Neoplastic Transformation by Notch Requires Nuclear Localization

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Notch proteins are plasma membrane-spanning receptors that mediate important cell fate decisions such as differentiation, proliferation, and apoptosis. The mechanism of Notch signaling remains poorly understood. However, it is clear that the Notch signaling pathway mediates its effects through intercellular contact between neighboring cells. The prevailing model for Notch signaling suggests that ligand, presented on a neighboring cell, triggers proteolytic processing of Notch. Following proteolysis, it is thought that the intracellular portion of Notch (N^{ic}) translocates to the nucleus, where it is involved in regulating gene expression. There is considerable debate concerning where in the cell Notch functions and what proteins serve as effectors of the Notch signal. Several Notch genes have clearly been shown to be proto-oncogenes in mammalian cells. Activation of Notch proto-oncogenes has been associated with tumorigenesis in several human and other mammalian cancers. Transforming alleles of Notch direct the expression of truncated proteins that primarily consist of N^{ic} and are not tethered to the plasma membrane. However, the mechanism by which Notch oncoproteins (generically termed here as N^{ic}) induce neoplastic transformation is not known. Previously we demonstrated that N1^{ic} and N2^{ic} could transform E1A immortalized baby rat kidney cells (RKE) in vitro. We now report direct evidence that N1^{ic} must accumulate in the nucleus to induce transformation of RKE cells. In addition, we define the minimal domain of N1^{ic} required to induce transformation and present evidence that transformation of RKE cells by N1^{ic} is likely to be through a CBF1-independent pathway.

Notch proteins are cell surface transmembrane-spanning receptors which mediate critically important cellular functions through direct cell-cell contact. Interaction between Notch and its proposed ligand, Delta or Serrate/Jagged, initiates a signaling cascade that governs cell fate decisions such as differentiation, proliferation, and apoptosis in numerous tissue types (2, 9, 27, 57, 58, 69). There are four mammalian Notch genes, Notch1/TAN-1, Notch2, Notch3, and Notch4/Int-3, which are expressed in both overlapping and distinct patterns throughout mammalian tissues (11, 12, 17, 22, 45, 49, 71, 86, 90, 91, 93). Multiple genes for each ligand (6, 29, 38, 53, 60, 62, 64, 77, 87) as well as genes for numerous modulators of Notch signaling (3, 5, 13, 19, 25, 34, 35, 37, 55, 88, 94) have been identified. The growing array of elements in the Notch pathway confers extensive complexity on this signaling system.

Substantial evidence indicates three members of the mammalian Notch family are involved in the generation of neoplasia (8, 17, 50, 54, 67, 70, 74, 93). TAN-1 was identified as a chromosomal translocation, t(7;9)(q34;q34.3), involving the T-cell receptor β locus and the human Notch1 gene in T-cell acute lymphoblastic leukemias (17). This translocation resulted in constitutive expression of aberrant Notch1 transcripts that consist primarily of the intracellular domain. Mouse bone marrow infected with retroviral vectors expressing analogous truncated Notch1/TAN-1 failed to demonstrate transformation in vitro but, when reconstituted into irradiated syngeneic mice, gave rise to T-cell neoplasms with a latency of 11 to 40 weeks (3, 67).

Our lab has demonstrated that an E1A-immortalized baby rat kidney cell line (RKE) can be directly transformed by

ectopic expression of constitutive alleles of Notch1 and Notch2 (N1^{ic} and N2^{ic}), indicating these proteins have a direct role in the transformation of cells. Furthermore, N1^{ic} has the ability to transform primary baby rat kidney cells in cooperation with E1A (8).

Genetic evidence indicates Notch1 can cooperate with cellular proto-oncogenes to accelerate tumorigenesis in different tissue types. Retroviral insertion into the Notch1 locus has led to acceleration of tumorigenesis in mouse mammary tumor virus (MMTV^P)/c-myc transgenic mice and in MMTV/neu transgenic mice (14, 26). Both insertional events led to expression of aberrant truncated transcripts encoding intracellular sequences of Notch1.

Notch2 and Notch4 have also been implicated in neoplasia. Recombinant feline leukemia virus isolated from a thymic lymphoma was found to contain sequences derived from the feline Notch2 gene (74). Evidence that Notch4 is involved in neoplasia has been derived through studies of MMTV insertional mutagenesis. The *int-3* locus was initially identified as a common site of integration for MMTV (24). Proviral insertion in this locus led to expression of Notch4 intracellular sequences. The resulting aberrant Notch4 proteins have been shown to contribute to the generation of mammary carcinoma in the mouse as well as directly transform mouse mammary epithelia in vitro (22, 23, 70, 80).

In all of the described incidences of Notch genes associated with tumorigenesis, the common theme is the constitutive expression of truncated Notch proteins consisting primarily of the intracellular domain that are not tethered to the plasma membrane. Once deleted of extracellular sequences, Notch proteins are thought to be constitutively active; when expressed in both vertebrates and invertebrates, they localize primarily in the nucleus (1, 8, 18, 20, 35, 43, 52, 63, 75, 82).

A current model of Notch signal transduction suggests that ligand binding to the extracellular domain induces proteolytic

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cleavage that releases the intracellular domain (N^{ic}) which then translocates to the nucleus, where it alters gene expression (2, 20, 36, 44, 75, 81, 83). The mechanism by which Notch influences gene expression is not well understood. In one model, Notch is believed to directly control transcription via interactions with CBF1 [c-promoter binding factor, Su(H) (Suppressor of Hairless), or RBP-J_κ]. CBF1 is a sequence-specific DNA binding protein that functions to repress transcription of cellular genes (15, 85). Ligand-activated Notch proteins are thought to bind to CBF1 in the nucleus, displace a complex of corepressors, and, in turn, lead to activation of target gene transcription (10, 30, 31, 33, 35, 40, 47, 48, 66, 84). However, recent evidence indicates that Notch participates in cellular functions that are independent of CBF1 activation, implying alternative effectors of the Notch signaling pathway (56, 61, 65, 78, 89).

The relevance of Notch nuclear translocation in signal transduction remains controversial (2). A recent report has demonstrated evidence of nuclear accumulation of endogenous Notch upon ligand stimulation in mouse cortical neurons. However, these authors were not able to define a strict correlation between Notch signaling and nuclear translocation (76).

Indirect evidence that Notch might function within the nucleus has been demonstrated in *Drosophila*, where ligand-activated Notch-GAL4-VP16 fusion proteins were found to activate transcription of an upstream activation sequence-*lacZ* transgene containing GAL4 DNA binding sites, presumably through proteolytic cleavage and subsequent nuclear translocation (51, 81).

Using the in vitro RKE transformation assay, we report that N1^{ic} must accumulate within the nucleus to elicit biological function. In addition, we define the minimal domain of N^{ic} able to induce transformation of RKE cells, and we report that transformation is likely to be independent of CBF1 activation.

MATERIALS AND METHODS

Plasmids, PCR mutagenesis, and expression constructs. Human N^{ic} deletion mutants were generated with high-fidelity PCR using a full-length human Notch1 expression vector as the template. Two 5' cloning primers were designed to initiate translation at either N^{ic} residue 1759 or residue 1848. Each contains a *Bam*HI restriction site upstream of the initiator codon and the following sequences: N^{ic} 1759 AAAGGATCCACCATGGCAGCGCAAGCGCCGGCGCAGTCAT; and N^{ic} 1848, AAAGGATCCACCATGGCAGCGCAACCTGGATGCGCTGACCTG (*Bam*HI site is shown in italics, initiator methionine is in bold, and N^{ic} sequences are underlined). Each of the 5' primers was paired with the following list of 3' cloning primers to generate C-terminal truncations. Each primer contains an *Xho*I restriction site (shown in italics), N^{ic} sequences are underlined, and the numbers indicate the C-terminal residue: N^{ic} 2556, GCGCCTCGAGCTTG AAGCGCTCCGGGATGCG; N^{ic} 2444, GCGCCTCGAGCAGCAGTCTGCTGGCTCGG; N^{ic} 2358, GCGCCTCGAGGCTGGCAGCAAGGCTACT; N^{ic} 2202, GCGCCTCGAGGTCCACGGCGAGAGCAT; N^{ic} 2171, GCGCCTCGAGGCTTCCACAGGCCAGGCTTT; N^{ic} 2120, GCGCCTCGAGCACCAGGTTGTACTCGTCCAG; N^{ic} 2095, GCGCCTCGAGGTCCATATGATCCGTGAT; and N^{ic} 1991, GCGCCTCGAGGGCATTCCAGGTCTGTGGC. PCR products were generated with Vent polymerase (New England Biolabs) using the following cycling parameters: denature at 94°C for 1 min, anneal at 58°C for 1 min, and extend at 72°C for 2 min (25 cycles). PCR products were ligated into pBp283, a pBabe-puro derived retroviral vector containing a C-terminal Myc epitope tag (EQKLISEEDL). Sequences for all constructs were verified by automated sequencing. Inserts containing the Myc epitope tag were subsequently subcloned into pcDNA3.1 (Invitrogen).

Site-directed mutagenesis of N^{ic} was accomplished using the QuickChange mutagenesis system (Stratagene) according to the manufacturer's protocol. The following primer combinations were used to introduce point mutations within the fourth ankyrin repeat: N^{ic}ANKm1 top, CGCATGCATGATGCGACGGCGCCA GCGATCTGGCTGCC; N^{ic}ANKm1 bottom, GGCAGCCAGGATCGCTGGC GCGCTCGCATCATGATGCG; N^{ic}ANKm2 top, GACGCCACTGATCTGG AATTCCGCTGGCGTGGAGG; and N^{ic}ANKm2 bottom, CTCCACGGCCA GGCGGAATTCCAGGATCAGTGGCGTC. Substituted codons are in bold. A 10-amino-acid deletion of N^{ic} residues 2105 to 2114 was engineered using the primers: N^{ic}Δ2105-2114 top (CGCGACATCGCACAGGAGGACGAGTACAAC CTGGTG) and N^{ic}Δ2105-2114 bottom (CACCAGGTTGTACTCGTCTCTCTGT GCGATGTCGCG. 5' and 3' boundaries of the deletion are in bold.

A

B

FIG. 1. Schematic representation of N^{ic} deletion constructs used in this study. (A) Constructs were designed to delete apparent secondary structure motifs, namely, C-terminal PEST, OPA, and ANK domains and a putative NLS. N-terminal deletions (N^{ic}ΔR) were designed to remove putative NLSs and the binding site for CBF1 (RAM domain). All constructs have been engineered with a C-terminal Myc epitope tag to facilitate detection (m). The N terminus begins at residue 1759 in N^{ic} constructs and at residue 1848 in N^{ic}ΔR constructs. Indicated are the residue numbers from full-length human Notch1, which form the C-terminal boundaries of all deletion constructs. Transformation potential refers to the ability of these constructs to elicit focus formation. (B) Verification of N^{ic} deletion mutant expression in transiently transfected Bosc23 cells. Bosc23 cells were transfected with the indicated N^{ic} expression vector as described in Materials and Methods. Cell lysates were analyzed for protein expression by Western blotting against the Myc epitope with 9E10. Indicated above each lane is the residue number that forms the C-terminal boundaries for each deletion construct (top, N^{ic} deletion constructs; bottom, N^{ic}ΔR deletion constructs). Molecular mass markers are indicated to the left.

N^{ic} constructs containing either a nuclear export signal (NES) or a nuclear localization signal (NLS) were constructed by subcloning N^{ic} fragments into modified pBp283 vectors. To generate pBp283NES, oligonucleotides encoding the following amino acids were annealed and ligated into pBp283: LALKLA GLDLEQKLISEEDL (NES sequence is in bold and Myc epitope is underlined [92]). pBp283NLS was constructed to encode the following residues:

PKKKRKVEQKLISEEDL (NLS sequence is in bold, and Myc epitope is underlined [39]).

Cell culture and transformation assays. Generation of baby rat kidney cells immortalized with E1A (RKE cells) has been previously described (8). RKE, HeLa, Bosc23 (68), and 293T cells were incubated at 37°C in 5.5% CO₂ and propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml (Life Technologies). For focus formation assays 10⁶ RKE cells were seeded onto 100-mm-diameter dishes 12 h prior to transfection; 10 µg of pBp283 expression construct DNA was diluted in 1.5 ml of OptiMEM (Life Technologies) containing 15 µl of Lipofectamine (Life Technologies) and incubated with cells for 8 h at 37°C in 5.5% CO₂ in a total volume of 6.5 ml of OptiMEM. At 48 h posttransfection, cells were trypsinized and split; half of the cells were selected in puromycin (2 µg/ml) to score transfection efficiency, while the remaining half were maintained in culture and fed fresh medium twice per week. After 2 weeks, the puromycin-selected colonies were fixed in methanol and stained with a solution of 0.5% methylene blue in 70% isopropanol, and drug-resistant colonies were counted. After 4 weeks, the focus assay plates were processed as described above and foci were counted. Focus formation efficiency was calculated as the number of foci formed divided by the number of puromycin-resistant colonies for each assay.

Clonal lines of transformed RKE cells were established by trypsin dissociation of foci isolated with cloning cylinders. Isolated cells were propagated in DMEM supplemented with the appropriate selectable marker (2 µg of puromycin per ml or 400 µg of neomycin per ml). Polyclonal cell lines for mutant constructs were established by transfection of expression vector DNA and subsequent drug selection. After 2 weeks, drug-resistant colonies were pooled and propagated under selection conditions.

Transformed RKE cells were assayed for anchorage-independent growth using the following protocol. A total of 5.0×10^3 cells from the indicated stable cell lines were suspended in 6 ml of DMEM containing 20% FBS and 0.35% low-melting-point agarose. The suspension was overlaid onto a 6-ml base of DMEM, 10% FBS, and 0.7% low-melting-point agarose in 60-mm-diameter dishes. Plates were incubated at 37°C in 5.5% CO₂ for 3 weeks and then photographed with a Zeiss IM-35 inverted microscope at a magnification of $\times 100$.

Protein expression analysis. For transient expression, 3.0×10^6 Bosc23 or 293T cells were transfected with 5 µg of the indicated plasmid DNA using calcium phosphate precipitation. At 48 h posttransfection, cell lysates were prepared in standard lysis buffer (150 mM NaCl, 50 mM HEPES [pH 7.4], 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 1% NP-40) supplemented with the protease inhibitors Pefabloc (2 mM), leupeptin (5 µg/ml), and aprotinin (2 µg/ml). Proteins were separated on sodium dodecyl sulfate (SDS)-8% polyacrylamide gels and immobilized on a nitrocellulose membrane (Schleicher & Schuell) by wet transfer, and equal loading was verified by staining with Ponceau S. Notch proteins were detected with the indicated antibodies using standard immunoblotting procedures. Proteins were visualized with enhanced chemiluminescence (ECL kit; Amersham) and exposure to X-ray film.

Indirect immunofluorescence (IF). Cells expressing the indicated N^{ic} mutant were seeded at 5.0×10^4 cells per well on four-chamber glass slides (LabTech) 12 h prior to processing. Unless noted otherwise, all steps were performed at 4°C. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then fixed for 30 min in 3% paraformaldehyde. Cells were washed in PBS supplemented with 3% FBS, permeabilized for 5 min in 0.2% Triton X-100, and washed in PBS-3% FBS. Cells were incubated for 1 h at room temperature in a blocking solution consisting of PBS-3% FBS and 0.5% Tween 20. Where indicated, either 9E10 (1:500) or bTAN15A (1:2) diluted in PBS-3% FBS was added to each chamber and incubated with rocking at room temperature for 1 h. Slides were washed in PBS-3% FBS and were then incubated with an appropriate Cy3-conjugated secondary antibody (1:1,000 dilution) for 30 min at room temperature. Following final washes, cells were mounted in 70% glycerol containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (DAPI; 0.1 µg/ml), and N^{ic} proteins were visualized and photographed on a Zeiss Axiophot fluorescent microscope with a Hamamatsu digital camera at a magnification of $\times 400$.

Luciferase reporter gene assay. A luciferase reporter plasmid (8x-CBF1-luc) containing eight copies of the CBF1 DNA binding consensus sequence (CGTG GGAA) cloned into the pGL2 reporter vector (Promega) (21) (provided by P. D. Ling, Baylor College of Medicine) was used to analyze the ability of N^{ic} deletion constructs to interact with CBF1 within cells. For luciferase activity, 2.5×10^5 HeLa cells were seeded in six-well plates and were cotransfected with 400 ng of 8x-CBF1-luc, 400 ng of RL-TK (renilla luciferase control plasmid; Promega), and 800 ng of the indicated expression plasmid. Cells were transfected using 8 µl

of Lipofectamine in a total volume of 2 ml of OptiMEM; 48 h posttransfection, cells were lysed and luciferase and renilla light units were measured in a Xylux Femptomaster FB 12 luminometer according to manufacturer's suggested protocol for the Dual Luciferase assay (Promega). Luciferase values were corrected for transfection efficiency by dividing luciferase light units by renilla light units (expressed as relative luciferase units).

Immunoprecipitation and GST-CBF1 pull-down from stable N^{ic}-expressing lysates. Whole cell lysates were made from stable RKE cell lines expressing the indicated mutant construct. Approximately 2×10^7 cells were washed twice in ice-cold PBS, scraped into centrifuge tubes, collected by centrifugation, and lysed on ice for 20 min in 1 ml of standard lysis buffer. Cell debris was cleared by centrifugation for 30 min at $10,000 \times g$ in a Heraeus benchtop 4°C centrifuge. The resulting supernatant was divided into two 500-µl aliquots. To determine protein expression, one aliquot was immunoprecipitated with N^{ic} polyclonal antiserum 925, a rabbit polyclonal antiserum directed against residues 1759 to 2095 of human N^{ic}. Immune complexes were collected on protein A-Sepharose beads, washed in standard lysis buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on an 8% gel, and N^{ic} proteins were detected by Western blotting against the Myc epitope. The other 500-µl aliquot was precleared for 2 h with 10 µg of glutathione S-transferase (GST)-Sepharose beads. Beads were removed following brief centrifugation, and the lysates were then split into two equal aliquots (250 µl); one received approximately 10 µg of GST-CBF1 fusion protein-bound beads, and the other received approximately 10 µg of GST-bound beads. Complexes were formed at 4°C with constant rocking for 8 h. Following incubation, beads were washed five times in standard lysis buffer, and protein complexes were separated by SDS-PAGE (8% gel) N^{ic} proteins were detected by Western blotting against the Myc epitope.

RESULTS

Construction and expression of human Notch1 deletion mutants. Previously we reported that ectopic expression of N^{ic} can transform primary baby rat kidney cells in cooperation with E1A or a cell line immortalized by E1A (RKE). Moreover, cells transformed by N^{ic} exhibit anchorage-independent growth and are tumorigenic in nude mice (8).

To determine the functional domains in N^{ic} that are necessary and/or sufficient for neoplastic transformation, we generated a series of N- and C-terminal deletion mutants of N^{ic} (see Materials and Methods for details; Fig. 1A). The boundaries of these deletion mutants were designed to systematically eliminate primary sequence motifs in N^{ic}. N^{ic}Δ2444 is deleted for the entire PEST region at the C-terminal tail of N^{ic}. N^{ic}Δ2358 is deleted of sequences that encode both the PEST and OPA motifs. N^{ic}Δ2202 is deleted of 156 additional residues N terminal to the OPA motif and terminates adjacent to a putative NLS. An additional C-terminal truncation (N^{ic}Δ2095) removes all 461 amino acids C terminal to the ankyrin repeat (ANK) domain, leaving the ANK domain sequences intact. The sole N-terminal deletion (N^{ic}ΔR) removes two putative NLSs and the binding site for the mammalian Su(H) homologue CBF1 (RAM domain). Deletion of the RAM domain has previously been shown to abolish the ability of N^{ic} to interact with CBF1 (4, 31, 75, 84). C-terminal deletions were made in the context of both the full-length N^{ic} and the N-terminally deleted form of N^{ic}. All deletion mutants encode a C-terminal Myc epitope tag for detection with 9E10.

To verify that these constructs expressed stable proteins of the anticipated size, expression plasmids were transiently transfected into the retroviral packaging cell line Bosc23. Western blot analysis of cellular lysates from transfected Bosc23 cells revealed that all deletion constructs expressed the predicted size protein (Fig. 1B).

FIG. 2. Deletion analysis maps the minimal N^{ic} transforming domain. (A) N^{ic} deletion construct focus assay plates (top row) with corresponding puromycin selection plates (bottom row); (B) N^{ic}ΔR deletion construct focus assay plates (top row) with corresponding puromycin selection plates (bottom row). Transfected plasmid DNA is indicated above each set of plates (N^{ic} and N^{ic}ΔR mutant constructs are diagrammed in Fig. 1A). Four weeks posttransfection, the plates were fixed and stained, and foci were counted as described in Materials and Methods. (C) Expression of N^{ic} mutant proteins in RKE cell lines. Transformed RKE cell lysates were immunoprecipitated with N^{ic} polyclonal antiserum 925; proteins were detected by Western blotting with 9E10. (D) N^{ic}-transformed RKE cell lines exhibit anchorage-independent growth in soft agar. Stable RKE cell lines were suspended in 0.35% agarose and monitored for growth; 3 weeks after plating, colonies were photographed at a magnification of $\times 100$. (A) Parental RKE cell line; (B) RKE cell line expressing GFP; (C) N^{ic}; (D) N^{ic}ΔR; (E) N^{ic}Δ2444; (F) N^{ic}ΔR-2444; (G) N^{ic}Δ2202; (H) N^{ic}ΔR-2202.

TABLE 1. Focus formation in RKE cells transfected with N^{ic} or N^{ic} mutant derivatives

Construct	Total foci formed ^a	Total puromycin-selected colonies ^b	% Focus formation ^c
Vector	5	1,610	0.3
N ^{ic}	523	1,116	46.9 ± 1.1
N ^{ic} ANKm1	0	883	0.0
N ^{ic} ANKm2	1	862	0.2
N ^{ic} Δ2105–2114	0	628	0.0
N ^{ic} Δ2444	514	998	51.5 ± 6.4
N ^{ic} Δ2444NES	39	298	13.1 ± 1.4
N ^{ic} Δ2444NLS	318	578	55.1 ± 1.9
N ^{ic} Δ2358	684	1,019	67.1 ± 4.9
N ^{ic} Δ2202	349	807	43.2 ± 5.0
N ^{ic} Δ2171	132	729	18.1 ± 2.1
N ^{ic} Δ2120	145	828	17.5 ± 0.6
N ^{ic} Δ2095	1	884	0.1
N ^{ic} Δ1991	2	928	0.2
N ^{ic} ΔR	268	830	32.3 ± 1.3
N ^{ic} ΔR-2444	395	948	41.7 ± 0.5
N ^{ic} ΔR-2444NES	5	172	3.0
N ^{ic} ΔR-2444NLS	171	304	56.0 ± 6.6
N ^{ic} ΔR-2358	670	1,169	57.3 ± 5.6
N ^{ic} ΔR-2202	343	1,015	33.8 ± 3.5
N ^{ic} ΔR-2095	0	1,147	0.0
N ^{ic} ΔR-1991	2	1,278	0.2

^a Total number of foci scored 4 weeks after transfection of RKE cells with expression vector DNA in at least three independent experiments.

^b Total number of drug-resistant colonies scored 2 weeks after selection in puromycin in at least three independent experiments.

^c Calculated as total foci formed/total puromycin-resistant colonies for each experiment. Mean ± standard deviation for at least three independent experiments is shown.

The 354 C-terminal amino acid residues encoding the OPA and PEST domains are dispensable for neoplastic transformation. To assay for focus formation, plasmids encoding N^{ic} C-terminal deletion mutants expressed from a retroviral long terminal repeat were transfected into RKE cells. At 48 h post-transfection, cells were harvested and split into two plates. Half of the cells were plated into medium containing puromycin (2 μg/ml) to select for the linked drug resistance marker (Fig. 2A, bottom row). Cells were maintained in culture for 2 weeks until drug-resistant colonies were detected. These plates were used to control for total number of transfected cells and to determine transformation efficiency for each deletion construct (Table 1). The remaining transfected cells were plated and monitored for focus formation as described in Materials and Methods (Fig. 2A, top row).

Deletion of 354 C-terminal residues flanking the putative NLS (N^{ic}Δ2202) did not greatly affect ability of N^{ic} to induce foci. Cells transfected with N^{ic}Δ2202 formed foci with similar efficiency as cells transfected with N^{ic} (Table 1); however, N^{ic}Δ2202-induced foci were consistently smaller than N^{ic}-induced foci (Fig. 2A). We found that a C-terminal deletion that removes both the OPA and PEST domains (N^{ic}Δ2358) resulted in an increase from 46.9% to 67.1% in the efficiency of focus formation compared to full-length N^{ic} (Table 1). Although more efficient in transformation, the foci resulting from transfection of RKE cells with N^{ic}Δ2358 are indistinguishable in appearance from N^{ic}-induced foci (Fig. 2A, top row). Removal of the PEST domain (N^{ic}Δ2444) had no effect on N^{ic}-induced focus formation (Table 1; plate not shown). In contrast, N^{ic}Δ2095 resulted in a complete loss of transformation when transfected into RKE cells (Fig. 2A). Transfection efficiencies for these constructs were essentially equal as determined by selection in puromycin (Fig. 2A, bottom row).

FIG. 3. The TFD is sensitive to mutation. (A) Schematic diagram of additional mutations designed within the TFD (see text). All constructs have been engineered with a C-terminal Myc epitope tag to facilitate detection (m). The N terminus begins at residue 1759 in all N^{ic} mutant constructs; C-terminal boundaries of deletion mutants (N^{ic}Δ2171 and N^{ic}Δ2120) are indicated. Full-length mutant constructs terminate at residue 2556. Letters (AxAXA in N^{ic}ANKm1 and EF in N^{ic}ANKm2) correspond to amino acid substitutions made within the fourth ankyrin repeat; a 10-amino-acid deletion (N^{ic}Δ2105–2114) is represented by a gap in full-length N^{ic}. Transformation potential is indicated as + for strong, +/- for weak, and - for negative. (B) Focus assay plates stained 4 weeks after transfection of RKE cells with the indicated N^{ic} mutant constructs.

The RAM domain of N^{ic} is not required for focus formation or growth in semisolid medium. We determined if deletion of amino-terminal NLSs and the binding site for the transcriptional regulatory protein CBF1 (RAM domain) had any consequence on the ability of N^{ic} to transform RKE cells. N^{ic} constructs comprised of both C-terminal and N-terminal deletions were assayed for focus formation (Fig. 2B, top row). Each N^{ic}ΔR mutant was approximately 70 to 85% as efficient in focus formation as their cognate N^{ic} deletion clone which contains the RAM domain (Table 1). The growth characteristics of N^{ic}ΔR-induced foci are equivalent to N^{ic} deletion constructs pictured in Fig. 2A.

To determine protein expression and the ability of the transformed cells to form colonies in semisolid media, individual foci were isolated for each transforming N^{ic} mutant. Figure 2C shows the results of Western blot analysis of cellular lysates

FIG. 4. All N^{ic} deletion mutants exhibit nuclear staining regardless of neoplastic properties or NLS deletions. (A) Polyclonal RKE cell lines for each indicated N^{ic} deletion mutant immunostained with 9E10 and visualized with a Cy3-conjugated secondary antibody (left); nuclei are stained with DAPI (right). (B) Nuclear localization is not sufficient for focus formation. Shown are polyclonal RKE cell lines for each indicated N^{ic} deletion or site-directed mutant. N^{ic}Δ2171 and site-directed mutant RKE cell lines (N^{ic}ANKm1, N^{ic}ANKm2, and N^{ic}Δ2105–2114) are immunostained with 9E10 and 15A, respectively, and visualized with a Cy3-conjugated secondary antibody (left); nuclei are indicated by staining with DAPI (right).

from transformed cell lines. All isolated foci expressed proteins of the predicted size, indicating that expression of N^{ic} deletion constructs are responsible for the observed transformed phenotype.

Cells from transformed RKE lines were seeded into semi-solid media to assay for anchorage-independent growth. Results obtained from this analysis revealed that all of the N^{ic} and N^{ic}ΔR deletion mutants that formed foci were equally capable of forming colonies in soft agar (Fig. 2D). The parental RKE cells and cells stably expressing green fluorescent protein (GFP) failed to form colonies (Fig. 2D). No apparent difference in colony size was observed between N^{ic} and N^{ic}ΔR mutant constructs. Furthermore, RKE cells expressing N^{ic}ΔR were tumorigenic when injected into nude mice (data not shown).

The results obtained from focus formation analysis and soft agar assays indicate that the minimal transformation domain of N^{ic} consists of 355 amino acids encompassing residues 1848 through 2202. These sequences encode the ANK domain and 107 additional C-terminal residues. The minimal transforma-

tion domain will subsequently be referred to as the TFD (transformation domain).

N^{ic} TFD is sensitive to mutation. To define elements within the TFD that are required for focus formation, we engineered C-terminal deletion mutants in the region between N^{ic}Δ2202 and N^{ic}Δ2095. In addition, we constructed a small internal deletion and two site-directed mutations within the context of N^{ic} (Fig. 3A). N^{ic}Δ2171, which is deleted of only 31 N-terminal amino acid residues from N^{ic}Δ2202, was severely impaired for focus formation (Fig. 3B). Transfection of RKE cells with this construct resulted in only 18.1% efficiency of focus formation (Table 1). However, the size of foci generated with N^{ic}Δ2171 was greatly reduced compared to that of N^{ic}Δ2202 (Fig. 3B; compare to Fig. 2A). N^{ic}Δ2120, which expresses a mutant protein missing an additional 51 amino acids N terminal to 2171, resulted in a phenotype identical to that of N^{ic}Δ2171 (Table 1; focus plate not shown).

N^{ic}Δ2105–2114 has a 10-amino-acid deletion (2105-RMHHDVRL-2114) located between N^{ic}Δ2120 (a weakly transforming

FIG. 5. Deletion of the RAM domain from N^{ic} constructs results in loss of CBF1-responsive reporter activity and abolishes binding. (A) HeLa cells were transiently transfected with the indicated N^{ic} expression vector (0.8 μ g), 8x-CBF1-luc plasmid (0.4 μ g), and RL-TK plasmid (0.4 μ g). At 48 h posttransfection, luciferase values were determined for each construct and normalized as described in Materials and Methods (given as relative luciferase units). Shown are the results from one experiment done in triplicate which are representative of multiple assays performed with each deletion construct. (B) N^{icAR} mutant constructs do not physically associate with GST-CBF1 fusion polypeptide. Cellular lysates were made from stable RKE cell lines expressing the indicated N^{ic} construct. Lysates were incubated with either GST-CBF1 beads (lanes P) or GST beads (lanes C). N^{ic} deletion constructs were detected by Western blotting against the Myc epitope with 9E10. An equivalent amount of each lysate was immunoprecipitated with anti-Notch1 polyclonal antiserum 925 and detected by Western blotting against the Myc epitope (shown in Fig. 2C). Molecular mass markers are shown to the left.

construct) and N^{icΔ2095} (a nontransforming construct). Deletion of these 10 amino acids from N^{ic} is sufficient to eliminate transforming activity as determined by the focus assay (Fig. 3B). The requirement for an intact ANK domain was determined by making two separate mutants (N^{icANKm1} and N^{icANKm2}) that encode amino acid substitutions in the fourth ANK repeat (40, 42, 43, 79). N^{icANKm1} has a three-amino-acid substitution that changes the N^{ic} primary sequence from 1995-DGTTPLI-2001 to 1995-DATAPAI-2001. In N^{icANKm2}, amino acids 2003 to 2005 are changed from 2002-LAAR-2006 to 2002-LEFR-2006. Both of these ANK mutants were incapable of transforming RKE cells (Fig. 3B).

Transforming and nontransforming mutants of N^{ic} localize to the nucleus. Subcellular localization of N^{ic} constructs in RKE cells was determined by IF in polyclonal cell lines expressing N^{ic} mutant constructs. For IF, cells were seeded onto plastic chamber slides and N^{ic} mutant proteins were detected using 9E10 and visualized with a Cy3-conjugated secondary antibody as described in Materials and Methods (Fig. 4).

Previously we reported that N^{ic} is primarily localized to the nuclei of RKE cells (8). Analysis of the C-terminal deletion mutants revealed that all mutant proteins resided primarily within the nucleus to the same extent as N^{ic} (Fig. 4A). In contrast, deletion of both N-terminal putative NLSs and the RAM domain (N^{icAR}) resulted in an increase of cytoplasmic staining, though each N^{icAR} construct retained prominent nuclear immunoreactivity.

When subcellular localization was examined for weakly transforming and nontransforming N^{ic} mutants, we observed that all N^{ic} mutant proteins localized to the nucleus regardless of their ability to induce focus formation in RKE cells (Fig. 4B).

Activation of CBF1 is not required for transformation by Notch proteins. Activation of CBF1-dependent transcription is considered to be an important component of the Notch signal transduction pathway. We analyzed the requirement of CBF1-mediated transcriptional activation using transient transfection assays in HeLa cells (Fig. 5A). Transfection of 8x-CBF1-luc with a GFP expression plasmid or wild-type Notch1 resulted in low basal activity. In contrast, transfection of a N^{ic} expression vector resulted in a 45-fold increase in luciferase activity over that obtained with GFP (expressed as relative luciferase units in Fig. 5A). Transfection of vectors expressing deletion mutants N^{icΔ2202} and N^{icΔ2120} resulted in a 30- and 6-fold increases of luciferase activity compared to the GFP control.

Deletion of the RAM domain in N^{icAR} mutants abolished their ability to activate CBF1-mediated transcription from the 8x-CBF1 reporter (Fig. 5A). All N^{icAR} mutants tested in this assay exhibited luciferase activities at basal levels, demonstrating loss of functional association with CBF1.

To establish that N^{icAR} constructs do not physically associate with CBF1, we tested for protein-protein binding interactions

FIG. 6. Site-directed mutations within the TFD abrogate CBF1 reporter activity without affecting CBF1 binding. (A) HeLa cells were transfected with the indicated N^{ic} mutant construct (see text), and luciferase activity was measured as described in Materials and Methods. (B) Mutations within the TFD do not affect CBF1 binding interactions. Lysates were made from transiently transfected Bosc23 cell lines expressing the indicated N^{ic} constructs and were incubated with either GST-CBF1 beads (lanes P) or GST beads (lanes C). N^{ic} deletion constructs were detected by Western blotting against the Myc epitope with 9E10 (top). Then 10% of each Bosc23 lysate was analyzed for protein expression by Western blotting with 9E10: N^{ic}, lane 1; N^{ic}ΔR, lane 2; N^{ic}ANKm1, lane 3; N^{ic}ANKm2, lane 4 and N^{ic}Δ2105-2114, lane 5 (bottom). Molecular mass markers are shown to the left.

using a GST pull-down assay. Whole cell lysates prepared from transformed RKE cells were incubated with either GST or GST-CBF1 adsorbed to glutathione-agarose beads and processed as described in Materials and Methods. Using a GST-CBF1 fusion protein, we could affinity precipitate only N^{ic} deletion proteins that encode the RAM domain (Fig. 5B). Deletion of the RAM domain completely abolished the ability of N^{ic}ΔR proteins from transformed RKE cells to physically interact with GST-CBF1. To control for the presence of Notch proteins in this assay, an equivalent amount of each lysate was immunoprecipitated with the anti-Notch1 polyclonal antiserum 925 and detected by Western blotting against the Myc epitope (Fig. 2C).

CBF1 binding to N^{ic} is not sufficient for transformation. Mutational analysis of the TFD revealed that site-directed

mutations in the fourth ANK repeat (N^{ic}ANKm1 and N^{ic}ANKm2) and a 10-amino-acid deletion (N^{ic}Δ2105-2114) resulted in transformation-defective N^{ic} proteins (Fig. 3). Since each of these constructs encodes the RAM domain, we determined if these N^{ic} site mutants maintained molecular interactions with CBF1. Previous studies have shown that the N^{ic}ANKm1 and N^{ic}ANKm2 point mutations ablate N^{ic} mediated transcriptional activation of a CBF1-responsive reporter (35, 40, 48). As predicted, co-transfection of N^{ic}ANKm1 and N^{ic}ANKm2 expression plasmids with 8x-CBF1-luc resulted in negligible reporter activity in HeLa cells (Fig. 6A). Similarly, when assayed for CBF1 reporter activity, N^{ic}Δ2105-2114 also failed to appreciably activate transcription from the reporter gene (Fig. 6A).

To determine if loss of reporter activity was due to a failure of N^{ic} mutants to physically associate with CBF1, we assayed for protein binding using the GST-CBF1 pull-down experiment (Fig. 6B). Whole cell lysates were prepared from Bosc23 cells transiently transfected with either N^{ic}ANKm1, N^{ic}ANKm2, or N^{ic}Δ2105-2114 and assayed for GST-CBF1 binding as described in Materials and Methods. In contrast to deletion of the RAM domain, N^{ic}ANKm1, N^{ic}ANKm2, and N^{ic}Δ2105-2114 mutants bound CBF1 in GST pull-down experiments as well as N^{ic} (Fig. 6B). This result indicates that binding CBF1 is not sufficient to either activate transcription from a reporter or induce focus formation.

Nuclear localization is required for neoplastic transformation. We showed that N^{ic} and all deletion mutants of N^{ic} are primarily localized to the nucleus of RKE cells, regardless of

FIG. 7. Nuclear localization is required for N^{ic} -induced transformation of RKE cells. (A) Derivatives of both $N^{ic}\Delta 2444$ and $N^{ic}\Delta R-2444$ containing either a PKI-like nuclear export sequence (+NES) or the SV40 large T NLS (+NLS) were constructed and assayed for focus formation in RKE cells as described in Materials and Methods. The transfected expression vector is indicated above each plate. (B) Expression was documented from transiently transfected 293T cells for each indicated construct. Cell lysates were collected and subjected to SDS-PAGE (8% gel), and proteins were visualized by Western blotting with 9E10. Molecular mass markers are shown to the left.

their ability to transform RKE cells (Fig. 4). To address the significance of nuclear localization in N^{ic} -mediated transformation, we used a mechanism to efficiently control subcellular localization of N^{ic} proteins. We created a series of $N^{ic}\Delta 2444$ and $N^{ic}\Delta R-2444$ constructs that encode at their C termini either a cyclic AMP-dependent protein kinase inhibitor (PKI)-like NES (amino acids LALKLAGLDL [92]) or the simian virus40 large T NLS (amino acids PKKKRKV [39]) followed by the Myc epitope tag. Addition of an ectopic NES should result in active export of $N^{ic}\Delta 2444$ and $N^{ic}\Delta R-2444$ from the nucleus to the cytoplasm. The NLS should not have any effect on nuclear localization of $N^{ic}\Delta 2444$ but should rescue the loss of nuclear localization associated with the Δ RAM mutation in $N^{ic}\Delta R-2444$.

To test the ability of these constructs to induce focus formation, we transfected plasmids expressing either the NES or NLS derivatives into RKE cells and performed the focus assay. The ability of $N^{ic}\Delta 2444$ to form foci in RKE cells was significantly diminished by addition of the NES (Fig. 7A, top row: +NES, Table 1). In contrast, addition of the NLS had no effect on transformation by $N^{ic}\Delta 2444$ (Fig. 7A, top row, +NLS; Table 1). When combined with the N-terminal RAM domain dele-

tion in $N^{ic}\Delta R-2444$, the effect of the NES was more pronounced, resulting in a dramatic loss of focus formation (Fig. 7A, bottom row, +NES; Table 1). Addition of an NLS to $N^{ic}\Delta R-2444$ increased transformation efficiency to that observed with $N^{ic}\Delta 2444$ (Fig. 7A, bottom row, +NLS; Table 1). This indicates that the reduction in transformation efficiency associated with the Δ RAM Notch mutants is a result of decreased nuclear localization and not with a loss of CBF1 binding.

NES constructs show reduced levels of N^{ic} in the nucleus. Subcellular localization of +NES and +NLS constructs was determined by IF in polyclonal RKE cell lines. Addition of the NES to $N^{ic}\Delta 2444$ resulted in more diffuse total cell distribution compared to the exclusively nuclear localization of $N^{ic}\Delta 2444$; however, it retained prominent nuclear staining (Fig. 8A, +NES). Addition of the NLS had no effect on subcellular localization of $N^{ic}\Delta 2444$ (Fig. 8A, +NLS). Analysis of $N^{ic}\Delta R-2444$ subcellular localization revealed an increase in cytoplasmic staining compared to $N^{ic}\Delta 2444$ (Fig. 8A). Addition of the NES to $N^{ic}\Delta R-2444$ resulted in a complete exclusion of the protein from the nucleus (Fig. 8A, +NES). In contrast, addition of the NLS to $N^{ic}\Delta R-2444$ rescued the decrease in nuclear localization associated with the RAM domain deletion (Fig. 8A, +NLS).

To authenticate that the $N^{ic}\Delta R-2444$ NES construct was being exported via the nuclear export machinery, we treated RKE cell lines with the nuclear export inhibitor leptomycin B (Fig. 8B) (46). Treatment of the $N^{ic}\Delta R-2444$ NES expressing polyclonal cell line with leptomycin B resulted in nuclear accumulation of the protein, whereas treatment with vehicle had no effect on subcellular localization as determined by indirect IF (Fig. 8B, center column, +NES). Subcellular localization of $N^{ic}\Delta R-2444$ and the NLS derivative was not affected by treatment with either leptomycin B or vehicle (Fig. 8B, right and left columns). Taken together, these results indicate the NES derivatives are functioning through the NES pathway and fail to proficiently transform RKE cells due to a lack of nuclear localization.

To determine if localization of $N^{ic}\Delta R-2444$ NES and $N^{ic}\Delta 2444$ NES was truly reflective of the subcellular distribution of these proteins and not a result of selecting polyclonal RKE cell lines, we performed IF on transiently transfected HeLa cells. HeLa cells were transfected with the indicated expression vectors, and subcellular localization of the N^{ic} proteins was determined as described above (Fig. 8C). As we demonstrated for RKE cells, $N^{ic}\Delta 2444$ NES retained prominent nuclear localization and to a lesser extent cytoplasmic localization. The subcellular distribution of $N^{ic}\Delta 2444$ NES was now remarkably similar to that of $N^{ic}\Delta R-2444$, indicating that addition of an NES is equivalent to removal of the RAM-NLS (Fig. 8C). Addition of an NES to $N^{ic}\Delta R-2444$ resulted in the complete redistribution of the protein to the cytoplasm, consistent with the results obtained with polyclonal RKE lines.

DISCUSSION

N^{ic} functions in the nucleus. Nuclear accumulation is a hallmark of ectopically expressed N^{ic} proteins in diverse cellular systems from invertebrates to vertebrates (2, 57, 58). This property of Notch proteins has confounded interpretations of where within the cell this plasma membrane-spanning receptor exerts its influence. Previously, we have shown that permanently tethering N^{ic} to the plasma membrane as a CD8- N^{ic} fusion protein abolishes N^{ic} transformation activity in RKE cells (8). *Drosophila* expressing myristylated- N^{ic} derivatives that predominantly localize to membranes also have a loss of N^{ic} activity (81). These results support the hypothesis that Notch does not function at the membrane and must be released from the membrane to signal. However, neither of these

approaches was able to discriminate between cytoplasmic or nuclear signaling events required for N^{ic} activity. To resolve the issue of nuclear versus cytoplasmic signaling, we engineered N^{ic} proteins that encode a functional NES at the C terminus. Our hypothesis was that if nuclear localization was required for transformation, addition of an NES to N^{ic} would block nuclear accumulation and thus N^{ic} would fail to transform RKE cells. Our data clearly demonstrate that active export of N^{ic} molecules from the nucleus to the cytoplasm results in loss of transforming activity.

Analysis of the subcellular localization of N^{ic} Δ 2444NES in a polyclonal RKE cell line revealed that this protein retained prominent nuclear localization. When transfected into RKE cells, this molecule is capable of eliciting focus formation, albeit at a lesser efficiency than N^{ic} Δ 2444. However, the N^{ic} Δ R-2444NES clone is completely excluded from the nucleus and is severely compromised in the transformation assay. One explanation for the incomplete penetrance of the N^{ic} Δ 2444NES clone is that this clone retains a strong NLS within the RAM domain that cannot be completely overcome by the addition of a single NES. Recent reports have demonstrated that in cases where proteins are actively exported out of the nucleus, there is a balance between NLS and NES strength (16, 28). This is likely the case in our situation since removal of a strong NLS in the RAM domain in N^{ic} Δ R-2444NES results in a complete loss of nuclear staining. Moreover, analysis of subcellular localization in polyclonal RKE cells might overemphasize nuclear localization. Since we know that N^{ic} Δ 2444NES is capable of transforming RKE cells (at a low level), it is reasonable to suspect that polyclonal lines contain a large percentage of cells that have selected for active nuclear N^{ic} Δ 2444NES proteins. In support of this explanation, transient expression of this clone in HeLa cells reveals a whole cell distribution of N^{ic} Δ 2444NES more intense than what we observe in the polyclonal RKE line (Fig. 8C). Taken together, these results strongly establish that neoplastic transformation of RKE cells by N^{ic} requires nuclear localization.

Recently, we have characterized chimeras of N^{ic} and the hormone binding domain of the estrogen receptor (Notch^{ic}-ER) that requires stimulation with 4-hydroxytamoxifen (OHT) to induce transformation of RKE cells (C. Ronchini and A. J. Capobianco, submitted for publication). In the absence of OHT, the Notch^{ic}-ER chimeras are diffusely distributed throughout the cell and are unable to induce transformation. When stimulated with OHT, Notch^{ic}-ER proteins induce transformation of RKE cells and the proteins accumulate in the nucleus. This supports our argument that nuclear accumulation of N^{ic} is required for its biological effects.

Identification of the N^{ic} transformation domain. Deletion analysis of N^{ic} demonstrates that the C-terminal sequences encoding the PEST and OPA domains are not required for transforming activity. Deletions of these sequences from mouse Notch1 have previously been shown to have no effect of N^{ic} inhibition of granulocytic differentiation or myogenesis (7, 43, 59). Our data also indicate that deletion of the OPA and PEST domains results in an increase in transforming activity, indicating that these domains might mediate negative regulation of N^{ic} within RKE cells.

We have defined the TFD as amino acids 1848 to 2202 of human Notch1. This domain consists primarily of the ankyrin repeats and an additional 107 C-terminal residues. Furthermore, we have shown that the 107 amino acids juxtaposed to the ANK domain are sensitive to mutation, indicating that their integrity as a domain is necessary. Notch molecules corresponding to the TFD are endowed with intrinsic signaling ability in other systems (7, 43, 56, 59, 72, 73, 82), indicating the

conserved role of the ANK domain in N^{ic}-mediated biological effects.

C-terminal deletion and site-directed mutagenesis have implicated the region located immediately downstream of the ANK domain as a region important for transformation. C-terminal deletion of 31 residues into the TFD (N terminal to amino acid 2202) greatly attenuated focus formation, without affecting nuclear localization. Furthermore, a site-directed deletion of 10 amino acids within this region was sufficient to destroy the ability of N^{ic} to transform RKE cells. These results indicate important functional interactions are likely to occur within this region.

Previously we reported that N2^{ic} was considerably weaker as a transforming molecule than N1^{ic} in the focus assay (8). It is likely that sequences within the TFD account for this difference. Sequence comparison between the TFD of Notch1 and the corresponding region in Notch2 reveals that these proteins share approximately 66% identity, considering similarity it is 74%. Interestingly, this region has been reported to mediate differences in signaling between Notch1 and Notch2 in granulocyte differentiation by granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), in that GM-CSF signals through N2 and G-CSF signals through N1 to inhibit differentiation (7).

CBF1 activation is not a critical target for N^{ic}-induced transformation of RKE cells. CBF1-dependent versus independent signaling pathways is a topic of considerable debate within the Notch field (2, 40, 61). Based on published reports, we engineered an N^{ic} mutant with an N-terminal deletion that removes both primary and secondary CBF1 binding sites (N^{ic} Δ R) within the RAM domain (31, 32, 48, 61, 84). Consistent with these reports, we demonstrate that N^{ic} Δ R mutants no longer retain the ability to interact with CBF1 from analysis of protein-protein interaction and CBF1 reporter assays. N^{ic} Δ R constructs retain the ability to induce transformation when expressed in RKE cells even though they are unable to interact with CBF1. Based on this evidence, we conclude that transformation of RKE cells by N^{ic} does not require direct activation of CBF1. Moreover, we have demonstrated that the ability of N^{ic} to bind CBF1 is not sufficient to induce transformation. We analyzed several Notch constructs that had point mutations within the ANK domain. These ANK mutants were defective for transformation and for the ability to activate the CBF1 reporter in HeLa cells. These constructs all contained the RAM domain and were able to bind to CBF1 in a GST pull-down experiment (Fig. 6B). Similar ANK mutants have been reported to abolish N^{ic} activation of CBF1-responsive reporter assays (35, 40, 48) and fail to inhibit muscle differentiation *in vitro* (40, 43).

Several lines of evidence indicate that the C terminus of N^{ic} encodes an intrinsic transcriptional activation domain. These observations were primarily made by experiments utilizing either LexA- or GAL4-N^{ic} fusion proteins (31, 41, 48). Data from these reports define the N^{ic} transcriptional activation domain as sequences between the OPA domain and the C-terminal putative NLS (C terminal to amino acid 2202) (48). Our CBF1 reporter data confirm that the C-terminal portion of N^{ic} does contain a transcriptional activation domain, but with one consideration. We show that reporter activity decreases with C-terminal truncations between the OPA domain and the NLS (amino acid 2202), which is in agreement with the above reports. However, our minimal transforming domain (amino acids 1848 to 2202) does not contain the C-terminal residues that have been attributed to the intrinsic transcriptional activation potential of N^{ic}. While not arguing against a role for N^{ic} in transcriptional activation, our data instead imply

FIG. 8. Exclusion from the nucleus correlates with loss of transforming activity. (A) Indicated polyclonal RKE cell lines were processed for IF as described in the text. +NES, clone containing an NES; +NLS, clone containing an NLS. Panels are arranged so that the +NES and +NLS derivatives of the indicated construct follow. N^{IC} constructs are visualized with Cy3 (left); nuclei are stained with DAPI (middle); merged Cy3 and DAPI images are pictured at the right (BOTH). (B) Exclusion from the nucleus depends on active nuclear export. Indicated polyclonal RKE cell lines were plated onto microscope slides and incubated with either the nuclear export inhibitor leptomycin B at 1 ng/ml (+LMB) or with vehicle (−LMB) for 2 h prior to processing for IF. (C) Subcellular localization in transiently transfected HeLa cells. Labels are as indicated for panel A. +NES versions of the construct indicated are to the right.

FIG. 8—Continued.

that amino acids within the TFD (i.e., N-terminal to amino acid 2202) appear to be directly responsible for transcriptional activation.

We have concluded that CBF1 activation is not a requirement for transformation of RKE cells. However, we support the hypothesis that a transcriptional activation domain is required for Notch activity. All of our data indicate that the RAM domain is solely responsible for mediating the interaction with CBF1 and that deletion of this domain does not affect the ability of N^{ic} to transform cells. How can we justify the apparent correlation between loss of CBF1 activity and transformation for the ANK mutants and the 10-amino-acid deletion in the TFD? If we assume that the CBF1 reporter assay in HeLa cells is simply reflective of transcriptional activation and

not of a CBF1 function in RKE cells (i.e., similar to a GAL4 fusion experiment), then we can conclude that the correlation is between transcriptional activation and transformation of RKE cells. Furthermore, we suggest that the transcriptional activation required for transformation is not mediated through CBF1.

A recent report describing in vitro transformation of a mouse mammary epithelial cell line (HC11) by intracellular Notch1 concluded that transformation required the N-terminal putative NLS and the RAM domain (14). Data concerning subcellular localization of N^{ic} constructs of functional association with CBF1 within HC11 cells were not presented. However, the authors did attempt to show activation of a potential cellular target of N^{ic} via CBF1 in N^{ic}-transformed HC11 cells.

The ERBB2 promoter contains CBF1 binding sites and can be activated by N^{ic} expression in reporter assays (10). The study by Dievart et al. was unable to detect elevated ERBB2 message (14), indicating N^{ic} may not target CBF1 repressed genes within HC11 cells to induce transformation. Alternatively, the requirement for CBF1 might be cell type specific in that immortalized mammary epithelial cells but not RKE cells require CBF1 activity. One could imagine that in RKE cells, expression of E1A somehow provides a redundant function for CBF1 activity, and therefore additional CBF1 activity from interaction with N^{ic} is not necessary.

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