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Neoplastic Transformation by Truncated Alleles of Human *NOTCH1/TAN1* and *NOTCH2*

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The *Notch* genes of *Drosophila melanogaster* and vertebrates encode transmembrane receptors that help determine cell fate during development. Although ligands for Notch proteins have been identified, the signaling cascade downstream of the receptors remains poorly understood. In human acute lymphoblastic T-cell leukemia, a chromosomal translocation damages the *NOTCH1* gene. The damage apparently gives rise to a constitutively activated version of NOTCH protein. Here we show that a truncated version of NOTCH1 protein resembling that found in the leukemic cells can transform rat kidney cells in vitro. The transformation required cooperation with the E1A oncogene of adenovirus. The transforming version of NOTCH protein was located in the nucleus. In contrast, neither wild-type NOTCH protein nor a form of the truncated protein permanently anchored to the plasma membrane produced transformation in vitro. We conclude that constitutive activation of NOTCH similar to that found in human leukemia can contribute to neoplastic transformation. Transformation may require that the NOTCH protein be translocated to the nucleus. These results sustain a current view of how Notch transduces a signal from the surface of the cell to the nucleus.

The *Notch* genes are members of an evolutionarily conserved family that help to determine cell fate during development (for a review, see reference 2). *Drosophila melanogaster* possesses a single *Notch* gene, whereas mammals possess at least four such genes termed *Notch1/TAN1*, *Notch2*, *Notch3*, and *Notch4/Int-3* (2, 6, 7, 11, 21, 37, 40, 43, 47). In both vertebrates and invertebrates, *Notch* genes are expressed throughout the development of the embryo in uncommitted proliferative cells (2, 9, 22, 29, 46). Later in development and in adult life, expression of *Notch* continues in the proliferative layers of mature tissues (20, 24, 47). These patterns of expression suggest that Notch proteins function in maintenance of the proliferative capacity of immature cells.

Notch proteins are transmembrane receptors with molecular weights of ca. 300,000 (17, 44) (see Fig. 1). The extracellular domain of the receptor is composed of ca. 1,750 amino acids, which include 36 tandem repeats of a sequence resembling epidermal growth factor and three repeats of a motif designated as lin-12 repeats. The cytoplasmic domain comprises a sequence of ca. 750 amino acids with no apparent enzymatic activity but containing six tandem copies of an ankyrin-like repeat (CDC10/ANK), a region rich in glutamine (OPA), and a region rich in glutamate, serine, and threonine (PEST). The first and second of these cytoplasmic motifs are thought to mediate protein-protein interactions, and the third may target the proteins for degradation.

The mechanism of signaling downstream of Notch receptors remains uncertain. The prevailing hypothesis holds that a proteolytic cleavage releases the cytoplasmic portion of Notch, which then translocates to the nucleus and participates in the activation of genes whose products inhibit differentiation (2, 10, 16, 18, 26, 38). For example, the cytoplasmic portion of Notch interacts with the transcription factor C-promoter binding factor-1 (CBF-1; also known as RBP-j kappa), leading to activation of the HES-1 gene (16, 18, 39). The product of the HES-1 gene is an inhibitor of muscle differentiation (34). Thus, the activity of Notch inhibits differentiation, and this in turn permits continued cellular proliferation. The evidence for this scheme remains provisional. For example, there has been no direct demonstration of a nuclear form of Notch following ligand stimulation. However, in certain mammalian cells, en-

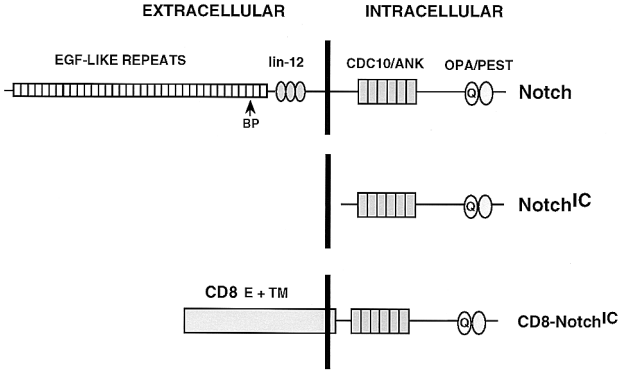

FIG. 1. Structure of *NOTCH* alleles. The diagram illustrates the molecular constructs used in this study. Wild-type (Notch) and activated ($Notch^{IC}$) constructs were made for both *NOTCH1* and *NOTCH2* alleles. CD8-$Notch1^{IC}$ was constructed only with *NOTCH1*. Features of the structure of NOTCH are indicated as follows: EGF-LIKE REPEATS, 36 tandem repeats of an EGF-like sequence found in all Notch family proteins; lin-12, three cysteine-rich repeats found in all members of the Notch family; BP, approximate position of the chromosomal breakpoint in the t(7;9)(q34;q34.3) chromosomal translocation found in certain T-ALLs; CDC10/ANK, six tandem copies of the ankyrin-like repeat; OPA, glutamine-rich region; PEST, a region rich in proline-glutamate-serine-threonine; CD8 E + TM, extracellular and transmembrane sequences of CD8.

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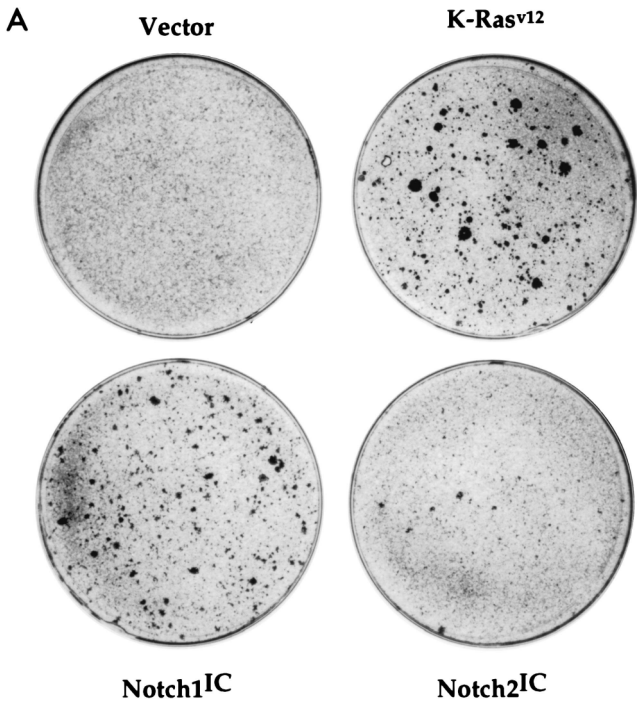
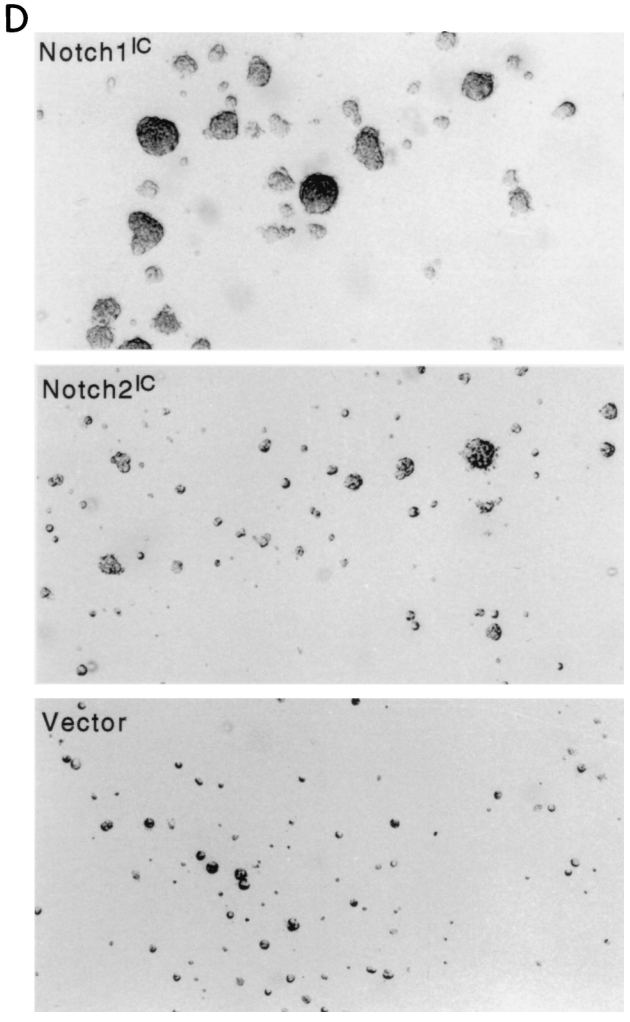


FIG. 2. Transformation by *NOTCH* alleles. (A) Focus assay. RKE cells were transfected with the indicated expression plasmid by the Lipofectamine method. Cells transfected with the retroviral vector pBabe-puro served as the control (vector). Notch1^{IC}, cells transfected with an activated allele of *NOTCH1*. Notch2^{IC}, cells transfected with an activated allele of *NOTCH2*. As a comparison for focus formation, cells were transfected with a K-*Ras*^{V12} expression plasmid (K-Ras^{v12}). (B) Photomicrographs of *NOTCH1*^{IC}-transformed cells. A single focus of cells transformed by *NOTCH1*^{IC} was photographed at an original magnification of ×160 (left) and ×100 (right) with a Zeiss IM35 inverted microscope. (C) Photomicrographs of cell monolayers at saturation density. Cells were plated at approximately the same density (10^6 cells/100-mm plate) and maintained in culture. Photographs were taken 1 week after cells grew to confluence. Vector, pBabe-puro-transfected cells selected with puromycin. Notch1^{IC}, an isolated clone of *NOTCH1*^{IC}-transformed cells. (D) Growth of *NOTCH*-transformed cells in soft agar. The cells were maintained in culture for approximately 5 weeks and then photographed at X40 with a Zeiss IM35 inverted microscope. Vector, as for panel C; Notch1^{IC}, RKE-*NOTCH1*^{IC} cells; Notch2^{IC}, RKE-*NOTCH2*^{IC} cells.



dogenous Notch proteins have been detected in the nucleus (1, 47). Furthermore, ectopic expression of the intracellular portion of Notch leads to nuclear localization in both vertebrates and invertebrates (1, 9, 10, 16, 18, 23, 26, 38, 47).

Notch function has also been implicated in the generation of neoplasia. In human T-cell acute lymphoblastic leukemia (T-ALL), the chromosomal translocation t(7;9)(q34;q34.3) joins a portion of *NOTCH1*/*TAN1* to the T-cell receptor β locus (7) (see Fig. 1). This translocation generates aberrant NOTCH proteins that lack most of the extracellular domain and are therefore thought to be constitutively active (3, 7, 31). Notch2 has also been implicated in leukemogenesis. Infection of cats with replication-competent feline leukemia virus (FeLV) yielded T-cell leukemia that harbored recombinant FeLV that had transduced a portion of the feline Notch2 gene (33). The transduced gene encodes a Notch2 protein analogous to those expressed in human T-ALL. However, it has not been demonstrated that this recombinant FeLV is itself oncogenic. Additional evidence to support a role for *Notch* genes in neoplasia has been derived from the study of insertional mutagenesis by mouse mammary tumor virus, which leads to mammary carcinomas. Among the genes affected by such mutations is *Int-3*, a *Notch* gene family member now termed *Notch4* (11, 32, 40). The mutations result in aberrant expression of truncated Int-3 proteins comprising only the intracellular portion of the molecule (32). The truncated proteins are again thought to be constitutively active.

Pear et al. have explored the tumorigenicity of human *NOTCH1* by introducing a potentially activated allele of the gene into mouse bone marrow cells (27). Although the recipient cells failed to display transformation in vitro, transfer of the cells to mice led to eventual T-cell leukemia. We now demonstrate that an activated allele of *NOTCH* can directly transform rat kidney cells in vitro. The transformation requires cooperation with the E1A oncogene of adenovirus and gives rise to tumorigenic cells containing NOTCH protein in their nuclei. In contrast, wild-type *NOTCH1* is inactive in this assay, as is a chimeric allele of *NOTCH1* whose product is permanently anchored to the plasma membrane. We conclude that a constitutively activated form of NOTCH protein can transform cells in vitro and that transformation probably requires translocation of the protein to the nucleus.

MATERIALS AND METHODS

Cells and transformation assays. Baby rat kidney (BRK) cells were prepared from 5-day-old Fisher rats as described previously (41). E1A-immortalized BRK (RKE) cells were generated by transfection of BRK cells with a vector that expresses E1A from the cytomegalovirus early-promoter/enhancer and selection

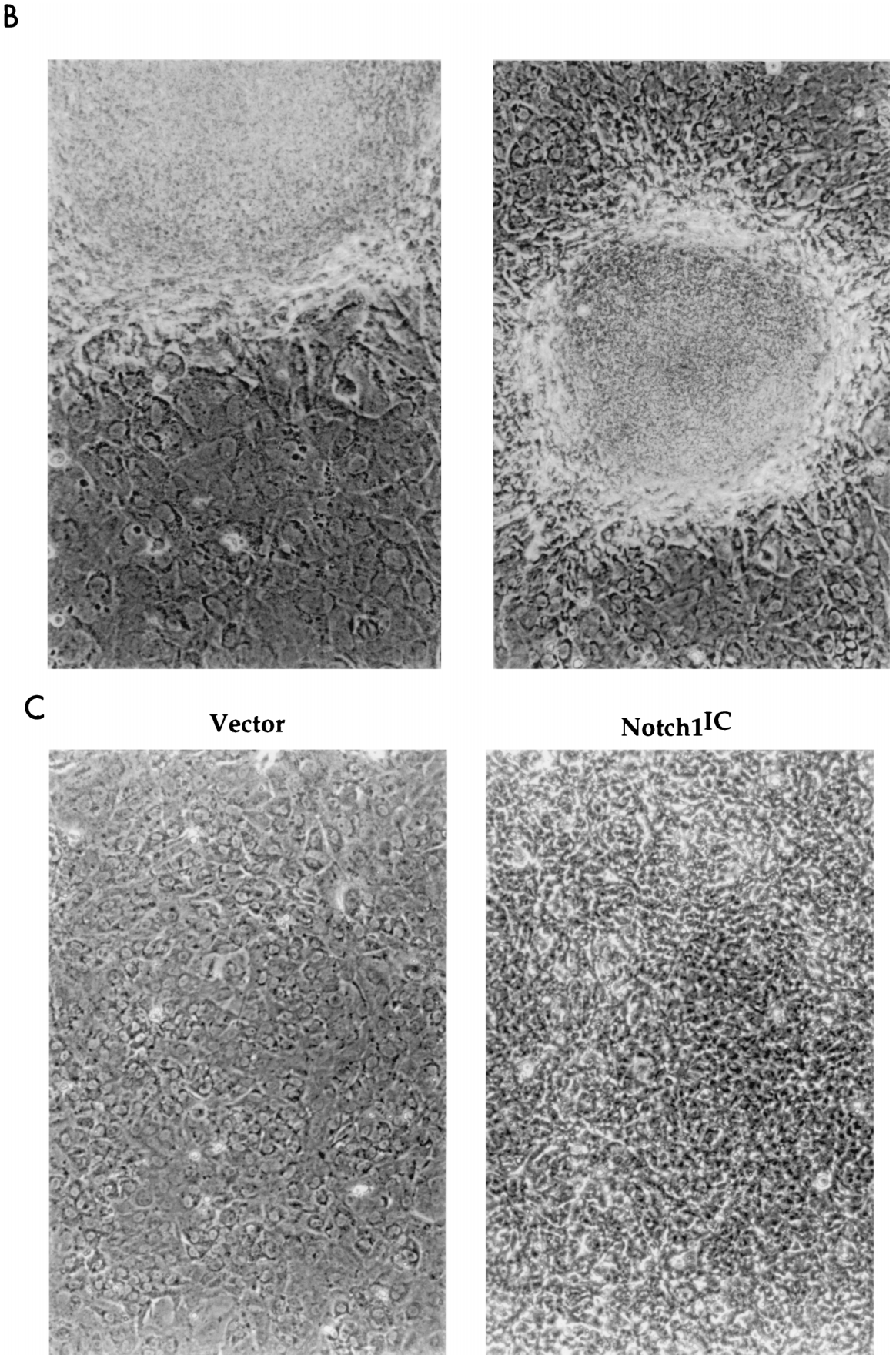


FIG. 2–*Continued.*

TABLE 1. Summary of transformation by NOTCH proteins

Cell type[a]	Plasmid	No. of foci[b]
RKE	Vector	0
	NOTCH1	0
	NOTCH1^{IC}	29–53
	NOTCH2	0
	NOTCH2^{IC}	1–7
	K-*Ras*^{V12}	20–36
BRK	Vector	0
	E1A	0
	K-*Ras*^{V12}	0
	NOTCH1	0
	NOTCH1^{IC}	0
	E1A + K-*Ras*^{V12}	24–36
	E1A + *NOTCH1*^{IC}	28–39
	K-*Ras*^{V12} + *NOTCH1*^{IC}	0

[a] RKE, E1A-immortalized baby rat kidney cell line. BRK, primary baby rat kidney cells.

[b] For RKE assays, the number of foci given is a range derived from more than six independent assays. For the BRK assays, the range is derived from three independent assays.

of immortalized colonies. These colonies were pooled, expanded in culture, and then stored as aliquots in liquid nitrogen. The cells were used for transformation within 2 to 10 passages after thawing. All cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. When indicated, either puromycin (2 μg/ml) or neomycin (400 μg/ml) was used for selection.

DNA was transfected into cells with Lipofectamine (Bethesda Research Laboratories) as specified by the manufacturer. In our assays, we used 30 μg of Lipofectamine per 10 μg of DNA in a volume of 8 ml.

For focus formation assays, approximately 5×10^5 cells were seeded onto a 100-mm tissue culture plate and transfected with plasmid DNA. The cells were maintained in culture and fed twice weekly until foci were observed. The cells were fixed in 100% methanol and stained with a solution of 0.5% methylene blue in 70% isopropanol. Transformed cell lines were established by picking isolated foci by using cloning rings and trypsin disruption. Isolated foci were then propagated in culture under standard conditions.

For growth in semisolid medium, approximately 10^4 cells were seeded into 7 ml of DMEM containing 10% fetal bovine serum and 0.35% low-melting-temperature agarose (FMC Corp.). This suspension of cells was then overlaid onto a base of 7 ml of DMEM containing 0.7% agarose. The cells were maintained in culture until colonies were visible and counted.

To analyze the ability of cell lines to escape contact inhibition and grow to a higher saturation density, approximately 10^6 cells were seeded onto a 100-mm tissue culture plate and allowed to grow to confluency. The cells were scored for the loss of contact inhibition 1 to 2 weeks later. For the nontransforming alleles, cell lines were established by transfection of plasmid DNA and subsequent selection based on drug resistance. Cell lines were then established by pooling drug-resistant colonies.

TABLE 2. Growth in soft agar and tumorigenicity of *NOTCH*-transformed cells

Clone[a]	Growth in soft agar	No. of tumors in nude mice[b]
RKE	−	0/4
RKE-V1	−	0/4
RKE-V2	−	ND[c]
RKE-cN1	+	3/3
RKE-cN2	+	4/4
RKE-cN3	+	ND
RKE-cN4	−	ND
RKE-cN5	+	4/4
RKE-cN6	+	4/4
RKE-cN2-1	−	ND
RKE-cN2-2	+	3/3
RKE-KR1	+	ND
RKE-KR2	+	ND

[a] RKE, parental cell line; V1 and V2, two independently derived cell lines carrying only the expression vector; cN1 to cN6, *NOTCH1*^{IC} clones 1 to 6; cN2-1 and cN2-2, *NOTCH2*^{IC}-derived cell lines; KR1 and KR2, K-*Ras*^{V12}-transformed RKE cell lines. All cells were derived from parental RKE cells.

[b] Number of tumors/number of sites injected.

[c] ND, not done.

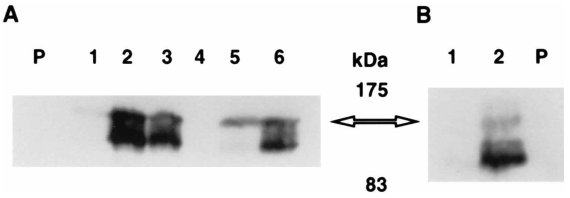


FIG. 3. Expression of NOTCH^{IC} proteins in transformed cells. (A) Western blot analysis of cell lysates from *NOTCH1*^{IC}-transformed clones. P, parental RKE cells. Lanes 1 to 6, clones of *NOTCH1*^{IC}-transformed cells. The arrowhead indicates the position of the NOTCH1^{IC} protein. (B) Western blot analysis of cell lysates from *NOTCH2*^{IC}-transformed clones. P, parental RKE cells. Lane 1, cell line that did not maintain a transformed phenotype; lane 2, clone of *NOTCH2*^{IC}-transformed cells. The arrowhead indicates the position of the NOTCH2^{IC} protein.

Plasmids. Recombinant DNA was manipulated by standard methods. Expression plasmids for wild-type human *NOTCH1*, *NOTCH2*, *NOTCH1*^{IC}, and *NOTCH2*^{IC} were constructed in pCDNA as described previously (37, 47). *NOTCH1*^{IC} and *NOTCH2*^{IC} were also expressed from the retroviral vector pBabe-puro. The *NOTCH1*^{IC} and *NOTCH2*^{IC} constructs express amino acids 1758 to 2556 and 1701 to 2471, respectively, and do not code for any of the transmembrane sequences. *CD8-NOTCH1*^{IC} was constructed by fusing the cytoplasmic portion of *NOTCH1*^{IC} (amino acids 1758 to 2556) to the extracellular and transmembrane domains of *CD8*. Human *CD8* was a kind gift from D. Littman (25). The *CD8-NOTCH1*^{IC} fusion was then inserted into the *Sna*bT and *Sal*1 sites of pBabe-puro. K-*Ras*^{V12} was expressed from the retroviral vector pZip-neo and was a gift from Channing Der (15). E1A was expressed from the vector pcDNA and was a kind gift from Eileen White (5).

Assay for tumorigenicity. Tumorigenicity assays were performed by injecting 0.1 ml of a cell suspension containing 10^6 cells in DMEM subcutaneousley into athymic nude mice (BALB/c byj; Charles River). The cells were injected into two sites on the flank of each mouse. The mice were monitored twice weekly and sacrificed when tumor growth was obvious. Tumors were scored as the number of tumor-positive sites per the number of sites injected for mice that survived.

Antibodies. Monoclonal antibodies were prepared against human NOTCH1 (bTAN15A) and human NOTCH2 (bhN6D) as described elsewhere (47). Tissue culture supernatants containing these antibodies were used at a 1:10 dilution.

Analysis of NOTCH proteins. Cells (10^6) were seeded onto 100-mm tissue culture plates. Approximately 48 h later, cell extracts were prepared by solubilization of cells in 1× sample buffer. The samples were boiled for 10 min and centrifuged for 10 min at top speed in a bench top microcentrifuge. Equal volumes of lysate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose by standard procedures. Proteins were detected by immunoblotting with anti-NOTCH monoclonal antibody bTAN15A or anti-NOTCH2 monoclonal antibody bhN6D and visualized by enhanced chemiluminescence (Amersham).

Indirect immunofluorescence. Cells were grown on glass slides in DMEM. To prepare the cells for detection, they were fixed in 100% methanol at −20°C for 10 min and washed three times in phosphate-buffered saline. The cells were preincubated for 1 h at room temperature in phosphate-buffered saline supplemented with 3% calf serum and 0.05 Tween 20 to block nonspecific binding. They were then incubated for 1 h with an anti-NOTCH monoclonal antibody (bTAN15A) and detected with a CY3-conjugated secondary antibody (Jackson Laboratory).

RESULTS

Structure of *NOTCH* alleles. Figure 1 illustrates the forms of *NOTCH1* used in this study: the wild-type allele, truncated alleles that encode only the intracellular portion of NOTCH (*NOTCH1*^{IC}/*NOTCH2*^{IC}), and a chimera in which the intracellular domain of the transmembrane protein CD8 was replaced by NOTCH1^{IC} (CD8-*NOTCH1*^{IC}). We presumed that the NOTCH^{IC} proteins would be constitutively active because they are analogous to the products of gain-of-function alleles in *D. melanogaster* (31). The chimera with CD8 should be anchored to the plasma membrane and unresponsive to ligands for NOTCH. The various alleles were expressed from plasmids

that used the promoter/enhancer of either cytomegalovirus or murine leukemia virus (see Materials and Methods for details).

Figure 1 also illustrates the location of the translocation break point within *NOTCH1*, found in examples of human T-ALL. The translocation results in the production of aberrant NOTCH1 proteins that are approximately the size of the cytoplasmic portion of the protein and therefore potentially active in the absence of ligand. Some of these proteins may retain the transmembrane domain of NOTCH, and others do not (3).

***NOTCH^{IC}* but not wild-type *NOTCH* transforms cells in vitro.** To determine if NOTCH proteins could transform cells in culture, we first had to identify a suitable indicator cell line. We therefore screened a variety of cell cultures and discovered that ectopic expression of *NOTCH1^{IC}* apparently transformed baby rat kidney cells that had been previously immortalized by E1A (designated RKE).

Foci of transformed cells developed within 3 to 4 weeks of introducing *NOTCH1^{IC}* into RKE cells (Fig. 2A). The number and appearance of these foci were similar to those obtained by transfecting RKE cells with a K-*Ras*^{V12} expression vector (Fig. 2A; Table 1). In contrast, transformation by *NOTCH2^{IC}* was far less efficient (Fig. 2A; Table 1). Microscopic examination of the *NOTCH1^{IC}*-induced foci revealed typical characteristics of transformed cells, including altered cell morphology and multilayered growth (Fig. 2B and C). No foci were detected with RKE cells transfected with a control vector that did not carry a *NOTCH* gene. Similarly, the wild-type genes *NOTCH1* and *NOTCH2* failed to transform RKE cells (Table 1 and data not shown).

Foci were isolated from both *NOTCH1^{IC}*- and *NOTCH2^{IC}*-transfected RKE cells. Five of six *NOTCH1^{IC}* foci could be propagated as transformed cell lines. We also established one *NOTCH2^{IC}*-transformed cell line from the two foci isolated. The transformed cell lines established by both *NOTCH1^{IC}* and *NOTCH2^{IC}* grew to a much higher saturation density than did the vector-transfected cells (Fig. 2C).

All the *NOTCH^{IC}*-transformed cell lines tested were able to form colonies in soft agar (Fig. 2D; Table 2). Approximately 40% of the *NOTCH1^{IC}*-transformed cells that were seeded in the semisolid media grew to form colonies. However, there appeared to be a restriction on the size of the colonies. That is, the colonies formed slowly and did not attain a size similar to that of RKE cells transformed by K-*Ras*^{V12} (K-*Ras* data not shown). The *NOTCH2^{IC}* line showed significantly less vigorous growth in soft agar: a smaller percentage of the cells formed colonies (approximately 11%), and the colonies formed were much smaller (Fig. 2D). The parental RKE line and a vector-transfected line showed no growth in semisolid medium (Fig. 2D; Table 2) (data not shown). From these data, we conclude that the truncation represented by the NOTCH^{IC} proteins results in a constitutive activity that is capable of transforming cells. The analogous truncation of NOTCH2 is much less active in transformation in vitro.

Cells transformed by *NOTCH^{IC}* alleles form tumors in nude mice. To examine whether the *NOTCH^{IC}*-transformed cells were tumorigenic, we tested clonal cell lines derived from transformed foci for the ability to form tumors in nude mice (Table 2). As a control, cells were transfected with the retroviral vector pBabe-puro and then selected for the drug resistance marker. Polyclonal cell lines of drug-resistant colonies were then tested in nude mice (Table 2, RKE-V1 and RKE-V2). All of the mice that were injected with cells transformed by either *NOTCH1^{IC}* or *NOTCH2^{IC}* were positive for tumors by 12 weeks. The diameters of the tumors varied from approximately 0.5 to over 2 cm. The larger tumors were all significantly vascularized and exhibited some invasion through the body wall (data not shown).

Expression of *NOTCH^{IC}* correlates with the transformed phenotype. Western blot analysis revealed that all of the transformed clones propagated in culture expressed NOTCH1^{IC} or NOTCH2^{IC} proteins (Fig. 3). Cells that failed to maintain a transformed phenotype when propagated in culture contained no detectable NOTCH protein (Fig. 3A, lane 4; Fig. 3B, lane 1). The level of NOTCH1^{IC} expression in the individual transformed clones varied. The varied level of expression did not reflect any obvious phenotypic differences among these lines. NOTCH1^{IC} and NOTCH2^{IC} proteins appeared in the Western blots as two major bands (Fig. 3). The more slowly migrating bands correspond to the predicted sizes of NOTCH1^{IC} and NOTCH2^{IC} proteins. We do not know the origin or significance of the more rapidly migrating bands. However, they appear to be stable proteins that are observed consistently in cells expressing *NOTCH^{IC}* alleles. It is possible that these proteins represent products of specific proteolysis; alternatively, they may be stable products of nonspecific degradation. It is notable that the more rapidly migrating forms of the protein predominated in cells expressing *NOTCH2^{IC}*. Perhaps this predominance accounts for the feeble transformation by that allele.

***NOTCH1^{IC}* collaborates with E1A to transform primary cells.** Since the indicator line we used was an E1A-immortalized rat kidney cell line, it is possible that *NOTCH1^{IC}* cooperated with E1A in the transformation of these cells. To test this possibility, primary BRK cells were cotransfected with *NOTCH1^{IC}* and either E1A or K-*Ras*^{V12}. The cells were maintained in culture for approximately 6 weeks, fixed, and stained for analysis. Indeed, *NOTCH1^{IC}* alone was not able to transform primary BRK cells (Table 1). However, when cotransfected with E1A, *NOTCH1^{IC}* effectively transformed the BRK cells. The cooperative transformation was as effective as that displayed by E1A and K-*Ras*^{V12}. In contrast, no foci were observed when *NOTCH1^{IC}* was cotransfected with K-*Ras*^{V12}.

Membrane-bound forms of NOTCH are not transforming. Binding of ligand to the Notch receptor is thought to initiate a proteolytic processing that yields a cytoplasmic protein approximately the size of NOTCH^{IC} (2, 10, 16, 19, 23). The processed form is then thought to be released from the plasma membrane and translocated to the nucleus. We wanted to determine if membrane-bound forms of the NOTCH^{IC} protein could transform cells. To test this issue, a chimeric protein was made by fusing NOTCH1^{IC} to the extracellular and transmembrane domains of CD8 (Fig. 1, bottom). The resulting fusion protein should be inserted into the plasma membrane and should not be responsive to NOTCH ligands.

The *CD8-NOTCH1^{IC}* construct failed to elicit any foci in cultures of RKE cells. To explore other parameters of transformation, we derived polyclonal lines of cells expressing various constructs; the lines were obtained by selecting for the puromycin resistance marker and pooling clones of resistant cells. We observed no morphological changes in cell lines expressing either *CD8-NOTCH1^{IC}* or wild-type *NOTCH*. We next tested to see whether the polyclonal cell lines could escape contact inhibition and grow to a higher saturation density. Cells (10^6) were seeded onto a 100-mm tissue culture plate and maintained in culture until 1 week after they had reached confluency. The *NOTCH1^{IC}*-expressing cells readily escaped contact inhibition, forming a dense mat of cells (Fig. 4A). In contrast, contact inhibition was maintained in cells carrying wild-type *NOTCH*, *CD8-NOTCH1^{IC}*, or the vector alone (Fig. 4A).

***NOTCH* and *CD8-NOTCH1^{IC}* are absent from the nucleus.** We used Western blotting to authenticate the presence of NOTCH proteins in the various cell lines. Cells expressing

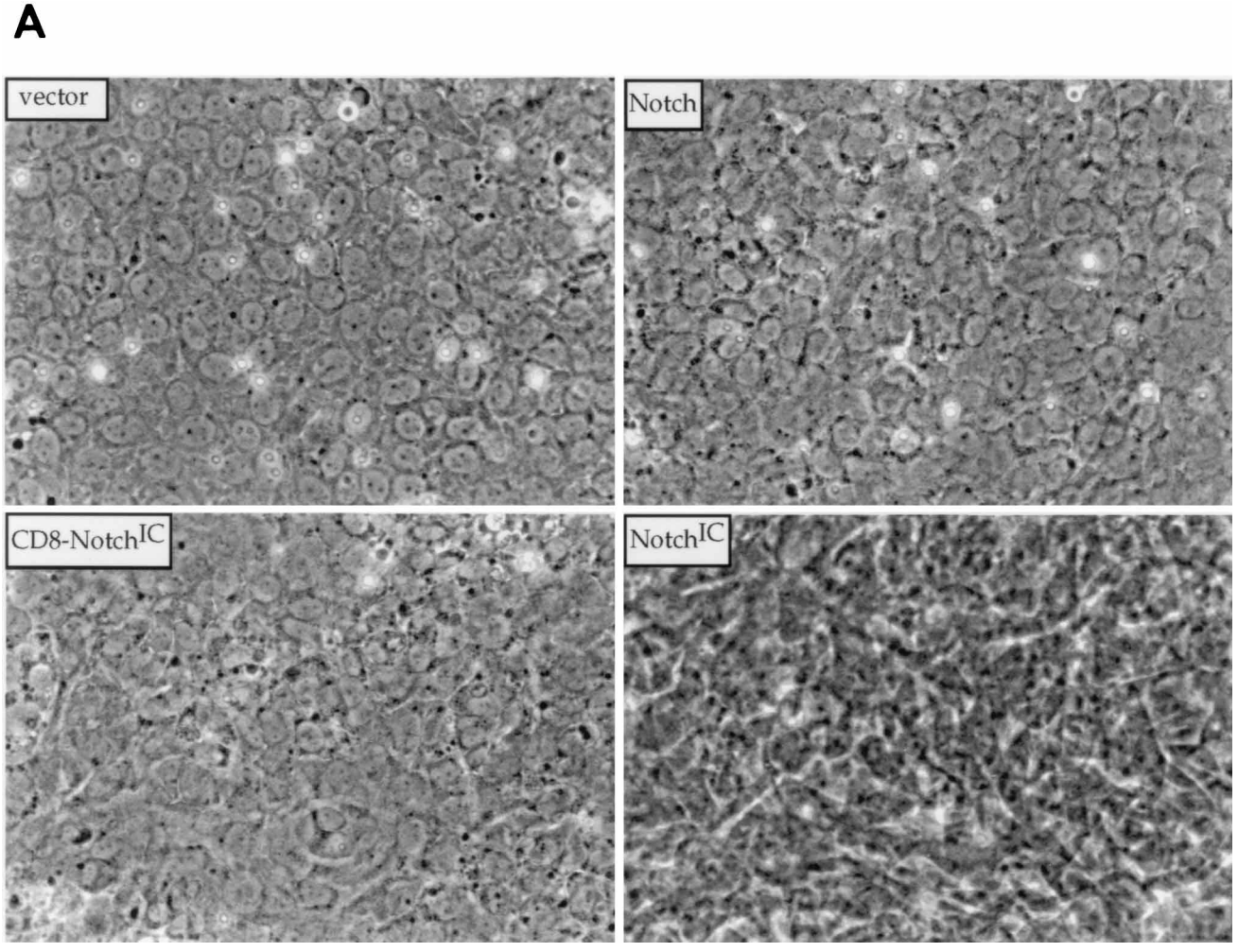


FIG. 4. Membrane-bound forms of NOTCH are not transforming. (A) Photomicrograph of cell monolayers at saturation density. Cells were transfected with the indicated plasmids, and pools of transfected cells were selected for the appropriate drug resistance marker. Cells were plated at 10^6 cells per 100-mm plate and maintained in culture. The cells were photographed 1 week after growing to confluence. Vector, cells transfected with pBabe-puro. Notch, cells expressing wild-type *NOTCH1*. CD8-Notch^{IC}, cells expressing *CD8-NOTCH1^{IC}* chimera. Notch1^{IC}, cells expressing *NOTCH1^{IC}*. (B) Western blot analysis of cells lysates from the cells depicted in panels A and C. Samples were prepared and analyzed as for Fig. 3. control, lysate from pBabe-puro vector-transfected cells; Notch1^{IC}, lysate from *NOTCH1^{IC}*-expressing cells; CD8-Notch^{IC}, lysate from *CD8-NOTCH1^{IC}*-expressing cells; Notch, lysate from wild-type *NOTCH*-expressing cells. Numbers on the left correspond to the molecular weight markers, in thousands. (C) Indirect immunofluorescence of expressed NOTCH proteins. Labels are the same as for panel A.

either *NOTCH1^{IC}* or *CD8-NOTCH1^{IC}* contained NOTCH proteins of the appropriate sizes (Fig. 4B). The CD8-NOTCH1^{IC} protein is approximately 25 kDa larger than the NOTCH1^{IC} protein, which is consistent with the electrophoresis data (Fig. 4B). In contrast, cells expressing wild-type *NOTCH1* contained a protein much shorter than anticipated, approximately the size of NOTCH1^{IC}. The size of this protein has been noted previously and attributed to rapid protein processing (4, 47) (see Discussion).

We used immunofluorescence to examine the locations of NOTCH proteins in stable cell lines (Fig. 4C). Wild-type NOTCH protein and the CD8-NOTCH1^{IC} chimera displayed a staining pattern consistent with localization to the cell periphery and the endoplasmic reticulum-Golgi, with complete absence of detectable nuclear staining. In contrast, NOTCH1^{IC} was localized primarily to the nucleus of the cell. The vector-transfected cells served as a control and showed no immunoreactivity with the anti-NOTCH antiserum (Fig. 4C).

DISCUSSION

Transformation by NOTCH proteins. The results of genetic analysis of *D. melanogaster* indicate that removal of the extracellular and transmembrane domains from the Notch protein creates a constitutively activated version of the receptor (30). Here

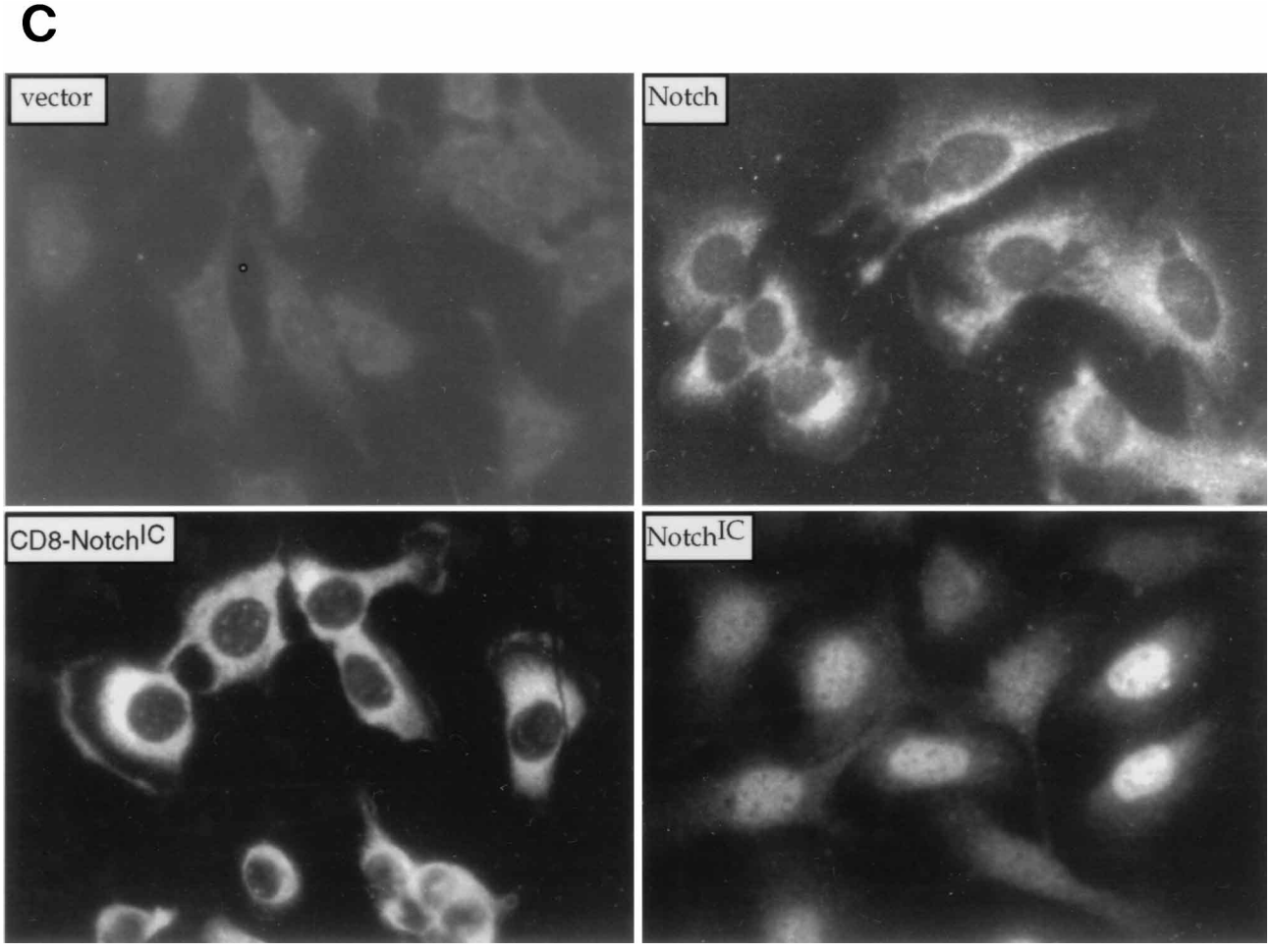


FIG. 4–*Continued.*

we have shown that analogous forms of human *NOTCH* genes (*NOTCH1*^{IC} and *NOTCH2*^{IC}) transform cells in culture. Moreover, the transformed cells are tumorigenic when injected into nude mice. In contrast, wild-type *NOTCH* does not transform cells. These results demonstrate that a truncated NOTCH protein displays properties of a dominant activated protein and may play a direct role in the generation of neoplasia. It is possible that the wild-type receptor will also transform cells if subjected to sustained stimulation by ligand. We have not explored this possibility. The assay for transformation described here will facilitate structure-function analysis of the NOTCH protein.

An activated allele of *NOTCH* has also been introduced into mouse bone marrow cells in vitro (27). The recipient cells did not display immediate transformation. However, when they were used to reconstitute the bone marrow of irradiated mice, they gave rise to T-cell leukemia after a latent period of 12 to 40 weeks. The protracted latency presumably reflects the requirement for additional, spontaneous genetic events.

Our work provides a clue to the nature of those additional events. Transformation of rat kidney cells by *NOTCH*^{IC} required the cooperation of E1A. The product of E1A inactivates the protein encoded by the tumor suppressor gene *RB-1* (45). Thus, any genetic lesions that lead to a deficiency of *RB-1* function might cooperate with *NOTCH* in neoplastic transformation. Such lesions are common in human tumors (for a review, see reference 32).

Activation of the *Myc* proto-oncogene can also cooperate with *Notch* in tumorigenesis (12). In this instance, a dominant lesion reproduces the effect of a deficiency in *RB-1*. Overexpression of *Myc* might serve to inactivate the RB-1 protein by increasing the levels of G1 cyclin-CDK complexes, resulting in ungoverned progression through the cell cycle (35, 36). It is notable that both *Myc* and E1A can elicit apoptosis when acting alone in cells (8, 28). Genes, such as E1B, that cooperate with E1A in transformation do so by blocking apoptosis (5, 28). The possibility that *Notch* does the same deserves examination.

Subcellular localization and transformation by NOTCH. The mechanism by which Notch functions remains poorly understood. It is thought that upon ligand activation, Notch is proteolytically processed to release the cytoplasmic domain from its membrane anchor. The released form is then translocated to the nucleus. Our work provides additional support for this view. The only forms of NOTCH that transformed cells in vitro were those that both resembled the intracellular product of ligand stimulation and were found in the nuclei of cells.

We also tested the importance of nuclear localization by anchoring NOTCH1^{IC} to the plasma membrane with the transmembrane domain of CD8. The chimeric protein remained in the plasma membrane and could not transform cells in vitro. A trivial explanation of the failure of this chimera to transform cells is that we have destroyed the functional integrity of the NOTCH sequences by fusing them to CD8. Although we can-

not formally rule out this possibility, it seems unlikely. The domain of NOTCH required for transformation is intact within the CD8 fusion and would presumably transform cells if cleaved out of that fusion. Such cleavage does not occur, and as a result, the protein remains anchored to the plasma membrane, providing a sufficient explanation for the lack of transformation. This view is sustained by the failure of wild-type NOTCH to transform cells in the absence of ligand. The protein product of unactivated wild-type NOTCH remains cytoplasmic in both our study and those of others (4). Thus, we have obtained a clear correlation between the biological function and nuclear localization of NOTCH protein.

Pear et al. have reported results contrary to those reported here (27). They used an expression construct that encodes the NOTCH signal peptide fused to amino acids 1704 to 2555. The resulting protein encodes the entire cytoplasmic domain, transmembrane-spanning sequences, and sequences just extracellular to the transmembrane domain. When this construct is introduced into mouse bone marrow cells and transplanted into irradiated recipient animals, the animals develop T-cell leukemia. In these leukemic cells, the NOTCH proteins are primarily cytoplasmic. We cannot presently account for this apparent discrepancy with our results. However, Rohn et al. have shown that a version of Notch2 similar to that used by Pear et al. displays exclusively nuclear localization (33).

In cells that were transfected with wild-type *NOTCH*, we detected only a protein approximately the size of $NOTCH^{IC}$ (Fig. 4B). This product of wild-type *NOTCH1* has been noted before and attributed to rapid processing of the 300-kDa wild-type protein (47). Since this form of NOTCH is processed, why is it excluded from the nucleus and why is it nontransforming in cells? Recently, it has been demonstrated that NOTCH proteins are translated as full-length precursors and rapidly processed in the *trans*-Golgi network to form a heterodimer of the two fragments in the plasma membrane (4). Since the cytoplasmic portion of NOTCH remains tethered to the plasma membrane, other signals (such as ligand binding) must be required to activate the signaling cascade. This would explain why the processed form of full-length NOTCH that we see is not in the nucleus and is not transforming.

Biochemical functions of Notch. What is the function of Notch in the nucleus, and how does it contribute to transformation? Several reports have shown that Notch relieves transcriptional repression of genes mediated by CBF-1 (14, 16), perhaps by physically binding CBF-1 and acting as a transcriptional activation domain (14, 16, 42). We do not yet know the importance of this interaction in mediating transformation by NOTCH. However, the ability to bind CBF-1 may not be sufficient for transformation by NOTCH. Although the NOTCH2 protein also binds CBF-1 (39), it transforms RKE cells only feebly. We therefore presume that transformation requires additional actions by NOTCH.

Additional support for a more complex mechanism has been obtained with the EBNA-2 protein of Epstein-Barr virus. EBNA-2 binds CBF-1 and serves as a transcriptional activation domain, inducing transcription from promoters containing CBF-1 binding sites (13, 42). However, we have been unable to transform RKE cells with EBNA-2, sustaining the view that relief of repression by CBF-1 is not sufficient to transform cells and that other signaling events are activated by NOTCH. It remains likely, however, that *NOTCH* genes transform cells by altering the pattern of transcription.

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