Human deltex is a conserved regulator of Notch signalling

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A fundamental cell-fate control mechanism regulating multicellular development is defined by the Notch-signalling pathway[1]. Developmental and genetic studies of wild type and activated Notch-receptor expression in diverse organisms suggest that Notch plays a general role in development by governing the ability of undifferentiated precursor cells to respond to specific signals[1,2]. Notch signalling has been conserved throughout evolution and controls the differentiation of a broad spectrum of cell types during development[1–3]. Genetic studies in *Drosophila* have led to the identification of several components of the Notch pathway[1]. Two of the positive regulators of the pathway are encoded by the suppressor of hairless [*Su(H)*] and deltex (*dx*) genes[5–8]. *Drosophila dx* encodes a ubiquitous, novel cytoplasmic protein of unknown biochemical function[9]. We have cloned a human deltex homologue and characterized it in parallel with its *Drosophila* counterpart in biochemical assays to assess deltex function. Both human and *Drosophila* deltex bind to Notch across species and carry putative SH3-binding domains. Using the yeast interaction trap system, we find that *Drosophila* and human deltex bind to the human SH3-domain containing protein Grb2 (ref. 10). Results from two different reporter assays allow us for the first time to associate deltex with Notch-dependent transcriptional events. We present evidence linking deltex to the modulation of basic helix-loop-helix (bHLH) transcription factor activity.

A human cDNA fragment encoding a peptide with similarity to a region of the *Drosophila* deltex protein was identified from the Expressed Sequence Tag data base (dbEST; ref. 11). We performed PCR to amplify a relevant fragment from a human fetal brain cDNA library and used it as a probe to isolate corresponding cDNA clones. Sequence analysis revealed the deduced amino-acid sequence of the human deltex homologue (DTX1) (Fig. 1*a*). Sequence similarity between *Drosophila* deltex and DTX1 extends throughout the open reading frame (Fig. 1*a*). *Drosophila* deltex domain I, which is necessary and sufficient to bind to the ankyrin repeats of Notch as well as sufficient to rescue a loss-of-function *deltex* allele[8] (amino acids 46–204 of *Drosophila* deltex), is well conserved in DTX1 (Fig. 1*a*, filled rectangle, Fig. 1*b*, shaded rectangle). The most conserved region is the carboxy-terminal region of the deltex proteins (domain III), which includes the RING-H2 zinc-finger motif[8] (Fig. 1*a*, open circles, Fig. 1*b*, filled rectangle). Sequence comparisons reveal similarity between a region that includes part of the RING-H2 zinc-finger domain of deltex and the N-terminal domain of a protein encoded by *PMS2LH*, a member of the hPMS2-related gene family (Fig. 1*c*; refs 12,13). PMS2 is a mutL homologue, a protein associated with hereditary non-polyposis colon cancer[12].

Drosophila deltex was shown to interact with the ankyrin repeat region of the Notch intracellular domain[8]. Using the yeast interaction trap system[30], we found that this property of deltex is conserved in the vertebrate counterpart. We detected interactions between DTX1 and the ankyrin repeats derived from human Notch-1and Notch-2 proteins (Table 1, pLEXDTX1/pJGhNotch-1 and pLEXDTX1/pJGhNotch-2)[14,15]. The ability of deltex to interact with Notch is conserved across species, since we also detected interactions between DTX1 and *Drosophila* Notch (Table 1, pLEXDTX1/pJGDNotch).

The *Drosophila* deltex domain II has a proline-rich region similar to the consensus sequence of SH3-domain binding sites[8]. While domain II is the least-conserved portion of the protein, the proline-rich motif is repeated several times in the human sequence (Fig. 1*a*,*b*). We detected interaction between deltex and an arbitrarily selected SH3-domain protein, human Grb2 (ref. 10), in the yeast two-hybrid system (Table 2). It remains to be seen if deltex interacts with an SH3 protein *in vivo*.

The expression of deltex in humans and *Drosophila* is ubiquitous, as judged by northern blots (data not shown). *In situ* hybridization and immunocytochemistry in tissues revealed uniform deltex expression for *Drosophila* (data not shown). However, *in situ* hybridization of embryonic human tissue indicates that the distribution of *DTX1* mRNA is not uniform (Fig. 2). We detected *DTX1*mRNA in several embryonic tissues, including the nervous system, blood vessels, pancreas, lung, adrenal gland, digestive tube and muscles. The most intense hybridization signal was localized in blood vessels (Fig. 2*a*, bv), a tissue that has been associated with pathologies caused by Notch signalling malfunction in humans[16]. *DTX1* expression appeared to be frequently, but not always, associated with epithelial-mesenchymal interaction sites. Weak expression was detected in epithelial cells of the developing pancreas (Fig. 2*b*) and alveolar

Table 1 • Interaction of DTX1 and ankyrin repeats of human Notch proteins

Coexpressed constructs	Media: Galactose	Media: Glucose
pLEXhDx-1/pJGhNotch-1	732 (35)	4 (1)
pLEXhDx-1/pJGhNotch-2	195 (25)	11 (5)
pLEXhDx-1/pJGDNotch	892 (184)	14 (4)
pLEXhDx-1/pJGHairless	61 (13)	20 (2)
pLEXhDx-1/pJG	38 (1)	21 (5)
pLEX/pJGhNotch-1	47 (13)	13 (5)
pLEX/pJGhNotch-2	45 (7)	19 (8)
pLEX/pJGDNotch	69 (18)	20 (5)
pLEX/pJGHairless	59 (4)	39 (14)
pLEX/pJG	60 (10)	32 (4)

In the yeast interaction trap system, the transcriptional activity of a β-galactosidase reporter gene is an indicator of protein-protein interactions between two proteins co-expressed in yeast[30]. Standard deviations are shown in parentheses. pLEXDTX1 contains the entire open reading frame (ORF) of *DTX1*. pJGhNotch-1 and pJGhNotch-2 contain ankyrin repeats of human Notch-1 (amino acids 1826–2147) and Notch-2 (amino acids 1772–2084), respectively[14,15]. pJGDNotch contains the ankyrin repeats of *Drosophila* Notch (amino acids 1827–2259). pJGHairless contains the entire ORF of *hairless*[24].

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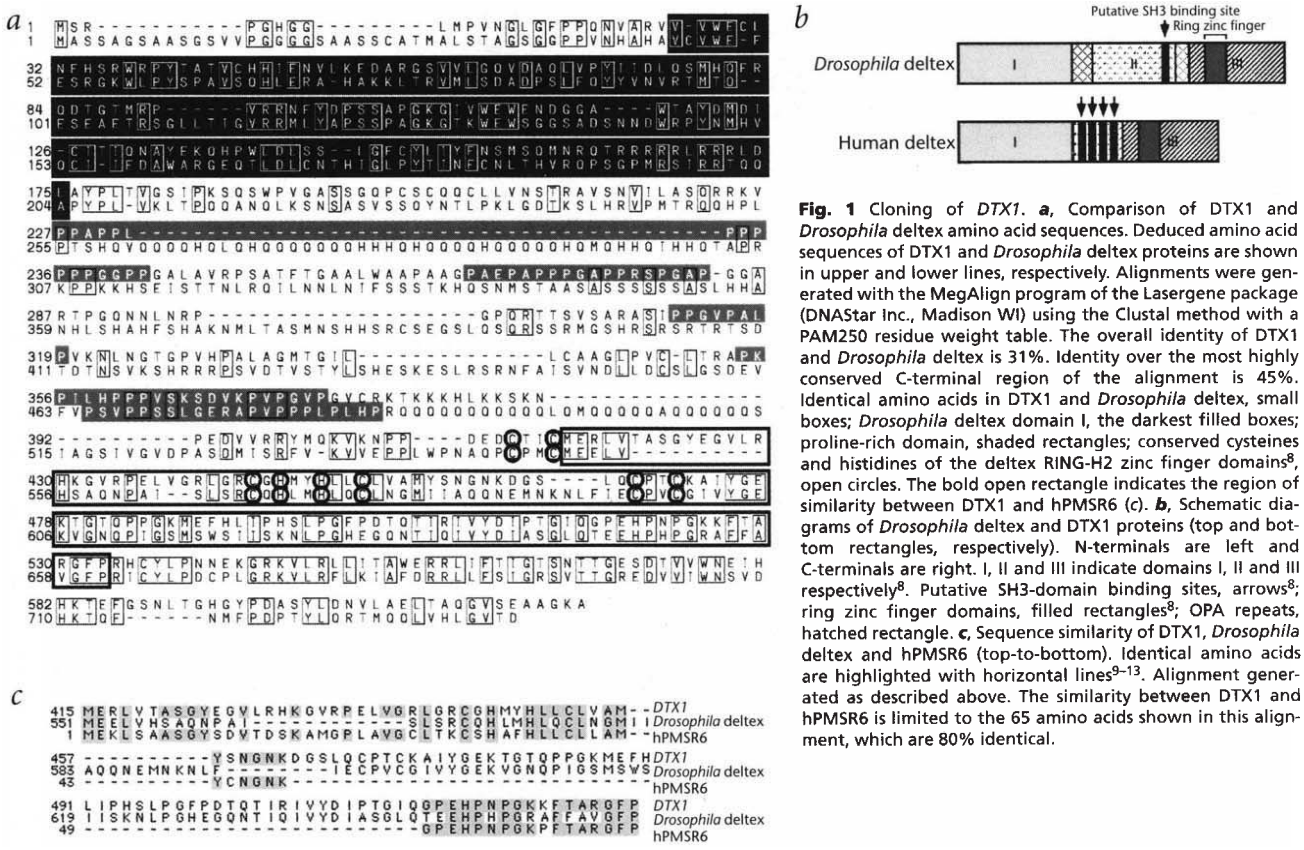

Fig. 1 Cloning of *DTX1*. ***a***, Comparison of DTX1 and *Drosophila* deltex amino acid sequences. Deduced amino acid sequences of DTX1 and *Drosophila* deltex proteins are shown in upper and lower lines, respectively. Alignments were generated with the MegAlign program of the Lasergene package (DNAStar Inc., Madison WI) using the Clustal method with a PAM250 residue weight table. The overall identity of DTX1 and *Drosophila* deltex is 31%. Identity over the most highly conserved C-terminal region of the alignment is 45%. Identical amino acids in DTX1 and *Drosophila* deltex, small boxes; *Drosophila* deltex domain I, the darkest filled boxes; proline-rich domain, shaded rectangles; conserved cysteines and histidines of the deltex RING-H2 zinc finger domains[8], open circles. The bold open rectangle indicates the region of similarity between DTX1 and hPMSR6 (*c*). ***b***, Schematic diagrams of *Drosophila* deltex and DTX1 proteins (top and bottom rectangles, respectively). N-terminals are left and C-terminals are right. I, II and III indicate domains I, II and III respectively[8]. Putative SH3-domain binding sites, arrows[8]; ring zinc finger domains, filled rectangles[8]; OPA repeats, hatched rectangle. ***c***, Sequence similarity of DTX1, *Drosophila* deltex and hPMSR6 (top-to-bottom). Identical amino acids are highlighted with horizontal lines[9–13]. Alignment generated as described above. The similarity between DTX1 and hPMSR6 is limited to the 65 amino acids shown in this alignment, which are 80% identical.

ducts of the lung (Fig. 2*c*), whereas the signal was undetectable in the surrounding parenchyma. Similarly, in the digestive tube, the hybridization signal was observed only in mucosal epithelium (Fig. 2*d*). In the spinal cord, expression was restricted to distinct cells, whereas transcripts were evident in all radial glial cells (Fig. 2*e*). A similar distribution of expressing and non-expressing cells was seen in dorsal root ganglia (Fig. 2*f*). In the adrenal gland, *DTX1* mRNA was more abundant in the cortical area (Fig. 2*g*) than in the medulla (Fig. 2*g′*). Transcripts were also detected in the forming muscles (Fig. 2*h*).

Ectopic expression of *Drosophila* deltex results in mutant phenotypes similar to those induced by the constitutive activation of the Notch receptor in *Drosophila*, whereas genetic interactions suggest that it acts as a positive regulator of Notch signalling[8]. In an attempt to uncover biochemical properties of deltex, we tested whether Notch and deltex share downstream target genes, and examined functional interactions between the activated Notch receptor and deltex. Previous analyses have established that transcription of the bHLH genes of the *Enhancer of split* [*E(spl)*] locus depends on Notch activation and the existence of Su(H) binding sites in their promoter regions[17–19].

Eastman *et al.*[19] have developed a reporter-based assay which monitors the transcriptional activity of the *E(spl)mγ* promoter. *mγ* is one of the *bHLH* genes in the *E(spl)* complex and its transcription *in vivo* is dependent upon Notch signalling. In this assay, the activity of a chloramphenicol acetyl transferase (CAT) reporter driven by the *mγ* promoter can be measured in *Drosophila* S2 tissue culture cells. S2 cells do not express endogenous Notch[19]. *mγ* transcription can be significantly enhanced by the presence of high levels of the truncated form of the Notch receptor (ICN; ref. 19), a form known to result in ligand-independent activation of the receptor[20]. Neither low levels of ICN nor human or *Drosophila* deltex alone stimulate transcription of the reporter (Fig. 3*a*). In contrast, co-expression of deltex with ICN resulted in substantial enhancement of transcription from the *mγ* promoter (Fig. 3*a*). We also note that similar experiments involving the coexpression of *Drosophila* deltex with *Drosophila* full-length Notch in S2 cells shows an approximate twofold induction of CAT activity compared with the expression of full-length Notch alone (data not shown). These findings demonstrate that deltex can synergistically modulate the ability of ICN to regulate the promoter activity of the *mγ* gene and are compatible with the genetic behaviour of deltex, which acts as a positive regulator of the pathway[8].

As the activated Notch receptor construct ICN is nuclear[2,21], we were interested in comparing the subcellular localizations of deltex and ICN. In *Drosophila* S2 tissue culture cells, ICN and deltex

Table 2 • *Drosophila* deltex and DTX1 interact with human Grb2

Co-expressed constructs	Media: Galactose	Media: Glucose
pLEXhDx-1/pJGhGrb2	403 (17)	8 (2)
pLEXhDDeltex/pJGhGrb2	786 (244)	92 (24)
pLEXHairless/pJGhGrb2	19 (7)	5 (1)
pLEXDNotch/pJGhGrb2	13 (1)	7 (1)
pLEXhDx-1/pJG	7 (1)	4 (2)
pLEXDDeltex/pJG	97 (20)	54 (8)
pLEXHairless/pJG	11 (3)	4 (1)
pLEXDNotch/pJG	15 (1)	8 (1)

Standard deviations are shown in parenthesis. pLEXDTX1 contains the entire open reading frame (ORF) of *DTX1*. pLEXDdeltex contains the entire ORF of Drosophila deltex. pJGDNotch contains the ankyrin repeats of *Drosophila* Notch (aa 1827-2259). pJGHairless contains the entire ORF of *Hairless*[24]. pJGhGrb2 contains an entire ORF of a human *Grb2*.

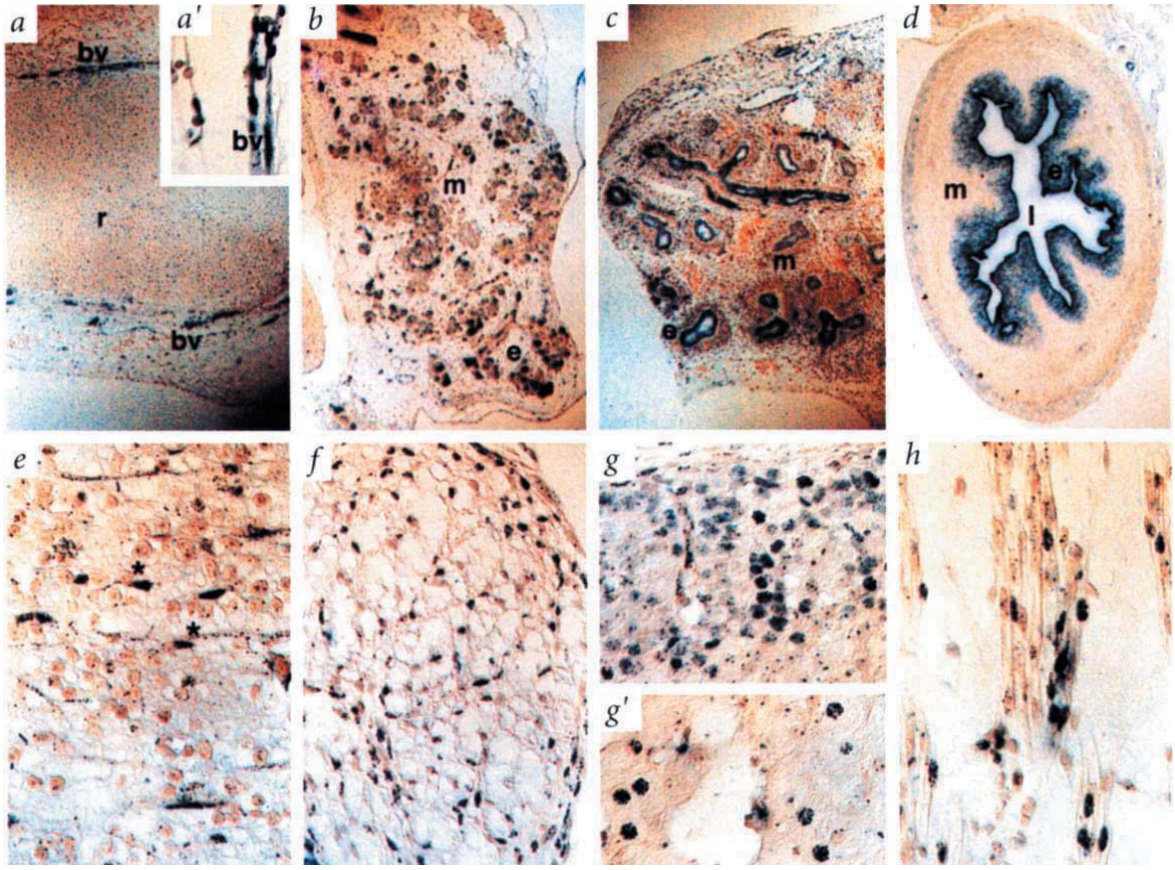

Fig. 2 *DTX1* expression in the human embryo. Photomicrographs of *in situ* hybridizations of tissue sections. ***a***, Sagittal section. Observe *DTX1* expression in blood vessels (bv) around the forming ribs (r). ***a'***, High magnification. Note expression of *DTX1* in endothelial cells. ***b–d***, Expression of the gene in epithelial cells (e) of the pancreas (*b*), lung (*c*) and digestive tube (*d*). ***e***, Scattered cells in the neural tube express *hdeltex-1*. Note expression in radial glia cells (asterisks). ***f***, Expression of *DTX1* in dorsal root ganglia. ***g***, ***g'***, Expression of *DTX1* during adrenal gland development; cortical area (*g*) and medula (*g'*). ***h***, note the expression in muscles. m, mesenchyme; l, lumen.

(Fig. 3*b,i,ii*) localize to the cell nucleus and cytoplasm, respectively. As previously shown, the ability of deltex to bind to the intracellular portion of Notch can be demonstrated[8] (Fig. 3*b,iii*). Co-expression of ICN and *Drosophila* deltex, however, does not detectably affect the individual subcellular localization of these proteins, even though it has been previously shown that deltex co-localizes with full-length Notch in the cytoplasm or membrane[5]. Thus, the lack of co-localization with ICN suggests that deltex does not augment ICN-dependent signalling *via* direct interaction between ICN and deltex in the nucleus. Given that previous studies have shown deltex to influence the interactions between Notch and Su(H) (ref. 8), it is reasonable to assume that the modulation of the *mγ*promoter by deltex may be exerted *via* the activity of endogenous Su(H). In spite of the documented interplay between Notch, Su(H) and deltex, we cannot exclude the possibility that deltex may manifest its activity through an unidentified effector protein. Notwithstanding the value of these *in vitro* assays we are mindful of the fact that they involve overexpression of the relevant proteins in cell cultures. The functional relevance of any results thus obtained must be corroborated by *in vivo* analysis.

The control of bHLH expression by Notch signalling affords the pathway the ability to affect indirectly the activity of a broad spectrum of genes. Notch-controlled HLH proteins can interact with other HLH factors in the cell, consequently influencing their ability to control transcription. Observations linking Notch activation with *bHLH* transcription have been also reported in vertebrates. For example, the promoter activity of *Hes1*, a gene homologous to the *Drosophila E(spl)* bHLH genes, was shown to be cooperatively activated by ICN and the Notch transcriptional effector RBP-Jk, the mammalian homologue of Su(H) (ref. 22). In turn, Hes1 is known to associate with the mammalian bHLH transcription factors E47, MyoD and MASH1, inhibiting their activity[23].

We extended our observations in *Drosophila* cells by establishing a connection between deltex and E47. The transcriptional activity of an E47-responsive CAT reporter plasmid in Baby Hamster Kidney (BHK) cells was enhanced by the expression of the E47 protein. The action of E47, however, was effectively suppressed when it was co-expressed with either an activated form of the human Notch-2 paralogue (intracellular Notch-2) or DTX1 (Fig. 3*c*). Notch-2, lacking the ankyrin repeats which contain the binding site of deltex[8], has no effect on E47 activity in this assay (Fig. 3*c*). Although our experiments identify E47 as a functional target of DTX1, there is no evidence to suggest that DTX1 directly interacts with E47.

Although we could not rescue a *Drosophila* deltex mutation with transgenes expressing *DTX1* (K.M. and S.A.-T., unpublished data), structural and functional analyses indicate that deltex and *DTX1* are functional homologues. The biochemical activity of deltex revealed by the present study is consistent with the notion that, at least in part, Notch exerts its action by controlling the activity of bHLH factors[25,26]. In *Drosophila*, as well as in mammals, bHLH proteins are capable of forming heterodimers, each of which has distinct specificities[25]. Therefore, a network of

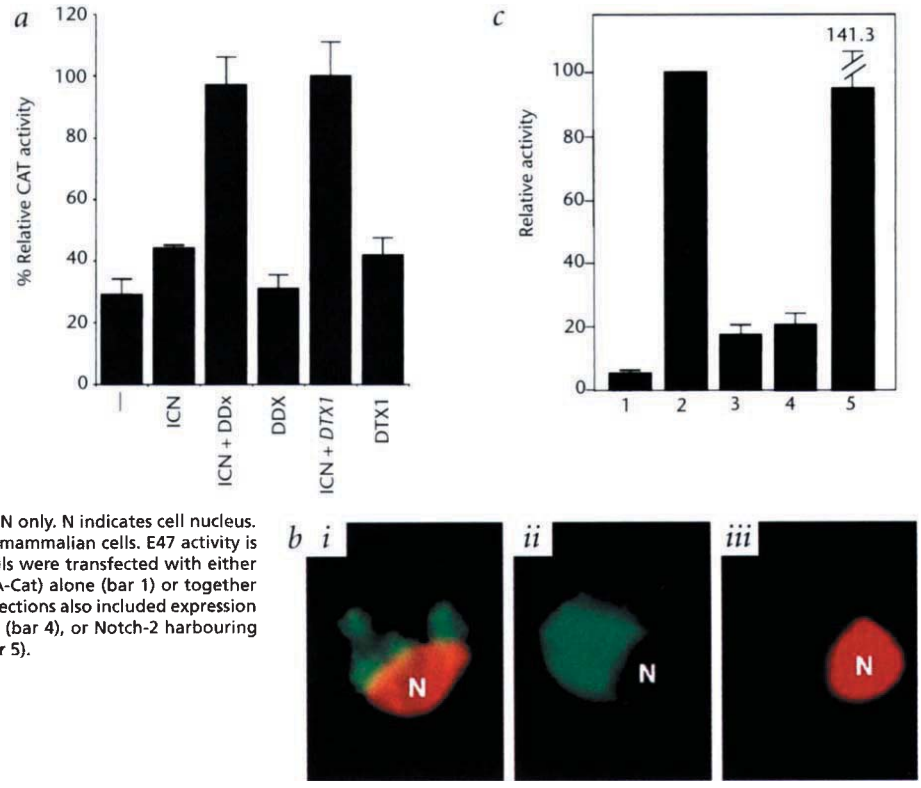

Fig. 3 Biochemical activities and subcellular localization of deltex. ***a***, DTX1 and *Drosophila* deltex augment activated form of Notch dependent transcriptional activation of the *E(spl)* gene, *mγ*, in *Drosophila* tissue culture cells. *Drosophila* S2 cells were transfected alone with a reporter vector (mγ1.2 CAT) containing 1.2 kb of the *mγ* upstream region in front of the CAT gene or co-transfected with mγ1.2 CAT and another plasmid containing a cDNA fragment encoding either the nuclear form of Notch (ICN), *Drosophila* deltex (DDx), or DTX1 (hDx-1), under the control of the metallothionine promoter[19]. ***b***, Co-expression of the intracellular domain of Notch does not affect cytoplasmic localization of deltex. *Drosophila* deltex and/or the intracellular domain of Notch (ICN) were expressed in *Drosophila* cultured cells under the control of the hsp70 promoter. ICN (red) and deltex (green) were detected by mouse anti-Notch monoclonal antibody and rat anti-deltex monoclonal antibody, respectively. (i), cells co-expressing ICN and deltex; (ii), cells expressing deltex only ; (iii), cells expressing ICN only. N indicates cell nucleus. ***c***, Modulation of Notch signalling by DTX1 in mammalian cells. E47 activity is inhibited by Notch-2 and by DTX1. NIH3T3 cells were transfected with either an E47 responsive CAT reporter ([E5+E2]4TATA-Cat) alone (bar 1) or together with an E47 expression vector (bars 2-5). Transfections also included expression vectors for intracellular Notch-2 (bar 3), DTX1 (bar 4), or Notch-2 harbouring an internal deletion of the ankyrin repeats (bar 5).

bHLH factors may have very broad biochemical action. For example, E47 as a homodimer is involved in B-cell differentiation, whereas it forms heterodimers with MyoD during muscle development[27–28]. A system that controls the activity of a variety of bHLH genes would have a pleiotropic action and could explain how Notch signalling results in the control of downstream targets that influence the developmental state of a broad spectrum of undifferentiated cells.

Methods

Isolation of hdx-1 cDNA. Using the similarity between the C-terminal region of *Drosophila* deltex and the translated cDNA fragment EST T05200 (ref. 11), we designed PCR primers to amplify fragments from a human fetal brain cDNA library (Invitrogen).

***In situ* hybridization.** Embryonic tissues were obtained from the archives of the Yale Department of Pathology. Age (8–12 gestational weeks) was estimated from fetal foot length. Embryos were fixed in 10% buffered formalin and embedded in paraffin. 4–6 mm thick sections were used for *in situ* hybridization. Two fragments of *DTX1* cDNA were used, corresponding to the 5′ (550 bp) and 3′ (250 bp) untranslated regions of human deltex-1. The 5′ fragment was derived from *Eco*RI/*Sma*I digestion (0–573) and the 3′ fragment was derived from *Pst*I/*Eco*RI digestion (2354–end). After linearization of the plasmid vector (*Pst*1 and *Eco*RI), single-stranded digoxigenin-labelled (Boehringer) antisense (pT3 and pT7 respectively) *DTX1* riboprobes were synthesized following the manufacturer's instructions. *In situ* hybridization on paraffin-embedded sections was performed as described[29].

Yeast-interaction trap assay. The yeast interaction trap assay was performed as described previously[30]. Constructs designed to produce fusion proteins with a DNA binding domain of LexA and with an acidic transcription-activation domain are referred to as pLEX and pJG, respectively[30]. Interaction was monitored by measurement of β-galactosidase activity in liquid cultures grown on galactose. Basal activity was measured when cells were grown on the equivalent glucose medium. Assays of β-galactosidase activity were performed on three independent transformants and are shown in arbitrary units as described previously[8].

***DTX1* expression constructs.** Three tandem copies of oligonucleotides encoding a Myc-epitope were ligated in-frame with an artificially generated *Bam*HI site located at the C-terminal end of *DTX1* cDNA[6]. The Myc-tagged *DTX1* cDNA was cloned into the *Xho*I/*Eco*RV sites of pCMV-NeoPoly2 vector.

Cell culture assays. The E47 repression assay and the *E(spl) mγ* reporter assay were carried out as previously described (refs 27 and 19, respectively). Procedures for transfection of *Drosophila* cells and immunostaining were described previously[8].

Acknowledgements

We thank R. Brent for his help with the two-hybrid system and the members of the Artavanis lab for comments on the manuscript. K.M. was supported by a long-term fellowship from the International Human Frontier Science Program Organization and a Howard Hughes Medical Institute postdoctoral fellowship. D.S.E. is supported by a Howard Hughes Medical Institute postdoctoral fellowship. S.A.T. is supported by the Howard Hughes Medical Institute, an NIH grant, and the Donaghue Medical Research Foundation.

Received 3 July, 1997; accepted 16 March, 1998.

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