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RBP-L, a Transcription Factor Related to RBP-Jκ

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RBP-Jκ is a sequence-specific DNA binding protein which plays a central role in signalling downstream of the Notch receptor by physically interacting with its intracellular region. Although at least four *Notch* genes exist in mammals, it is unknown whether each Notch requires a specific downstream signalling molecule. Here we report isolation and characterization of a mouse *RBP-Jκ*-related gene named *RBP-L* that is expressed almost exclusively in lung, in contrast to the ubiquitous expression of *RBP-Jκ*. For simplicity, we propose to call *RBP-Jκ* *RBP-J*. The RBP-L protein bound to a DNA sequence almost identical to that of RBP-J. Surprisingly, RBP-L did not interact with any of the known four mouse Notch proteins. Although we found that RBP-L and EBNA-2 cooperated in transcriptional activation, they did not show significantly strong protein-protein interaction that can be detected by several in vivo and in vitro assays. This is again in contrast to physical association of RBP-J with EBNA-2. Several models to explain functional interaction between RBP-L and EBNA-2 are discussed.

RBP-Jκ is a 60-kDa DNA binding protein recognizing a consensus sequence (C/T)GTGGGAA although it has no typical DNA binding motif (19, 23, 38, 52). The structure of the RBP-Jκ protein is strongly conserved during evolution among nematode, fruit fly, mouse, and human (1, 5, 13, 38).

We and others have shown that the *Drosophila RBP-Jκ* gene is identical to *Suppressor of Hairless* [*Su(H)*] (14, 46), a member of the neurogenic gene family including *Notch*, *Delta*, *Enhancer of split* [*E(spl)*], and *Hairless*. Genetic analyses have shown that the neurogenic genes, including *Su(H)*, participate in lateral inhibition to single out a sensory mother cell from its precursor cells during peripheral nervous system development (2, 40). RBP-Jκ/Su(H) binding sites were identified in the 5′-flanking regions of the *E(spl)* complex [*E(spl)-C*] neurogenic genes of *Drosophila melanogaster*, and the transactivation of the *E(spl) m8* enhancer promoter by Su(H) was demonstrated in the Schneider cell line (16). Transactivation of the *E(spl)-C* promoters by Su(H) was also demonstrated in vivo by using transgenic flies carrying *E(spl)-C* promoter–β-galactosidase gene constructs (3, 15, 16, 33).

A breakthrough in the elucidation of the RBP-Jκ function in vertebrates came from studies on transcriptional regulation of human DNA tumor viruses. The adenovirus capsid protein polypeptide IX (pIX) promoter contains the RBP-Jκ target-like TGGGAAAGAA sequence between the SP1 binding site and the TATA box. Repression of the *pIX* promoter by RBP-Jκ was shown by in vitro as well as in vivo experiments (9). Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA-2) is essential for transformation of primary human B lymphocytes (7, 21, 58) and acts as a transcriptional activator of latent viral as well as cellular genes by interacting with RBP-Jκ (18, 22, 54, 61). Thus, RBP-Jκ is essential to B-lymphocyte transformation by EBV.

RBP-Jκ knockout mice die before 10.5 days of gestation and show severe developmental defects in somites and neural tube (41). RBP-Jκ mutant embryos express a posterior mesodermal marker, FGFR1. Their defective somites express the *Mox1* gene but fail to express the myogenin gene at 9.0 days of gestation. These phenotypes are similar to those of *Notch1*$^{-/-}$ mutant embryos (8, 49), suggesting that mammalian Notch and RBP-Jκ may also have functional interaction.

The Notch receptor consists of a large extracellular region with epidermal growth factor-like repeats and an intracellular region with cdc10/ankyrin repeats. The membrane-proximal region called the RAM domain of the intracellular region has been shown to interact directly with RBP-Jκ/Su(H) (25, 51). Furthermore, the truncated Notch containing the total intracellular region can transactivate the promoter of *HES-1*, the mammalian homolog of *Hairy* and *Enhancer of split*, which has RBP-Jκ binding sites (26). Since the truncated form of Notch has been shown to be involved in the development of T-cell leukemias in mice and humans (10, 42), RBP-Jκ is presumably involved in transformation of both T and B lymphocytes.

Intracellular events occurring after interaction of Notch with its ligand(s) are not understood. Two models were proposed to explain the direct association between the surface receptor Notch and the nuclear DNA binding protein RBP-Jκ. The first model assumes that RBP-Jκ is associated with the Notch receptor in a steady state and is released from Notch by ligand binding, resulting in migration of free RBP-Jκ to the nucleus as a signal transducer (11). The other model assumes that ligand binding induces a proteolytic cleavage of the receptor at a membrane-proximal site of the intracellular region, releasing the intracellular fragment which is directly involved in transcriptional events after migration to the nucleus as a signal transducer (26, 29, 35, 51). In either case, the Notch/RBP-Jκ signal transduction pathway takes the simplest and shortest route from the surface to the nucleus.

In mammals, there are at least four Notch family members (10, 12, 30, 31, 47, 48, 53, 55, 56) that can interact with RBP-Jκ (28). In addition there are multiple ligands for the Notch receptor (4, 36). Embryonic expression profiles of these *Notch* genes display distinct, although partially overlapping, patterns (30, 57). Multiple Notch receptors may have RBP-Jκ as a common signalling molecule that is expressed ubiquitously in different developmental stages (20). Alternatively, specific RBP-Jκ-related molecules may be involved in signal transduction of individual Notch receptors.

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To assess this possibility, we looked for *RBP-Jκ*-related genes and found a novel mouse gene homologous to *RBP-Jκ*. The protein encoded by this gene, named RBP-L, binds to a DNA sequence almost identical to that of RBP-Jκ. Unexpectedly, however, this protein does not associate with any of known Notch members. Nonetheless, RBP-L shows cooperative transactivation activity with EBNA-2.

MATERIALS AND METHODS

Cloning and sequencing of *RBP-L* cDNA clones. A cDNA fragment (nucleotides 990 to 1237) was amplified by PCR from an adult mouse lung cDNA pool, using gene specific primers designed from an expression sequence tag (34), and used to screen C57BL/6 adult mouse lung cDNA libraries. Hybridization and isolation of clones were done under standard conditions (45). Insert cDNAs of isolated clones were subcloned into pBluescript II KS (Stratagene) and subjected to sequencing by the dideoxy-chain termination method with an automatic DNA sequencer (Applied Biosystems).

Northern blot analysis. Total RNA was isolated with Trizol (GIBCO). Poly(A)$^+$ RNA was prepared with oligotex-dT30 latex beads (TaKaRa). Two micrograms of poly(A)$^+$ RNA was electrophoresed on an 0.8% agarose gel and transferred to Hybond N-fp nylon membranes (Amersham). cDNA probes of *RBP-L* (nucleotides 969 to 1562) and β-actin (nucleotides 183 to 427) were obtained by reverse transcription (RT)-PCR from appropriate cDNA pools. Hybridizations were done under standard conditions (45).

RT-PCR. cDNA was synthesized from 5 μg of total RNA in 25 μl of each reaction mixture, using Superscript II reverse transcriptase (GIBCO). An *RBP-L* DNA fragment (nucleotides 969 to 1562) was amplified by PCR from 1 μl of each cDNA sample mixed with 30 pmol each of gene-specific primers, 4 μl of 2.5 mM deoxynucleoside triphosphates, 5 μl of 10× PCR buffer (TaKaRa), and 0.5 μl of *Taq* polymerase (TaKaRa) in a 50-μl volume; PCR consisted of 30 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C. The PCR products were separated on an agarose gel, blotted, and hybridized with an appropriate cDNA fragment. As a control, PCR products with β-actin primers (nucleotides 183 to 427) were analyzed by ethidium bromide staining.

CASTing and EMSA. CASTing (cyclic amplification and selection of targets) was performed with the oligonucleotide R76 as described previously (43). The RBP-L protein tagged C terminally with a gene 10 epitope peptide (Novagen) was produced in vitro with the T3-coupled TNT reticulocyte lysate system (Promega) as specified by the manufacturer and used for coimmunoprecipitation of DNA with a mouse anti-T7 monoclonal antibody (Novagen). Binding-site selection using electrophoretic mobility shift assay (EMSA) was performed as described previously (52). The finally recovered DNA was digested with both *Eco*RI and *Bam*HI, subcloned into pBluescript II KS, and sequenced. EMSA was carried out as described by Hamaguchi et al. (19). The HES-1 probe is derived from the *HES-1* promoter sequence (nucleotides −90 to −51) (50). The O54 probe was previously described (60).

In vitro binding assay with GST fusion proteins. Expression of glutathione *S*-transferase (GST) fusion proteins and in vitro interaction assays were performed as described previously (51). Plasmids for expression of GST proteins fused to the Notch1, -2, -3, and -4 fragments were described previously (28, 51). A cDNA fragment encompassing the entire RBP-L protein was subcloned into pGEX4T-1 (Pharmacia). A C-terminal deletion construct of RBP-J, Δ1115Apa, was described previously (6) and subcloned into pGEX4T-1. ^{35}S-labeled RBP-J and RBP-L proteins were produced in vitro with pU1093-5 (60) and a plasmid generated by replacement of the RBP-J-coding sequence in pU1093-5 with an appropriate DNA fragment encoding the RBP-L protein. Plasmid pGa376-8, used for in vitro translation of ^{35}S-labeled EBNA-2 protein, was previously described (61).

Immunoprecipitation experiments. Immunoprecipitation was done as described previously (51). C-terminally gene 10 epitope-tagged RBP-J and RBP-L were expressed from the pEF-BOS vector (39). The EBNA-2 expression constructs are described below. Immunoprecipitation was carried out either with the anti-T7 antibody or with mouse anti-EBNA-2 monoclonal antibody PE2 (YLEM). Immune complexes were separated on a sodium dodecyl sulfate-polyacrylamide gel and analyzed by immunoblotting. The upper part of the gel was used to monitor EBNA-2 expression with PE2 and anti-mouse immunoglobulin G (IgG) antibodies, and the lower part was used to detect RBP-J or RBP-L expression with the anti-T7 antibody and anti-mouse IgG antibodies.

Plasmids for cotransfection assay. pGa981-6 was constructed as described below. A 50-bp oligonucleotide harboring both RBP-J binding sites of the *TP1* promoter, with a cleaved *Bam*HI site at the 5′ end and a cleaved *Bgl*II site at the 3′ end (uncleaved sequence; GGATCCCGACTCGTGGGAAAATGGGCGGA AGGGCACCGTGGGAAAATAGTAGATCT), was ligated as a hexamer into plasmid pGa50-7 (32) cleaved with *Bam*HI. The identical orientation of all monomers was confirmed by *Bam*HI and *Bgl*II digestion. TK-MH100×4-LUC (27) was kindly provided by K. Umesono. RBP-J was expressed by CDM8-RBP-2 (6). The authentic RBP-L protein was expressed from pEF-BOS. All GAL4 fusions were constructed on pGBT9 (Clontech) and subcloned into pEF-BOS. The wild-type EBNA-2 expression plasmid was constructed by ligation of the 5,076-bp *Hin*dIII (blunt ended)/*Bgl*II fragment of pU294-6 (60) into the expression vector pSG5 (Stratagene) cleaved with *Eco*RI (blunt ended) and *Bgl*II. Just in front of the translation start of EBNA-2, an *Eco*RI site was inserted by site-directed mutagenesis. The EBNA-2 mutant WW323SS, with a $W_{323}W_{324}$-to-$S_{323}S_{324}$ mutation, was described previously (59). The amino acid positions of the EBNA-2 constructs are indicated on the basis of the EBV strain B95-8 sequence, although our constructs are derived from strain M-ABA. The truncated Notch1 construct consisting of amino acids 1747 to 2531 (RAMIC) was described previously (51).

Luciferase cotransfection assay. COS7 cells were transfected by lipofection with a mixture of DNAs consisting of 400 ng of the reporter plasmid, 500 ng each of the effector plasmids, and 100 ng of pRSV-lacZ (17) as an internal control for transfection efficiency. The total amount of DNA was adjusted by adding pEF-BOS vector DNA. Luciferase and β-galactosidase assays were done as described previously (16). All experiments were repeated at least three times, and the averages of more than three independent experiments with standard deviations are shown in the figures.

Nucleotide sequence accession number. The accession number of the *RBP-L* sequence is Y10926.

RESULTS

Cloning of an *RBP-Jκ*-like gene. To find *RBP-Jκ*-related genes, we searched a database of expressed sequence tags (34) and identified a rat partial sequence displaying a high level of identity to the *RBP-Jκ* gene. We therefore isolated a mouse cDNA fragment corresponding to this rat sequence by PCR, screened a mouse adult lung cDNA library by using this cDNA as a probe, and finally obtained a full-length cDNA containing an open reading frame that encodes a 57-kDa protein of 515 amino acids (Fig. 1A).

The overall structure of the deduced protein bears close similarity to the RBP-Jκ homologs. The RBP-Jκ-like protein exhibits 48% overall identity to RBP-Jκ. Although RBP-Jκ does not possess any known DNA binding motif, mutagenesis analyses have revealed two critical regions (N and C) for DNA binding (6). Interestingly, the N region between residues 218 and 227 of the RBP-Jκ-like protein is identical to the equivalent regions of the RBP-Jκ homologs (Fig. 1B). The other DNA binding domain, between residues 290 and 326, has 59% identity to that of RBP-Jκ (Fig. 1B). The amino acid sequence from 230 to 268 of the RBP-Jκ-like protein corresponding to the integrase-related motif conserved in the RBP-Jκ homologs is 48% identical to that of RBP-Jκ (Fig. 1B).

A striking feature of the isolated *RBP-Jκ*-like gene cDNA structure is the absence of the poly(A) signal. Although we determined the sequences of 38 independent cDNA clones, we failed to find any clone with poly(A) signal-like sequences. Nonetheless, many clones contain poly(A) at the 3′ end starting from various sites (Fig. 1A).

Expression of the *RBP-Jκ*-like gene. Several adult tissues were examined for the presence of *RBP-Jκ*-like gene transcripts by Northern blot analysis and RT-PCR (Fig. 2). By Northern blot hybridization of poly(A)$^+$ RNAs from adult tissues, only lung mRNA was shown to contain a detectable level of *RBP-Jκ*-like transcripts of approximately 2, 4, and 7 kb (Fig. 2A). As all of the isolated poly(A)$^+$ cDNA clones possess 3′ untranslated regions of less than 205 bp (Fig. 1A), these poly(A)$^+$ clones appear to be derived from the 2-kb transcript. We also isolated a poly(A)$^-$ clone with an 808-bp 3′ untranslated region (Fig. 1A). This clone could be derived from the longer transcripts. It is possible that these 4- and 7-kb transcripts possess the poly(A) signal, and we isolated the cDNA clones derived from abundant 2-kb transcripts whose poly(A) tails begin at aberrant sites by unknown reasons.

To examine scarce expression of *RBP-Jκ*-like transcripts in other tissues, we used RT-PCR analysis in combination with Southern blot hybridization and detected specific bands corresponding to the *RBP-Jκ*-like transcript at much lower levels in spleen and brain (Fig. 2B). Taken together, the results indicate

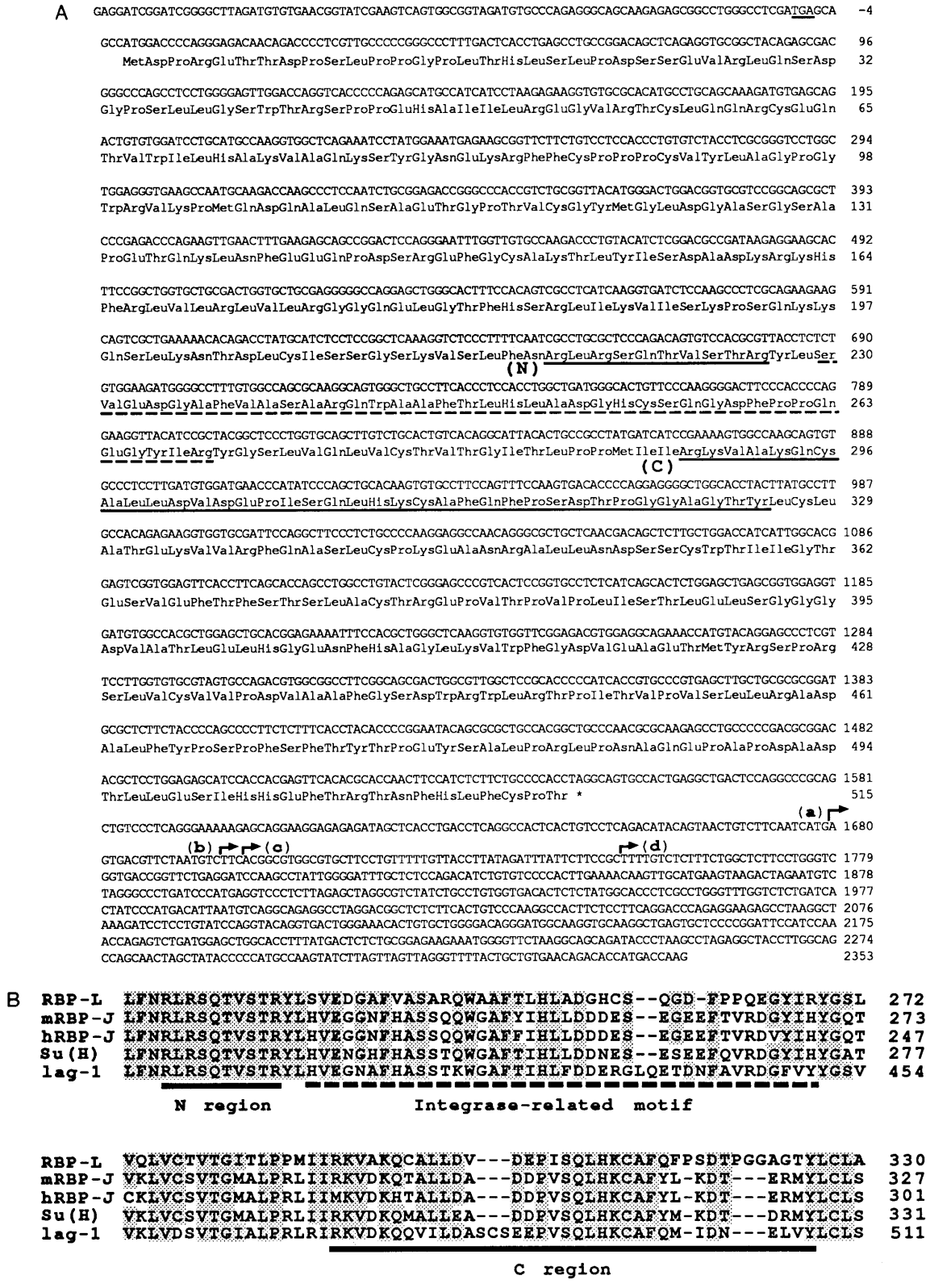

FIG. 1. Primary structure of the *RBP-Jκ*-like gene. (A) Nucleotide and deduced amino acid sequences of the *RBP-Jκ*-like gene. The asterisk indicates the predicted termination codon. An in-frame stop codon at the 5′-flanking region is indicated by a bar under the nucleotide sequence. Two putative DNA binding domains (N and C) are underlined. The integrase-related motif is indicated by a dashed underline. Arrows (a, b, c, and d) mark the positions of various poly(A) starting sites. Of the four sites, the poly(A) startings are most frequently observed at the b site. (B) Sequence comparison of the RBP-Jκ-like protein with the RBP-Jκ homologs in the DNA binding domains and the integrase-related motif. The mouse, human, *Drosophila* and *Caenorhabditis elegans* RBP-Jκ homologs are designated mRBP-J, hRBP-J, Su(H), and lag-1, respectively. Amino acid identities are shaded. Two DNA binding domains (N and C) are indicated by solid underlines. The integrase-related motif is indicated by a dashed underline.

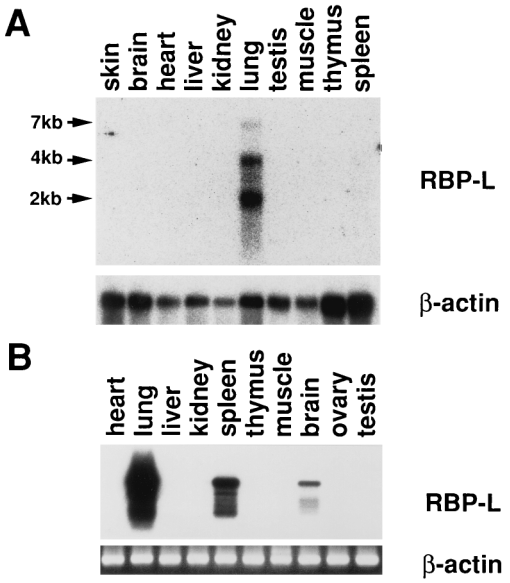

FIG. 2. The *RBP-Jκ*-like gene displays a restricted expression pattern. (A) The *RBP-Jκ*-like transcripts are expressed specifically in adult lung. Shown are Northern blot analyses of various adult mouse tissues with the *RBP-Jκ*-like (upper panel) and β-actin (lower panel) gene probes. The 2-, 4-, and 7-kb transcripts are indicated by arrows. (B) The *RBP-Jκ*-like transcripts are also expressed weakly in adult spleen and brain. Expression was analyzed by RT-PCR with gene-specific primers of the *RBP-Jκ*-like (upper panel) and β-actin (lower panel) genes from adult mouse tissue cDNAs.

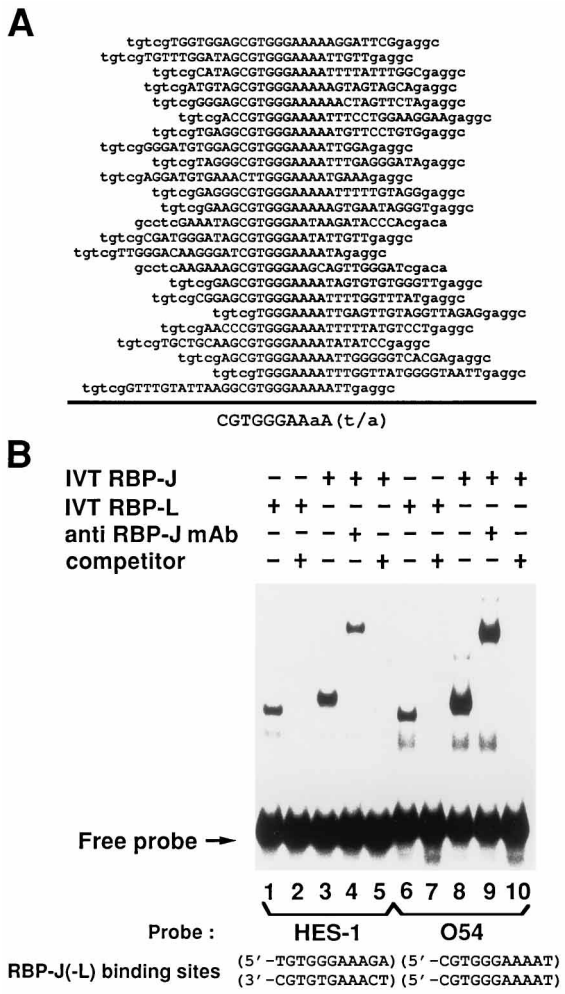

FIG. 3. Recognition sequence of RBP-L. (A) The target DNA sequence of RBP-L is closely related to that of RBP-J. Shown is alignment of oligonucleotides selected by CASTing. Capitals show nucleotides in the random sequence region, and lowercase letters show those in the primer region. Nucleotides conserved at 23 or more of 24 sequences and less conserved flanking nucleotides are shown at the bottom as capital and lowercase letters, respectively. (B) RBP-L binds to RBP-J binding sites of RBP-J target genes. EMSAs were performed with the in vitro-translated (IVT) RBP-L and RBP-J proteins and the ^{32}P-labeled synthetic HES-1 and O54 oligonucleotide probes. The probes were mixed with RBP-L (lanes 1, 2, 6, and 7) or RBP-J (lanes 3 to 5 and 8 to 10) in the presence (lanes 2, 5, 7, and 10) or absence (lanes 4 and 9) of excess cold probes. RBP-J complexes were supershifted by anti-RBP-J monoclonal antibody (mAb) K0043 (44).

that *RBP-Jκ*-like gene expression is highly restricted to adult lung, in contrast to the ubiquitous expression of *RBP-Jκ*. We therefore propose to call the new *RBP-Jκ*-like gene *RBP-L* and the original *RBP-Jκ* gene *RBP-J*.

DNA binding property of RBP-L. The strong homology of the DNA binding domains of RBP-L with those of RBP-J prompted us to perform CASTing experiments. To select oligonucleotides recognized by RBP-L, we used in vitro-translated RBP-L tagged C terminally with the gene 10 epitope sequence and an anti-T7 monoclonal antibody which recognizes the gene 10 epitope sequence. After each round of selection, we monitored the probe recovery to follow the progress of selection. However, the level of recovered DNA was so low that enrichment of the target DNA could not be estimated even after six rounds of selection. Therefore, the PCR-amplified DNA after the fourth selection was subjected to EMSA using in vitro-translated RBP-L. This assay detected a weak but specific retarded band corresponding to the complex with the RBP-L protein. This shifted band was excised, amplified, and subjected to another cycle of selection using EMSA. After four cycles of CASTing and an additional two cycles of enrichment by EMSA, recovered DNA was cloned into a plasmid vector and sequenced (Fig. 3A). The oligonucleotide sequences of 24 independent clones clearly demonstrate that an octanucleotide sequence, CGTGGGAA, appears to be most significantly conserved. This consensus sequence is identical to the target sequence, (C/T)GTGGGAA, recognized by RBP-J (52).

To address whether RBP-J and RBP-L recognize the same target gene, we examined several DNA probes for the ability to form complexes with RBP-L in EMSA (Fig. 3B). Both RBP-L and RBP-J strongly bound to the O54 probe, whose sequence is derived from the EBV terminal protein 1 (*TP1*) gene promoter (60) and contains two direct repeats of the RBP-L-recognizing consensus sequence. RBP-L also bound to the HES-1 probe containing two inverted repeats of the RBP-J recognition sequence in the *HES-1* gene promoter (3, 33, 50).

Furthermore, we found that RBP-L binds to the m8 probe, derived from the *Drosophila E(spl) m8* gene promoter sequence, which was previously shown to bind RBP-J (52). To identify the minimum core recognition sequence of RBP-L, we introduced a series of point mutations into the m8 probe and examined these probes for binding to RBP-L by EMSA (Table 1). The nine probes, named m8-5A, -5T, -6A, -78C, -9C, -10T, -10G, -11C, and -11G, have nucleotide substitutions in the heptamer sequence GTGGGAA, and these replacements almost completely prevented binding. The effects of the mutations were more severe for RBP-L than for RBP-J. On the other hand, the binding activities were diminished but not

TABLE 1. Binding of m8 mutant probes to the RBP-J and RBP-L proteins[a]

DNA probe	Sequence	Relative binding (%)	
		RBP-J	RBP-L
	1 5 10 15		
m8	GATCGGCACT**GTGGGAA**CGGAA	100	100
m8-2T	GATCGGCTCTGTGGGAACGGAA	66	37
m8-5A	GATCGGCACTATGGGAACGGAA	23	0.3
m8-5T	GATCGGCACTTTGGGAACGGAA	0	0
m8-6A	GATCGGCACTGAGGGAACGGAA	0	0.15
m8-78C	GATCGGCACTGTCCGAACGGAA	0	0
m8-9C	GATCGGCACTGTGGCAACGGAA	0	0
m8-10T	GATCGGCACTGTGGGTACGGAA	2	0
m8-10G	GATCGGCACTGTGGGGACGGAA	2.5	0
m8-11C	GATCGGCACTGTGGGACCGGAA	16	0
m8-11G	GATCGGCACTGTGGGAGCGGAA	72	0.45
m8-12G	GATCGGCACTGTGGGAAGGGAA	32	45
m8-12T	GATCGGCACTGTGGGAATGGAA	58	37
m8-13C	GATCGGCACTGTGGGAACCGAA	88	77

[a] End-labeled synthetic oligonucleotide probes were used for EMSA. Relative binding of RBP-L to the mutant probes was determined by measuring radioactivity of the shifted band with an Image Analyzer as described in Materials and Methods and is shown as percentage relative to m8 probe binding. Binding activities of RBP-J were taken from reference 52. Boldface letters show the heptamer motif, and mutated nucleotides are underlined.

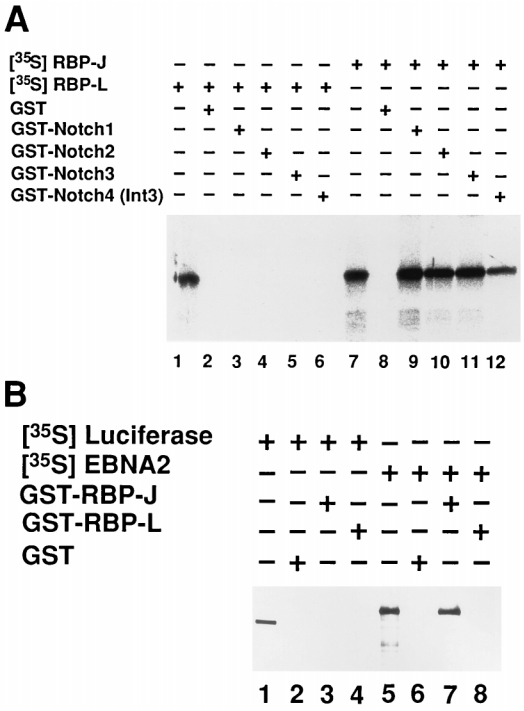

FIG. 4. The RBP-L protein does not interact with either mouse Notch family members or EBNA-2. (A) RBP-L does not bind to the RAM domains of mouse Notch family members. GST (lanes 2 and 8) or GST-RAMs of mouse Notch1 (lanes 3 and 9), Notch2 (lanes 4 and 10), Notch3 (lanes 5 and 11), and Notch4 (lanes 6 and 12) were incubated with in vitro-translated radiolabeled RBP-L (lanes 2 to 6) or RBP-J (lanes 8 to 12) and subjected to GST pull-down. ^{35}S-labeled RBP-L and RBP-J before incubation with the beads were loaded as size markers (lanes 1 and 7). (B) RBP-L does not bind to EBNA-2 in vitro. GST (lanes 2 and 6), GST–RBP-J (Δ1115Apa) (lanes 3 and 7), and GST–RBP-L (lanes 4 and 8) were incubated with in vitro-translated radiolabeled full-length EBNA-2 (lanes 5 to 7) or luciferase (lanes 2 to 4) and subjected to GST pull-down. ^{35}S-labeled luciferase and EBNA-2 before incubation with the beads were loaded as size markers (lanes 1 and 5).

abolished if mutations were introduced outside the heptamer sequence. Therefore, we conclude that RBP-L recognizes the core heptamer sequence GTGGGAA more strictly than RBP-J.

Interaction with EBNA-2 and Notch. The transcriptional regulation of RBP-J is modulated by the protein-protein interaction with EBNA-2 and the truncated Notch protein. We therefore examined whether the RBP-L protein can interact with these transcriptional regulators by using in vitro binding assays (Fig. 4). Notch1, -2, -3, and -4 fragments containing the membrane-proximal RAM domain previously described as the interaction region with RBP-J (28, 51) were fused to a GST protein. Bacterially expressed GST fusion proteins were immobilized on glutathione-agarose beads and incubated with in vitro-translated ^{35}S-labeled full-length RBP-J or RBP-L. These GST-fused RAM domains failed to show significant interaction with the RBP-L protein, although all of them bound to RBP-J (Fig. 4A). To rule out the possibility that RBP-J and RBP-L can bind to distinct regions on the Notch protein, we performed an additional GST pull-down experiment using a GST protein fused to a Notch1 intracellular region from residues 1751 to 2170 containing the entire six ankyrin repeats and the RAM domain (51) and did not find any interaction with RBP-L, indicating that RBP-L does not interact significantly with either the ankyrin repeats or the RAM domain (data not shown).

We next examined EBNA-2 for its ability to interact with RBP-L by using a GST fusion protein containing full-length RBP-L and in vitro-translated ^{35}S-labeled full-length EBNA-2 (Fig. 4B). C-terminally truncated RBP-J (Δ1115Apa), which contains the entire region of interaction with EBNA-2 (6), linked to the GST protein was used as a positive control and found to specifically interact with EBNA-2. In contrast, GST–RBP-L did not display any augmented coprecipitation of ^{35}S-labeled EBNA-2 compared with that of a negative control, ^{35}S-labeled luciferase. Thus, we concluded that RBP-L does not have significant physical interaction with EBNA-2 in vitro.

Effects of RBP-L on transcriptional events. In spite of the absence of interaction with Notch family members and EBNA-2, RBP-L could still be involved in the transcriptional activation by EBNA-2 or the truncated form of Notch in collaboration with cellular factors. To address this possibility, we tested the transcriptional function of RBP-L in the presence of EBNA-2 or the truncated Notch1, using luciferase cotransfection assays in COS7 cells (Fig. 5A). Transcription from the reporter plasmid pGa981-6, which contains 12 copies of the RBP-J/-L binding site from the EBV *TP1* promoter linked to the β-globin gene promoter, was monitored by determining luciferase activity. Cotransfection of this reporter with plasmids expressing EBNA-2 and the intracellular region of Notch1 (RAMIC [51]) increased the levels of luciferase activity about 14- and 400-fold, respectively. This activity is probably mediated by endogenous RBP-J. We introduced an additional RBP-J or RBP-L expression plasmid into these settings. Introduction of the RBP-J expression plasmid strongly suppressed the transcription augmented by the activator EBNA-2 or Notch1 but did not alter the steady-state transcription level by itself. This repression effect could result from the competition with endogenous DNA-bound RBP-J for binding to EBNA-2, Notch1, or other unknown cofactors. On the other hand, we found that the addition of RBP-L remarkably augmented the EBNA-2 activity approximately 10-fold. Furthermore, the addition of RBP-L caused slight but significant enhancement of transactivation by the truncated Notch1. However, RBP-L did not exhibit any significant effect by itself.

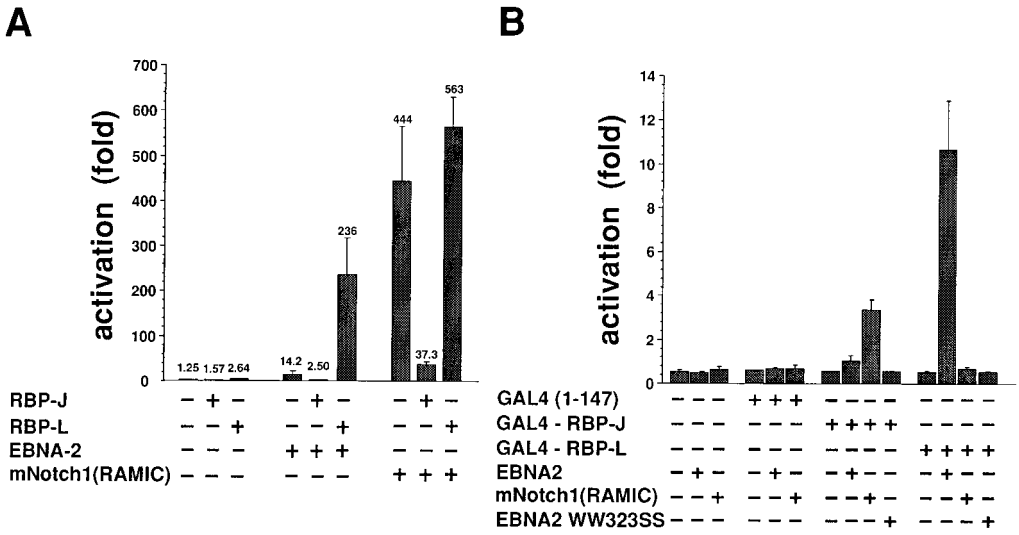

FIG. 5. RBP-L exhibits strong transcriptional stimulation activity with EBNA-2. (A) RBP-L strongly stimulates EBNA-2-dependent activity from *TP1* promoter-derived RBP-J and -L binding sites. COS7 cells were transiently cotransfected with the reporter pGa981-6 and indicated effectors together with pRSV-lacZ. In each case, luciferase activity was normalized to the β-galactosidase activity. Fold activations represent the ratios between individual normalized luciferase activities on pGa981-6 and on a control reporter plasmid without the RBP-J and -L binding sequence. Standard deviations are shown at the top of columns by vertical lines. (B) GAL4(1-147)–RBP-L stimulates transcription with EBNA-2 from GAL4 binding sites of TK-MH100×4-LUC. The experiments were the same as for panel A except that RBP-J and RBP-L were replaced by their GAL4(1-147) fusion constructs.

RBP-J is known to be expressed ubiquitously, and COS7 cells also express RBP-J but not RBP-L (data not shown). To avoid effects of endogenous RBP-J on the reporter assays, we used GAL4 fusion constructs encoding RBP-J or RBP-L fused N terminally to the GAL4 DNA binding domain (amino acids 1 to 147) with reporter plasmid TK-MH100×4-LUC, which contains four copies of a GAL4 binding site named MH100 (27) about 110 bp upstream of the TATA box of the herpes simplex virus thymidine kinase promoter (Fig. 5B). GAL4–RBP-J stimulated transcription of the reporter strongly with the truncated Notch1 but slightly with EBNA-2. On the other hand, the truncated Notch1 did not display any activity with GAL4–RBP-L, in agreement with the absence of interaction between RBP-L and Notch1 in the in vitro GST pull-down experiment (Fig. 4A). However, we found strong transcriptional activation by GAL4–RBP-L in combination with EBNA-2 (Fig. 5B). It is noteworthy that this transcriptional cooperation of GAL4–RBP-L with EBNA-2 was not shown with an EBNA-2 mutant, WW323SS, bearing amino acid substitutions which specifically interfere with EBNA-2 interaction with RBP-J (59). Consistent with this observation is that EBNA-2 deletions in the RBP-J interaction domain abolished the cooperation with GAL4–RBP-L in transcriptional activation although comparable stable protein expression of the wild-type and mutant EBNA-2 constructs was confirmed in immunoblot analysis (data not shown). The requirement of the RBP-J interaction domain of EBNA-2 for transcriptional cooperation with RBP-L suggests possible involvement of RBP-J. We therefore examined recombinant or endogenous RBP-J for the ability to interact with RBP-L in GST pull-down assays, yeast two-hybrid analyses, and in vivo immunoprecipitation experiments, but no significant interaction was detected (data not shown).

To address the possibility that RBP-L is modulated in the cell by some cofactor or posttranslational modification and forms complexes with EBNA-2 in vivo, we next performed coimmunoprecipitation experiments (Fig. 6). The EBNA-2 expression plasmid was introduced into COS7 cells together with a plasmid expressing RBP-J or RBP-L tagged C terminally with the gene 10 epitope. The EBNA-2 WW323SS mutant, which is unable to interact with RBP-J, was used as a negative control. Cells were lysed 24 h after transfection and subjected to immunoprecipitation using the anti-T7 or an anti-EBNA-2

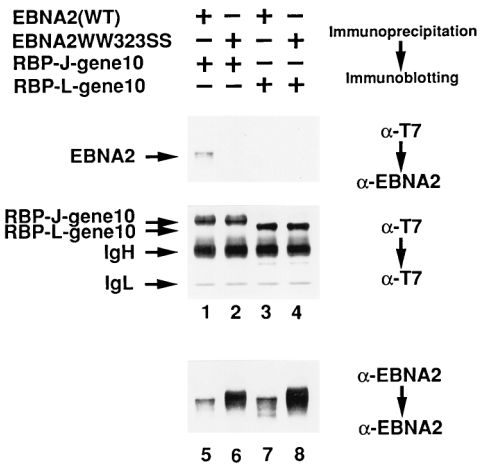

FIG. 6. RBP-L does not bind to EBNA-2 in vivo. Extracts of COS7 cells overexpressing T7 gene 10 epitope-tagged RBP-J (lanes 1, 2, 5, and 6) or RBP-L (lanes 3, 4, 7, and 8) together with either wild-type EBNA-2 [EBNA(WT); odd-numbered lanes] or the EBNA-2 mutant WW323SS (even-numbered lanes) were separated into two aliquots and subjected to immunoprecipitation either with the anti-T7 monoclonal antibody (α-T7) for coimmunoprecipitation analysis (lanes 1 to 4) or with the anti-EBNA-2 monoclonal antibody (α-EBNA2) for confirmation of EBNA-2 and WW323SS expression (lanes 5 to 8). The immune complexes purified with protein A-Sepharose beads were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The gel of immunoprecipitates with the anti-T7 antibody was cut horizontally into two. The upper part of the gel was used to monitor EBNA-2 coprecipitation, and the lower part was used to confirm precipitation of RBP-J or RBP-L. The anti-T7 antibody used in the immunoprecipitation step was detected by anti-mouse antibodies used in the immunoblotting and is represented by IgH (heavy chain) and IgL (light chain).

monoclonal antibody, and then the immunoprecipitates were analyzed by Western blotting. Immunoprecipitation using the anti-EBNA-2 monoclonal antibody was performed to confirm that comparable amounts of EBNA-2 are expressed in different samples. Wild-type EBNA-2 but not the WW323SS mutant was coprecipitated with RBP-J by the anti-T7 monoclonal antibody. However, neither wild-type EBNA-2 nor the WW323SS mutant was coprecipitated with RBP-L, indicating that RBP-L did not form a stable complex with EBNA-2 in vivo or in vitro. This conclusion was confirmed in the yeast two-hybrid assay (data not shown).

DISCUSSION

Here we report the isolation of a novel mouse gene named *RBP-L* whose protein product exhibits structural homology with the RBP-J protein, suggesting the presence of the RBP-J protein family. The high degree of structural similarity suggests that RBP-L also functions as a transcription factor. In fact, RBP-L binds to DNA by recognizing the same nucleotide sequence as RBP-J (Fig. 3). However, RBP-L differs from RBP-J in the following important aspects. The *RBP-L* gene is expressed in a tissue-restricted manner, in contrast to the ubiquitous expression of *RBP-J*. Unexpectedly, the RBP-L protein does not interact with Notch1, -2, -3, or -4. In agreement with this observation, the truncated Notch1 does not display significant transcriptional cooperation with RBP-L. On the other hand, RBP-L exhibits strong transcriptional stimulation cooperativity with EBNA-2 in spite of the absence of physical interaction with EBNA-2.

Involvement of the RBP family in Notch signalling pathway. RBP-J/Su(H) has been well established as a key molecule in the Notch signalling pathway. RBP-J physically interacts with the RAM domains of all four Notch receptors (28). In addition, the RAMIC portion of not only Notch1 but also Notch4, the most distant member of the Notch family, transactivates the *TP1* promoter in collaboration with RBP-J (28), suggesting that ubiquitously expressed RBP-J may be involved in the signalling pathways of all four Notch receptors. If this is so, it is puzzling how different Notch receptors can deliver specific signals, because expression profiles of Notch1, -2, -3, and -4 overlap at least partially and the known ligands of the Notch receptor appear to bind many, if not all, Notch receptors.

Although we had expected that other members of the RBP family might be found as more specific partners for different Notch receptors, the newly found RBP-L does not show any physical or functional interaction with the four Notch receptors. In agreement with this, Southern blot hybridization analysis revealed strong and discrete cross-hybridization signals in human DNA, weak signals in cow, chick, frog, and fish DNAs, and no signal in yeast, nematode, and fruit fly DNAs (data not shown), indicating that the *RBP-L* gene is less conserved than the *RBP-J* and *Notch* genes. RBP-L may interact within as yet unidentified Notch receptor that is specifically expressed in lung. Alternatively, RBP-L may have an entirely different partner for its function.

Functional interaction of RBP-L with EBNA-2. RBP-L exhibits transactivation activity in collaboration with EBNA-2 in spite of the absence of a direct physical interaction. The following three models can be proposed to explain this puzzle (Fig. 7). The first model assumes that EBNA-2 is recruited to the promoter by an unknown cofactor that binds to both RBP-L and EBNA-2 and forms the ternary complex on the promoter (Fig. 7A). Although no protein complex containing both EBNA-2 and RBP-L was detected in the coimmunoprecipitation experiment (Fig. 6), this complex could be too unstable to be detected under our experimental conditions. As shown in Fig. 5B, the EBNA-2 WW323SS mutant lacking the interaction activity with RBP-J loses transcriptional activity with GAL4–RBP-L, suggesting the possibility that endogenous RBP-J tethers EBNA-2 to DNA-bound RBP-L. However, we failed to detect any interaction between RBP-J and RBP-L. The other explanation is that the EBNA-2 mutant has lost affinity not only for RBP-J but also for the unknown cofactor. In the second model, RBP-L is assumed to associate with a putative constitutive corepressor to which EBNA-2 competes for binding with DNA-bound RBP-L, resulting in sequestering of the corepressor from the promoter (Fig. 7B). The third model assumes that collaboration of EBNA-2 with endogeneous RBP-J induces expression of a coactivator which transregulates other genes, including the reporter gene in cooperation with RBP-L (Fig. 7C). Possible involvement of endogeneous RBP-J in this event agrees with but is not directly supported by the observation that the EBNA-2 WW323SS mutant is unable to cooperate with GAL4–RBP-L (Fig. 5B). This model predicts that the EBNA-2–RBP-J complex should possess a target gene specificity different from that of the Notch–RBP-J complex because the truncated Notch1 did not display significant activity with GAL4–RBP-L (Fig. 5B). Although we and others have shown that EBNA-2 and Notch can transactivate some, if not all, common promoters (28, 37), the present study suggests that EBNA-2 and Notch1 regulate genes in different manners.

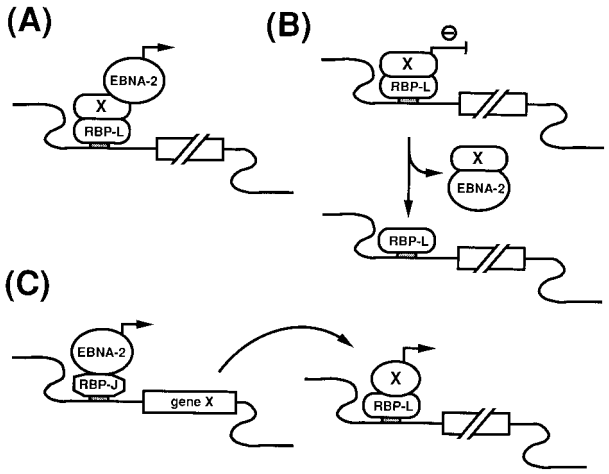

FIG. 7. Three models to explain cooperative transcriptional activity of RBP-L with EBNA-2. The recruitment model (A), the competition model (B), and the transactivation model (C) are schematically presented to explain functional interaction between RBP-L and EBNA-2 in transcriptional regulation. Putative cellular factors are represented by X. In model A, the cofactor X is assumed to recruit EBNA-2 to the promoter by tethering EBNA-2 to DNA-bound RBP-L. In model B, a constitutive corepressor X is assumed to be sequestered from the promoter by EBNA-2 competing with RBP-L for binding to the X protein. In model C, EBNA-2 is assumed to induce expression of a cellular coactivator X in collaboration with endogenous RBP-J, and the resulting X gene product transactivates other genes in the presence of RBP-L.

Possible function of RBP-L in vivo. Although in vivo function of RBP-L is unknown, it is likely that RBP-L plays some role in transcriptional regulation in view of its similarity to RBP-J in structure and binding DNA sequence. Expression of RBP-L is strictly confined to lung, with much less amounts in brain and spleen, suggesting its role in regulation of lung-specific functions. RBP-L may cooperate with an unidentified lung-specific Notch family member or an unknown host homolog of EBNA-2. In any case, it will be interesting to test the

possibility that there are more RBP-J-related proteins whose expression is restricted to limited tissues.

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