Evi9 Encodes a Novel Zinc Finger Protein That Physically Interacts with BCL6, a Known Human B-Cell Proto-Oncogene Product

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Evi9 is a common site of retroviral integration in BXH2 murine myeloid leukemias. Here we show that *Evi9* encodes a novel zinc finger protein with three tissue-specific isoforms: Evi9a (773 amino acids [aa]) contains two C_2H_2-type zinc finger motifs, a proline-rich region, and an acidic domain; Evi9b (486 aa) lacks the first zinc finger motif and part of the proline-rich region; Evi9c (239 aa) lacks all but the first zinc finger motif. Proviral integration sites are located in the first intron of the gene and lead to increased gene expression. Evi9a and Evi9c, but not Evi9b, show transforming activity for NIH 3T3 cells, suggesting that *Evi9* is a dominantly acting proto-oncogene. Immunolocalization studies show that Evi9c is restricted to the cytoplasm whereas Evi9a and Evi9b are located in the nucleus, where they form a speckled localization pattern identical to that observed for BCL6, a human B-cell proto-oncogene product. Coimmunoprecipitation and glutathione *S*-transferase pull-down experiments show that Evi9a and Evi9b, but not Evi9c, physically interact with BCL6, while deletion mutagenesis localized the interaction domains in or near the second zinc finger and POZ domains of Evi9 and BCL6, respectively. These results suggest that *Evi9* is a leukemia disease gene that functions, in part, through its interaction with BCL6.

Retroviral integration in murine hematopoietic cells can lead to the generation of leukemias by enhancing expression of cellular proto-oncogenes or by disrupting expression of tumor suppressor genes. Retroviral proviruses in murine leukemias thus provide powerful genetic tags for identifying leukemia disease genes (15).

One mouse strain that develops a high incidence of retrovirally induced leukemia is BXH2. More than 95% of BXH2 mice die of retrovirally induced myeloid leukemia by 1 year of age (2). A number of disease genes have been identified in BXH2 leukemias by proviral tagging. They include a tumor suppressor gene, *Nf1* (neurofibromatosis type 1); a gene with homology to the lymphoid-restricted type II membrane protein Jaw1 *Mrvi1* (Mrv integration site 1); a gene encoding a hematopoietic cell growth and differentiation factor, *Myb* (myeloblastosis oncogene); three homeobox genes, *Hoxa7* (homeobox A7), *Hoxa9* (homeobox A9), and *Meis1* (myeloid ecotropic viral integration site 1); and a gene with homology to the *Usp8* (ubiquitin-specific protease 8) oncogene and to genes encoding various cell cycle regulatory proteins, *Evi5* (ecotropic viral integration site 5) (5, 21, 32). At least three of these genes are proven or suspected human disease genes: *NF1* and *HOXA9* are causally associated with myeloid leukemia, and *EVI5* is associated with stage 4S neuroblastoma (5, 30), validating the usefulness of this approach for identifying human disease genes.

Although proviral tagging has successfully identified a number of leukemia disease genes, it appears that there are many more to be identified. For example, whereas seven disease genes have been identified in BXH2 leukemias to date, as many as 65% of BXH2 leukemias do not have a virally induced mutation in one of these genes. The expectation is that continued proviral tagging in BXH2 leukemias will identify additional disease genes and, based on previous studies, that a number of these genes will represent human disease genes.

In previous studies, we identified a new common site of proviral integration site in BXH2 leukemias that we called ecotropic viral integration site 9 (*Evi9*) (26). Proviral integrations at *Evi9* are rare and were found in only 2 of 205 (1%) BXH2 leukemias tested. Chromosome and physical mapping studies showed that *Evi9* is located near the *c-rel* proto-oncogene on chromosome 11 (but did not encode c-Rel) and suggested that *Evi9* may represent a new leukemia disease gene. Here we show that this is likely to be the case by demonstrating that *Evi9* encodes a novel zinc finger protein which transforms NIH 3T3 cells in vitro and binds to another zinc finger protein, BCL6, which itself is a known human B-cell proto-oncogene product.

MATERIALS AND METHODS

BXH2 mice and leukemic cell line. The BXH2 recombinant inbred strain was obtained from the Jackson Laboratory (Bar Harbor, Maine) and maintained at the NCI-Frederick Cancer Research and Development Center. BXH2 leukemic cell lines have been previously described (19).

Genomic and cDNA cloning. *Evi9* ecotropic proviral integration sites from BXH2 leukemia cell lines B162 and B139 locus were isolated according to previously described methods (26). A murine bacterial artificial chromosome (BAC) clone was purchased from Research Genetics after PCR-based screening for positive clones. Lambda phage clones derived from a 129/Sv mouse genomic

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library (Stratagene) were also isolated by hybridization. Exon trapping was performed using BAC clone B413N15 and the pSPL3 vector (Life Technology) according to the manufacturer's protocol. The trapped exons were used as a probe to screen an 11-day mouse cDNA library (Clontech). Positive clones were purified, subcloned into pBluescript, and sequenced.

RNA isolation, Northern blots, and reverse transcription-PCR (RT-PCR). Poly(A)$^+$ RNA was isolated from frozen leukemia cell suspensions using a FastTrack 2.0 kit (Invitrogen). Total RNA was extracted from mouse normal tissues or cultured cell suspensions by the RNAzol method (TelTest). Two micrograms of poly(A)$^+$ RNA was fractionated by electrophoresis in 1.0% agarose gels containing formaldehyde and transferred to Hybond N+ membrane (Amersham). Mouse multiple-tissue Northern blots were purchased from Clontech. Membranes were hybridized and washed according to the method of Church and Gilbert (4). AA53, which is a 1.5-kb cDNA fragment which corresponds to nucleotides 838 to 2346 of *Evi9*, was used as a probe.

First-strand cDNA was synthesized from 1 μg of total RNA by using random hexamers and Superscript II reverse transcriptase (Life Technologies) in a total volume of 20 μl. The mixture was diluted to 50 μl, and 2 μl was subjected to PCR using a temperature cycling protocol of 94°C for 30 s, 60°C for 1 min, and 72°C for 4 min (35 cycles). PCR products were separated by electrophoresis through a 1.2% agarose gel, transferred to a Hybond N+ membrane, and hybridized with the AA53 probe. The PCR primers used were *Evi9* RACE1 (5′-TGAACCGA GCCGTCGTCCGCACG-3′) and S2 (5′-TCCATCCGAAAACTGCCAC-3′).

Antibodies. Rabbit polyclonal antisera were raised against the recombinant Evi9 proteins (Ab1 for amino acids [aa] 80 to 208 and Ab2 for aa 613 to 663). Anti-BCL6 antibodies were described previously (27). Anti-PML and anti-Sp100 antibodies were kind gifts from Thomas Sternsdorf. Anti-Myc epitope antibody 9E10 (Boehringer Mannheim) was also used.

Plasmid constructs. Three cDNA isoforms of *Evi9* were subcloned into the pCDNA3.1 expression plasmid (Invitrogen). Green fluorescent protein (GFP)-tagged constructs were generated by insertion of the isoforms in frame into the EGFPN1 vector (Clontech). Truncated proteins for glutathione *S*-transferase (GST) fusion constructs were generated by PCR of the full-length cDNA clone. The Myc-tagged BCL6 expression construct was a kind gift from Tohru Miki, and PML and Sp100 constructs were gifts of Thomas Sternsdorf. GST-BCL6 fusion constructs were described previously (25).

Transformation assays. NIH 3T3 fibroblasts were seeded on six-well culture dishes at low cell density 18 h prior to transfection. pCDNA expression plasmids bearing each isoform of *Evi9* were transfected into NIH 3T3 cells, using Lipofectamine and Opti-MEM (Life Technology). Transfectants were first selected with G418, and 2×10^4 cells of G418-resistant fibroblasts were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.3% agar in 3-cm-diameter dish. The cells were cultured at 37°C under 5% CO_2, and colonies were counted after 3 weeks. The cells were plated in triplicate each for three independent experiments.

Transient transfection and immunofluorescence. COS7 and HeLa cells were seeded on poly-L-lysine-coated culture slides (Falcon) 18 h prior to transfection. Transfection of expression plasmids was carried out using Lipofectamine and Opti-MEM. After overnight culture, cells were fixed in methanol for 30 min at −20°C, washed three times with phosphate-buffered saline, blocked with 10% normal goat serum, and incubated with primary antibodies. Positive signals were visualized with fluorescein isothiocyanate or rhodamine. Nuclear DNA was counterstained with Hoechst 33342. The cells were examined by confocal laser-scanning microscopy (Olympus) or Carl Zeiss Axiophoto II fluorescence microscopy.

Coimmunoprecipitation and in vitro binding experiments. A total of 10^7 B162, Raji, or Jurkat cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 1 mg of leupeptin per ml, 1 mg of aprotinin per ml). The cell suspensions were sonicated and clarified by centrifugation. Immunoprecipitation was carried out by using anti-Evi9 antibody Ab1 or preimmune serum and then by adding protein A-Sepharose (Pharmacia). Immunoprecipitates were Western blotted with the anti-BCL6 antibody by the enhanced chemiluminescence method (Amersham).

For in vitro binding experiments, bacterially expressed GST fusion proteins were purified according to methods previously described (33). In vitro-translated Evi9 and BCL6 proteins were prepared by using pSP64 plasmids bearing cDNAs, the TnT coupled reticulocyte lysate system (Promega), and [^{35}S]methionine. Similar quantities of GST or GST fusion proteins immobilized on glutathione-Sepharose beads were incubated with in vitro-translated proteins in buffer A (50 mM Tris [pH 7.5], 0.5 mM EDTA, 0.4 M NaCl, 5 mM $MgCl_2$, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol) for 1 h at 4°C with gentle agitation. Bound proteins were then washed four times in 1 ml of buffer A, eluted in SDS sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Nucleotide sequence accession numbers. The sequence data reported have been submitted to the GenBank database under accession no. AF051525, AF169036, and AF169037.

RESULTS

***Evi9* transcript identification.** Genomic restriction analysis of DNA from two BXH2 myeloid leukemias with *Evi9* proviral integrations (B139 and B162) showed that the proviral integrations are located about 1 kb apart from each other (Fig. 1A). A 90-kb BAC clone (B413N15) which covers the integration site was then isolated and exon trapped. Two exons of 171 bp (exon 1) and 198 bp (exon 2) were identified (Fig. 1A). Sequence analysis showed that the proviral integrations are located within the first intron of the gene in the reverse transcriptional orientation (Fig. 1A), a location and orientation frequently found for retroviral integrations that activate gene expression through an enhancer mechanism (15).

Hybridization of adult and embryonic tissue Northern blots using the two exons as a probe identified four major *Evi9* transcripts 9.5, 7.4, 3.2, and 1.5 kb in size. In the adult, the highest *Evi9* expression was found in the brain and spleen with lower levels in the testis and lung (Fig. 1B). In the embryo, little expression of *Evi9* was detected at day 7. However, by day 11 high levels of *Evi9* expression were observed, which continued until day 17, the last embryonic stage examined.

Northern blots containing RNA from a number of BXH2 myeloid leukemia cell lines were also examined for *Evi9* expression. In the two cell lines containing viral integrations at *Evi9*, high expression of *Evi9* was observed (Fig. 1C). In contrast, cell lines like B160, B114, and B112, which lack proviral integrations at *Evi9*, do not express *Evi9* at detectable levels. These results are consistent with the hypothesis that proviral integrations at *Evi9* upregulate the expression of *Evi9*. In a few cell lines like B132 and B119, which do not have proviral integrations at *Evi9*, high expression of *Evi9* was also observed. While the significance of this result is unclear, it is possible that these cells lines contain proviral integrations at *Evi9* that were undetected because they lie outside the region screened for proviral integrations or, alternatively, there are other mechanisms for upregulating *Evi9* expression in leukemic cells besides proviral integration.

***Evi9* encodes a novel Krüppel-like zinc finger protein.** *Evi9* cDNAs were identified by screening an 11-day mouse embryonic cDNA library, using exons 1 and 2 as the probe. Sequence analysis of the positive clones yielded a 3,002-bp continuous sequence containing a 2,319-bp open reading frame, 273 bp of 5′ untranslated region (UTR) sequence, and 410 bp of 3′ UTR sequence (Fig. 2). The deduced amino acid sequence contains both single and double Cys$_2$-His$_2$-type zinc finger motifs, indicating that *Evi9* encodes a member of the Krüppel-like family of zinc finger proteins. Outside of these zinc finger domains, *Evi9* has no homology to anything in current databases, indicating that it encodes a novel protein. Known structures such as a KRAB box, POZ domain, or FAX domain, sometimes seen in Krüppel-type zinc finger proteins (1, 10, 17, 29, 41), are missing. The only other notable features of the Evi9, beside the zinc fingers, are a 186-aa proline-rich region located between the two zinc finger motifs and an acidic domain located downstream of the second zinc finger motif. The presence of an acidic domain suggests that Evi9 may act as a transcriptional activator in some cell contexts (24). As shown in Fig. 1B and C, *Evi9* has large, alternatively spliced transcripts. Sequence analysis of multiple clones revealed that there are additional 3′ untranslated sequences which contribute larger transcripts (data not shown).

Multiple *Evi9* isoforms. *Evi9* cDNA sequencing suggested that *Evi9* may be alternatively spliced. RT-PCR experiments confirmed this prediction and identified two alternatively spliced *Evi9* isoforms designated *Evi9b* and *Evi9c* (Fig. 3A).
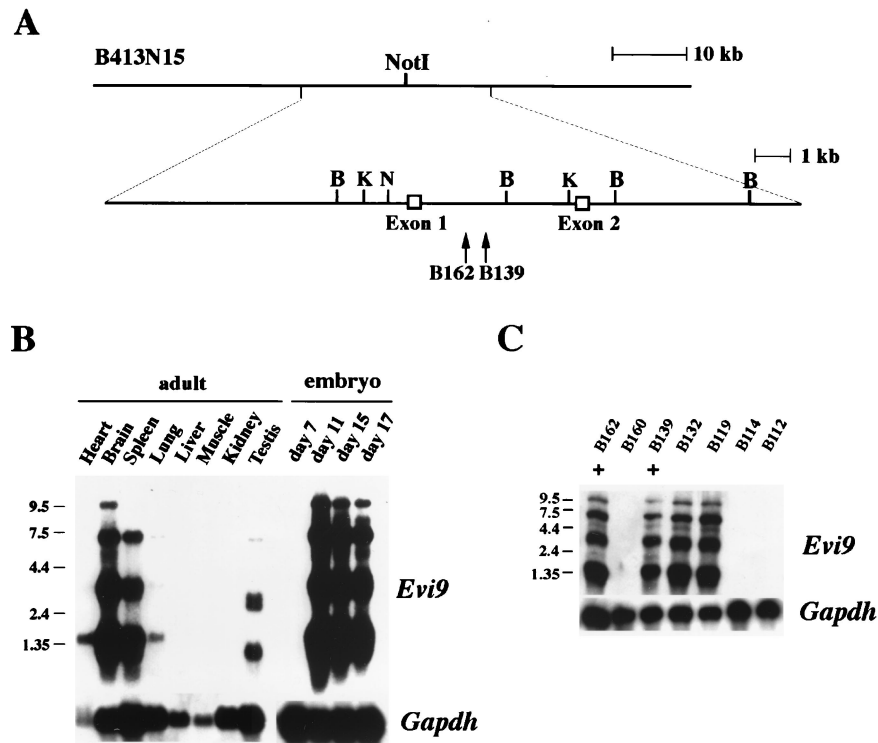
FIG. 1. Identification of *Evi9* transcripts. (A) Partial genomic structure of the 5′ region of *Evi9*. Exons 1 and 2 were trapped from the 90-kb BAC clone B413N15. B; *Bam*HI, K; *Kpn*I, N; *Not*I. Locations of the proviral integration sites in BXH2 leukemias B139 and B162 are shown by arrows. (B and C) *Evi9* expression in normal adult and embryonic mouse tissue (B) and BXH2 leukemias (+ indicates leukemias with proviral integration at *Evi9*) (C). The blots were probed with *Gapdh* to normalize for RNA loading. Sizes of molecular weight markers in kilobases are shown on the left of each panel.

Evi9b is predicted to encode a 487-aa protein from an alternative translation initiation site. This protein is missing the first zinc finger motif and part of the proline-rich region of Evi9a (the full-length protein). *Evi9c* is predicted to encode a 239-aa protein that is missing all but the first zinc finger motif of Evi9a.

RT-PCR expression analysis showed that *Evi9* isoform expression varies according to cell type (Fig. 3B). In the testis, kidney, and spleen, all three isoforms are expressed at similar relative levels. The same is true for BXH2 leukemia cell lines such as B119, B162, and B139. In other tissues like the cerebrum and intestine, expression of *Evi9a* is enriched, while in the cerebellum and thymus, expression of *Evi9b* is enriched. In other tissues, one or more isoforms appear to be missing. For example, only *Evi9a* appears to be expressed in the stomach, while *Evi9b* is expressed exclusively in the lung, being absent in bone marrow, heart, and brain stem. These results suggest that the *Evi9* isoforms have cell-type-specific functions.

Evi9 transforms NIH 3T3 fibroblasts. Since the enhanced expression of *Evi9* by retroviral integration in BXH2 leukemias suggested that *Evi9* might act as a dominant oncogene, the oncogenic potential of the various *Evi9* isoforms was determined in an NIH 3T3 cell transformation assay. Each *Evi9* isoform was inserted into a pCDNA3.1 expression vector and transfected into NIH 3T3 cells. NIH 3T3 cells transfected with *Evi9a* formed significantly increased anchorage-independent colonies in soft agar compared to cells transfected with vector alone (Fig. 4). In addition, *Evi9c* showed weak transforming activity for NIH 3T3 cells that was additive with the transforming activity of *Evi9a*. In contrast, *Evi9b* did not show transforming activity for NIH 3T3 cells when assayed alone or in com-

bination with *Evi9a* or *Evi9c*. These results support the hypothesis that *Evi9* functions as a dominant oncogene.

Evi9 isoforms have different subcellular localization patterns. The subcellular localization of the different Evi9 isoforms was assessed by transient expression of each isoform followed by immunofluorescence using isoform-specific antibodies (Fig. 5). Evi9a and Evi9b, which are similar in their C-terminal regions, are located exclusively in the nucleus (Fig. 5A and C). In addition, both isoforms are distributed within spherical nuclear structures, 50 to 500 nm in diameter. In contrast, Evi9c, which lacks most of the C-terminal region of the protein, is localized predominantly in the cytoplasm (Fig. 5E). The nuclear localization of endogenous Evi9a and/or Evi9b proteins was also observed in B162 leukemia cells. The spherical nuclear localization was again noted, though the structures were more irregular in distribution and size (Fig. 5G).

Evi9a colocalizes with BCL6. The speckled hybridization pattern of Evi9a and Evi9b suggested that they are located within nuclear bodies or other subnuclear compartments (8, 11, 20, 33). Many proteins are known to localize to these nuclear compartments. Among these proteins, three—PML, Sp100, and BCL6—are involved in hematopoietic disease (7, 8, 36). To determine whether any of these proteins colocalize with Evi9a or Evi9b, expression constructs of these three proteins were cotransfected into HeLa cells along with a GFP-tagged *Evi9a* expression construct (Fig. 6). The nuclear localization of GFP-tagged Evi9a was identical to that of native Evi9a (indicating that the GFP tag did not cause Evi9a to become mislocalized [Fig. 5A and 6A]) as well as to BCL6 (Fig. 6A to C) but not to PML or Sp100 (Fig. 6D to I). Evi9a,

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       GCCATTTTTTTCATCTCTCTCTCTCCCTCTATCCCTCTTCTCTCTTCCTCTCTCTCTTTTGTTTCCTTAAAAAAAAAAAAAGCCATGACGGCTCTCCCACA    100
       ATTCATCTTCCCTGCGCCATCTTTGTATTATTTCTAATTTATTTTGGATGTCAAAAGGCACTGATGAAGAATGTTCTCTGGAGTCTCCTTCTTTCTAACC    200
       CGGCTCTCCCGATGTGAACCGAGCCGTCGTCCGCACGCCGCCGCCGCCGCCGCCGCCCGCCCCGCAGCCCACCATGTCTCGCCGCAAGCAAGGCAAACCC    300
                                                                                    M  S  R  R  K  Q  G  K  P
  1                                  Exon 1
       CAGCACTTAAGCAAACGGGAATTCTCGGCCGAACCTCTTGAAGCCATTCTTACAGATGATGAACCAGACCATGGCCCGTTGGGAGCTCCAGAAGGGGACC    400
 10    Q  H  L  S  K  R  E  F  S  P  E  P  L  E  A  I  L  T  D  D  E  P  D  H  G  P  L  G  A  P  E  G  D
Exon 2                                                                                              Exon 2
       ACGACCTTCTCACCTGTGGGCAGTGCCAGATGAATTTCCCACTGGGGGACATTCTTATTTTTATCGAGCACAAACGGAAACAATGCAATGGCAGCCTCTG    500
 43    H  D  L  L  T  C  G  Q  C  Q  M  N  F  P  L  G  D  I  L  I  F  I  E  H  K  R  K  Q  C  N  G  S  L  C
       CTTAGAAAAAGGTGTGGATAAGCCGCCTTCCCCTTCTCCCATCGAGATGAAAAAGGCATCCAATCCTGTGGAGGTTGGCATCCAGGTCACGCCAGAGGAT    600
 77    L  E  K  G  V  D  K  P  P  S  P  S  P  I  E  M  K  K  A  S  N  P  V  E  V  G  I  Q  V  T  P  E  D
       GACGATTGTTTATCAACGTCATCTAGAGGAATTTGCCCCAAACAGGAACACATAGCAGATAAACTTCTGCACTGGAGGGGCCTGTCCTCTCCTCGGTCTG    700
110    D  D  C  L  S  T  S  S  R  G  I  C  P  K  Q  E  H  I  A  D  K  L  L  H  W  R  G  L  S  S  P  R  S
       CACACGGAGCTCTAATCCCCACGCCCGGGATGAGTGCAGAATATGCCCCGCAGGGTATTTGTAAAGATGAGCCCAGCAGCTACACATGTACAACTTGCAA    800
143    A  H  G  A  L  I  P  T  P  G  M  S  A  E  Y  A  P  Q  G  I  C  K  D  E  P  S  S  Y  T  C  T  T  C  K
       ACAGCCATTCACCAGTGCATGGTTTCTCTTGCAACACGCACAGAACACTCATGGATTAAGAATCTACTTAGAAAGTAACACGGAAGTCCCCTGACCCCG    900
·177   Q  P  F  T  S  A  W  F  L  L  Q  H  A  Q  N  T  H  G  L  R  I  Y  L  E  S  E  H  G  S  P  L  T  P
       CGGGGTTGGTATCCCTTCAGGACTAGGTGCAGAATGTCCTTCCCAGCCACCTCTCCATGGGATTCATATTGCAGACAATAACCCCTTTAACCTGCTAAGAA   1000
210    R  V  G  I  P  S  G  L  G  A  E  C  P  S  Q  P  P  L  H  G  I  H  I  A  D  N  N  P  F  N  L  L  R
       TACCAGGATCAGTATCGAGAGAGGCTTCCGGCCTGGCAGAAGGGCGCTTTCCACCCACTCCCCCCCTGTTTAGTCCACCACCGAGACATCACTTGGACCC   1100
243    I  P  G  S  V  S  R  E  A  S  G  L  A  E  G  R  F  P  P  T  P  P  L  F  S  P  P  P  R  H  H  L  D  P
       CCACCGCATAGAGCGCCTGGGGGCGGAAGAGATGGCCCTGGCCACCCATCACCCGAGTGCCTTTGACAGGGTGCTGCGGTTGAATCCAATGGCTATGGAG   1200
277    H  R  I  E  R  L  G  A  E  E  M  A  L  A  T  H  H  P  S  A  F  D  R  V  L  R  L  N  P  M  A  M  E
       CCTCCCGCCATGGATTTCTCTAGGAGACTTAGAGAGCTGGCAGGGAACACGTCTAGTCCACCGCTGTCCCCAGGCCGGCCCAGTCCTATGCAAAGGTTAC   1300
310    P  P  A  M  D  F  S  R  R  L  R  E  L  A  G  N  T  S  S  P  P  L  S  P  G  R  P  S  P  M  Q  R  L
       TGCAACCATTCCAGCCAGGTAGCAAGCCACCCTTCCTGGCGACGCCCCCCCTCCCTCCTCTGCAATCCGCCCCTCCTCCCTCCCAACCCCCGGTCAAGTC   1400
343    L  Q  P  F  Q  P  G  S  K  P  P  F  L  A  T  P  P  L  P  P  L  Q  S  A  P  P  P  S  Q  P  P  V  K  S
       CAAGTCATGCGAGTTCTGCGGCAAGACGTTCAAATTTCAGAGCAACTTGGTGGTTCACCGACGCAGCCATACTGGTGAGAAGCCCTATAAGTGCAACCTG   1500
377    K  S  C  E  F  C  G  K  T  F  K  F  Q  S  N  L  V  V  H  R  R  S  H  T  G  E  K  P  Y  K  C  N  L
       TGCGACCACGCGTGCACACAGGCCAGCAAGCTGAAGCGTCACATGAAGACACACATGCACAAATCGTCCCCCATGACAGTCAAGTCCGACGATGGCCTCT   1600
410    C  D  H  A  C  T  Q  A  S  K  L  K  R  H  M  K  T  H  M  H  K  S  S  P  M  T  V  K  S  D  D  G  L
       CCACAGCCAGCTCCCCGGAACCTGGTACCAGCGACCTGGTGGGCAGCGCCAGCAGTGCGCTCAAGTCAGTGGTGGCCAAGTTCAAGAGTGAGAACGACCC   1700
443    S  T  A  S  S  P  E  P  G  T  S  D  L  V  G  S  A  S  S  A  L  K  S  V  V  A  K  F  K  S  E  N  D  P
       CAACTTGATCCCAGAGAACGGGGATGAGGAGGAGGAGGAGGACGACGAGGAAGAAGAAGAAGAGGAGGAAGAGGAGGAGGAGGAGCTGACGGAGAGCGAG   1800
477    N  L  I  P  E  N  G  D  E  E  E  E  E  D  D  E  E  E  E  E  E  E  E  E  E  E  E  E  E  E  E  L  T  E  S  E
       AGGGTGGACTACGGCTTCGGGCTGAGCCTGGAGGCTGCACGCCACCATGAGAACAGCTCTCGGGGCGCAGTGGTGGGCGTGGGCGACGAGGGCCGCGCCC   1900
510    R  V  D  Y  G  F  G  L  S  L  E  A  A  R  H  H  E  N  S  S  R  G  A  V  V  G  V  G  D  E  G  R  A
       TGCCCGATGTCATGCAGGGCATGGTGCTCAGCTCCATGCAGCACTTCAGCGAGGCCTTCCACCAGGTCCTGGGCGAAAAGCATAAGCGTAGCCACCTGGC   2000
543    L  P  D  V  M  Q  G  M  V  L  S  S  M  Q  H  F  S  E  A  F  H  Q  V  L  G  E  K  H  K  R  S  H  L  A
       CGAGGCCGAGGGCCATAGGGACACTTGTGATGAAGACTCGGTGGCCGGTGAGTCAGACCGCATAGACGATGGCACTGTTAATGGTCGTGGCTGCTCCCCC   2100
577    E  A  E  G  H  R  D  T  C  D  E  D  S  V  A  G  E  S  D  R  I  D  D  G  T  V  N  G  R  G  C  S  P
       GGCGAATCGGCTTCGGGGGGTCTGTCCAAAAAGCTGCTGCTGGGGTAGCCCCAGCTCGCTGAGCCCCTTCTCCAAGCGCATCAAGCTGGAGAAGGAGTTTG   2200
610    G  E  S  A  S  G  G  L  S  K  K  L  L  L  G  S  P  S  S  L  S  P  F  S  K  R  I  K  L  E  K  E  F
       ACCTGCCCCCGGCCGCGATGCCTAACACGGAGAACGTGTATTCGCAGTGGCTCGCTGGCTATGCGGCCTCCAGGCAGCTCAAAGATCCCTTCCTTACTTT   2300
643    D  L  P  P  A  A  M  P  N  T  E  N  V  Y  S  Q  W  L  A  G  Y  A  A  S  R  Q  L  K  D  P  F  L  T  F
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677    G  D  S  R  Q  S  P  F  A  S  S  E  H  S  S  E  N  G  S  L  R  L  S  T  P  P  G  E  L  D  G  G
       ATCTCAGGGCGCAGCGGCACAGGAAGTGGAGGGAGCACGCCCCATATTAGTGGTCCGGGCCCGGGCAGGCCCAGCTCAAAAGAGGGCAGACGCAGCGACA   2500
710    I  S  G  R  S  G  T  G  S  G  G  S  T  P  H  I  S  G  P  G  P  G  R  P  S  S  K  E  G  R  R  S  D
       CTTGTCCTTCACACACCCCCGTTCGGCGTAGTACCCCGCGAGCTCAAGATGTGTGGCAGTTTTCGGATGGAAGCTCAAGAACCCTTAAGTTCTGAGAAAC   2600
743    T  C  P  S  H  T  P  V  R  R  S  T  P  R  A  Q  D  V  W  Q  F  S  D  G  S  S  R  T  L  K  F  *
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       AA
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FIG. 2. Nucleotide and deduced amino acid sequence of *Evi9* (GenBank accession no. AF051525). Zinc finger motifs are in bold. The first and second exons shown in Fig. 1A are boxed. A short acidic-rich region is doubly underlined. Two potential poly(A) signal sequences in the 3' UTR are singly underlined.
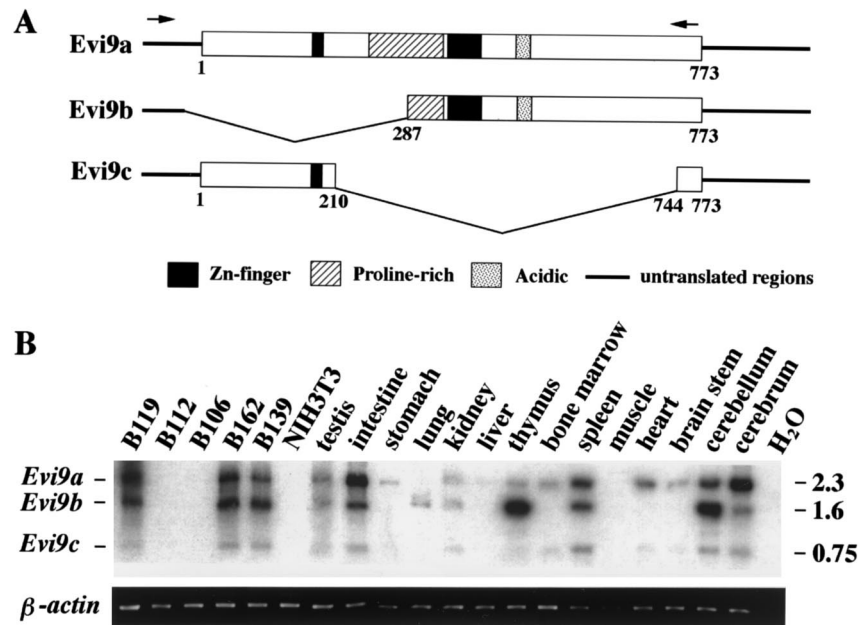
FIG. 3. Three Evi9 isoforms are expressed in a tissue-specific fashion. (A) Protein structures of three Evi9 isoforms. Arrows indicate the locations of PCR primers used for RT-PCR. (B) Expression patterns of three Evi9 isoforms (upper panel) in various tissues and BXH2 leukemia cell lines were analyzed by RT-PCR. RT-PCR products were gel fractionated, transferred to a nylon membrane, and hybridized with probe AA53. Sizes of PCR products in kilobases are shown on the right. β-Actin (lower panel) was amplified from the same samples to check for RNA quality.

without a GFP tag, also colocalized with BCL6, indicating that Evi9a binds to BCL6 in its native state (Fig. 6J to L). These results show that Evi9a (and Evi9b) and BCL6 colocalize within the nucleus.

Evi9a and BCL6 directly interact. To test for a direct interaction between Evi9 and BCL6, cellular extracts from B162 cells, in which *Evi9* is upregulated by retroviral integration, were subjected to immunoprecipitation using an anti-Evi9 antibody. Northern blot analysis showed that *BCL6* is expressed in B162 cells and in the majority of BXH2 leukemia cell lines examined (data not shown). The immunoprecipitated material was then immunoblotted with an anti-BCL6 antibody. These experiments clearly showed that Evi9a is coimmunoprecipitated along with BCL6 (Fig. 7A). The interaction between

FIG. 4. Anchorage-independent growth of NIH 3T3 cells expressing three different isoforms of Evi9. Numbers of colonies per 10^4 cells were determined after 3 weeks of growth in soft agar. Values are the means (±standard deviation) of three experiments.

Evi9a and BCL6 was also observed in the Raji B-lymphocytic cell line (Fig. 7B); however, no interaction was detected in the Jurkat T-cell lymphocytic cell line, which does not express *BCL6* (Fig. 7B).

GST pull-down assays were used to confirm that these two proteins directly interact. Purified GST-tagged BCL6 protein, or GST alone, was immobilized on glutathione-Sepharose beads and incubated with in vitro-translated Evi9a, Evi9b, or Evi9c, and the bound proteins were analyzed by SDS-PAGE. Consistent with immunolocalization and immunoprecipitation studies, Evi9a and Evi9b were found to physically interact with BCL6 (Fig. 8A). On the other hand, Evi9c, which is found in the cytoplasm, did not bind to BCL6 (Fig. 8A).

Deletion mutagenesis was carried out to map the protein interaction domains. Among the Evi9a deletion mutants tested for the ability to bind to BCL6, only the mutant containing the second zinc finger motif (aa 372 to 486) bound to BCL6 (Fig. 8B and C). This suggested that the BCL6 interaction domain is located in, or very near, the second zinc finger motif of Evi9. Likewise, the Evi9 interaction domain was localized within, or very near, the POZ domain of BCL6 (Fig. 8D and E).

DISCUSSION

We show here that the *Evi9* common retroviral integration site in BXH2 myeloid leukemias encodes three isoforms (Evi9a, Evi9b, and Evi9c) of a novel C_2H_2-zinc finger protein that does not show homology with any protein in current databases. Retroviral integration at *Evi9* leads to increased *Evi9* expression without altering the size of *Evi9* transcripts, suggesting that retroviral integration increases *Evi9* expression by an enhancer activation mechanism (15).

The upregulated expression of *Evi9* in myeloid leukemogenesis suggests that *Evi9* might act as a dominant oncogene. This possibility was confirmed using an NIH 3T3 transformation assay, which showed that Evi9a and Evi9c, but not Evi9b, have
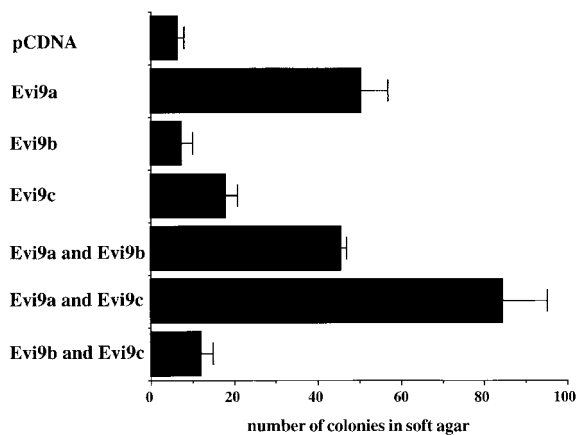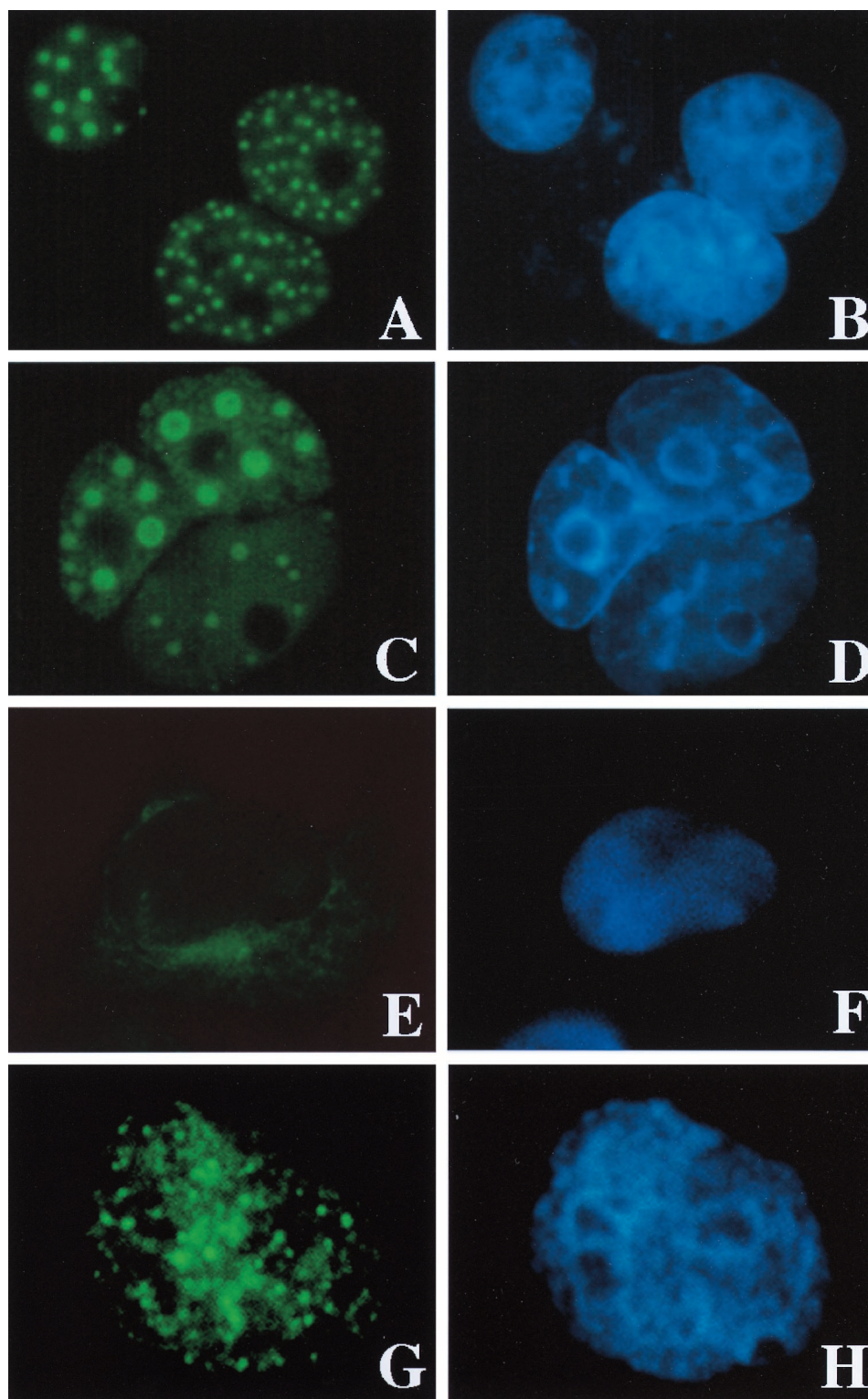
FIG. 5. Subcellular localization of Evi9 isoforms. Evi9a (A and B), Evi9b (C and D), and Evi9c (E and F) mammalian expression vectors were introduced into COS7 cells, and the protein was detected using polyclonal Evi9 antibody Ab1 (Evi9a and Evi9c) or Ab2 (Evi9a and Evi9b). Endogenous Evi9 protein (Evi9a and Evi9b) was also detected in B162 leukemia cells using the Ab2 antibody (G and H). Primary antibodies were visualized with fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin G (A, C, E, and G). DNA was stained with Hoechst 33342 (B, D, F, and H).
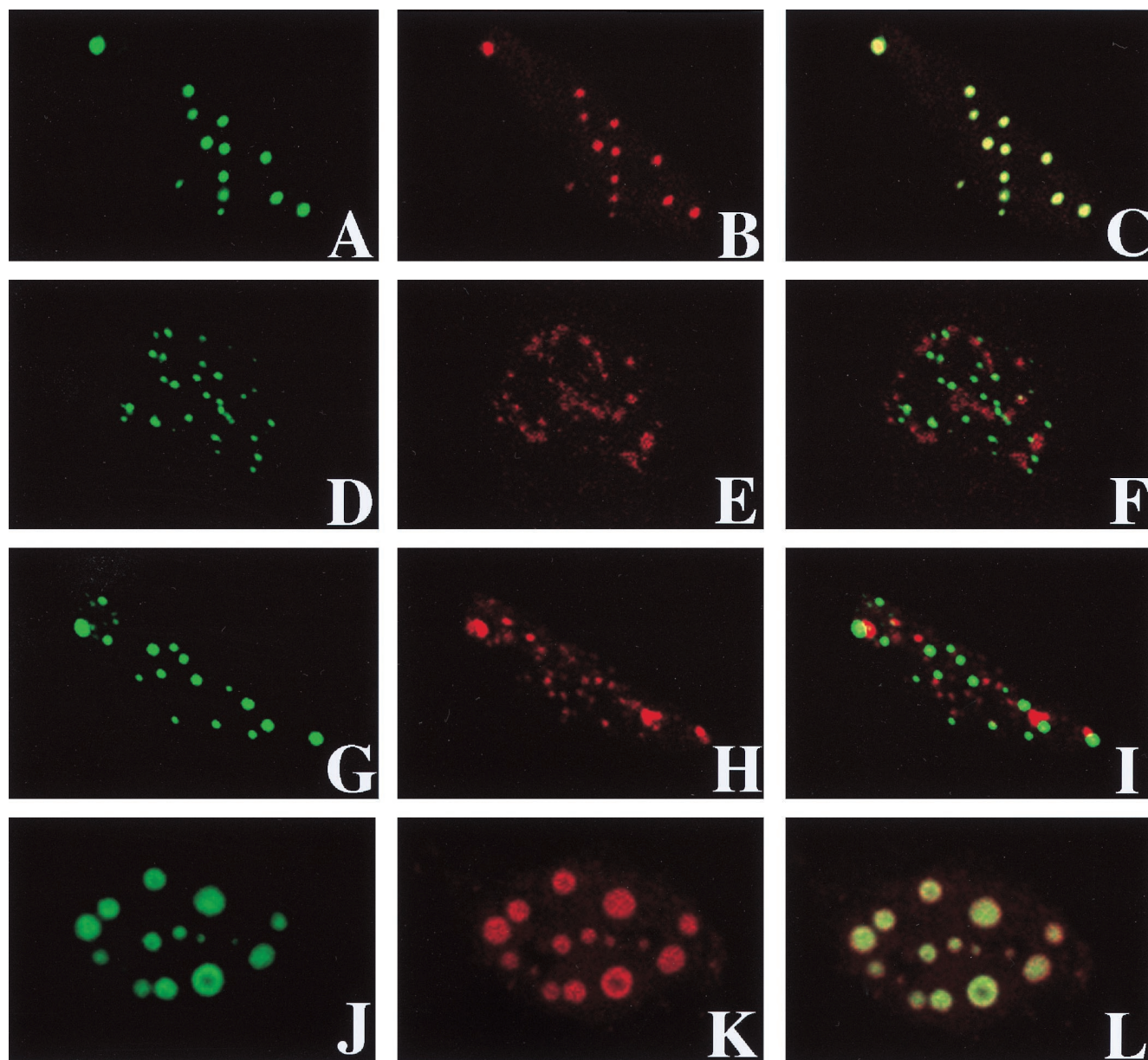
FIG. 6. Evi9 colocalizes with BCL6 but not with PML or Sp100. GFP-tagged Evi9a was cotransfected along with expression constructs of BCL6 (A to C), PML (D to F), or Sp100 (G to I) into HeLa cells. Evi9 localization was detected as GFP signals in panels A, D, and G. Polyclonal antisera against BCL6 (B), PML (E), or Sp100 (H) were also used, and the signals were detected using rhodamine-conjugated anti-rabbit immunoglobulin G. Colocalization was evaluated by merging the different images (C, F, and I). Colocalization of Evi9a and BCL6 was also confirmed by using nontagged Evi9a and Myc-tagged BCL6 constructs with anti-Evi9 Ab1 antisera (J) and anti-Myc epitope antibody (K). Colocalization was noted by merging the images (L).

transforming potential. These findings also suggested that the N-terminal region of Evi9, including a single zinc finger motif that is deleted in Evi9b, may be important for the oncogenic potential of Evi9. While Evi9a is predominantly localized in the nucleus, Evi9c is cytoplasmic. It is not clear at this point how Evi9c transforms NIH 3T3 cells, given its cytoplasmic localization. Perhaps the nucleus contains a small amount of Evi9c which is not detectable by immunohistochemistry, and it is this Evi9c that is important for cellular transformation. Alternatively, Evi9c may function in the cytoplasm by an as yet undefined mechanism.

The nucleus is not an amorphous structure but has many subnuclear functional domains (37). The speckled nuclear lo-

calization pattern of the larger of the two Evi9 isoforms (Evi9b and Evi9c), which contain the C-terminal region, suggested that Evi9 might interact with subnuclear structures such as nuclear bodies. In an attempt to identify interacting partners for Evi9, we tested PML (9, 18, 38), Sp100 (37), and BCL6/ LAZ3 (16, 23, 40) for the ability to interact with Evi9 since all three protein are involved in hematopoietic disease and are located in subnuclear structures. PML and Sp100 are located within what is called the PML oncogenic domain (POD), which seems to play some role in transcriptional regulation (11). Our findings, however, showed that Evi9 is not localized to PODs, and it is thus very likely that Evi9 nuclear speckles belong to a functionally distinct subdomain. Instead, we find that BCL6,
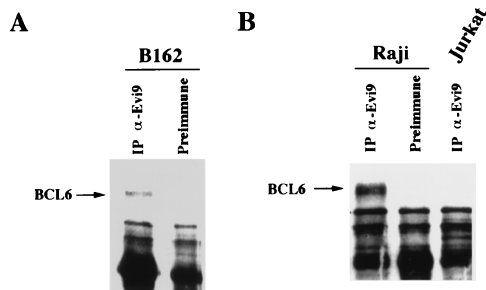
FIG. 7. Interaction of Evi9 and BCL6 in leukemia cell lines. (A) Cellular extracts of B162 leukemia cells were immunoprecipitated (IP) with anti-Evi9 antibody Ab1 or preimmune serum followed by immunoblotting using anti-BCL6 antibody. (B) A similar experiment showed interaction of Evi9 and BCL6 in the B-lymphocytic cell line Raji. No interaction was observed in the T-lymphocytic cell line Jurkat, which does not express BCL6.

which is known to show POD-independent nuclear dots (7), localizes and physically interacts with Evi9.

The *BCL6* gene is located at human chromosome 3q27 and was found to be involved in diffuse large B-cell lymphoma (16, 23, 40). *BCL6* encodes a zinc finger protein which functions as a sequence-specific transcriptional repressor (3, 31), and recent studies have suggested that the repressor function of BCL6 might result from its interaction with components of a corepressor complex containing N-CoR, SMRT, and histone deacetylase via its N-terminal POZ domain (14, 39). Similar

interactions have been found between corepressors and PLZF, another POZ/zinc finger protein which is fused to retinoic acid receptor in acute promyelocytic leukemia with a t(11;17) chromosome translocation (6, 12, 39). Unlike the case for *BCL6*, very weak expression of *PLZF* is found in most BXH2 leukemia, with or without retroviral integrations at *Evi9* (data not shown), suggesting that PLZF is not a major partner for Evi9 in BXH2 leukemias.

The interaction of transcription factors with corepressor complexes results in target gene repression through histone deacetylation and modulation of chromatin assembly (22). Our preliminary experiment show that Evi9 exhibits repressional potential for transcription (Y. Saiki et al., unpublished data), suggesting that the association of Evi9 and BCL6 may lead to recruitment of BCL6 and a corepressor complex to Evi9 target genes. Alternatively, Evi9 may bind the DNA target cooperatively with POZ/zinc finger proteins, and Evi9 may be recruited to the corepressor complexes by these partner proteins. Some cofactors may be required to increase the DNA binding specificity of Evi9, since it is likely that Evi9 can bind only a hexanucleotide sequence with its two C-terminal zinc fingers (28).

The biological significance of Evi9 and BCL6 interactions in leukemogenesis remains to be clarified. BCL6 is only weakly expressed in NIH 3T3 cells (data not shown), and coexpression of BCL6 did not alter the transforming activity of Evi9 against NIH 3T3 cells (data not shown). In addition, the shortest
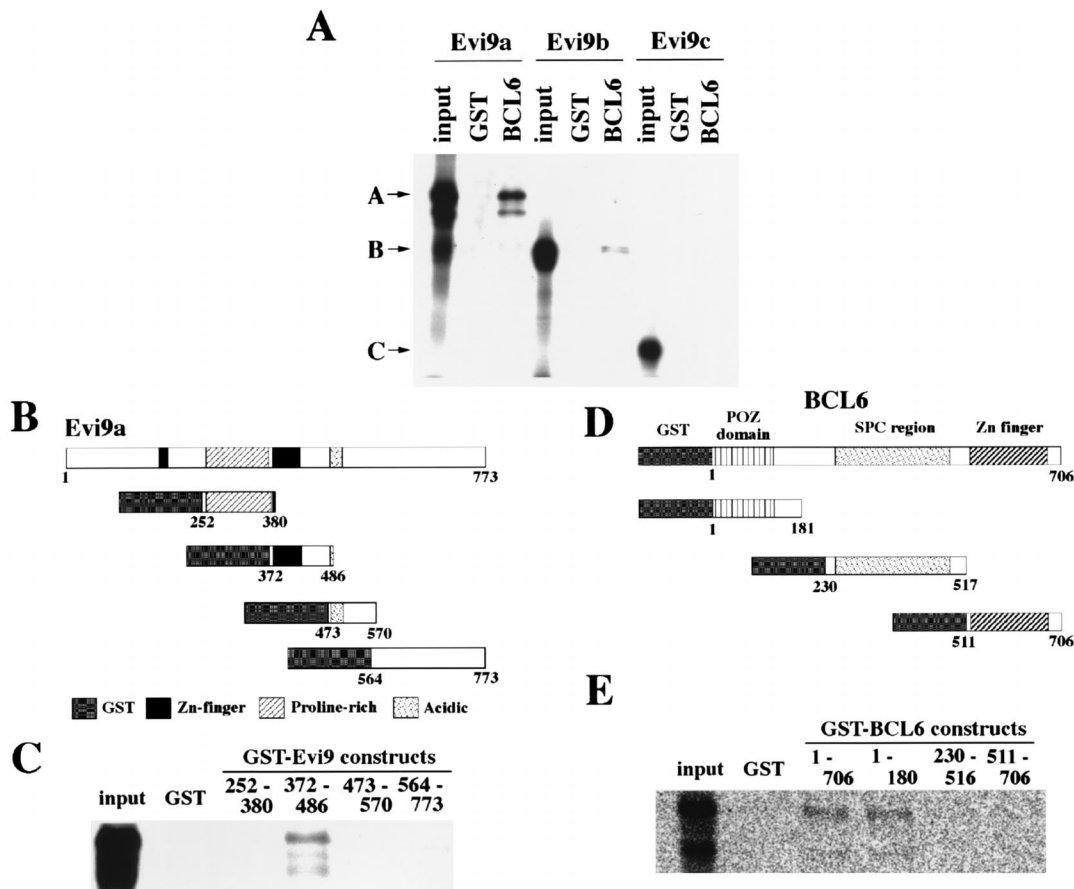

FIG. 8. Direct interaction of Evi9 and BCL6 in vitro. (A) GST pull-down assays were performed using in vitro-translated Evi9 isoforms and the full-length GST-BCL6 fusion protein. Evi9a and Evi9b show binding with BCL6, whereas Evi9c does not. (B) GST fusion constructs of Evi9. (C) The Evi9 fusion construct 372–486, which contains the second zinc finger motif, shows interaction with in vitro-translated BCL6 protein. (D) GST fusion constructs of BCL6. (E) The BCL6 fusion constructs 1–706 (full-length protein) and 1–180 show interaction with Evi9.

isoform of Evi9 (Evi9c), which does not interact with BCL6, also shows transforming activity for NIH 3T3 cells. These data suggest that there are two major possibilities. The first is that Evi9 and BCL6 interaction is not functionally important in leukemia cells; the second is that the interaction is important in leukemia cells but not in NIH 3T3 cells. Given the facts that Evi9 is expressed in the nucleus and is associated with BCL6 in leukemia cells, the latter possibility is more feasible. Experiments to test the functional importance of this interaction in leukemia cells are under way.

Currently we are investigating the possible involvement of *EVI9* in human hematopoietic disease. The human homologue of *Evi9* is located at chromosome 2p13 (T. Nakamura and M. Yoshida, unpublished data), where chromosomal abnormalities associated with human malignant lymphoma have been found (13). We are also making a targeted mutation of *Evi9* using embryonic cell knockout technology in order to better understand the biological relevance of the *Evi9* gene.

ACKNOWLEDGMENTS

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