

Roles of the ankyrin repeats and C-terminal region of the mouse Notch1 intracellular region

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ABSTRACT

The Notch intracellular region (RAMIC) interacts with a DNA binding protein RBP-J to activate transcription of genes that inhibit cell differentiation. The RAM domain and ankyrin (ANK) repeats of mouse Notch1 RAMIC were shown to be responsible for RBP-J binding and necessary for transactivation. The C-terminal portion of Notch1 RAMIC has also been suggested to be important for transactivation. Using GAL4 fusion constructs, we identified a novel transactivation domain (TAD) between the ANK repeats and the PEST sequence of mouse Notch1. The C-terminal half of mouse Notch2 RAMIC also exhibited TAD activity. Unexpectedly, the RBP-J chimeric protein with the Notch1 TAD failed to activate transcription but the activity was recovered by addition of either the RAM domain or ANK repeats. The results suggest that the activity of Notch1 TAD is repressed by fusion with RBP-J because of the presence of a RBP-J-associated co-repressor(s), which could be displaced by either the RAM domain or ANK repeats. Taken together, mouse Notch1 RAMIC can experimentally be separated into three functional domains: the RAM domain and ANK repeats for RBP-J binding and co-repressor displacement and the C-terminal TAD.

INTRODUCTION

The Notch receptor is a cell surface transmembrane protein that plays an important role in cell fate determination (1,2). The Notch family consists of one member in *Drosophila* (3,4), two (GLP-1 and LIN-12) in nematodes (5,6) and four in mammals (7–11). Notch signal triggered by interaction with the ligand blocks differentiation of stem (or progenitor) cells and keeps them in a proliferative state. Expression of the intracellular region of Notch can mimic the Notch signal because its overexpression causes an antineurogenic phenotype in *Drosophila* (12,13) and suppresses neurogenesis and myogenesis of mammalian precursor cells (14,15). The intracellular region of mammalian Notch was initially isolated as an oncogenic form. N-terminally truncated human Notch1 (TAN-1) was identified in T cell acute lymphoblastic leukemia/lymphoma (16). Subsequently, neoplastic transformation

of various cells by the intracellular regions of mouse Notch1 (17) and of human Notch1 and Notch2 (18) were also reported.

A nuclear DNA binding protein Suppressor of Hairless [Su(H)], and its mammalian homolog RBP-J, function downstream of Notch signaling (19). Notch and Su(H) activate transcription of *Enhancer of split* [E(spl)] complex genes in *Drosophila* (20–22). The intracellular region of mammalian Notch binds to RBP-J in the nucleus (see below) and activates transcription through the RBP-J recognition motifs (YGTGGGAA) (23) in the promoter region of HES-1, a mammalian antineuronal basic helix–loop–helix factor structurally related to *Drosophila* hairy and E(spl) proteins (24), or in TP1 and C promoters, which are called Epstein–Barr virus nuclear antigen 2 (EBNA2)-responsive elements (25–28). The two observations, i.e. physical interaction between the intracellular region of the transmembrane receptor (Notch) and the nuclear protein (RBP-J) and transactivation of genes by the intracellular region of Notch suggest an attractive model for Notch signaling, in which interaction of the Notch extracellular region with the ligand induces a proteolytic cleavage of the Notch intracellular region, resulting in its translocation to the nucleus and interaction with RBP-J (19,24,29–33).

The Notch intracellular region, designated RAMIC, contains several functional motifs. The RAM domain was originally isolated in yeast two-hybrid screening as a molecule that associates with mouse RBP-J (34). The RAM domain consists of 100 amino acids between the transmembrane region and the ankyrin (ANK) repeats of mouse Notch1 and includes a nuclear localization signal (NLS) near the region critical for RBP-J binding (34,35). It has been considered that the RAM domain is important for transactivation activity of RAMIC (36,37) because it is a primary binding domain to RBP-J. The RAM domain was also shown to displace a putative co-repressor from RBP-J (38). Interactions between the RAM domain and Su(H)/RBP-J are evolutionarily conserved (34,39–41) and all the RAM domains of mouse Notch family members are capable of binding to RBP-J *in vitro* (36,42). However, it remains to be seen whether signals transmitted by the four Notch receptors are identical or not.

Another motif important for Notch function is the ANK repeats, which are highly conserved among Notch proteins of various species and are thought to mediate protein–protein interactions. In fact, the ANK repeats of *Drosophila* Notch interact with a cytoplasmic protein Deltex (43). Missense mutations (M1 and M2) in the ANK repeats of mouse Notch1 show loss-of-function phenotypes (14,38). A missense mutation

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in human Notch3 causes a type of stroke and dementia (44), showing their crucial role in Notch function. Although how the ANK repeats are involved in transactivation remains elusive, it has recently been shown that the ANK repeats of mammalian Notch1 interact weakly with RBP-J and therefore the Notch1 RAMIC construct devoid of the RAM domain (i.e. IC) can activate transcription only weakly (35,38). Another role of the ANK repeats in transactivation was indicated in a study of *Caenorhabditis elegans*; the GLP-1 ANK repeats are not only involved in interaction with LAG-1, a nematode homolog of RBP-J, but also act as an autonomous transactivation domain (TAD) (40). In *Xenopus*, the ANK repeats of Notch1 fused to Su(H) were shown to be constitutively active in inducing the expression of *ESR-1*, a gene related to *E(spl)* (41). However, there is no direct evidence demonstrating that the ANK repeats of mammalian Notch possess an autonomous TAD activity.

Downstream of the ANK repeats, there is the second NLS that is found in all mouse Notch proteins except Notch4. The glutamine-rich OPA sequence is conserved between *Drosophila* Notch and mammalian Notch1, but its role in Notch signaling is obscure. The C-terminal PEST sequence found in all Notch proteins seems to be associated with high rates of protein turnover. Deletion analyses have suggested that the C-terminal portion containing the OPA and PEST sequences is important for transactivation activity of RAMIC or IC (38,45), but the precise role of the C-terminal portion has not been elucidated.

In this study, we investigated the region of mouse Notch1 responsible for autonomous transactivation function and localized such activity in the 200 amino acid residues downstream of the second NLS. Moreover, our results suggest that not only the RAM domain but also the ANK repeats seem to be important for relieving the repression of RBP-J, probably by displacing a co-repressor(s).

MATERIALS AND METHODS

Cell culture

COS7, SV40-transformed monkey kidney cells and NIH 3T3 murine fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin. C2C12 murine myoblasts were maintained in DMEM containing 15% FBS, 100 U/ml penicillin and 2 mM L-glutamine.

Construction of Notch and RBP-J expression plasmids

Mouse Notch1 intracellular region constructs of pEFBOSneo were described previously (38) and its related constructs prepared newly for this study are derived from them. GAL4-mouse Notch1 IC fusions were constructed in pBluescript II KS(+) (Stratagene). The *HindIII*-*PstI* fragment of the GAL4 DNA binding domain (amino acids 1-147) excised from pGBT9 (Clontech) was cloned into KS(+) and subsequently the sequence between the *KpnI* and *ClaI* sites was replaced by an adaptor (5'-CTCTAGAACTAGTAT-3') containing *XbaI* and *SpeI* sites [KS(+)-GAL4] for rapid cloning into the *XbaI* site of pEFBOSneo vector. A fragment of Notch1 IC (full-length, 1848-2531) was excised from the yeast expression plasmid (34) with *EcoRI* and then fused, in-frame, downstream of the GAL4 DNA binding domain. GAL4-Notch1 deletion constructs were prepared by ligating *EcoRI*-*XhoI* (1848-2193), *XhoI*-*EcoRI* (2194-2531, Notch1-IC-

Δ N), *XhoI*-*HindIII* (2194-2398), *XhoI*-*SacI* (2194-2293), *SacI*-*HindIII* (2294-2398) and *HindIII*-*EcoRI* (2399-2531) fragments C-terminal to the GAL4 DNA binding domain in-frame. Notch2-IC Δ N (2154-2352), Notch3-IC Δ N (2107-2304) and Notch4-IC Δ N (1817-1964) were obtained by PCR amplification. To amplify the Notch C-terminal fragments, the following primers were used: 5'-CCCGTCCGAATTCCTCGAGTCTCC-3' and 5'-GTGTGATAGGGAAATTCATCTC-3' for Notch2; 5'-CTCTGGACT-CACCACGGCCTTTC-3' and 5'-CAAGAACTTAGGCCATC-ACCTGC-3' for Notch3; 5'-CTGCTGGAATTCGCTGGACCG-AC-3' and 5'-AGTCCGGAGAATTCAGACTCG-3' for Notch4. The amplified fragments of Notch2 and Notch4 were digested with *EcoRI* (*EcoRI* sites shown in italic) and cloned into KS(+)-GAL4. The fragment of Notch3 was inserted into pGEM-T vector-Easy (Promega). It was treated with *EcoRI* and the digested fragment was cloned into KS(+)-GAL4. Sequences of the amplified fragments were confirmed using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) for the automated laser fluorescent sequencer (model 377A; Applied Biosystems). All the GAL4-Notch constructs were cloned into pEFBOSneo vector.

For generation of the chimeric constructs of RBP-J with Notch IC, an adaptor (5'-AGCTTGGATATCTGACTAACTAG-3') containing an *EcoRV* site (italic) and stop codons for three frames (underlined) was inserted in the *HindIII* site of pSG5-Flag-RBP-J-VP16 (46). *StuI*-*EcoRV* (1810-2079), *EcoRV*-*SnaBI* (2080-2531) and *StuI*-*SnaBI* (1810-2531) fragments from the plasmid pCS2+6MT-mNotchIC (14), respectively, were cloned into the above adaptor-containing plasmid.

Transient transfections, luciferase assays and reporter plasmids

Cells were plated in 3.5 cm dishes and co-transfected with plasmid DNA using LipofectAMINE reagent (Gibco BRL). Appropriate amounts of pEFBOSneo carrier DNA were included to make equivalent amounts of total DNA (1-2 μ g) for each transfection. Cells were harvested 40-48 h after transfection and luciferase activities in the cell extracts were measured according to the manufacturer's instructions (Toyo Ink Corp.) in a Berthold luminometer, LumatLB9501. Normalized luciferase activities (luciferase/ β -galactosidase ratio) from all the samples were then compared. Transactivation (fold) means the values when the control is calculated as 1. All the experiments were carried out in triplicate.

Reporter plasmid pGa50-7 contains the minimal β -globin promoter driving the luciferase gene. pGa981-6 was generated by inserting the hexamerized 50 bp EBNA2 response element of the TP-1 promoter in front of the minimal β -globin promoter of pGa50-7 (38). TK-MH 100 \times 4-luciferase is a TK-luciferase reporter plasmid driven by tandem GAL4 binding sites. A plasmid for normalization, pCMX-LacZ, was generated by replacing the *HindIII*-*BamHI* fragment of pCMX-VP16 (new) vector with the *HindIII*-*BamHI* fragment of the β -galactosidase coding region from plasmid p- β gal (Clontech).

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from COS7 cells were prepared according to the methods of Schreiber *et al.* (47) for GAL4 fusion proteins and of Lassar *et al.* (48) for RBP-J-IC fusion proteins. The probe containing a single GAL4 binding site is MH100 (49) and the

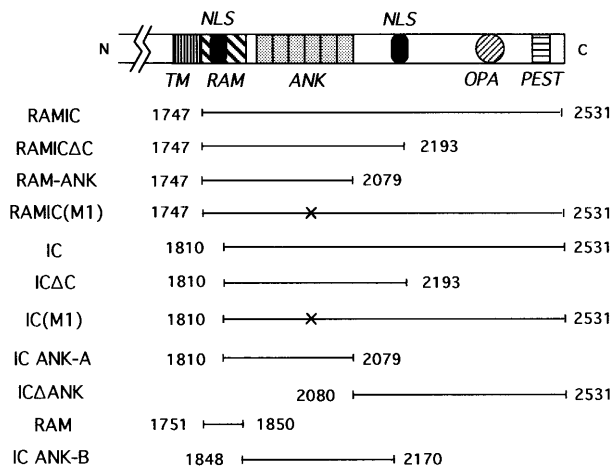


Figure 1. Schematic representation of the mouse Notch1 intracellular region (RAMIC) and its derivatives used in this study. Vertical hatched, diagonal hatched, dotted and horizontal hatched boxes indicate the transmembrane, RAM, ANK (CDC10/ankyrin repeats) and PEST regions, respectively. The diagonal hatched circle and black ellipses show the OPA and nuclear localization signals, respectively. The constructs used in this study are drawn as horizontal lines with amino acid numbers below the ends. The mutation site in the ANK repeats (M1) is indicated by a cross.

probe containing the RBP-J binding sites is O54 (28). The binding reaction for GAL4 fusion proteins contained 20 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM MgCl₂, 10 μM ZnSO₄, 10% glycerol, 0.1 mg/ml bovine serum albumin, 1 μg poly(dI-dC) and 0.5 ng ³²P-labeled probe. The binding reaction for RBP-J-IC fusion proteins contained 20 mM HEPES (pH 7.9), 50 mM NaCl, 1.5 mM MgCl₂, 5% glycerol, 1 μg poly(dI-dC) and 1 ng ³²P-labeled probe. The mixture in a final volume of 30 μl was incubated for 30 min at 20°C and loaded onto a native 4% polyacrylamide gel in a buffer (pH 7.5) containing 6.7 mM Tris, 3.3 mM NaOAc and 1 mM EDTA. After electrophoresis at 130 V for 2.5 h at 4°C, the gels were dried and analyzed using an Imaging Analyzer BAS1500 (Fuji Film).

Immunoblotting

Transfected cells were scraped into PBS (-), resuspended in 1× sample buffer, boiled for 5 min, subjected to 7–14% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane with Semi-Dry (Bio-Rad). Western blotting was performed with anti-myc monoclonal antibody (mAb, 9E10) for Notch-related proteins and with anti-GAL4 polyclonal antibodies (Santa Cruz Biotechnology) for GAL4 fusion proteins. For detection of RBP-J-related proteins, nuclear extracts (described above) and anti-FLAG mAb (M2; Eastman Kodak) were used. The membranes were incubated with the antibodies diluted in 1× PBS (-) containing 2% skimmed milk, washed with PBS (-) containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies against mouse IgG or rabbit IgG. Immunoreactive bands were developed using ECL detection reagent (Amersham) and visualized by exposure to X-ray films.

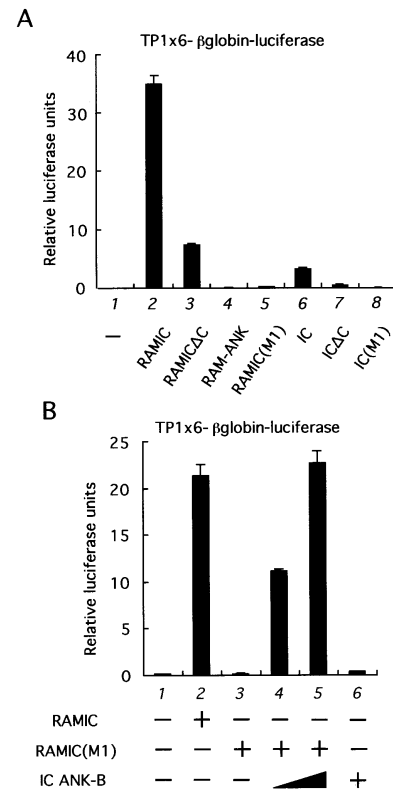


Figure 2. Transactivation activities of RAMIC, IC and their derivatives through endogenous RBP-J. (A) 0.4 μg of each derivative construct of pEFBOSneo-mouse Notch1 RAMIC or IC was co-transfected with 0.2 μg pGa981-6 and 0.1 μg pCMX-LacZ into COS7 cells. Comparable protein expressions of the constructs were confirmed by western blot analysis using anti-myc monoclonal antibody (data not shown). It should be noted that the ANK repeats not only in IC but also in RAMIC are critical for transactivation mediated by endogenous RBP-J. (B) The ANK repeats activate transcription when expressed *in trans* with RAMIC (M1). 0.15 μg pEFBOSneo-RAMIC or -RAMIC (M1), 0.2 μg pGa981-6 and 0.05 μg pCMX-LacZ were co-transfected into COS7 cells with increasing amounts (0, 0.3 and 1.5 μg) of pEFBOSneo-IC ANK-B (1848–2170). The abolished activity of RAMIC (M1) was restored to the wild-type level by co-expression of the construct containing the wild-type ANK repeats (IC ANK-B), which themselves do not show transactivation activity.

RESULTS

Effects of C-terminal deletions on transactivation activities of mouse Notch1 RAMIC and IC

C-terminally deleted RAMIC and IC constructs of mouse Notch1 were generated (Fig. 1) and their transactivation activities were examined using the luciferase reporter gene construct containing the hexamerized RBP-J binding motifs of the TP-1 promoter and the β-globin minimal promoter. RAMIC markedly activated transcription (Fig. 2A, lane 2) while IC showed one-tenth of the transactivation activity of RAMIC (lane 6), because IC lacks the RAM domain, as reported previously (38). Endogenously expressed RBP-J appears to be involved in transactivation by RAMIC and IC because their activities were inhibited by overexpression of a DNA binding-defective mutant of RBP-J (38; data not shown). The mutant (R218H) would compete with endogenous RBP-J for binding to RAMIC or IC. The activities of

RAMIC Δ C and IC Δ C, the C-terminally truncated forms of RAMIC and IC, were reduced to one-tenth and one-fifth of their parental forms, respectively (lane 3 versus lane 2, and lane 7 versus lane 6), confirming the previous reports that the C-terminal portions of RAMIC and IC play important roles in transactivation mediated by endogenously expressed RBP-J (38,45). The RAM-ANK construct containing only the two RBP-J binding domains (RAM and ANK) did not activate transcription at all (lane 4). When the reporter plasmid lacking the RBP-J binding sites was used, no constructs showed transactivation activity (38; data not shown).

A novel transactivation domain in the C-terminus of mouse Notch1

To examine whether mouse Notch1 contains an autonomous TAD, IC and its derivatives were C-terminally fused to the heterologous yeast GAL4 DNA binding domain and their transcriptional activities were determined using the GAL4-TK promoter (Fig. 3A). Comparable expression levels of all the fusion proteins in COS7 cells were confirmed by western blot analysis with polyclonal antibodies against the GAL4 DNA binding domain (data not shown).

Expression of GAL4-IC (full-length) activated transcription >60-fold as compared with GAL4 DNA binding domain alone (Fig. 3A, a). A GAL4 fusion protein with the N-terminal half of IC containing the ANK repeats did not exhibit any activity (Fig. 3A, b), but a fusion construct with the C-terminal half of IC transactivated twice as strongly as that with the full-length construct (Fig. 3A, c). The C-terminal half of IC was divided into the two regions containing either the OPA or PEST sequence and the TAD was mapped to the 200 amino acid residues containing the OPA sequence (amino acids 2194–2398, Fig. 3A, d). A further dissection of this region into two halves grossly reduced the activity (Fig. 3A, e and f), indicating that the OPA sequence alone is unable to mediate the transactivation function. While the M1 mutation in the ANK repeats of RAMIC or IC abolished its transactivation activity mediated by RBP-J (Fig. 2A, lane 5 or 8), GAL4-IC (M1) retained a strong transactivation activity (Fig. 3A, h). Basically, similar results were obtained using two other cell lines, NIH 3T3 and C2C12 cells.

DNA binding activity of these fusion proteins expressed in COS7 cells was examined by electrophoretic mobility shift assay (EMSA) using the respective nuclear extracts and the ³²P-labeled oligonucleotide probe containing a GAL4 binding site (Fig. 4, lanes 1–9). All GAL4 fusion proteins, except for h (lane 9), bound to the GAL4 binding site with comparable efficiencies. A subtle variation of the binding efficiencies cannot account for the difference in their transactivation activities observed (Fig. 3A). Despite its low DNA binding activity (Fig. 4, lane 9), the Notch1 IC h fusion construct has significant transactivation activity (Fig. 3A, h). It is of note that all ANK-containing constructs showed smears and aggregates in the wells (lanes 2, 3 and 9), probably because of homophilic interaction between the ANK repeats (39,40; data not shown).

TAD in other mouse Notch family members

The amino acid sequence of the TAD defined in mouse Notch1 (2194–2398) was compared with those of the corresponding regions (between the ANK repeats and PEST sequence) of the

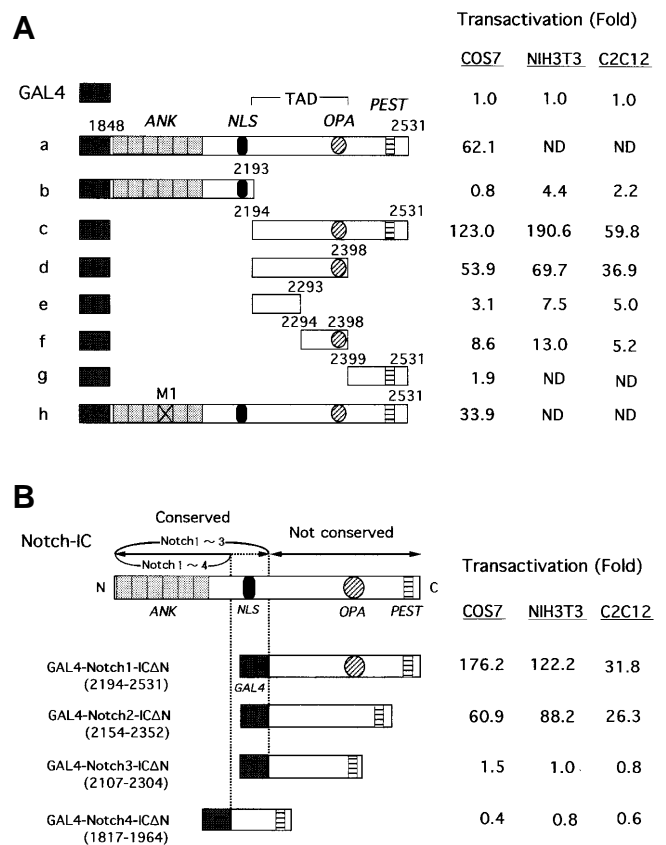


Figure 3. Transactivation domain (TAD) in the mouse Notch intracellular region. The heterologous yeast GAL4 DNA binding domain and the luciferase reporter plasmid containing four GAL4 binding sites were used to investigate the autonomous transactivation function of mouse Notch IC. 0.35 μ g each expression plasmid of pEFBOSneo was co-transfected into each type of cell (COS7, NIH 3T3 and C2C12 cells) together with 0.35 μ g TK-MH 100 \times 4-luciferase and 0.1 μ g pCMX-LacZ. In each case, the activity of the GAL4 alone is calculated as a value of 1. The normalized luciferase activity of each sample was compared with that of GAL4 alone. (A) Fusion constructs of the various mouse Notch1 IC regions with an N-terminally fused GAL4 DNA binding domain (a–h) are shown on the left. Numbers refer to amino acids of Notch1 IC fused to the GAL4 DNA binding domain. Their transactivation activities are represented on the right. ND, not done. Mouse Notch1 IC contains a novel autonomous TAD. (B) GAL4 fusion constructs with the individual Notch (mouse Notch1–4) C-termini downstream of the ANK repeats (GAL4-IC Δ N) are shown on the left. Numbers refer to amino acids of Notch1–4 IC Δ N fused to the GAL4 DNA binding domain. GAL4-Notch1-IC Δ N is identical to the deletion construct c shown in (A). Relative transcriptional activities are represented on the right. The C-termini of mouse Notch family members exhibit differential profiles of autonomous transactivation.

other mouse Notch family members. The TAD sequence of Notch1 and the corresponding region of Notch2 or Notch3 are less conserved (20%) than the RAM domain (40%) (42) or the ANK repeats (70%) (8). No significant similarity was found between the TAD sequence of Notch1 and the C-terminal portion downstream to the ANK repeats of Notch4.

Although the region homologous to the TAD of mouse Notch1 was not found in the intracellular regions of other mouse Notch family members, GAL4 fusion constructs were generated to determine whether their C-terminal portion contains an autonomous TAD (Fig. 3B). In COS7 cells, GAL4-Notch2-IC Δ N exhibited a significant transactivation activity, which was slightly less than

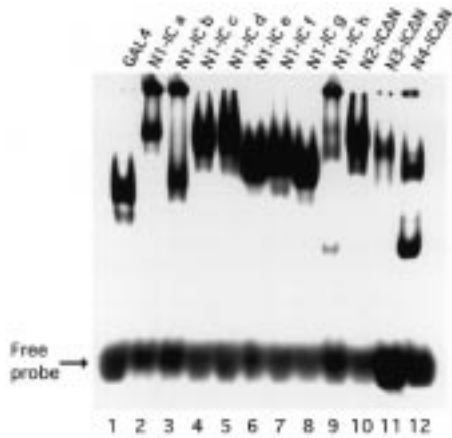


Figure 4. EMSA of products of GAL4–mouse Notch IC fusion constructs. Nuclear extracts were prepared from COS7 cells transiently transfected with the plasmid constructs indicated in Figure 3. Each extract (4 µg of protein) was incubated with a ³²P-labeled DNA probe containing a GAL4 binding site and the mixture was analyzed by native PAGE, as described in Materials and Methods. N1, N2, N3 and N4 represent Notch1, Notch2, Notch3 and Notch4, respectively.

that of GAL4–Notch1-ICΔN. The profiles of transactivation by the two constructs in two different cell lines (NIH 3T3 and C2C12 cells) were similar to those in COS7 cells, indicating that their activities are not cell type-dependent. On the other hand, no transactivation activity was displayed either by GAL4–Notch3-ICΔN or by GAL4–Notch4-ICΔN in all three cell lines. The absence of transactivation activities in the C-termini of mouse Notch3 and Notch4 is not due to inefficient expression of the fusion proteins because similar expression levels of all Notch-ICΔN fusion proteins were confirmed by western blot analysis (data not shown) and formation of comparable amounts of gel-shifted fusion complexes (Fig. 4, lanes 4 and 10–12).

Mouse Notch1 TAD is repressed by fusion with RBP-J

We next examined whether the fusion protein between RBP-J and the mouse Notch1 TAD showed transactivation activity (Fig. 5A) like the RBP-J–VP16 fusion protein (46). The fusion construct of RBP-J with the ANK repeats (RBP-J–IC ANK-A; Fig. 1) did not activate transcription through the RBP-J binding sites, in agreement with the result in the GAL4 system (Fig. 3, b). Unexpectedly, the chimeric protein with the TAD (RBP-J–ICΔANK; Fig. 1) also failed to activate transcription whereas the fusion construct of RBP-J with the whole IC containing both the ANK repeats and the TAD activated transcription ~10-fold, suggesting that the ANK repeats may have an unknown function to enhance TAD activity. Little transactivation activity of any construct was detected using the reporter plasmid lacking the RBP-J binding sites.

Comparable expression levels of these fusion proteins by transient transfection of COS7 cells were confirmed by western blot analysis with the monoclonal antibody (M2) against the N-terminally tagged FLAG epitope (Fig. 5B). To ensure that the absence of transactivation activity of the RBP-J chimeric protein with the ANK repeats or with the Notch1 TAD is not due to the inability to bind to DNA, we also performed EMSA using nuclear extracts prepared from transiently transfected COS7 cells and the ³²P-labeled 54 bp oligonucleotide probe, O54, containing the

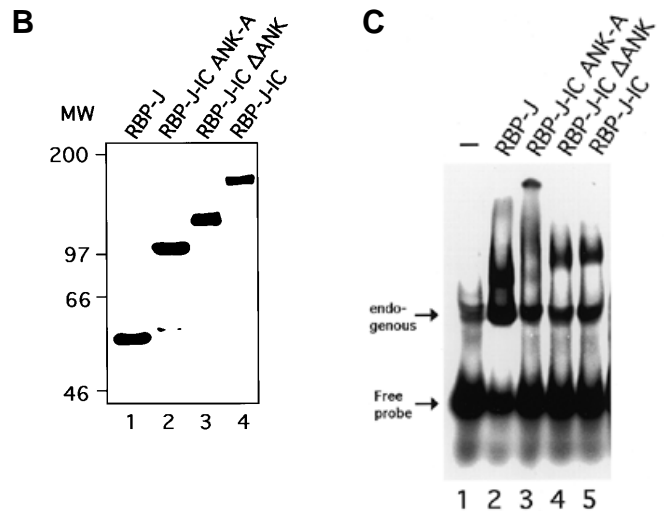
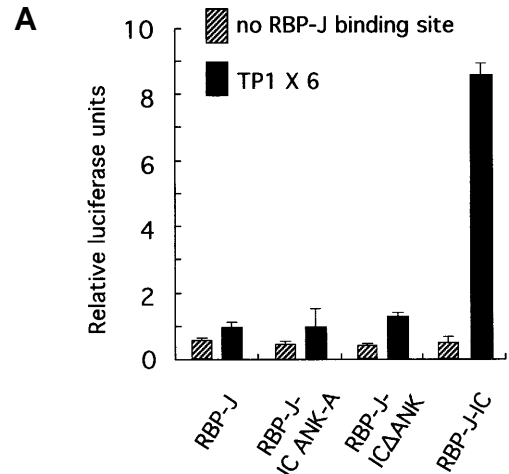


Figure 5. Mouse Notch1 TAD is repressed by fusion with RBP-J. (A) Parts of the mouse Notch1 IC region were C-terminally fused to human RBP3 (46). RBP-J–IC ANK-A (1810–2079) contains the ANK repeats. RBP-J–ICΔANK (2080–2531) contains the TAD. RBP-J–IC (1810–2531) contains the whole IC region. 0.3 µg each fusion construct of pSG5 was transfected together with 0.2 µg pGa981-6 and 0.05 µg pCMX-LacZ into COS7 cells. Potential activity of the mouse Notch1 TAD fused to RBP-J is silenced when the ANK repeat is missing. (B) Nuclear extracts from COS7 cells transiently transfected with the plasmid constructs of RBP-J–mouse Notch IC fusion were resolved by 7% SDS–PAGE and visualized by western blot analysis using the monoclonal antibody (M2) against the N-terminally tagged FLAG epitope. Numbers to the left of the panel show molecular mass in kilodaltons. (C) EMSA of products of RBP-J–mouse Notch1 IC fusion constructs. Each extract (4 µg of protein) was incubated with a ³²P-labeled DNA probe containing the RBP-J binding sites and the mixture was analyzed by native PAGE, as described in Materials and Methods.

RBP-J binding sites (Fig. 5C). It was reported that several RBP-J–DNA complexes were observed when O54 was used as probe (27,28) and only the major complex of them seems to be visualized using COS7 nuclear extracts containing endogenous RBP-J (Fig. 5C, lane 1). The overexpressed RBP-J and RBP-J–IC

fusion proteins gave rise to more than two complexes (Fig. 5C, lanes 2–5); one complex with the same mobility as that of endogenous RBP-J (Fig. 5C, lane 1) and more slowly migrating complexes with different mobilities. The RBP-J fusion with the ANK repeats showed smears and aggregates in the well (Fig. 5C, lane 3), as observed for all the GAL4-IC fusion proteins that contain the ANK repeats (Fig. 4, lanes 2, 3 and 9). Since all the fusion proteins showed comparable DNA binding efficiencies, their different transactivation activities are not due to their relative DNA binding efficiencies.

ANK repeats enhance TAD activity *in trans*

The ANK repeats of mouse (38) and human (35) Notch1 were shown to be a weak binding domain to RBP-J. Therefore, they are necessary for transactivation activity of IC (devoid of the RAM domain) through RBP-J and the M1 mutation in the ANK repeats of IC, which disrupts their interaction with RBP-J, results in abolition of its transactivation activity (Fig. 2A, lane 8). To investigate the function of the ANK repeats in transactivation other than RBP-J binding, the same M1 mutation was introduced in RAMIC. RAMIC (M1) failed to activate transcription mediated by endogenous RBP-J (Fig. 2A, lane 5, and B, lane 3), indicating that the ANK repeats have an indispensable function in transactivation mediated by RBP-J, even though the RAM domain is present. To our surprise, co-expression of the construct which primarily consists of the ANK repeats restored the abolished transactivation activity of RAMIC (M1) to the level of wild-type RAMIC (Fig. 2B, lanes 4 and 5), although the ANK construct alone did not activate transcription (Fig. 2B, lane 6). These results suggest that another important function, in addition to RBP-J binding, of the ANK repeats in transactivation was impaired by the M1 mutation of RAMIC and that expression of the ANK repeats *in trans* can complement the impaired function.

We have shown previously that the RAM domain of mouse Notch1, which itself does not have transactivation activity, synergizes with IC in transactivation mediated by RBP-J (38). The RAM domain also enhances the transactivation activity of the RBP-J-VP16 fusion protein (38; Fig. 6B, lanes 2–4), probably by displacing a putative co-repressor from RBP-J. Assuming that such a co-repressor binds to the RBP-J chimeric protein with the mouse Notch1 TAD (RBP-J-ICΔANK) and silences its potential activity, it is reasonable to expect that the addition of the RAM domain might relieve repression. In fact, the RAM domain activated transcription mediated by the chimeric protein with the Notch1 TAD in a dose-dependent manner (Fig. 6A, lanes 9 and 10).

Since the ANK repeats can rescue RAMIC (M1), we examined the effect of addition of the ANK repeats to the RBP-J chimeric protein with the mouse Notch1 TAD (RBP-J-ICΔANK) on transactivation activity. Addition of the ANK repeats to the RBP-J-TAD fusion protein activated transcription in a dose-dependent manner (Fig. 6A, lanes 11 and 12) and their effect was comparable with that of the RAM domain alone (compare lanes 9 and 10 with lanes 11 and 12). Increasing amounts of the RAM-ANK construct augmented transcription more efficiently (lanes 13 and 14). The addition of either RAM, ANK or RAM-ANK construct to the RBP-J-VP16 fusion protein also enhanced transcription (Fig. 6B, lanes 2–8), although the effect of RAM-ANK on the RBP-J-VP16 fusion protein was not so strong as that on the RBP-J-TAD fusion protein. Neither the

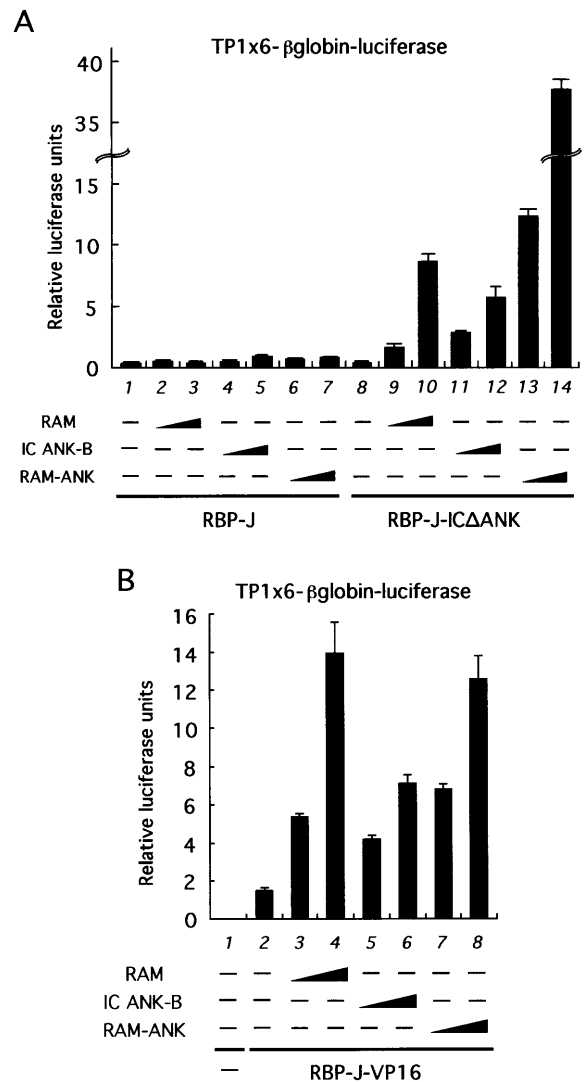


Figure 6. Roles of RAM and ANK in transactivation. (A) 0.3 μg pSG5-RBP-J or -RBP-J-ICΔANK, 0.2 μg pGa981-6 and 0.05 μg pCMX-LacZ were co-transfected into COS7 cells with increasing amounts (0, 0.25 and 1.25 μg) of pEFBOSneo-RAM (1751–1850) or -RAM-ANK (1747–2079) or -IC ANK-B (1848–2170). The ANK repeats as well as the RAM domain relieve the repression of the RBP-J-TAD (RBP-J-ICΔANK) fusion protein. The repression is more efficiently relieved by addition of the RAM-ANK construct. (B) 0.15 μg pSG5-RBP-J-VP16, 0.2 μg pGa981-6 and 0.05 μg pCMX-LacZ were co-transfected into COS7 cells with increasing amounts (0, 0.25 and 1.25 μg) of pEFBOSneo-RAM (1751–1850) or -RAM-ANK (1747–2079) or -IC ANK-B (1848–2170). Transactivation activity of the RBP-J-VP16 fusion protein is enhanced by addition of either RAM, ANK or RAM-ANK construct.

RAM (Fig. 6A, lanes 2 and 3), ANK (lanes 4 and 5) nor RAM-ANK (lanes 6 and 7) construct transactivated the control RBP-J protein, indicating that their effects on the augmentation of transcription are dependent on the C-terminally fused TAD of Notch1 or VP16.

DISCUSSION

We have identified a novel autonomous TAD between the ANK repeats and the PEST sequence in mouse Notch1, in contrast to previous reports that a strong TAD was absent from mouse

Notch1 or rat Notch2 RAMIC (36,37). This apparent contradiction could result from the different constructs used in the assays. The mouse Notch1 RAMIC construct used by Hsieh *et al.* (36) is truncated at residue 2293 and therefore lacks the C-terminal half (amino acids 2294–2398) of the TAD defined in this study. We showed that the N-terminal half (amino acids 2194–2293) of the TAD has very weak activity (Fig. 3A). RAMIC of rat Notch2 is highly homologous (>95% identity) to that of the mouse counterpart (10,11,50), whose C-terminal half exhibits a significant autonomous TAD activity (Fig. 3B). We suspect that their failure to identify the TAD was merely due to the use of C-terminally truncated forms of RAMIC of mouse Notch1 and rat Notch2.

The RAMIC construct without the autonomous TAD (RAMIC- Δ C) possesses transactivation activity weaker than RAMIC but stronger than IC (Fig. 2A), in agreement with reports that the C-terminally truncated RAMIC constructs of mouse Notch1 and rat Notch2 could activate transcription mediated by RBP-J (36,37). As the residual activity was abrogated by a further C-terminal deletion (Fig. 2A, lane 5), it is likely that the deleted region (amino acids 2080–2193 in mouse Notch1) between the ANK repeats and the TAD may act as another weak TAD only when it is tethered to RBP-J. Therefore, significant transactivation activities of mouse Notch3 and Notch4 RAMICs mediated by endogenous RBP-J (42; H.Kato and T.Honjo, unpublished data) are not surprising, although their C-terminal halves did not act as an autonomous TAD when fused to GAL4 (Fig. 3B).

A nuclear protein encoded by Epstein–Barr virus, EBNA2, also binds to RBP-J directly and activates transcription (25–28). EBNA2 contains an acidic TAD in the C-terminal end and it can be substituted by a similar acidic TAD of VP16 (51). It was demonstrated that VP16 is able to render RBP-J a transactivator when it is fused to RBP-J (38,46). Since the TAD of mouse Notch1 contains few acidic amino acids and fails to activate transcription when fused to RBP-J (Fig. 5A, lane 3), the Notch1 TAD is clearly distinguished from the TAD of EBNA2 or VP16. The autonomous TAD of mouse Notch1 is rich in glutamine, proline and serine/threonine (9, 14 and 19%, respectively) residues, which is reminiscent of other classical transcriptional activators. The C-terminus of Notch2, devoid of the glutamine-rich OPA sequence, exhibited an autonomous transactivation activity and contains many proline and serine/threonine (16 and 17%, respectively) residues in the region corresponding to the Notch1 TAD. However, the C-terminus of Notch3 did not display any activity despite the high contents of proline and serine/threonine (23 and 18%, respectively) residues in the corresponding region. No polypeptides showing sequence similarity to the TAD of mouse Notch1 or the corresponding region of Notch2 were found in protein databases, suggesting that they may be classified as a novel type of TAD. The mechanism by which the novel type of TAD activates transcription remains to be investigated.

We have already shown that the ANK repeats can associate with RBP-J because the M1 mutation in the ANK repeats of mouse Notch1 IC disrupts a weak interaction between IC and RBP-J (38). In this study, an intriguing possibility was suggested that the ANK repeats of mouse Notch1 may have another important function in addition to RBP-J binding. RAMIC (M1) has no ability to activate transcription mediated by endogenous RBP-J (Fig. 2B), although it still interacts with RBP-J through the RAM domain in the mammalian two-hybrid assay (data not shown). We showed that the ANK repeats molecule given *in trans* restored the transactivation activity abolished by the M1 mutation.

In addition, the ANK repeats as well as the RAM domain given *in trans* recovered the repressed TAD activity in the RBP-J–TAD (RBP-J–IC Δ ANK) fusion protein (Fig. 6A). These results suggest that the ANK repeats could displace a co-repressor from RBP-J to activate transcription, which is consistent with previous reports that the masking of an RBP-J repression domain is responsible for transactivation by Notch RAMIC (36,37) as well as by EBNA2 (52). It remains to be investigated whether the RAM domain and the ANK repeats compete with the same co-repressor or different ones for RBP-J binding.

The involvement of the ANK repeats of mouse Notch1 in autonomous transactivation function was investigated by a reporter assay using GAL4 fusion constructs (Fig. 3A), but their direct involvement was not revealed. We also showed that the ANK repeats themselves failed to activate transcription either when added to endogenous (Fig. 2B) or exogenous RBP-J (Fig. 6A) or when directly fused to RBP-J (Fig. 5A). Thus the ANK repeats of mouse Notch1 RAMIC do not seem to function as an autonomous TAD *per se*. However, we cannot completely exclude the possibility that the ANK repeats are indirectly involved in transactivation. The ANK repeats may recruit a putative co-activator. Alternatively, their association with RBP-J may lead to a conformational alteration to facilitate an interaction with the basal transcription machinery.

It was shown that the ANK repeats of *C.elegans* Notch GLP-1 act as an autonomous TAD when fused to GAL4 (40). GLP-1 interacts with EMB-5, a nematode homolog of yeast acidic nuclear protein which controls chromatin structure (53). Since the homology of the ANK repeats between GLP-1 and mouse Notch1 or Notch2 is only ~20% (5,6), these functional differences of the ANK repeats would have arisen during evolution. *Drosophila* Notch RAMIC or IC is shown to activate transcription when fused to GAL4 (31,32). To determine which portions of *Drosophila* and of *Xenopus* Notch possess an autonomous TAD will be helpful for understanding how the Notch proteins have evolved from invertebrates to vertebrates.

Taken together, we conclude that mouse Notch1 RAMIC can experimentally be separated into at least three functional domains: the RAM and ANK repeats for RBP-J binding and co-repressor displacement and a C-terminal TAD. Our results indicate that all three domains contribute to the full transactivation activity of mouse Notch1 RAMIC mediated by RBP-J. Identification and characterization of the co-repressor(s) of RBP-J are important to elucidate the complex mechanism of transcriptional regulation by Notch RAMIC. We have previously shown that a novel LIM protein, KyoT2, competes with mouse Notch1 RAMIC for RBP-J binding and represses RBP-J-mediated transcription by Notch1 RAMIC (54). Recently it has been reported that RBP-J targets TFIID and TFIIA to prevent activated transcription (55). We are currently investigating the possibilities that KyoT2 functions as a co-repressor of RBP-J *in vivo* and that TFIID and TFIIA are involved in transactivation by Notch RAMIC.

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