoding the DNA-binding domain of Gal4 (amino acids 1–147) was inserted between sequence encoding a 4 amino acid long translational start (MAGS; in the single-letter amino-acid code) and the intracellular domain of Notch (amino acids 1791–2703). Conditional expression of this Gal4–N^{intra} fusion protein was regulated by the heat-inducible *hsp70* promoter, and by an FRT–polyA–FRT cassette that was inserted between the promoter and the coding sequence. This polyadenylation signal blocked Gal4–N^{intra} expression from the leaky *hsp70* promoter. Expression of Gal4–N^{intra} therefore required the Flp-recombinase-induced deletion of this signal. The positions of FRT sequences (black dots) and polyadenylation signals (inverted arrows) are indicated. Cloning details are available in Supplementary material published with this paper on the internet. **(c,d)** Cuticular preparations from (c) hs>polyA>Gal4–N^{intra}/+ and (d) hs>Gal4–N^{intra}/+ adult flies that had been exposed to heat shock for 1 h at 37°C 6–9 h after puparium formation. (d) Strong microchaete-loss and macrochaete-double-socket phenotypes were specifically observed following Flp-recombinase-mediated deletion of the FRT–polyA–FRT cassette.

Figure 2



Transcriptional activity of Gal4–N^{intra}. *In situ* hybridization analysis of whole-mount embryos using a *lacZ* probe. **(a–f)** Lateral views of stage 10 embryos of the following genotypes: (a) m5–lacZ/+; (b) UAS–m5–lacZ/+; (c,d) UAS–m5–lacZ/hs>Gal4–N^{intra} (in panel c, the embryo was exposed to a 20 min heat shock at 37°C, followed by 20 min at 25°C; in panel d, no heat shock was applied); (e) *Dl^{revF10}* UAS–m5–lacZ/*Dl^{9P39}* hs>Gal4–N^{intra}; **(f)** *Su(H)^{SF8}* FRT40A/*Su(H)^{AR9}* UAS–m5–lacZ; hs>Gal4–N^{intra}/+ mutant embryos derived from maternal *Su(H)* germ-line clones [8]. In (c–f), the FRT–polyA–FRT cassette was removed by Flp-mediated recombination in males carrying a β2-tubulin–*flp* transgene [9] (the frequency of excision was 0.6). The faint, out-of-focus, striped pattern seen in (b) is due to the expression of UAS–m5–lacZ in the tracheal pit anlagen (see Figure 4a). The abbreviations are: wt, wild type; +hs, heat shock applied; –hs, no heat shock applied.

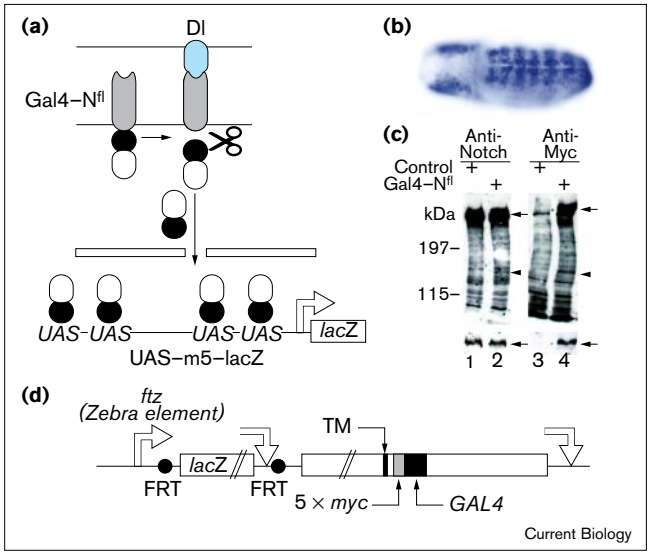
positions of the Su(H)-binding sites through Gal4 should bypass the requirement for *Su(H)* activity. We studied the expression of UAS–m5–lacZ in *Su(H)*-mutant embryos derived from germ-line clones, and expressing Gal4–N^{intra}. Unexpectedly, no significant *lacZ* expression was detected in these embryos (Figure 2f), showing that Gal4–N^{intra} required *Su(H)* activity to regulate transcription. As all known Su(H)-binding sites have been deleted in the UAS–m5–lacZ construct, this suggests that Su(H) may not only target N^{intra} to the DNA but also have an additional function. For instance, Su(H) may be required to protect processed Notch from degradation [13], or participate in transcriptional activation together with processed Notch.

If signalling involves Notch as a transcriptional co-activator for Su(H), then a nuclear form of Notch has to be generated following receptor activation at the membrane. To test whether Notch can be processed in response to ligand binding, the nuclear-activity assay described above was used to examine whether a proteolytic fragment, similar to Gal4–N^{intra} and active in the nucleus, can be generated from a membrane-bound form of Notch (Figure 3a). Intracellular cleavage has been suggested to occur within either the transmembrane domain or the first 10 amino acids of the intracellular domain [3]. A full-length Notch, containing

a Myc-epitope-tagged version of the Gal4 DNA-binding domain 27 amino acids carboxy terminal to the transmembrane domain, that is, downstream of the putative cleavage site, was engineered (Gal4–N^{fl}; Figure 3d). Expression of Gal4–N^{fl} was regulated by a *fushi tarazu* (*ftz*) promoter. In the parental ftz>lacZ>Gal4–N^{fl} transgenic lines, *lacZ* was expressed in the neuroectoderm of stage 9–10 embryos (Figure 3b). The FRT–lacZ–FRT cassette was then removed by Flp-mediated recombination, and stable ftz>Gal4–N^{fl} lines were established [9].

The unprocessed form of Notch is the major immunoreactive species detected by western blot analysis in stage 9–11 embryos using C17-9C6 anti-Notch antibody that has been raised against the intracellular form of Notch (Figure 3c, arrow in lanes 1,2). Full-length (300–320 kDa) Gal4–N^{fl} was specifically detected using anti-Myc antibodies in ftz>Gal4–N^{fl} embryos (Figure 3c, lane 4). A novel 130–140 kDa species that reacted with both anti-Notch and anti-Myc antibodies was seen in ftz>Gal4–N^{fl} but not in control embryos (Figure 3c, arrowhead in lanes 2,4). This species might correspond to the membrane-bound fragment of the functional heterodimeric receptor [14]. The intensities of the 300–320 kDa endogenous Notch and Gal4–N^{fl} bands relative to the novel 130–140 kDa band were similar (Figure 3c, lanes 2,4). We conclude that Gal4–N^{fl}, like endogenous Notch, mainly accumulated as an unprocessed form, and that Gal4–N^{fl} was no more abundant than endogenous Notch.

Figure 3



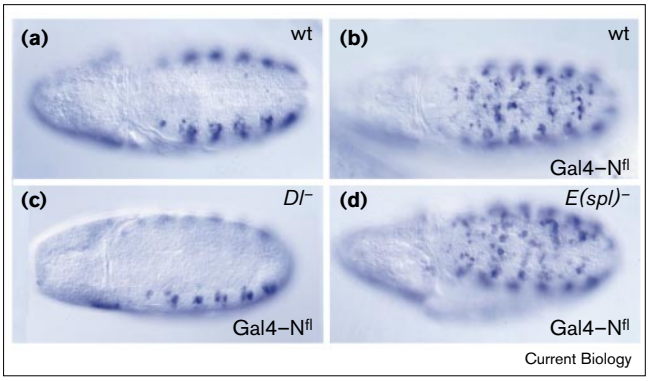
A nuclear-activity assay for Notch intracellular processing: structure and expression of Gal4–N^{fl}. **(a)** Strategy used to detect Notch intracellular processing: binding of Dl to Gal4–N^{fl} (see panel d) induces the release of an intracellular fragment that is structurally similar to Gal4–N^{intra} from the plasma membrane, which, following nuclear translocation, activates UAS–m5–lacZ transcription. **(b)** *In situ* hybridization analysis of a stage 10 ftz>lacZ>Gal4–N^{fl} embryo (dorsal view) showing *lacZ* expression driven by the *ftz* promoter. **(c)** Western blot analysis of Notch and Gal4–N^{fl} in UAS–m5–lacZ (control; lanes 1,3) and ftz>Gal4–N^{fl} (lanes 2,4) stage 9–11 embryos, using anti-Notch (C17-9C6; lanes 1,2) and anti-Myc (9E10; lanes 3,4) antibodies. The unprocessed 300–320 kDa Notch and Gal4–N^{fl} bands are indicated by arrows (a shorter exposure of this part of the gel is shown at the bottom of the panel). The position of a processed 130–140 kDa Gal4–N^{fl} form is indicated by an arrowhead. **(d)** Diagram of the ftz>lacZ>Gal4–N^{fl} construct. DNA fragments encoding five Myc epitope tags and the DNA-binding domain of Gal4 (amino acids 1–147) were inserted, within a cDNA encoding full-length Notch (amino acids 1–2703), at a point that was 27 codons 3′ to the sequence encoding the transmembrane domain (TM; amino acids 1746–1765), to generate Gal4–N^{fl}. Expression of Gal4–N^{fl} was regulated by *ftz* regulatory sequences (nucleotides −669 to +70). An FRT–lacZ–polyA–FRT cassette was inserted between the *ftz* promoter and the Gal4–N^{fl} coding sequence.

Next, we examined whether Gal4–N^{fl} activated transcription from the *E(spl)-m5* promoter in a UAS-dependent manner. The UAS–m5–lacZ reporter construct was not expressed in the ventral neuroectoderm at stage 10 (Figure 4a). Transcriptional activation occurred in a subset of the cells expressing Gal4–N^{fl} (Figure 4b). Thus, Gal4–N^{fl}, like Gal4–N^{intra} (Figure 2d), regulated transcription from the *E(spl)-m5* promoter in a UAS-dependent manner. This implied that a Gal4-containing proteolytic fragment must be released from the membrane and translocated into the nucleus *in vivo*.

As Gal4-containing fragments might be produced by proteolysis of Gal4–N^{fl} that is unrelated to Notch signalling, it was essential to verify that Gal4–N^{fl}-mediated transcription required activation of the receptor at the membrane. We thus examined the expression of the UAS–m5–lacZ reporter gene in *Dl*-mutant embryos expressing Gal4–N^{fl}. No *lacZ* expression was detected in the ventral neuroectoderm in these embryos (Figure 4c). This contrasts with the Gal4–N^{intra}-mediated transcription, which was shown to be *Dl* independent (Figure 2e). Reduced UAS–m5–lacZ expression in *Dl*-mutant embryos might, however, result from neurogenic cell-fate transformations associated with loss of *Dl* activity. To investigate this possibility, transcriptional activation by Gal4–N^{fl} was examined in embryos carrying a deletion of the *E(spl)* gene complex. These neurogenic embryos are defective in their response to Notch signalling [4], but not in transducing the signal. Figure 4d shows one such mutant embryo at late stage 10 in which Gal4–N^{fl} protein activated UAS–m5–lacZ transcription. This indicates that neurogenic cell-fate transformations do not prevent Gal4–N^{fl} nuclear activity *per se*. We conclude that transcriptional activation by Gal4–N^{fl} is *Dl* dependent.

The nuclear-activity assay described in this report suggests that a ligand-dependent, intracellular processing of an engineered Notch protein, Gal4–N^{fl}, occurs *in vivo*. We suggest that the binding of Dl to Gal4–N^{fl} might modify Gal4–N^{fl} such that it becomes available as a substrate for

Figure 4



Transcriptional activity of Gal4–N^{fl}. *In situ* hybridization analysis of whole-mount embryos using a *lacZ* probe. **(a–d)** Dorsal views of stage 10 embryos of the following genotypes: (a) UAS–m5–lacZ/+; *lacZ* expression at tracheal pit anlagen served as an internal control for staining; (b) UAS–m5–lacZ/ftz>Gal4–N^{fl}; (c) *Dl*^{9P39} UAS–m5–lacZ/*Dl*^{revF10} ftz>Gal4–N^{fl}; (d) UAS–m5–lacZ Df(3R)b$^{32.2}$/ftz>Gal4–N^{fl} Df(3R)b$^{32.2}$. Expression of Gal4–N^{fl} induced the expression of *lacZ* in the ventral neuroectoderm (compare panels a and b). (c) Expression of *lacZ* was not detected in the neuroectoderm of *Dl*-mutant embryos. In contrast, *lacZ* expression was similar in mutant embryos carrying a deletion in the *E(spl)* complex, and wild-type embryos (compare panels b and d). Df(3R)b32.2 removes all basic helix-loop-helix genes in the *E(spl)* complex. UAS–m5–lacZ expression was transient in *E(spl)*-mutant embryos, however. This is probably because the expression of *Dl* is dramatically reduced in *E(spl)*-mutant embryos from stage 11 onwards [15].

an intracellular proteolytic activity. This indirect assay did not allow us to detect processed Gal4–N^{fl}, however, nor to determine its structure and the position of the cleavage site. Still, detection of a nuclear activity implies that the cleavage site must reside between the Gal4 DNA-binding domain and the transmembrane domain. Consistent with this hypothesis, an activated version of murine Notch1 is processed intracellularly at a conserved valine residue (Val1744, which corresponds to Val1763 in *Drosophila*) located at the carboxy-terminal extremity of the transmembrane domain [13]. Val1763 and flanking sequences are present in Gal4–N^{fl}.

Although the fate of the endogenous receptor could not be investigated by this indirect assay, our results suggests that Notch signalling involves intracellular processing of the receptor upon ligand activation, nuclear translocation of the processed form, and, together with Su(H), transcriptional activation by this intracellular domain of Notch. This proposed mechanism has important implications for signal specificity, signal thresholds, signal integration and signalling dynamics. For instance, ending of signalling might require the proteolytic degradation of processed nuclear Notch.

The demonstration that intracellular processing can mediate signalling now awaits genetic analysis of this proteolytic activity. The nuclear-activity assay described in this study may be extremely useful in identifying mutations specifically affecting this process.

Supplementary material

Cloning details are published with this paper on the internet.

Acknowledgements

We thank S. Artavanis-Tsakonas, M. Haenlin, P. Heitzler, B. Holmgren, S. Jarriault, M. Muskavitch, L. Seugnet and E. Wimmer for plasmids, antibodies and flies. We thank A. Israël, F. Logeat and R. Kopan for sharing results prior to publication. We also thank members of our laboratory and of A. Israël's group for critical reading of the manuscript, and E. Dequier for his help during initial stages of this work. This work was supported by grants from the CNRS, MENESR, FRM, ARC (ARC 1828) and LNC-Comité de Paris.

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Supplementary material

Indirect evidence for *Delta*-dependent intracellular processing of Notch in *Drosophila* embryos

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Current Biology 8 June 1998, **8**:771–774

Materials and methods

Cloning details

UAS–m5–lacZ. The four 7 bp long core Su(H)-binding sites within the *E(spl)-m5* regulatory sequence were substituted by site-directed PCR mutagenesis by optimized Gal4-binding sites corresponding to the *Sca*I site. The mutated *E(spl)-m5* regulatory sequence (nucleotides –897 to +20) was subcloned into pCasperβgal as an *Eco*RI–*Kpn*I fragment to generate UAS–m5–lacZ.

hs>polyA>Gal4–N^{intra}. A *Bam*HI–*Aat*II PCR product encoding the DNA-binding domain of Gal4 (amino acids 1–147) was generated using the two following primers: 5′-GGGACGTCCAGATCTAATC-GATACAGTCAACTG-3′ and 5′-GCGGATCCATGAAGCTACT-GTCTTC-3′, ligated to an *Aat*II–*Xba*I fragment encoding the intracellular domain of Notch (amino acids 1791–2703; from plasmid pN^{intra}AatII; gift of L. Seugnet) and inserted into pT7βlink opened by *Bam*HI and *Xba*I. The first 4 amino acids (MAGS) of the resulting Gal4–N^{intra} are provided by the pT7βlink vector. The *Xba*I Gal4–N^{intra} fragment was then subcloned into pCasper–hsp70 together with a blunt-ended *Hind*III–*Sal*I fragment encoding a FRT–polyA–FRT cassette purified from pGem4–polyA–FRT (gift of B. Holmgreen).

ftz>lacZ>Gal4–N^{fl}. A PCR product corresponding to the *ftz* promoter region (nucleotides –669 to +70), was amplified from genomic DNA using the two following primers: 5′-GCGAATTCTTGGCCAC-GAGGGC-3′ and 5′-GCACTAGTATCGGATGTGTATTGCTAG-3′. An *Aat*II–*Bam*HI PCR fragment encoding the 5 × Myc epitope tag, amplified from the mNotchΔE plasmid (gift of A. Israël) using the two following primers: 5′-GCGGATCCGAGGTCGCCCAAGCTC-3′ and 5′-GCGTCGACGTCGATTTAAAGCTATGGAG-3′, was ligated to a *Bgl*II–*Aat*II fragment encoding Gal4 (amino acids 1–147). The resulting *Aat*II–*Aat*II 5 × Myc–Gal4 fragment was inserted at the *Aat*II site of a full-length Notch cDNA clone (pN*Xba*; gift of L. Seugnet) to generate Gal4–N^{fl}. The sequences encoding the *ftz* promoter and the Gal4–N^{fl} were subcloned as *Eco*RI–*Spe*I and *Spe*I–*Not*I fragments into pC>AB (gift of E. Wimmer), to generate pCftz>Gal4–N^{fl}.

The sequence of all PCR products was verified by sequencing.