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Truncated Mammalian Notch1 Activates CBF1/RBPJk-Repressed Genes by a Mechanism Resembling That of Epstein-Barr Virus EBNA2

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The Notch/Lin-12/Glp-1 receptor family participates in cell-cell signaling events that influence cell fate decisions. Although several Notch homologs and receptor ligands have been identified, the nuclear events involved in this pathway remain incompletely understood. A truncated form of Notch, consisting only of the intracellular domain (NotchIC), localizes to the nucleus and functions as an activated receptor. Using both an in vitro binding assay and a cotransfection assay based on the two-hybrid principle, we show that mammalian NotchIC interacts with the transcriptional repressor CBF1, which is the human homolog of *Drosophila* Suppressor of Hairless. Cotransfection assays using segments of mouse NotchIC and CBF1 demonstrated that the N-terminal 114-amino-acid region of mouse NotchIC contains the CBF1 interactive domain and that the cdc10/ankyrin repeats are not essential for this interaction. This result was confirmed in immunoprecipitation assays in which the N-terminal 114-amino-acid segment of NotchIC, but not the ankyrin repeat region, coprecipitated with CBF1. Mouse NotchIC itself is targeted to the transcriptional repression domain (aa179 to 361) of CBF1. Furthermore, transfection assays in which mouse NotchIC was targeted through Gal4-CBF1 or through endogenous cellular CBF1 indicated that NotchIC transactivates gene expression via CBF1 tethering to DNA. Transactivation by NotchIC occurs partially through abolition of CBF1-mediated repression. This same mechanism is used by Epstein-Barr virus EBNA2. Thus, mimicry of Notch signal transduction is involved in Epstein-Barr virus-driven immortalization.

The Notch/Lin-12/Glp-1 receptor family is highly conserved from worms to vertebrates. These large, transmembrane proteins are characterized by extracellular domains containing tandem epidermal growth factor (EGF)-like repeats followed by cysteine-rich Notch/Lin-12 repeats and intracellular domains containing cdc10/ankyrin repeats and a PEST sequence (3, 9, 14, 17, 55, 56, 60, 61). Notch/Lin-12/Glp-1 participate in intercellular signaling events that mediate cell fate specification. *Drosophila* Notch was categorized as a neurogenic gene on the basis of the observation that loss-of-function mutations cause hypertrophy of the neural tissue at the expense of epidermal structures (2). However, subsequent genetic analyses showed that Notch is required for the normal development of all three germ layers (12, 25).

Although many Notch receptor ligands (8, 13, 18, 26, 28, 37, 42, 45, 52, 57) and Notch homologs (3, 9, 14, 17, 55, 56, 60, 61) have been identified in different species, the intracellular events regulated by Notch signaling remain incompletely understood. The *Drosophila* proteins Deltex and Suppressor of Hairless [Su(H)] are the only two factors known so far which interact directly with the intracellular domain of Notch (19, 22, 59). Deltex is a cytoplasmic protein that becomes associated with the Notch intracellular cdc10/ankyrin repeats upon ligand binding to the extracellular EGF repeats (6, 15). Deltex functions genetically as a positive regulator but has yet to be characterized biochemically (6, 15, 22, 59). Su(H) and its homologs are ubiquitously expressed and highly conserved nuclear proteins (1, 41, 48). Su(H) is required for Notch signal reception during lateral inhibition in *Drosophila melanogaster*, and mutant alleles display a neurogenic phenotype in the peripheral nervous system (21, 47, 48). The evidence suggests that Su(H) plays a central role in Notch signaling (2). CBF1/RBPJk, the human homolog of Su(H) (1, 21, 23, 27, 39, 41, 48), is a transcriptional repressor (16, 30) which binds to the DNA sequence GTGGGAA (38, 53).

It has been postulated that in *D. melanogaster*, Notch functions by preventing nuclear translocation of Su(H) (19). Here we present evidence for an alternative mechanism whereby a truncated form of Notch, NotchIC, regulates gene expression controlled by CBF1, the human homolog of Su(H). NotchIC, which expresses only the intracellular domain, functions phenotypically as a ligand-activated receptor with an effect similar to that of gain-of-function alleles (2). This activated form of Notch is found in the nucleus, and its overexpression diverts cell fate determination (10, 20, 35, 36, 43, 44, 49, 51). Our data imply that NotchIC acts by targeting DNA-bound CBF1 within the nucleus and abolishing CBF1-mediated repression through masking of the repression domain. We have shown previously that the Epstein-Barr virus (EBV) immortalizing protein EBNA2 (11, 24, 58) also utilizes CBF1 tethering and masking of repression to upregulate expression of CBF1-repressed B-cell genes (23, 27, 30, 39, 54, 63). This study links EBV immortalization to the Notch signal transduction pathway.

MATERIALS AND METHODS

Plasmid constructions. A modified SG5 vector (Stratagene, La Jolla, Calif.) containing the hemagglutinin (HA) epitope at the 5′ translation start site was obtained from E. Cheng (Johns Hopkins Medical School), and sequences containing the EBNA2 transcriptional activation domain and nuclear localization

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signal (NLS) (40) (E2TANLS; EBNA2 amino acids [aa] 425 to 487) were introduced downstream of and in frame with the HA epitope. Different segments of mouse NotchIC (mNotchIC) were then generated by PCR and inserted either between the HA epitope and E2TANLS or after the Gal4 DNA binding domain (aa1 to 147) [Gal4(1-147)]. Four copies of the wild-type (GATCTGGTGTA AACACGCCGTGGGAAAAAATTTATG) or mutant (GATCTGGTGTAAA CACGGGCTTGGAAAAAATTTATG) CBF1 binding elements were cloned in front of a simian virus 40 promoter-driven luciferase reporter construct, GL2pro (Promega, Madison, Wis.) to generate 4xwtCBF1Luc and 4xmtCBF1Luc, respectively. All constructs were sequenced. All mNotchIC-containing plasmids were shown to be expressing comparable amounts of the appropriately sized proteins by immunoblot analysis with either an anti-HA (Berkeley Antibody, Berkeley, Calif.) or anti-Gal4 (Upstate Biotechnology) antibody. The Gal4-CBF1 plasmids and the 5xGal4TKCAT and TKLuc reporter plasmids have been described elsewhere (30). An oligonucleotide encoding the Flag epitope (Flag system; Eastman Kodak Co., New Haven, Conn.) was cloned into the *Bam*HI site of the expression vector SG5 to create pJH253. The CBF1 open reading frame was then introduced into the *Bg*lII site of pJH253 to generate Flag-CBF1.

CAT and luciferase assays. HeLa cells were maintained in Dulbecco modified Eagle medium plus 10% fetal calf serum. Unless otherwise noted, cotransfected HeLa cells received 6 μg of 5xGal4TKCAT, 1 μg of the indicated Gal4-CBF1, mNotchIC, or mNotchIC-E2TANLS expression plasmid, and 1 μg of TKLuc as an internal control for transfection efficiency. Transient transfection, chloramphenicol acetyltransferase (CAT), and luciferase assays were performed as described previously (38). All assays were repeated three times.

Coimmunoprecipitation and in vitro translation. mNotchIC (aa 1751 to 2294) and firefly luciferase were translated in vitro from a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-labeled methionine. Equal amounts of the in vitro translation products were incubated for 1 hr at 4°C in 1 × phosphate-buffered saline (PBS)–0.1% Nonidet P-40 with approximately equal amounts of either HA-tagged human CBF1 which was purified by DNA affinity chromatography (27) from recombinant vaccinia virus-infected HeLa cells, HA-tagged *Drosophila* TATA-binding protein (TBP) (29) which was overexpressed in *Escherichia coli* and purified by S-Sepharose chromatography, or HA-tagged human TAF250 (7) which was purified from a cleared lysate of SF9 cells infected with a recombinant baculovirus. Reaction mixtures were immunoprecipitated with the anti-HA monoclonal antibody 12CA5 (Babco). Precipitates were washed three times with 1 × PBS–0.1% Nonidet P-40 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide gel).

Cos1 cells were transfected with 8 μg each of Flag-CBF1 and the different HA-mNotchIC-E2TANLS constructions. At 48 h after transfection, the cells were lysed in 500 μl of isotonic lysis buffer (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.2], 1 mM EGTA [pH 8.0], 0.2% Nonidet P-40). One hundred microliters of the extract was kept for direct analysis of the proteins present; 400 μl of the extract was incubated with anti-Flag monoclonal antibody M2 (Eastman Kodak) overnight at 4°C, after which 100 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) was added. After incubation for 3 h at 4°C, these samples, along with the samples for direct analysis, were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The individual proteins were detected with the anti-HA monoclonal antibody and enhanced chemiluminescence (Amersham, Little Chalfont, England). The membrane was then stripped and reprobed with the anti-Flag monoclonal antibody.

RESULTS

Notch, Su(H), and the Notch signaling pathway are all highly conserved from *D. melanogaster* to humans. We first demonstrated that the Notch-Su(H) interaction was retained in mammals, using a coimmunoprecipitation assay. mNotchIC used in the following experiments consists of aa 1751 to 2294 (14). This region encompasses the sequences between the transmembrane domain and the PEST motif and includes the six tandem cdc10/ankyrin repeats and the two putative NLSs (35, 36, 50). In vitro-translated ^{35}S-labeled mNotchIC and control ^{35}S-luciferase were incubated with purified HA-tagged CBF1 and with two purified control proteins, HA-TBP and HA-TAF250. The addition of an anti-HA antibody resulted in coprecipitation of HA-CBF1 and mNotchIC. Coprecipitation was not observed with any of the control proteins (Fig. 1).

To determine which domains were required for protein-protein contacts, a cotransfection assay based on the two-hybrid principle was used. CBF1 and mNotchIC were each expressed as chimeric proteins with CBF1 fused to the Gal4 DNA binding domain and mNotchIC fused to a heterologous transactivation domain. Segments of CBF1, the human homolog of Su(H), were expressed from previously described constructions (30) as chimeric proteins containing aa 1 to 147 of yeast Gal4 (Fig. 2A). Segments of mNotchIC were expressed as chimeras in which the transactivation domain and NLS (E2TANLS) provided by aa 425 to 487 of the EBV EBNA2 protein were fused to the carboxy terminus of mNotchIC (Fig. 2A) (40). The E2TANLS domain lies outside the CBF1 interaction domain of EBNA2, which is located between aa 252 and 425. Cotransfection into HeLa cells of 5xGal4TKCAT, intact mNotchIC-E2TANLS, and intact Gal4-CBF1 constructions resulted in an approximately 165-fold activation of the CAT target, indicating in vivo interaction between mNotchIC and human CBF1 (Fig. 2B). Cotransfection of the Gal4-CBF1 constructions illustrated in Fig. 2A revealed that activation of the CAT reporter by mNotchIC-E2TANLS was dependent on the presence of CBF1 aa 179 to 361 (Fig. 2B). Interestingly, this represents the minimal transcriptional repression domain and EBNA2 interaction domain of CBF1 (30). In a complementary set of experiments, the 5xGal4TK CAT reporter was cotransfected with the construction expressing the intact Gal4-CBF1 fusion protein and the different mNotchIC-E2TANLS constructions. Surprisingly, the six tandem cdc10/ankyrin repeats were not essential for the in vivo interaction between mNotchIC and CBF1. The interaction mapped to a domain within the N-terminal 114 aa (aa 1751 to 1864) of the intracellular domain immediately preceding the cdc10/ankyrin repeats (Fig. 2C).

To confirm this mapping data, a coimmunoprecipitation assay was performed. The four HA-tagged mNotchIC constructions containing full-length mNotchIC (mNotchIC-E2TANLS), the N-terminal 114 aa [mNotchIC(1751-1864)-E2TANLS], the cdc10/ankyrin repeats [mNotchIC(1865-2075)-E2TANLS], and the C terminus of the protein including a subset of the cdc10/ankyrin repeats [mNotchIC(1976-2294)-E2TANLS] were each transfected into Cos1 cells with a plasmid encoding Flag epitope-tagged CBF1. Cell extracts were analyzed by SDS-PAGE and immunoblotting with an anti-HA monoclonal antibody to detect the HA-tagged mNotchIC polypeptides (Fig. 3A and B). The membrane was then stripped and reprobed with the anti-Flag monoclonal antibody to detect Flag-CBF1 (Fig. 3C and D). These analyses showed that each of the NotchIC-E2TANLS proteins was expressed (Fig. 3A), that Flag-CBF1 was also present in each transfected extract (Fig. 3C), and that the Flag antibody was specific for the Flag epitope and did not react with cells transfected only with

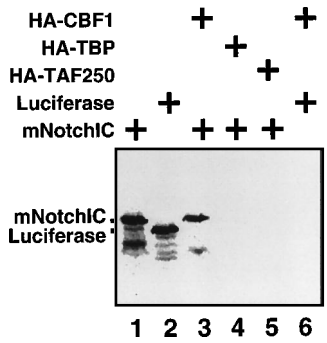

FIG. 1. Coimmunoprecipitation of purified HA-CBF1 with in vitro-translated mNotchIC. In vitro-translated ^{35}S-labeled mNotchIC (lanes 1 and 3 to 5) or ^{35}S-luciferase (control, lanes 2 and 6) was incubated with either HA-CBF1 (lanes 3 and 6), HA-TBP (control, lane 4), or HA-TAF250 (control, lane 5). Reactions were immunoprecipitated with an anti-HA monoclonal antibody and analyzed by SDS-PAGE. The positions of the radiolabeled in vitro-translated mNotchIC and luciferase are indicated.

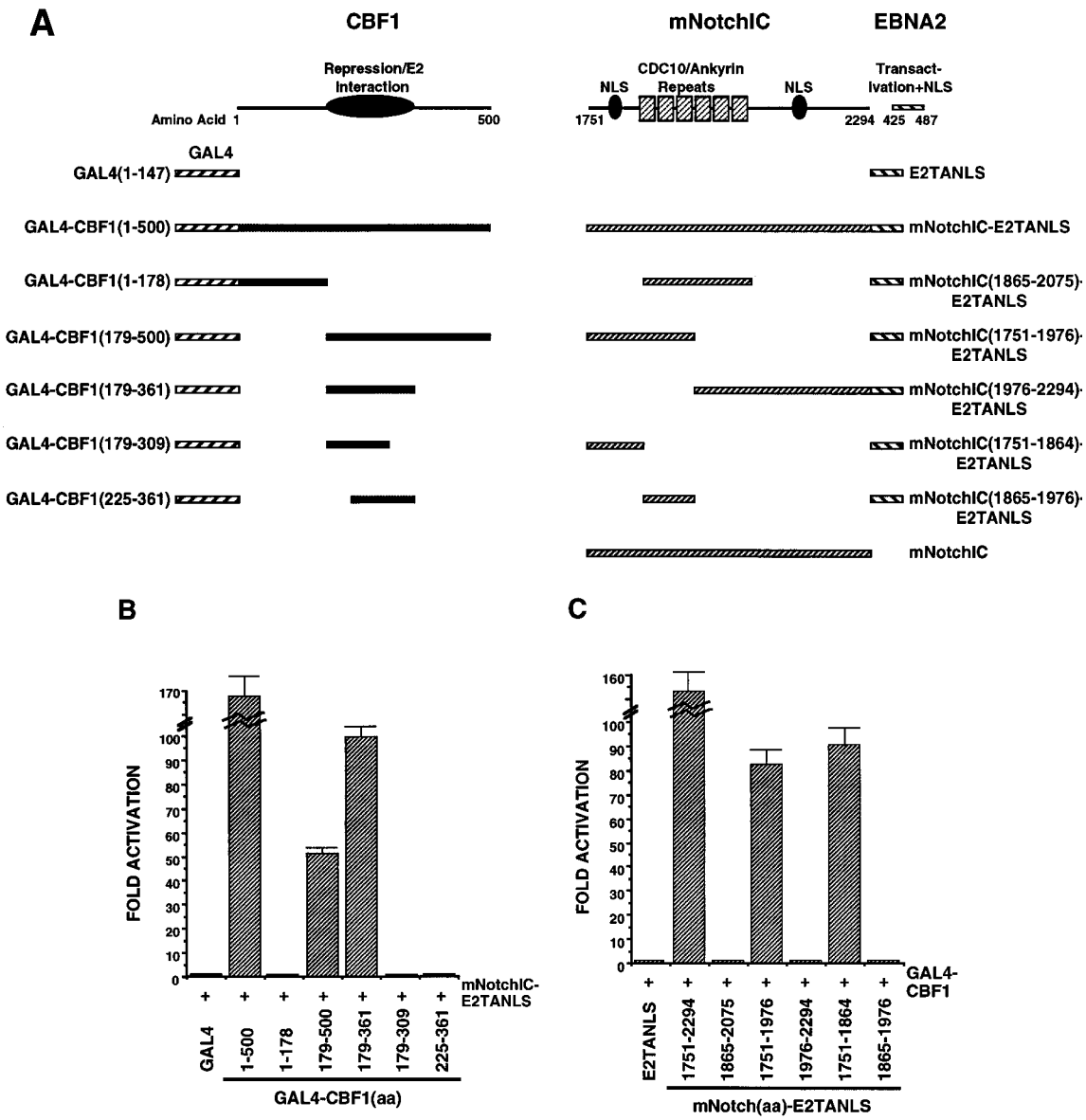

FIG. 2. Mapping of the CBF1 and mNotchIC interaction domains. (A) Diagrammatic representation of the constructions expressing Gal4-CBF1 fusion proteins (left) and mNotchIC-E2TANLS proteins (right) used in the mapping experiments. (B and C) Transient expression assays in HeLa cells which received 6 μg of 5xGal4TKCAT, 1 μg of the indicated Gal4-CBF1 or mNotchIC-E2TANLS expression plasmid, and 1 μg of TKLuc as an internal control for transfection efficiency. (B) mNotchIC-E2TANLS transactivated the CAT target only in the presence of CBF1 constructions containing aa 179 to 361, indicating that the CBF1 transcriptional repression-EBNA2 interaction domain also serves as the mNotchIC interaction domain. Fold activation was calculated relative to the expression seen in the absence of mNotchIC-E2TANLS for individual Gal4-CBF1 expression constructions. (C) Only those mNotchIC-E2TANLS constructions containing the N-terminal 114 aa (aa 1751 to 1864) could efficiently transactivate 5xGal4TKCAT in the presence of intact Gal4-CBF1. Thus, the minimal interaction domain of mNotchIC is located N terminal to the ankyrin repeats. Fold activation was calculated relative to that obtained with cotransfected intact Gal4-CBF1 plus vector (SG5). The average and standard deviation from three experiments are presented.

mNotchIC-E2TANLS (Fig. 3C, lane 5). The same cell extracts were also subjected to immunoprecipitation with the anti-Flag monoclonal antibody prior to SDS-PAGE separation and immunoblotting. The intact mNotchIC and the mNotchIC polypeptide containing the N-terminal 114 aa coprecipitated with CBF1, but no coprecipitation of the polypeptides containing the cdc10/ankyrin repeats or the C-terminus of mNotchIC was observed (Fig. 3B). Flag-CBF1 itself could be immunoprecipitated from each of the transfected extracts (Fig. 3D, lanes 1 to 4). Comparison of the amino acid sequence of this 114-aa mNotchIC domain with the equivalent regions of human Notch1 (TAN1) (17), zebra fish Notch (4), and *Xenopus* Notch (9) reveals several blocks of sequence identify and a high degree of similarity. The segment adjacent to the cdc10/ankyrin repeats is particularly well conserved in the vertebrate homologs (Fig. 4).

The targeting of mNotchIC to the minimal repression domain of CBF1 raised the possibility that truncated Notch modulates CBF1-mediated transcriptional repression. Transfection of mNotchIC with intact Gal4-CBF1 and the 5xGal4TKCAT reporter construction resulted in a 25-fold activation of CAT expression (Fig. 5), while expression from a control cotrans-

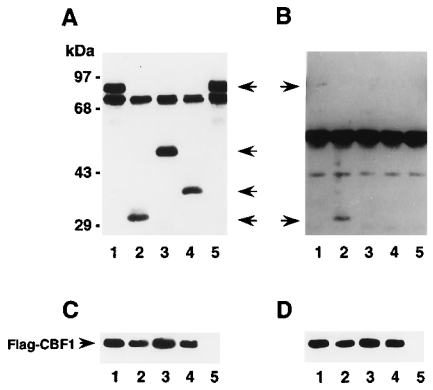

FIG. 3. Coimmunoprecipitation confirms that the N-terminal 114-aa region of mNotchIC is sufficient for interaction with CBF1. Extracts from Cos1 cells transfected with Flag-CBF1 and constructions expressing the indicated HA-tagged mNotchIC-E2TANLS polypeptides were subjected to SDS-PAGE, and the proteins present were detected by immunoblot analysis with either an anti-HA (A and B) or anti-Flag (C and D) monoclonal antibody. Cells were transfected as follows: lane 1, Flag-CBF1 and intact mNotchIC-E2TANLS; lane 2, Flag-CBF1 and mNotchIC(1751-1865)-E2TANLS; lane 3, Flag-CBF1 and mNotchIC(1976-2294)-E2TANLS; lane 4, Flag-CBF1 and mNotchIC(1865-2075)-E2TANLS; lane 5, intact mNotchIC-E2TANLS. (A) Direct analysis of extracts by using the anti-HA monoclonal antibody shows that each of the transfected HA-NotchIC-E2TANLS polypeptides is expressed. (B) Analysis of extracts immunoprecipitated with the anti-Flag antibody prior to SDS-PAGE and immunoblotted with the anti-HA antibody reveals coprecipitation of HA-tagged intact mNotchIC-E2TANLS (lane 1) and of the polypeptide representing the N-terminal 114 aa of mNotchIC [mNotchIC(1751-1864)-E2TANLS] (lane 2) but no coprecipitation of the mNotchIC polypeptides containing the C-terminal segment of the protein that includes a subset of the cdc10/ankyrin repeats [mNotchIC(1976-2294)-E2TANLS] (lane 3) or the cdc10/ankyrin repeat domain itself [mNotchIC(1865-2075)-E2TANLS] (lane 4). There was also no coprecipitation of intact mNotchIC in the absence of transfected Flag-CBF1 (lane 5). (C) Direct analysis with the anti-Flag antibody, demonstrating that each of the Flag-CBF1-transfected cell extracts (lanes 1 to 4) expresses Flag-CBF1. (D) Analysis of extracts immunoprecipitated with the anti-Flag antibody prior to SDS-PAGE and immunoblotting with the anti-Flag antibody, demonstrating that Flag-CBF1 could be immunoprecipitated from each of the Flag-CBF1-transfected cell extracts (lanes 1 to 4).

fected luciferase reporter gene lacking the Gal4 binding sites (TKLuc) was not affected (data not shown). The approximately 25-fold activation observed with mNotchIC was less than the transactivation induced by cotransfection of an mNotchIC-E2TANLS or EBV EBNA2 expression plasmid (Fig. 5). In an attempt to determine whether mNotchIC contained an identifiable transcriptional activation domain, the intact mNotchIC and different segments of mNotchIC (Fig. 2A) were expressed as fusions with the yeast Gal4 DNA binding domain and tested in cotransfection experiments for the ability to transactivate a 5xGal4TKCAT reporter gene. The control Gal4(1-147) plasmid itself induced a threefold activation of CAT expression. This activation was not observed when the NotchIC ankyrin repeat region (aa 1865 to 2075) was fused to Gal4(1-147). Of the Gal4-mNotchIC constructions, only the intact Gal4-mNotch IC (aa 1751 to 2294) and the construction containing the C-terminal half of mNotchIC (aa 2042-2294) showed any positive effect, producing activation 1.6- to 3.5-fold above that seen with the Gal4(1-147) control (Fig. 6).

The absence of a strong activation domain in NotchIC reinforced the idea that masking of CBF1-mediated repression contributed mechanistically to activation by NotchIC of targets

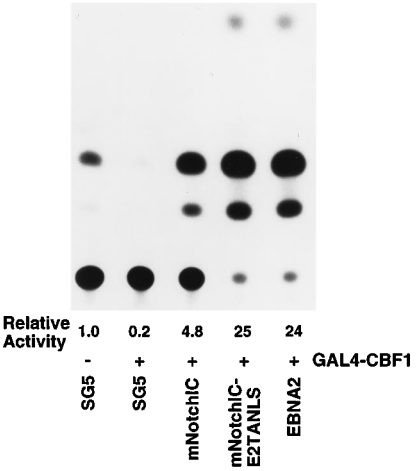

FIG. 5. mNotchIC transactivates reporter gene expression, as demonstrated by a transient expression assay showing transactivation of a 5xGal4TKCAT reporter by mNotchIC in the presence of cotransfected intact Gal4–CBF1. The activation obtained was fivefold less than that seen with mNotchIC carrying the fused EBNA2 transactivation domain (mNotchIC-E2TANLS) or with the co-transfected EBNA2 protein. The cell extract was diluted to quantitate the acetylation obtained in the presence of these latter two effectors.

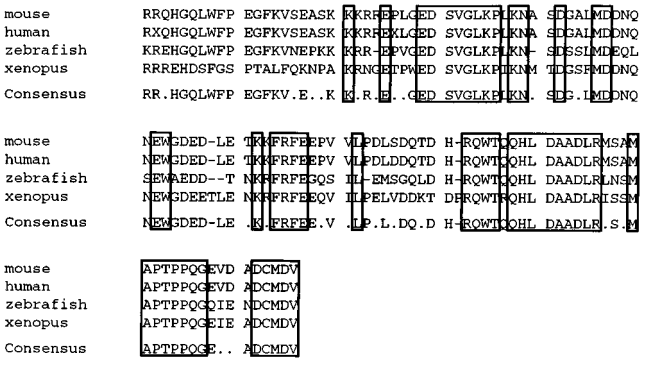

FIG. 4. Alignment of the 114-aa mNotchIC domain (aa 1751 to 1864) found to interact with CBF1 with the equivalent regions of the vertebrate homologs, human TAN1 (aa 1760 to 1873) (17), zebra fish Notch (aa 1752 to 1861) (4), and *Xenopus* Notch (aa 1755 to 1870) (9). Sequence alignment was carried out by using the GeneWorks program (IntelliGenetics Inc). Boxed sequences are identical in all four species.

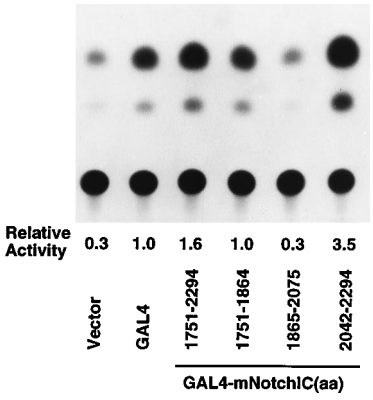

FIG. 6. mNotchIC lacks a strong transcriptional activation domain, as demonstrated by a transient expression assay examining the ability of cotransfected plasmids (1 μg) expressing Gal4(1-147)-mNotchIC fusion proteins to transactivate expression from a 5xGal4TKCAT target (3 μg). Weak transactivation was observed with chimeras containing the intact mNotchIC (aa 1751 to 2294) or the carboxy-terminal half of the protein (aa 2042 to 2294). Cotransfected TKLuc (0.5 μg) provided an intemal control for transfection efficiency.

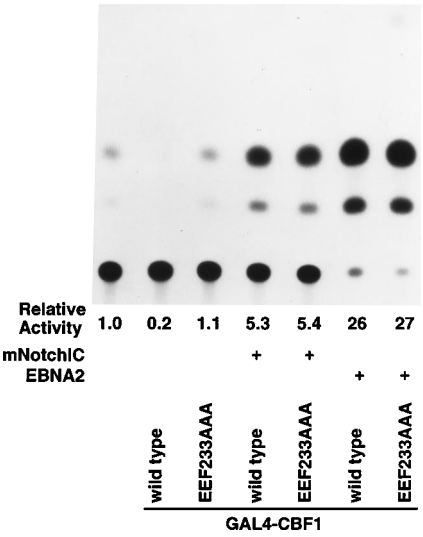

FIG. 7. A loss-of-repression mutant of CBFI provides evidence that NotchIC masks CBFI-mediated repression. A transient expression assay was performed; in this assay, HeLa cells received 6 μg of 5xGAL4TKCAT, 1 μg of wild-type or mutant GAL4–CBF1, a mNotchIC or EBNA2 expression plasmid, and 1 μg of TKLuc as an internal control for transfection efficiency. The activation profiles of mNotchIC and EBNA2 are identical. In each case, the fold activation was greater in the presence of the wild-type CBF1 than in the presence of the loss-of-repression mutant of CBF1, indicating that mNotchIC, like EBNA2, masks CBF1-mediated repression. CAT activity was calculated relative to that obtained with transfected CAT reporter alone. The cell extract was diluted to quantitate the acetylation obtained in the presence of the EBNA2 expression plasmid.

containing CBF1 binding sites. To provide experimental evidence for this mechanism, transactivation by NotchIC was compared with that of EBNA2 in the presence of cotransfected Gal4-CBF1 or Gal4-CBF1(EEF233AAA), a mutant form of CBF1 that has lost repression activity (30). The activation seen in the presence of the repression-minus CBF1 mutant is a measure of intrinsic activation ability, which in the case of NotchIC was fivefold in this assay (Fig. 7). The fold activation seen in the presence of the wild-type CBF1 was 25-fold, indicating that there is a second component to the activation in the normal setting. The activation profile of NotchIC was identical to that observed with EBNA2, which also produced fivefold-greater activation in the presence of the wild-type CBF1 compared with the CBF1 mutant. EBNA2 is known to mask the CBF1 repression domain (30). The data are consistent with a mechanism of NotchIC activation which combines masking of CBF1 repression with a weak activation function.

So far, the transactivation by mNotchIC has been shown in a setting in which the Notch protein was expressed as a chimera and targeted to Gal4 binding sites on the reporter plasmid. To ensure that the mechanism of transactivation by NotchIC was similar with endogenous cellular CBF1, mNotchIC was cotransfected with luciferase reporter constructions containing four upstream copies of either the wild-type or mutant CBF1 binding elements. Basal expression from the luciferase reporter containing the mutant binding sites was approximately threefold higher than that from the reporter containing the wild-type CBF1 binding sites (Fig. 8). In a dose-response assay, the mNotchIC expression plasmid transactivated the reporter containing the wild-type CBF1 binding sites (4xwtCBF1Luc) but not the reporter bearing the mutant sites (4xmtCBF1Luc) (Fig. 8). Again, the level of activation by the mNotchIC-E2TANLS fusion protein was greater than that observed with mNotchIC alone. As will be discussed, the data

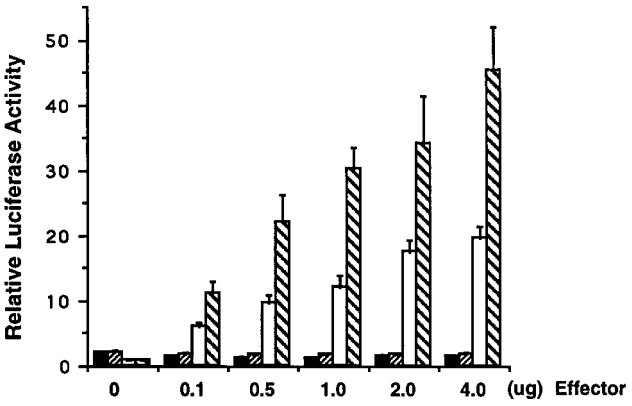

FIG. 8. CBF1 binding sites confer responsiveness to mNotchIC. Transient expression assays with HeLa cells were performed to compare the abilities of mNotchIC and mNotchIC-E2TANLS to transactivate expression in a dose-response assay from a luciferase reporter plasmid (5 μg) carrying either wild-type CBF1 binding sites (4xwtCBF1Luc) or mutated CBF1 sites (4xmtCBF1Luc). Wild-type CBF1 binding sites were necessary and sufficient to confer responsiveness to mNotchIC. Transactivation of expression from the wild-type CBF1 binding site containing target by mNotchIC-E2TANLS was again stronger than that observed with mNotchIC at all doses of effector plasmid. Luciferase activity was calculated relative to the expression obtained with cotransfected 4xwtCBF1Luc plus vector (SG5). The average and standard deviation from three experiments are provided. ■, 4xmtCBF1Luc plus mNotchIC; ▨, 4xmtCBF1Luc plus mNotchIC-#2TANLS; □, 4xwtCBF1Luc plus mNotchIC; ▧, 4xwtCBF1Luc plus mNotchIC-E2TANLS.

support a mechanistic model in which mNotchIC counters CBF1 repression by physical masking of the repression domain and potentially contributes an additional three- to fivefold effect through a weak, cryptic activation domain. A model summarizing the nature of the mNotchIC-CBF1 interaction as deduced from this study is presented in Fig. 9.

DISCUSSION

The Notch/Lin-12/Glp-1 transmembrane receptor proteins contain three highly conserved tandem repeats, EGF, Notch/

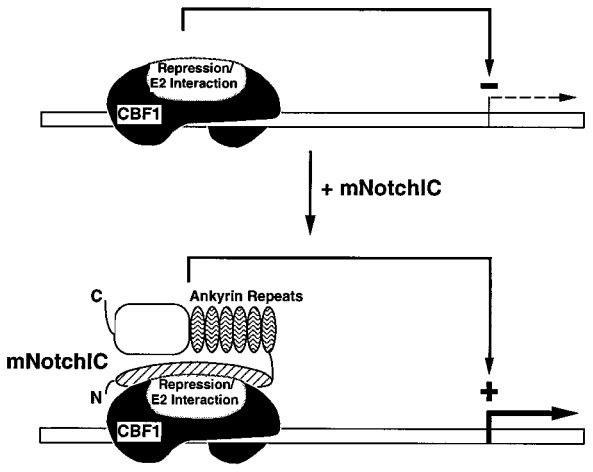

FIG. 9. Model illustrating the proposed mechanism of action of activated Notch. DNA-bound CBF1 is capable of repressing gene expression either directly or through a corepressor (30). The amino- terminal region of mNotchIC binds to CBF1, masking the repression domain. This physical masking, in combination with the introduction of a weak activation activity, leads to upregulation of expression from the downstream promoter. The action of mNotchIC is mimicked by the EBV EBNA2 protein, which also masks the CBFI repression domain but further amplifies the effects on gene expression by in by incorporating a strong transcriptional activation domain.

Lin-12, and cdc10/ankyrin, each of which is essential for the wild-type phenotype (15, 33–35). It was somewhat surprising to find that the minimal domain required for NotchIC interaction with CBF1 is located within the 114-aa region amino terminal to the six cdc10/ankyrin repeats. The CBF1 interacting region can be further narrowed down to the C-terminal 60 aa of the 114-aa domain by taking into account data on Su(H) and Notch interactions obtained by using an overlapping construction in a yeast interaction trap assay (19). Recently, Jarriault et al. (31) also provided evidence for NotchIC transactivation through CBF1/RBF2/RBPJk binding sites and showed that mutations in the fourth ankyrin repeat of Notch affected CBF1 binding. This finding correlates with the fact that point mutations introduced into the fourth ankyrin repeat of truncated Notch abolish the ability of NotchIC to suppress myogenesis (35). Interaction between NotchIC and *Drosophila* Su(H) is also abolished by the binding of Deltex to the ankyrin repeats of Notch. Thus, it had been anticipated that CBF1 binding would directly involve the ankyrin repeats. In view of the close proximity of the ankyrin repeats and the CBF1 interaction domain mapped in our experiments, competition for binding between Deltex and Su(H) may explain the Deltex effect. However, the combined data may also indicate that modification of the ankryin repeats, either through binding by Deltex or through mutagenesis, may exert a transmitted negative effect on the binding of CBF1. Both the CBF1 interaction domain and the six cdc10/ankyrin repeats were deleted concurrently in each of the published loss-of-function intracellular truncation mutants introduced into *Drosophila* Notch (20, 36, 44). It would therefore be of value to examine the effects on Notch signaling of mutations that specifically target only the CBF1 interaction. Three Notch homologs have been identified in mammals (2), but CBF1/RBPJk is so far the only identified mammalian homolog of Su(H) (1, 5, 21, 23, 27, 41, 48). It will be interesting to determine whether the other Notch family members also signal through CBF1. Moreover, CBF1 is central to the differentiation process, and identification of downstream genes under the regulation of CBF1 will further our understanding of Notch signaling.

Although truncated Notch can partially restore a wild-type phenotype to *D. melanogaster* carrying null mutations in the Notch gene, it remains unclear if NotchIC is generated directly as a consequence of physiological ligand binding to the Notch receptor. Evidence supporting such a mechanism includes the rapid processing of the 300-kDa intact Notch to a 100-kDa form and the detection of this 100-kDa species as the dominant Notch polypeptide in both human and insect cells (62). It has now been demonstrated, both here and recently by Jarriault et al. (31), that the interaction between mNotchIC and CBF1 leads to activation mediated through DNA-bound CBF1. CBF1 itself functions as a transcriptional repressor, producing a five- to eightfold repressive effect in cotransfection assays using Gal4-CBF1 and reporters containing Gal4 binding sites (30). Reporters such as 4xwtCBF1Luc exhibit approximately threefold-lower basal activity than versions containing four mutated CBF1 sites. Our results show that mNotchIC binds to the repression domain of CBF1. If masking of repression were the sole factor contributing to mNotchIC activation, then the expected activation effect would be five- to eightfold in the assays using reporters containing Gal4 binding sites and approximately threefold in the assays using reporters containing CBF1 binding sites. In each case, higher levels of activation were observed (25- to 30-fold and 18-fold, respectively). This observation is compatible with the existence of an additional weak activation domain within mNotchIC. Experiments to detect such a domain by using the traditional approach of creating Gal4 fusion proteins containing segments of mNotchIC were consistent with this interpretation in that constructions expressing the intact mNotchIC or the carboxy-terminal half of this polypeptide gave activation two- to fourfold above that observed with Gal4(1-147) but were not definitive because of the relatively small effects observed. On the other hand, these experiments eliminate the alternative explanation that the increased expression observed upon cotransfection of mNotchIC was due solely to the presence of a strong activation domain carried by mNotchIC. More definitive evidence for the presence of a weak activation domain within NotchIC comes from the fivefold activation produced by NotchIC in the presence of a repression-minus CBF1 mutant. The greater fold activation observed in the presence of wild-type CBF1 than in the presence of the mutant CBF1 also provides direct evidence that masking of CBF1 repression forms part of the mechanism of action of NotchIC.

Notch is believed to function in cell fate determination by locking cells into an immature state in which they can await the proper environmental cues for further differentiation. Deregulating this pathway by overexpression of the constitutively activated form of Notch not only diverts cell fate decisions but also is tumorigenic. For example, truncation of the Notch homologs TAN1 and int3 results in human acute T-cell lymphoblastic leukemia and mouse mammary tumors, respectively (17, 32, 46). Overexpression of Notch has also been detected in human cervical carcinoma (62). mNotchIC can translocate to the nucleus and transactivate CBF1-downregulated genes through a mechanism remarkably reminiscent of that used by the EBV EBNA2 protein. EBNA2 is a transcriptional activator that is essential for EBV-driven B-cell immortalization. EBNA2, which has a strong carboxy-terminal, negatively charged activation domain, is targeted to CBF1 binding sites in DNA by binding to the same minimal repression domain of CBF1 as NotchIC. These two proteins not only use the same strategy, mechanical masking of the repression domain of CBF1, but also have similar functions phenotypically in that EBNA2-initiated immortalization leads to a B cell that is locked into less differentiated, proliferative state. It has been postulated that EBNA2 may act by imitating the function of a cellular factor and, as would be typical of a viral regulatory protein, magnifying its effects. No cellular EBNA2 homolog has been identified. However, from a mechanistic and functional point of view, NotchIC can be considered a cellular equivalent of EBNA2.

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REFERENCES

1. **Amakawa, R., W. Jing, K. Ozawa, N. Matsunami, Y. Amaguchi, F. Matsuda, M. Kawaichi, and T. Honjo.** 1993. Human Jk recombination signal binding protein gene (IGKJRB): comparison with its mouse homologue. Genomics **17:**306–315.
2. **Artavanis-Tsakonas, S., K. Matsuno, and M. E. Fortini.** 1995. Notch signaling. Science **268:**225–232.
3. **Austin, J., and J. Kimble.** 1989. Transcript analysis of *glp-1* and *lin-12*, homologous genes required for cell interactions during development of C. elegans. Cell **58:**565–571.
4. **Bierkamp, C., and J. A. Campos-Ortega.** 1993. A zebrafish homologue of the Drosophila neurogenic gene Notch and its pattern of transcription during early embryogenesis. Mech. Dev. **43:**87–100.

5. **Brou, C., F. Logeat, M. Lecourtois, J. Vandekerckhove, P. Kourilsky, F. Schweisguth, and A. Israel.** 1994. Inhibition of the DNA-binding activity of *Drosophila* Suppressor of Hairless and of its human homolog, KBF2/RBP-J_k, by direct protein-protein interaction with *Drosophila* Hairless. Genes Dev. **8:**2491–2503.
6. **Busseau, I., R. J. Diederich, T. Xu, and S. Artavanis-Tsakonas.** 1994. A member of the Notch group of interacting loci, deltex encodes a cytoplasmic basic protein. Genetics **136:**585–596.
7. **Chen, J. L., L. D. Attardi, C. P. Verrijzer, K. Yokomori, and R. Tjian.** 1994. Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. Cell **79:**93–105.
8. **Chitnis, A., D. Henrique, J. Lewis, D. Ish-Horowicz, and C. Kintner.** 1995. Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature (London) **375:**761–766.
9. **Coffman, C., W. Harris, and C. Kintner.** 1990. *Xotch*, the *Xenopus* homolog of *Drosophila Notch*. Science **249:**1438–1441.
10. **Coffman, C. R., P. Skoglund, W. A. Harris, and C. R. Kintner.** 1993. Expression of an extracellular deletion of *Xotch* diverts cell fate in Xenopus embryos. Cell **73:**659–671.
11. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86:**9558–9562.
12. **Corbin, V., A. M. Michelson, S. M. Abmayr, V. Neel, E. Alcamo, T. Maniatis, and M. W. Young.** 1991. A role for the Drosophila neurogenic genes in mesoderm differentiation. Cell **67:**311–323.
13. **Couso, J. P., and A. M. Arias.** 1994. *Notch* is required for wingless signaling in the epidermis of drosophila. Cell **79:**259–272.
14. **del Amo, F. F., M. Gendron-Maguire, P. J. Swiatek, N. A. Jenkins, N. G. Copeland, and T. Gridley.** 1993. Cloning, analysis, and chromosomal localization of *Notch-1*, a mouse homolog of *Drosophila Notch*. Genomics **15:** 259–264.
15. **Diederich, R. J., K. Matsuno, H. Hing, and S. Artavanis-Tsakonas.** 1994. Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development **120:**473–481.
16. **Dou, S., X. Zeng, P. Cortes, H. Erdjument-Bromage, P. Tempst, T. Honjo, and L. D. Vales.** 1994. The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. Mol. Cell. Biol. **14:**3310–3319.
17. **Ellisen, L. W., J. Bird, D. C. West, A. L. Soreng, T. C. Reynolds, S. D. Smith, and J. Sklar.** 1991. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell **66:**649–661.
18. **Fehon, R. G., P. J. Kooh, I. Rebay, C. L. Regan, T. Xu, M. A. T. Muskavitch, and S. Artavanis-Tsakonas.** 1990. Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in Drosophila. Cell **61:**523–534.
19. **Fortini, M. E., and S. Artavanis-Tsakonas.** 1994. The suppressor of hairless protein participates in Notch receptor signaling. Cell **79:**273–282.
20. **Fortini, M. E., I. Rebay, L. A. Caron, and S. Artavanis-Tsakonas.** 1993. An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. Nature (London) **365:**555–557.
21. **Furukawa, T., S. Maruyama, M. Kawaichi, and T. Honjo.** 1992. The drosophila homolog of the immunoglobulin recombination signal-binding protein regulates peripheral nervous system development. Cell **69:**1191–1197.
22. **Gorman, M. J., and J. R. Girton.** 1992. A genetic analysis of *deltex* and its interaction with the *Notch* locus in *Drosophila melanogaster*. Genetics **131:** 99–112.
23. **Grossman, S. R., E. Johannsen, X. Tong, R. Yalamanchili, and E. Kieff.** 1994. The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J*k* recombination signal binding protein. Proc. Natl. Acad. Sci. USA **91:**7568–7572.
24. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340:**393–397.
25. **Hartenstein, A., A. Rugendorff, U. Tepass, and V. Hartenstein.** 1992. The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. Development **116:**1203–1220.
26. **Henderson, S. T., D. Gao, E. J. Lambie, and J. Kimble.** 1994. *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. Development **120:**2913–2924.
27. **Henkel, T., P. D. Ling, S. D. Hayward, and M. G. Peterson.** 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J_k. Science **265:**92–95.
28. **Henrique, D., J. Adam, A. Myat, A. Chitnis, J. Lewis, and D. Ish-Horowicz.** 1995. Expression of a Delta homologue in prospective neurons in the chick. Nature (London) **375:**787–790.
29. **Hoey, T., B. D. Dynlacht, M. G. Peterson, B. F. Pugh, and R. Tjian.** 1990. Isolation and characterization of the Drosophila gene encoding the TATA box binding protein, TFIID. Cell **61:**1179–1186.
30. **Hsieh, J. J.-D., and S. D. Hayward.** 1995. Masking of the CBF1/RBPJk transcriptional repression domain by Epstein-Barr virus EBNA2. Science **268:**560–563.
31. **Jarriault, S., C. Brou, F. Logeat, E. H. Schroeter, R. Kopan, and A. Israel.** 1995. Signalling downstream of activated mammalian Notch. Nature (London) **377:**355–358.
32. **Jhappan, C., D. Gallahan, C. Stahle, E. Chu, G. H. Smith, G. Merlino, and R. Callahan.** 1992. Expression of an activated *Notch*-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev. **6:**345–355.
33. **Kelley, M. R., S. Kidd, W. A. Deutsch, and M. W. Young.** 1987. Mutations altering the structure of epidermal growth factor-like coding sequences at the Drosophila *Notch* locus. Cell **51:**539–548.
34. **Kodoyianni, V., E. M. Maine, and J. Kimble.** 1992. Molecular basis of loss-of-function mutations in the *glp-1* gene of *Caenorhabditis elegans*. Mol. Biol. Cell **3:**1199–1213.
35. **Kopan, R., J. S. Nye, and H. Weintraub.** 1994. The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development **120:**2385–2396.
36. **Lieber, T., S. Kidd, E. Alcamo, V. Corbin, and M. W. Young.** 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. Genes Dev. **7:**1949–1965.
37. **Lindsell, C. E., C. J. Shawber, J. Boulter, and G. Weinmaster.** 1995. Jagged: a mammalian ligand that activates Notch 1. Cell **80:**909–917.
38. **Ling, P. D., J. J.-D. Hsieh, I. K. Ruf, D. R. Rawlins, and S. D. Hayward.** 1994. EBNA-2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1. J. Virol. **68:**5375–5383.
39. **Ling, P. D., D. R. Rawlins, and S. D. Hayward.** 1993. The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by cellular enhancer-binding protein. Proc. Natl. Acad. Sci. USA **90:**9237–9241.
40. **Ling, P. D., J. J. Ryon, and S. D. Hayward.** 1993. EBNA-2 of herpesvirus papio diverges significantly from the type A and type B EBNA-2 proteins of Epstein-Barr virus but retains an efficient transactivation domain with a conserved hydrophobic motif. J. Virol. **67:**2990–3003.
41. **Matsunami, N., Y. Hamaguchi, Y. Yamamoto, K. Kuze, K. Kangawa, H. Matsuo, M. Kawaichi, and T. Honjo.** 1989. A protein binding to the J kappa recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif. Nature (London) **342:**934–937.
42. **Mello, C. C., B. W. Draper, and J. R. Priess.** 1994. The maternal genes *apx-1* and *glp-1* and establishment of dorsal-ventral polarity in the early C. elegans embryo. Cell **77:**95–106.
43. **Nye, J. S., R. Kopan, and R. Axel.** 1994. An activated *Notch* suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. Development **120:**2421–2430.
44. **Rebay, I., R. G. Fehon, and S. Artavanis-Tsakonas.** 1993. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell **74:**319–329.
45. **Rebay, I., R. J. Fleming, R. G. Fehon, P. Cherbas, and S. Artavanis-Tsakonas.** 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell **67:** 687–699.
46. **Robbins, J., B. J. Blondel, D. Gallahan, and R. Callahan.** 1992. Mouse mammary tumor gene *int-3*: a member of the *notch* gene family transforms mammary epithelial cells. J. Virol. **66:**2594–2599.
47. **Schweisguth, F.** 1995. *Suppressor of Hairless* is required for signal reception during lateral inhibition in the *Drosophila* pupal notum. Development **121:** 1875–1884.
48. **Schweisguth, F., and J. W. Posakony.** 1992. Suppressor of hairless, the drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. Cell **69:**1199–1212.
49. **Smith, G. H., D. Gallahan, F. Diella, C. Jhappan, G. Merlino, and R. Callahan.** 1995. Constitutive expression of a truncated *INT3* gene in mouse mammary epithelium impairs differentiation and functional development. Cell Growth Differ. **6:**563–577.
50. **Stifani, S., C. M. Blaumueller, N. J. Redhead, R. E. Hill, and S. Artavanis-Tsakonas.** 1992. Human homologs of a Drosophila Enhancer of Split gene product define a novel family of nuclear proteins. Nat. Genet. **2:**119–127.
51. **Struhl, G., K. Fitzgerald, and I. Greenwald.** 1993. Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell **74:**331–345.
52. **Tax, F. E., J. J. Yeargers, and J. H. Thomas.** 1994. Sequence of *C. elegans lag-2* reveals a cell-signalling domain shared with *Delta* and *Serrate of Drosophila*. Nature (London) **368:**150–154.
53. **Tun, T., Y. Hamaguchi, N. Matsunami, T. Furukawa, T. Honjo, and M. Kawaichi.** 1994. Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. Nucleic Acids Res. **22:**965–971.
54. **Waltzer, L., F. Logeat, C. Brou, A. Israel, A. Sergeant, and E. Manet.** 1994. The human J kappa recombination signal sequence binding protein (RBP-J kappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements. EMBO J. **13:**5633–5638.
55. **Weinmaster, G., V. J. Roberts, and G. Lemke.** 1991. A homolog of *Drosophila Notch* expressed during mammalian development. Development **113:**199–205.
56. **Wharton, K. A., K. M. Johansen, T. Xu, and S. Artavanis-Tsakonas.** 1985. Nucleotide sequence from the neurogenic locus Notch implies a gene prod-

uct that shares homology with proteins containing EGF-like repeats. Cell **43:**567–581.
57. **Wilkinson, H. A., K. Fitzgerald, and I. Greenwald.** 1994. Reciprocal changes in expression of the receptor *lin-12* and its ligand *lag-2* prior to commitment in a C. elegans cell fate decision. Cell **79:**1187–1198.
58. **Woisetschlager, M., X. W. Jin, C. N. Yandava, L. A. Furmanski, J. L. Strominger, and S. H. Speck.** 1991. Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. Proc. Natl. Acad. Sci. USA **88:**3942–3946.
59. **Xu, T., and S. Artavanis-Tsakonas.** 1990. *deltex*, a locus interacting with the neurogenic genes, *Notch*, *Delta* and *mastermind* in *Drosophila melanogaster*. Genetics **126:**665–677.
60. **Yochem, J., and I. Greenwald.** 1989. *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. Cell **58:**553–563.
61. **Yochem, J., K. Weston, and I. Greenwald.** 1988. The *Caenorhabditis elegans lin-12* gene encodes a transmembrane protein with overall similarity to *Drosophila Notch*. Nature (London) **335:**547–550.
62. **Zagouras, P., S. Stifani, C. M. Blaumueller, M. L. Carcangiu, and S. Artavanis-Tsakonas.** 1995. Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc. Natl. Acad. Sci. USA **92:**6414–6418.
63. **Zimber-Strobl, U., L. J. Strobl, C. Meitinger, R. Hinrichs, T. Sakai, T. Furukawa, T. Honjo, and G. W. Bornkamm.** 1994. Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless. EMBO J. **13:**4973–4982.