JOURNAL OF VIROLOGY, Sept. 1997, p. 6796–6804 Vol. 71, No. 9
0022-538X/97/$04.00+0
Copyright © 1997, American Society for Microbiology

The Genes Encoding the Peripheral Cannabinoid Receptor and α-L-Fucosidase Are Located near a Newly Identified Common Virus Integration Site, *Evi11*

PETER J. M. VALK,[1] SAMANTHA HOL,[1] YOLANDA VANKAN,[1] JAMES N. IHLE,[2] DAVID ASKEW,[3] NANCY A. JENKINS,[4] DEBRA J. GILBERT,[4] NEAL G. COPELAND,[4] NICO J. DE BOTH,[5] BOB LÖWENBERG,[1] AND RUUD DELWEL[1]*

Institute of Hematology[1] and Department of Pathology,[5] Erasmus University Rotterdam, 3000 DR, Rotterdam, The Netherlands; Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38105[2]; Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, Ohio 45267[3]; and Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702[4]

Received 7 February 1997/Accepted 4 June 1997

A new common region of virus integration, *Evi11*, has been identified in two retrovirally induced murine myeloid leukemia cell lines, NFS107 and NFS78. By interspecific backcross analysis, it was shown that *Evi11* is located at the distal end of mouse chromosome 4, in a region that shows homology with human 1p36. The genes encoding the peripheral cannabinoid receptor (*Cnr2*) and α-L-fucosidase (*Fuca1*) were identified near the integration site by using a novel exon trapping system. *Cnr2* is suggested to be the target gene for viral interference in *Evi11*, since proviruses are integrated in the first intron of *Cnr2* and retroviral integrations alter mRNA expression of *Cnr2* in NFS107 and NFS78. In addition, proviral integrations were demonstrated within the 3′ untranslated region of *Cnr2* in five independent newly derived CasBrM-MuLV (mouse murine leukemia virus) tumors, CSL13, CSL14, CSL16, CSL27, and CSL97. The *Cnr2* gene encodes a seven-transmembrane G-protein-coupled receptor which is normally expressed in hematopoietic tissues. Our data suggest that the peripheral cannabinoid receptor gene might be involved in leukemogenesis as a result of aberrant expression of *Cnr2* due to retroviral integration in *Evi11*.

The identification of common virus integration sites (VISs) in retrovirally induced murine cancers has been proved successful for isolating transforming genes from hematopoietic malignancies and solid tumors (3, 30, 50). Cellular genes may become aberrantly expressed following proviral integration, through activation by viral promoter or enhancer sequences. Alternatively, proviral integration within the protein-coding region of a gene may result in the expression of an altered product (50). Abnormal expression of a proto-oncogene may provide the target cell with a growth abnormality and contribute to malignant transformation. A number of common VISs have been identified in murine retrovirally induced hematopoetic malignancies. These VISs are frequently designated ecotropic VISs (*Evi1* to *Evi10*), although other names have been used as well (4, 9, 30, 31, 34, 37, 41). Leukemia development in retrovirally induced leukemias is a multistep process which usually takes more then 3 months (3, 30, 50). Thus, in a retrovirally induced leukemia, one can predict more than one common VIS, resulting in aberrant expression of multiple genes. The frequency of a particular common VIS is usually low, i.e., below 5%, indicating that several of different genes may be responsible for retrovirus-mediated leukemogenesis in mice (3, 30, 50).

From a number of primary hematopoietic tumors, induced by Moloney murine leukemia virus (MoMuLV) or the wild mouse ecotropic retrovirus CasBrM-MuLV, in vitro cell lines have been established (3, 24). In these interleukin-3 (IL-3)-dependent cell lines, several common VISs and potential oncogenes, including *Evi1* (36), *Evi2* (9), and *His1* and *His2* (4), have been found. In the present study, we describe the identification of a new common VIS, designated *Evi11*, in two myeloid leukemia cell lines and five newly isolated CasBrM-MuLV-induced primary tumors.

Exon trapping has been used as an effective method for deducing mRNA-coding sequences from genomic DNA fragments (5, 10, 18, 23). We used a new exon trap vector, pEVRF0-ET, based on the rabbit β-globin (RβG) gene, which, in contrast to pSPL1 with an artificial intron (10), contains the natural intervening sequence 2 of the RβG gene (IVS2-β). This exon trap system was applied to the *Evi11* locus, and two genes, the peripheral cannabinoid receptor gene (*Cnr2*, formerly designated *Cb2* [S2]) and the α-L-fucosidase gene (*Fuca1*), were identified. The results of the studies presented here suggest that the hematopoietic receptor gene *Cnr2* is the candidate gene for viral interference in *Evi11*.

MATERIALS AND METHODS

Cell lines. Nine leukemic cell lines established in vitro from CasBrM-MuLV-induced primary tumors (NFS22, -36, -56, -58, -60, -61, -78, -107, and -124) (24) and 13 cell lines derived from MoMuLV-induced tumors (DA1, -2, -3, -7, -8, -13, -24, -25, -28, -29, -31, -33, and -34) (26) were cultured in RPMI 1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 ng/ml), 10% fetal calf serum (FCS), and 10% WEHI-3B cell conditioned medium (25). COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, penicillin, and streptomycin.

Primary tumors. Newborn NIH-Swiss mice were injected subcutaneous with cell culture supernatant of CasBrM-MuLV-producing NIH 3T3 cells (obtained from H. Morse III, National Cancer Institute, Frederick, Md.). Between 150 and 220 days after injection, the mice developed leukemias. All leukemic mice had enlarged (5- to 10-fold) spleens, and some had thymomas. Sixty CasBrM-MuLV-induced leukemias (CSL [CasBrM-MuLV Swiss leukemias]) were cryopreserved

* Corresponding author. Mailing address: Institute of Hematology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Phone: 31104087843. Fax: 31104362315. E-mail: Delwel@hema.fgg.eur.nl.

in liquid nitrogen. From these tumors, high-molecular-weight DNA was isolated (44) for Southern blot analysis.

Genomic cloning. From NFS107 cells, high-molecular-weight DNA was isolated as described previously (44). The DNA was partially digested with *Sau*3A and separated on 0.4% agarose. DNA fragments of 9 to 23 kb were isolated by electroelution and purified on NACS columns (Bethesda Research Laboratories, Bethesda, Md.). These fragments were then ligated into *Bam*HI EMBL3 phage arms and packaged (Stratagene, La Jolla, Calif.). DNA from 10^6 plaques was transferred to nitrocellulose filters and screened (44) with a [^{32}P]dATP-labelled 270-bp *Sma*I-*Pvu*II fragment isolated from the U_3 long terminal repeat (LTR) of MoMuLV (15). Probes were labelled by random priming (19). Filters were hybridized in 50% (vol/vol)–formamide–5% dextran sulfate sodium salt–5× SSPE (0.9 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 0.005 M EDTA [pH 7.7]–10× Denhardt's solution (0.2% [wt/vol] bovine serum albumin, 0.2% [wt/vol] Ficoll 400, 0.2% [wt/vol] polyvinylpyrrolidone)–0.1% (wt/vol) sodium dodecyl sulfate (SDS)–0.1 mg of sonicated salmon sperm DNA per ml. The blots were hybridized at 42°C overnight, washed for 15 min at 65°C in 2× SSPE–0.5% SDS and for 15 min at 65°C 0.5× SSPE, and analyzed by autoradiography. Phage DNA was isolated (44), and restriction maps of the different clones were prepared. DNA fragments adjacent to the VIS were isolated, subcloned in pBluescript II SK+ (Stratagene), and used for Southern blot analysis using DNAs of the different DA and NFS cell lines. Cosmid clones were isolated from a female (4 to 8 weeks) mouse liver genomic library (Stratagene catalog no. 946305) and a murine embryonic stem cell genomic library in cosmid pTBE, using standard techniques (44).

Southern and Northern blot analysis. Genomic DNA was isolated as described previously (16), digested with the appropriate restriction enzymes, and electrophoresed on a 0.6% agarose gel. Fragments were transferred to Hybond-N$^+$ (Amersham) nylon membranes with 0.25 M NaOH–1.5 M NaCl. Total RNA from cell lines was extracted with guanidinium isothiocyanate as described previously (44). Ten micrograms of total RNA was separated on a 1% agarose–6% formaldehyde gel and blotted with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto Hybond-N$^+$ nylon membranes (Amersham). Hybridization and washing procedures for Northern and Southern blots were identical to those described above. mRNA hybridization signals were quantitated by scanning of the Northern blots with a Vilber Lourmat camera and Bio-Profil V 4.6 software.

RNase protection. RNase protection was performed as described previously (44). Fragments were cloned into pBluescript II SK+ and linearized by using the proper enzymes, and RNA probes were synthesized by using T3 or T7 polymerase. For each incubation, 10 μg of RNA and radiolabelled RNA probe (15,000 cpm) was suspended in 30 μl of hybridization buffer [80% deionized formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M sodium acetate, 1 mM EDTA]. The samples were heated to 85°C for 5 min and then incubated for 16 h at an annealing temperature of 50°C. To these mixtures, 300 μl of RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 200 mM sodium acetate) and 1 U of RNase One (Promega, Leiden, The Netherlands) were added. After 1 h at 37°C, the reaction was stopped by the addition of 3.3 μl of 10% SDS and 20 μg of carrier tRNA. The reaction products were precipitated with ethanol, fractionated by electrophoresis in a 6% polyacrylamide–7 M urea gel, and analyzed by autoradiography. A radiolabelled *GAPDH* RNA fragment was used as a control (28).

Interspecific backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described previously (14). A total of 205 backcross mice were used to map the λ6.2 locus (see Results for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described elsewhere (29). All blots were prepared with Hybond-N$^+$ nylon membrane (Amersham). The probe, a 1.2-kb *Sal*I/*Sst*I fragment of mouse genomic DNA, was labelled with [α-^{32}P]dCTP by using a random prime labelling kit (Amersham); washing was done to a final stringency of 0.8× SSCP–0.1% SDS at 65°C. A 10.5-kb fragment was detected in *Hinc*II-digested C57BL/6J DNA, while 6.2- and 4.0-kb fragments were detected in *M. spretus* DNA. The presence or absence of the 6.2- and 4.0-kb *M. spretus*-specific *Hinc*II fragments, which cosegregated, were followed in backcross mice. The probes and restriction fragment length polymorphisms for the loci linked to the *Evi11* locus, including the Gardner-Rasheed feline sarcoma viral oncogene homolog (*Fgr*) and natriuretic peptide precursor type A gene (*Nppa*), have been described previously (11). Recombination distances were calculated as described by Green (22), using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of double and multiple recombination events across the chromosome.

Exon trapping. The exon trap vector was based on a eukaryotic expression vector pEVRF0, which had been designed for the analysis of mutant proteins (38). The vector contains pSP65 bacterial plasmid sequences, the human cytomegalovirus enhancer/promoter, the translation initiation region from the herpes simplex virus thymidine kinase gene, splicing and polyadenylation signals from the RβG gene, and the simian virus 40 origin of replication (46) (see Fig. 4A). The RβG fragment includes map position 905 (*Bam*HI) to position 2080 of the RβG gene. This fragment consists of IVS2-β flanked by thymidine kinase-RβG exon 2 and RβG exon 3. A 39-bp *Sma*I/*Hinc*II fragment from the polylinker of pBluescript II SK+ was cloned into the *Hinc*II site of the IVS2-β to introduce multiple cloning sites. Genomic DNA from cosmids 5 and 10 (see Fig. 2A) were partially digested with *Hpa*II and separated on a 1% agarose gel, and fragments of between 2 and 5 kb were isolated. These *Hpa*II fragments were ligated in the newly created *Cla*I site. COS cells were grown to ±50% confluency. DEAE-dextran (100 μg/ml) and 10 μg DNA in DMEM were added; after 2 h, this solution was replaced by 0.1 mM chloroquine in DMEM. Cells were incubated with this mixture for 3 h, and then the medium was refreshed by DMEM with 10% FCS. The cells were cultured for 2 to 3 days, and total RNA was isolated. Reverse transcriptase (RT)-PCR was carried out with primers 1A (5′-GGGGGATCTTGGTGGCGTG-3′) and 1B (5′-AGATCTCAGTGGTATT TGTGAGC3′). Subsequently, a nested PCR was performed with primers 2A (5′-CGTCTAGAGGAGTGAATTCTTTGC-3′) and 2B (5′-ATCCATGGAT CCTGAGAACTTCAG-3′). The PCR cycling conditions were as follows: for primers set 1A-1B, 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C (30 cycles); for primer set 2A-2B, 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C (30 cycles). Amplified fragments were visualized on a 2% agarose gel by ethidium bromide staining. Potential exons were subcloned into pBluescript II SK+ by digestion with *Bam*HI/*Eco*RI (see Fig. 4A). Border sequences of RβG exon 2 and RβG exon 3 were thereby included in the cloned product.

Sequence analysis. Nucleotide sequencing was performed by the method of Sanger and Coulsen (45). Fragments were cloned into pBluescript II SK+ and sequenced with T3, T7, or sequence-specific primers. Deduced sequences were analyzed by using the BLAST network service of the National Center for Biotechnology Information.

RT-PCR. RT reactions were performed for 1.5 h at 37°C with 3 μg of total RNA in 50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM $MgCl_2$–1 mM dithiothreitol–40 mM oligo$(dT)_{16}$–0.5 mM deoxynucleoside triphosphates–1 U of RNAguard (Pharmacia)–100 U of SuperScript RT (Gibco, Breda, The Netherlands). The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 150 μM deoxynucleoside triphosphates, and 2.5 U of *Taq* polymerase. PCR cycling started with 10 min at 94°C and ended with a 10-min extension at 72°C.

cDNA cloning by PCR. All RT reactions for the RACE (rapid amplification of cDNA ends) experiments were done with 2 μg of poly$(A)^+$ RNA. Poly$(A)^+$ RNA was purified by affinity chromatography using oligo(dT)-cellulose columns (Pharmacia). 3′ RACE experiments were performed by using an oligo(dT) adapter primer [5′-GTCGCGAATTCGTCGACGCG$(dT)_{15}$-3′] for first-strand synthesis. Subsequently, PCR was carried out with the adapter primer (5′-GTCGCGAATTCGTCGACGCG-3′) in combination with gene-specific primers (CBR15 [5′-CACGCTTAGTGATTTAGACT-3′; bp 2634 to 2653] and CBR16 [5′-GTATTTCAACATCAACTTGG-3′; bp 2654 to 2673]). Cycling parameters were 1 min at 94°C, 2 min at 57°C, and 3 min at 72°C (25 cycles). Products were cloned into pBluescript II SK+ and sequenced. A 5′-AmpliFINDER RACE kit (Clontech, Palo Alto, Calif.) was used to isolate additional 5′ cDNA sequences. The strategy for the 5′ RACE was as follows. A gene-specific primer (CBR3 [5′-GTGAAGGTCATGGTCACACT-3′; bp 517 to 536]) was used for first-strand synthesis, and then an anchor primer was ligated to the cDNA 3′ end. Subsequently, the anchor primer and nested primers (CBR9 [5′-CCGTTGGTCACTTCTGTCTC-3′; bp 199 to 218] and CBR10 [5′-GAGC TGTCCAGAAGACTGGG-3′; bp 138 to 158]) were used to amplify additional 5′ cDNA fragments. Cycling parameters for both primer sets were 1 min at 94°C, 1 min at 57°C, and 3 min at 72°C (25 cycles). The products that were obtained by 5′ RACE were cloned into pBluescript II SK+ and sequenced.

Nucleotide sequence accession number. The accession number for the murine *Cnr2* sequence is X93168.

RESULTS

Identification of a common integration site (*Evi11*) in cell lines NFS107 and NFS78. A *Sau*3A partial genomic library from NFS107 was prepared in EMBL3. The library was screened with a specific U_3 LTR probe from MoMuLV (15), and eight different LTR-positive clones were obtained (data not shown). The restriction map of one clone, λ6.2, is shown in Fig. 1A. From this phage clone, a nonviral, nonrepetitive 1.2-kb *Sst*I/*Sal*I probe F (Fig. 1A) was isolated and used for further analysis. With this probe, DNAs from 13 MoMuLV- and 9 CasBrM-MuLV-induced leukemic cell lines were screened by Southern analysis using the restriction enzymes *Pvu*II, *Sst*I, *Xba*I, *Kpn*I, *Pst*I, and *Hin*dIII. As shown in Fig. 1B, rearrangements were identified in NFS107 and in the leukemic cell line NFS78. Southern analysis also revealed that NFS78 had lost its normal allele (Fig. 1B, *Sst*I, *Kpn*I, *Pst*I, and *Hin*dIII). NFS107 and NFS78 are both IL-3 dependent and do not respond to granulocyte and granulocyte-macrophage colony-stimulatory factors (G-CSF and GM-CSF), stem cell factor, and IL-7 (data not shown). NFS107 cells express the my-

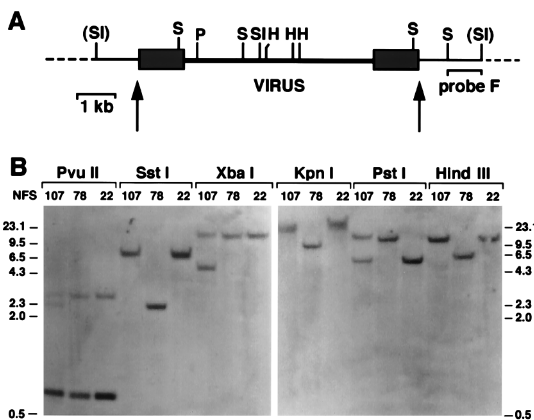

FIG. 1. Identification of common integration site *Evi11*. (A) Restriction enzyme map of virus integration site λ6.2 [SI, *Sal*I; S, *Sst*I; H, *Hin*dIII; P, *Pst*I; (SI), *Sal*I from EMBL3 phage arms]. (B) Rearrangement of the *Evi11* locus by Southern blot analysis of genomic DNA from retrovirus-induced murine leukemia cell lines NFS22, NFS78, and NFS107. Filters were hybridized to a 1.2-kb fragment (probe F) from the λ6.2 integration site. Sizes are indicated in kilobases.

eloid markers myeloperoxidase, *Mac*-1, and *Mac*-2 (27). NFS78 is a leukemic cell line with myeloblastic characteristics (24) and expresses the myeloid marker *Mac*-1 (27). Immunoglobulin heavy-chain genes of both cell lines are in germ line configuration. Southern blotting and genetic backcross analysis with a fragment derived from another integration site in NFS107 demonstrated that this integration had occurred in the recently identified common VIS, *Evi3* (data not shown and reference 31). NFS78 expresses another common VIS, *Evi1* (36). NFS22 (Fig. 1B) and 19 other cell lines did not show rearrangements in the *Evi11* locus after hybridization with probe E, F, or C (Fig. 2B). To obtain genomic DNA of the normal *Evi11* locus, two genomic cosmid libraries were screened with probe F (Fig. 1A and 2B). We isolated three cosmid clones, 32B, 5, and 10, covering a region of approximately 70 kb of the locus that we designated *Evi11*. A limited restriction map of *Evi11* is shown in Fig. 2A. The orientation of the viral DNA in NFS107 and NFS78 was deduced from restriction enzyme analysis of the normal genomic organization (cosmids 5 and 32B) and Fig. 1B, in combination with the restriction map of CasBrM-MuLV (accession no. X57540). In NFS78, retroviral DNA is oriented opposite the integrated viral DNA within the same locus in NFS107 (Fig. 2A). The distance between the two integrations is approximately 3 kb.

Chromosomal localization of *Evi11*. The chromosomal localization of *Evi11* was initially determined to be on murine chromosome 4 by fluorescence in situ hybridization (FISH) analysis (data not shown). A more exact positioning of *Evi11* was obtained by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2,100 loci that are well distributed among all mouse autosomes and the X chromosome (14, 14a). C57BL/6J and *M. spretus* DNAs were digested with several restriction enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms, using the mouse 1.2-kb *Sst*I/*Sal*I probe F (Fig. 1A). Cosegregating 6.2- and 4.0-kb *M. spretus*-specific *Hin*cII fragments were used to monitor the segregation of the *Evi11* locus in backcross DNAs. The mapping results indicated that *Evi11* is located in the distal region of mouse chromosome 4 (Fig. 3). Although 117 mice were analyzed for all three markers shown in the haplotype analysis (Fig. 3), up to 121 mice could be typed for one pair of markers. Each locus was analyzed in pairwise combinations for recombination frequencies, using

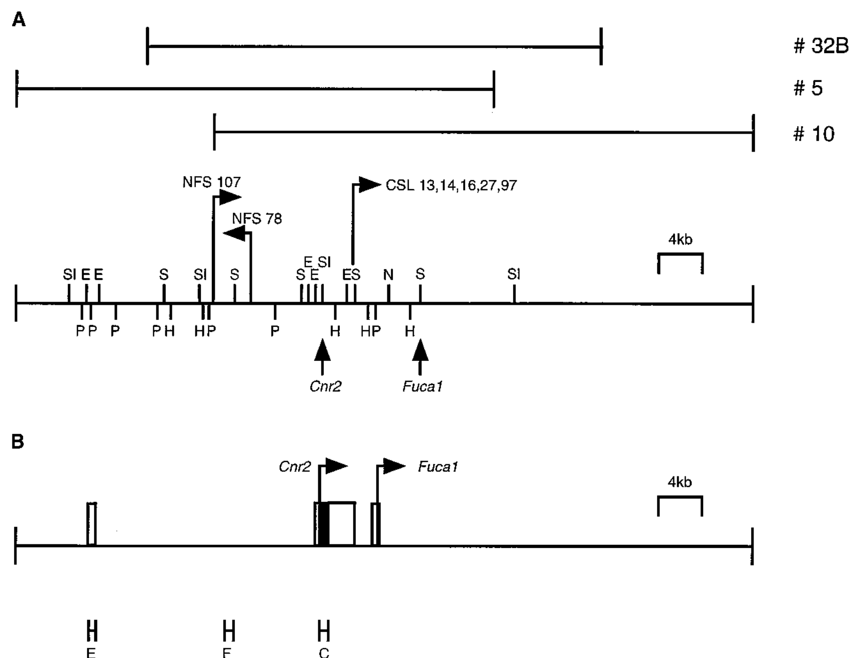

FIG. 2. Restriction enzyme map and genomic structure of *Evi11*. (A) Restriction pattern of *Evi11* based on Southern analysis with cosmid clones 5, 10, and 32B (N, *Not*I; SI, *Sal*I; E, *Eco*RI; S, *Sst*I; H, *Hin*dIII; P, *Pst*I). The locations of the trapped *Cnr2* fragment (*Cnr2*) and exon 3 of *Fuca1* (*Fuca1*) are indicated. Arrows indicate the locations and orientations of provirus in NFS78 and NFS107 as well as CSL13, CSL14, CSL16, CSL27, and CSL97. (B) Genomic structure of the *Evi11* locus. The location of the 5′ noncoding exon 1 and protein-coding exon 2 of *Cnr2*, the 5′ exon of α-L-fucosidase, and probes E, F, and C are depicted diagrammatically, with notation corresponding to that in panel A.

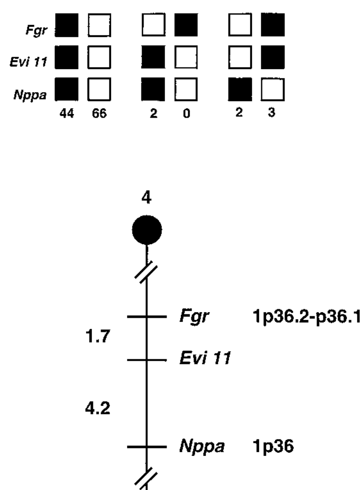

FIG. 3. *Evi11* maps in the distal region of mouse chromosome 4. *Evi11* was mapped to mouse chromosome 4 by interspecific backcross analysis. The segregation patterns of *Evi11* and flanking genes in 117 backcross animals are shown at the top. For some of the individual pairs of loci, more than 117 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The shaded boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed below each column. A partial chromosome 4 linkage map showing the location of *Evi11* in relation to linked genes is shown at the bottom. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes are shown to the right. References for human map positions can be obtained from GDB, a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, Md.).

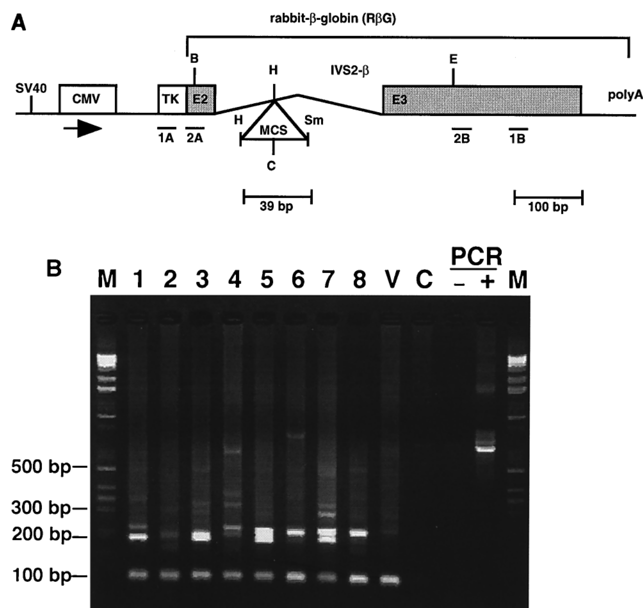

FIG. 4. Isolation of transcribed sequences from *Evi11* by exon trapping. (A) Exon trap vector pERVF0. Abbreviations not given in the text: SV40, simian virus 40 origin of replication; CMV, human cytomegalovirus enhancer/promoter region; TK, thymidine kinase translation initiation region; E2 and E3, exon 2 and exon 3 of the RβB gene; MCS, multiple cloning site (*Bam*HI [B], *Cla*I [C], *Eco*RI [E], *Hinc*II [H], *Sma*I [Sm], and primers 1A, 1B, 2A, and 2B). (B) Agarose gel electrophoresis of RT-PCR fragments (primers 2A and 2B) obtained from COS-1 cells transfected with the exon trap vector pERVF0. Lanes 1 to 8, ethidium bromide stainings of RT-PCR fragments obtained from transfections with pERVF0 containing *Evi11* genomic inserts; lane V, RT-PCR fragment from a transfection with empty vector; lane C, RT-PCR using RNA from nontransfected COS cells; M, 1-kb ladder (Boehringer).

the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere-*Fgr*-2/121-*Evi11*-5/119-*Nppa*. The recombination frequencies (expressed as genetic distances in centimorgans ± the standard error) are centromere-*Fgr*-1.7 ± 1.2-*Evi11*-4.2 ± 1.8-*Nppa*. No recombinations were observed between *Evi11* and the α-L-fucosidase gene. The distal half of mouse chromosome 4 shares a region of homology with human chromosome 1 (Fig. 3).

Identification of transcribed sequences in the *Evi11* locus by using exon trapping. To isolate coding sequences from the *Evi11* locus, an exon trapping system was designed (Fig. 4A) and applied to cosmid clones 5 and 10 (Fig. 2A). Partially *Hpa*II-digested fragments of cosmids 5 and 10, between 2 and 5 kb, were cloned into the *Cla*I site of the exon trap vector. These constructs were pooled in groups of six clones per COS cell transfection. Thirty transfections were performed. RT-PCR was carried out on RNA isolated from the transfected COS cells. Potential exons with sizes ranging from 50 to 300 bp were amplified from the different pools, using primer set 1A-1B followed by 2A-2B, and visualized by ethidium bromide staining. Eight examples of COS cell transfections are shown in Fig. 4B (lanes 1 to 8). Transfection of the exon trap vector without insert resulted in the isolation of a 97-bp fragment representing the fusion of exons 2 and 3 of the RβG gene (Fig. 4B, lane V). Potential exons were subcloned into *Bam*HI/*Eco*RI-digested (Fig. 4A) pBluescript II SK+ and sequenced. The DNA sequences were compared to those in the National Center for Biotechnology Information database. Among 16 clones analyzed, one exon was the mouse homolog of exon 3 of human *FUCA1* (32), a gene which by interspecific backcrossing had been shown to be located near *Evi11* (Fig. 3). Seven clones contained sequences that were homologous to part of the coding region of human *CNR2* (39). However, the *Cnr2* fragments were trapped between the two RβG exons in the reversed transcriptional orientation. Sequence analysis of these trapped fragments and of the human cDNA of *CNR2* demonstrated the presence of cryptic splice sites at the proper positions in the reversed orientation (see Fig. 6A). Eight clones contained cosmid vector sequences. The trapped fragments were radiolabelled and used for Northern analysis to examine the expression of the representing genes in the different cell lines. Two different mRNAs, of ±1.6 and ±4.0 kb, were identified (Fig. 5). Analysis using separate probes revealed that the ±1.6-kb signal represented α-L-fucosidase, and the *Cnr2* fragment detected the ±4.0-kb mRNA. In NFS78, NFS107, and control cell lines NFS22 and NFS36, variable levels of *Cnr2* mRNA were apparent (Fig. 5). In NFS78, *Cnr2* mRNA levels were higher than in control cell lines, whereas in NFS107, no *Cnr2* mRNA could be identified (Fig. 5 and Table 1). In fact, Northern analysis using RNAs from a panel of cell lines demonstrated variable levels of *Cnr2* mRNA expression in all DA and NFS lines (data not shown). Levels of *Fuca1* transcripts in the different cell lines were comparable (Fig. 5, Table 1, and data not shown). Although the levels of *Fuca1* mRNA in the different leukemic cell lines (Table 1 and data not shown) were less variable than the *Cnr2* mRNA levels, the Northern analysis was not conclusive with respect to altered gene expression caused by virus integration.

The *Cnr2* nucleotide sequence and exon structure. Southern blot analysis was carried out on the three *Evi11* cosmid clones

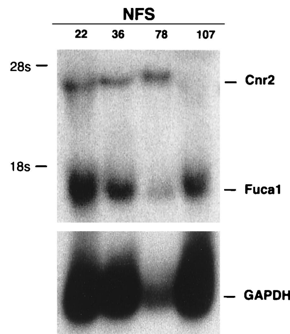

FIG. 5. Expression of *Cnr2* and *Fuca1* in retrovirally induced leukemias, determined by Northern analysis of NFS22, NFS36, NFS78, and NFS107. Total RNA was hybridized to a ^{32}P-labelled *Fuca1* exon 3 probe and a ^{32}P-labelled *Cnr2* fragment which had been obtained by exon trapping. The filter was subsequently hybridized with a ^{32}P-labelled *GAPDH* probe (28).

to determine the positions of the amplified fragments (Fig. 2A). The trapped *Cnr2* fragment was mapped on a 3-kb *Eco*RI/*Eco*RI fragment (Fig. 2). The localization was confirmed by the presence of a relatively rare *Sal*I site in this fragment. α-L-Fucosidase exon 3 is situated approximately 12 kb from the provirus in NFS78. Since *Cnr2* is located nearer the VIS than the α-L-fucosidase gene, and the gene encodes a hematopoietic receptor (39), which may be involved in monocyte/macrophage differentiation (40), we decided to determine the nucleotide sequence and genomic structure of *Cnr2*. Southern analysis with 5′ and 3′ cDNA probes of the human *CNR2* gene suggested that the entire protein-coding sequence was located within the 3-kb *Eco*RI/*Eco*RI fragment (Fig. 2). *Hpa*II and *Sau*3A fragments of the 3-kb *Eco*RI/*Eco*RI fragment were subcloned into, respectively, the *Cla*I and *Bam*HI sites of pBluescript II SK+ and sequenced (Fig. 6A). The merged nucleotide sequences of the 3-kb *Eco*RI/*Eco*RI fragment showed that the complete protein-coding sequence of the murine *Cnr2* receptor is present within this 3-kb fragment. The amino acid sequence of the murine *Cnr2* gene product is 82% homologous to that of the human *CNR2* gene product. The C-terminal end of the murine homolog lacks nine amino acids. An amino acid sequence comparison of murine *Cnr2*, human *CNR2* (39), and murine (accession no. U22948) and human (*CNR1* [21]) brain cannabinoid receptor 1 gene products is shown in Fig. 6B. Nucleotide sequencing of the *Cnr2* protein-coding region from NFS107 and NFS78 cDNA did not show any mutations. The 3′ noncoding sequence was isolated by 3′ RACE on poly(A)$^+$ from control cell line NFS22 by using two gene-specific primers (CBR15 and CBR16). Two fragments, which terminated at bp 2951 and 3756, were isolated. The 3′ untranslated regions (UTRs) appeared to be, respectively, 1,724 and 2,528 bp long, with putative polyadenylation signals (AAUAAA) at bp 2928 and 3743. This variability in 3′ UTR lengths has also been shown for the rat brain cannabinoid receptor (35). The *Cnr2* sequence identified so far is located in

TABLE 1. *Cnr2* and *Fuca1* mRNA levels in NFS cell lines

mRNA[a]	Level (% of *GAPDH* control)			
	NFS22	NFS36	NFS78	NFS107
Fuca1	15	12	18	7
Cnr2	10	4	36	0

[a] Identified by Northern blot analysis (Fig. 5).

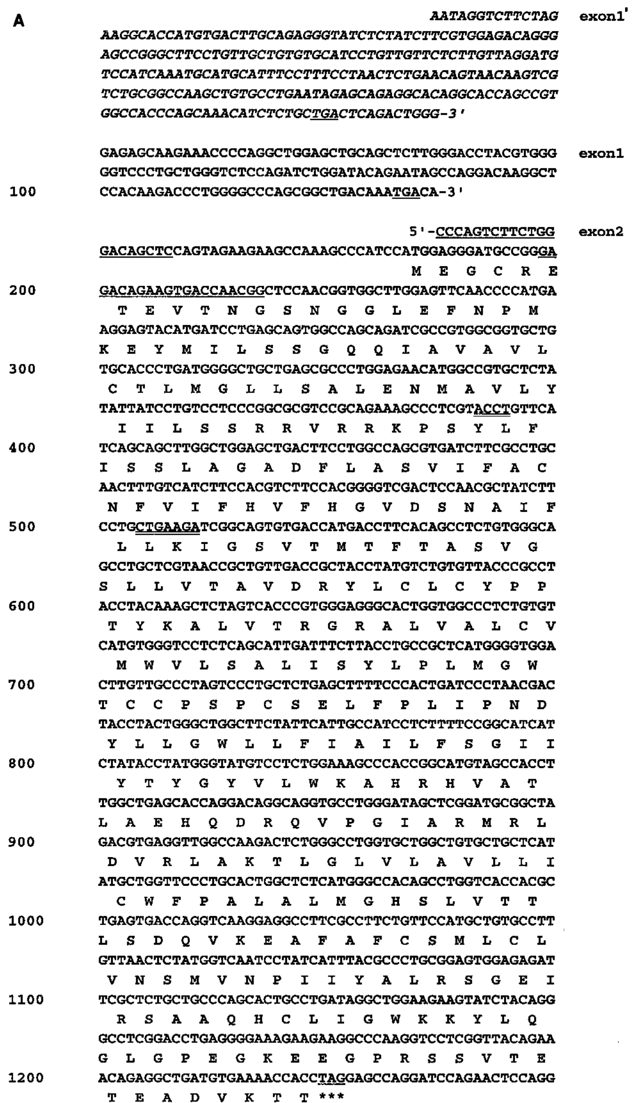

FIG. 6. Nucleotide and protein sequences of the mouse and human cannabinoid receptors. (A) Nucleotide and deduced protein sequences of mouse *Cnr2*. In-frame stop codons in the 5′ and 3′ UTRs and 5′ RACE primers CBR9 (bp 199 to 218) and CBR10 (bp 138 to 158) are underlined. The splice site of *Cnr2* is indicated by an arrow. Exon 1 of NFS78 is italicized. The cryptic splice sites located on the noncoding strand are double underlined. (B) Amino acid comparison of the murine *Cnr2* (MCB2), human *CNR2* (HCB2), mouse *Cnr1* (MCB1) and human *CNR1* (HCB1) gene products. Identities are on a black background, similarities are on a gray background, and the seven transmembrane domains are underlined.

one exon. Upstream of the ATG start site of mouse *Cnr2* on the 3-kb *Eco*RI/*Eco*RI fragment, a potential 3′ splice acceptor site was identified (Fig. 7A). To investigate whether 5′ exons, which could splice to this potential acceptor site, existed, 5′ RACE was carried out using poly(A)$^+$ RNA from the control cell line NFS22. Amplification with the anchor primer and CBR9, followed by a nested PCR with the anchor primer and CBR10, resulted in the isolation of a fragment of 179 bp (Fig. 6A). This fragment was cloned into pBluescript II SK+, and sequence analysis revealed that an exon of at least 121 bp was spliced to the predicted splice acceptor site. Since this exon introduces a stop codon in frame with the ATG start site, this exon is noncoding (Fig. 6A). Interestingly, Southern blot anal-

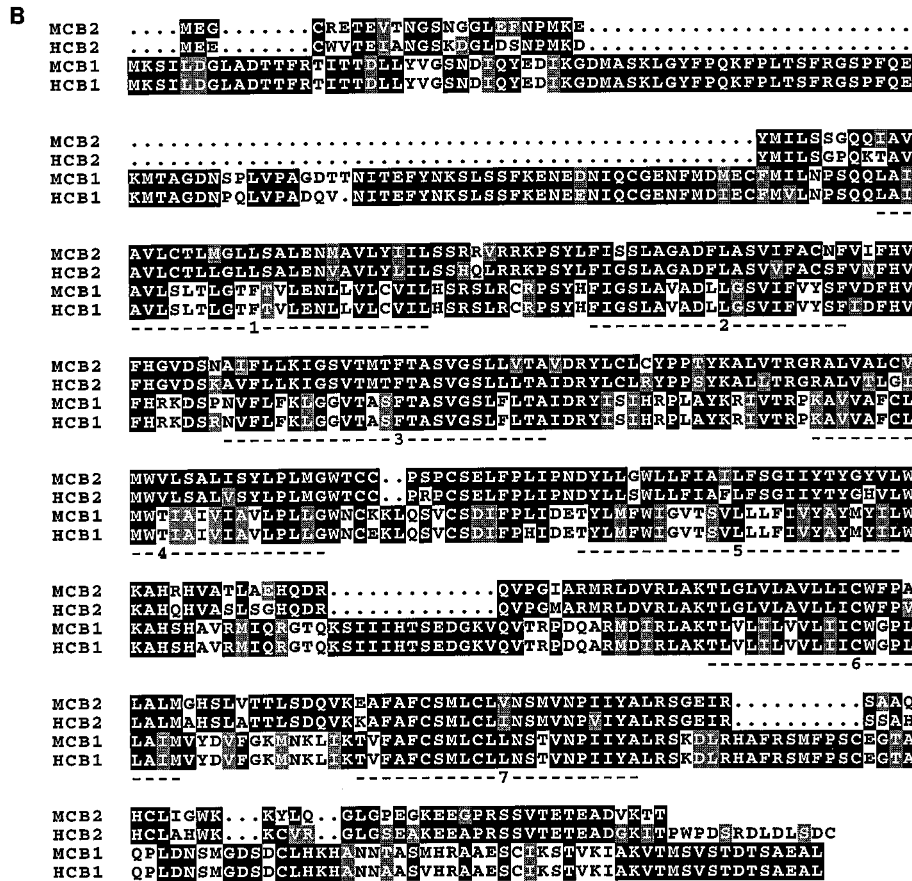

FIG. 6—*Continued.*

ysis demonstrated that exon 1 of *Cnr2* is located approximately 20 kb upstream of protein-coding exon 2. Thus, virus integrations in NFS107 and NFS78 have occurred in an intron of *Cnr2* (Fig. 7A).

***Cnr2* expression in leukemic cell lines.** Northern analysis demonstrated variable levels of *Cnr2* mRNA expression in the distinct cell lines studied (Fig. 5, Table 1, and data not shown) but was not conclusive with respect to the viral integrations. Since in NFS78 and NFS107, proviral DNA is integrated in the *Cnr2* intron, we verified with RNase protection whether the levels of expression of the two exons had been altered. A 195-bp *Eco*RI (anchor 5′ RACE)/*Nco*I fragment overlapping *Cnr2* exons 1 and 2 (Fig. 7A) was generated to protect both exons with RNase protection. RNase protection analysis revealed variable levels of full-length *Cnr2* mRNA in the control cell lines (NFS36, NFS56, NFS58, NFS61, and NFS124) (Fig. 7B, 187 bp [exon 1 + 2]). In NFS107, full-length *Cnr2* as well as 137-bp transcripts, representing exon 1, were detected. Thus, although no *Cnr2* transcripts could be detected in NFS107 by Northern blot analysis, *Cnr2* mRNA was demonstrated by using the more sensitive RNase protection technique. In NFS78, full-length *Cnr2* mRNA could not be demonstrated; however, a 50-bp fragment which corresponds to exon 2 mRNA was protected. This finding is in agreement with the Southern blot analysis (Fig. 1B), showing a normal and rearranged allele in NFS107 and only an abnormal allele in NFS78. 5′ RACE carried out on poly(A)$^+$ RNA of NFS78 resulted in fragments of different sizes. Sequencing (Fig. 6A) showed that in this cell line, another noncoding exon located downstream of provirus DNA was fused to the splice acceptor site of exon 2 of *Cnr2* (Fig. 7A). In an assay using a 680-bp probe specific for exons 1′ and 2 (Fig. 7A), two large fragments (600 and 430 bp) were protected in NFS78 only (Fig. 7C). The 600-bp band represents the complete exon 1′-plus-2 transcript, whereas the smaller band corresponds with a part of exon 1′ fused to exon 2. In the other cell lines, a band of 350 bp which represents exon 2 only is protected. Thus, the RNase protection analysis demonstrate altered aberrant *Cnr2* mRNA expression in both NFS107 and NFS78.

***Evi11* in CasBrM-MuLV-induced primary tumors.** To determine the frequency of *Evi11*/*Cnr2* retroviral insertions, Southern blot analysis was carried out with probe E, F, or C (Fig. 2B) and different restriction enzymes on high-molecular-weight DNA isolated from CasBrM-MuLV-induced splenic tumors. Interestingly, DNA rearrangements were observed within the *Evi11* locus in five cases, i.e., CSL13, CSL14, CSL16, CSL27, and CSL97, using probe C (Fig. 8), which corresponds to the protein-coding region of *Cnr2* (Fig. 2B). No rearrangements were detected in control primary tumors, e.g., CSL103 and CSL12. From the Southern blot analysis of DNA digested with multiple enzymes (*Sst*I, *Pst*I, and *Bam*HI), it was concluded that retroviral integrations in *Evi11* had occurred within the 3′ UTR of *Cnr2* in the same transcriptional orientation (Fig. 2B). The locations and orientations of the proviruses were confirmed by PCR analysis using *Cnr2*- and MuLV LTR-specific primers (data not shown).

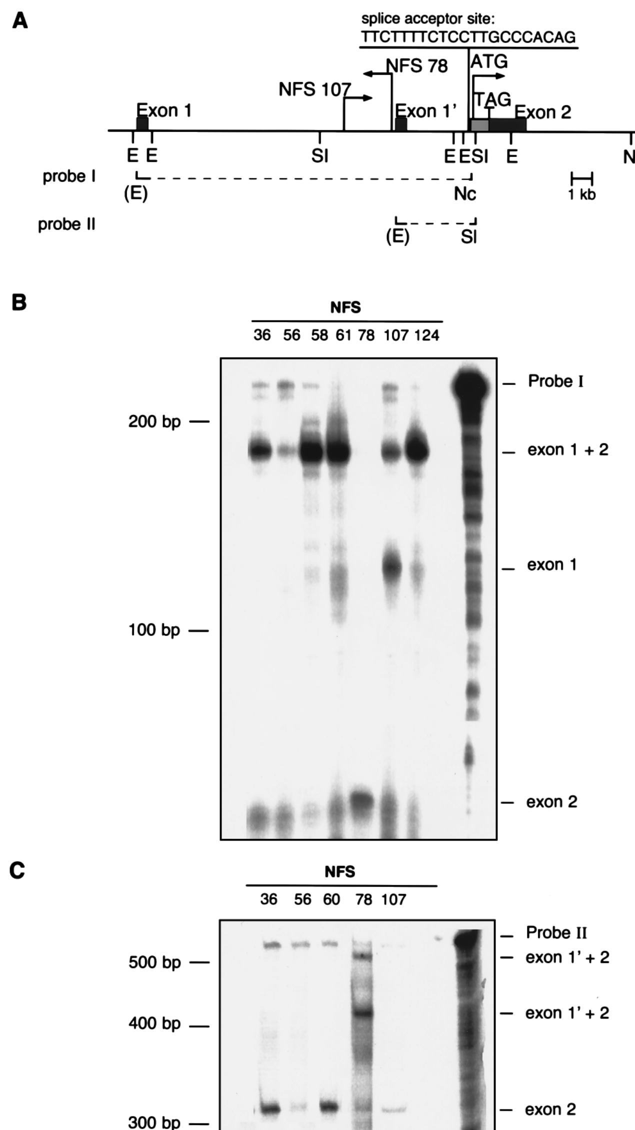

FIG. 7. *Cnr2* is aberrantly expressed in the murine myeloid leukemia cell lines NFS78 and NFS107. (A) Genomic organization of mouse *Cnr2*. The shaded box represents the open reading frame. The sites of proviral integration as well as the orientation are shown by arrows. Exon 1′ is the alternative exon which is fused to exon 2 in NFS78. The fragments and restriction sites used to generate RNA probes for RNase protection experiments are indicated [E, *Eco*RI; N, *Not*I; Nc, *Nco*I; SI, *Sal*I; (E), *Eco*RI site in anchor primer 5′ RACE]. (B) RNase protection on 10 μg of total RNA of a series leukemic cell lines, using an exon 1- and 2-specific probe (A). The full-length *Cnr2* protected fragment was 187 bp (exon 1 [137 bp] and exon 2 [50 bp]). (C) RNase protection on 10 μg of total RNA of a series leukemic cell lines, using an exon 1′- and 2-specific probe (A). The full-length protected fragment was 600 bp (exon 1′ [250 bp] and exon 2 [350 bp]). Another fragment of approximately 430 bp represents part of exon 1′ plus exon 2.

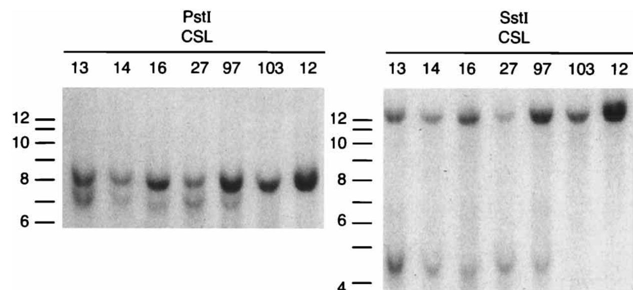

FIG. 8. Proviral integrations within the 3′ UTR of the *Cnr2* gene, determined by Southern blot analysis with probe C (Fig. 2B) on *Pst*I and *Sst*I-digested genomic DNA from CasBrM-MuLV-induced primary tumors. The locations and orientations of the retroviruses in *Evi11* are depicted in Fig. 2B.

DISCUSSION

Neoplasias induced by retroviruses that lack dominant-acting oncogenes have been shown to depend on proviral integrations into particular loci of the cellular genome (3, 30, 50). Integration of viral DNA independently in the same locus in different tumors might indicate loci that have a functional role in the multistep process of malignant transformation. In two murine IL-3-dependent myeloid leukemia cell lines (24), i.e., NFS107 and NFS78, and five primary tumors, i.e., CSL13, CSL14, CSL16, CSL27, and CSL97, induced in CasBrM-MuLV-infected NFS/N mice, a new common virus integration site, *Evi11*, was identified and cloned. NFS107 was initially chosen for this study because of the absence of rearrangements in loci of several known proto-oncogenes (27). Interestingly, however, among eight integration sites that we isolated from NFS107, one represented the recently identified common VIS *Evi3* (data not shown and reference 31). This finding might suggest that alterations in the expression of the *Evi11* and an *Evi3* proto-oncogene synergize to generate a malignantly transformed myeloid cell. Likewise, in NFS78 leukemia cells, provirus is integrated in the *Evi1* locus (36), which may suggest that aberrant expression of an *Evi11* proto-oncogene and *Evi1* cooperated in the development of the latter myeloid leukemia. Cooperation of genes involved in the initiation and/or progression of hematopoietic tumors has been shown by others (30, 41).

To isolate genes located near the VIS, we established an exon trapping/amplification system based on the expression vector pEVRF0 (38). This exon trap vector is related to the pSPL1-vector (10), which also uses the RβG gene exons 2 and 3. The main difference between pEVRF0 and pSPL1 is that the natural intervening sequence, IVS2-β, is used in pERVF0, whereas in pSPL1, IVS2-β is replaced by the human immunodeficiency virus *tat* intron. Thus, by using pERVF0, we wished to avoid the possibility of isolating false positives caused by a cryptic splice site present within the *tat* intron sequence (13, 42). With the novel exon trapping system, we identified two genes in *Evi11*. The α-L-fucosidase exon that was trapped is the mouse homolog of exon 3 of human *FUCA1* (20). The second fragment that was isolated from the *Evi11* locus encodes part of the murine cannabinoid receptor 2, based on a strong homology to human *CNR2* (39) and identity to the recently cloned mouse *Cnr2* (48). The isolated fragment of *Cnr2* was trapped in the incorrect transcriptional orientation because of the presence of cryptic 5′ and 3′ splice sites on the noncoding strand (Fig. 6A). Thus, although apparently exons can be isolated efficiently by using pERVF0, it is also clear that false positives may be isolated by using exon trapping procedures. Since sequences derived from the cosmid vector were also trapped, it appears useful to eliminate vector DNA before cloning genomic DNA into the pERVF0 vector.

Two genes, *Fuca1* and *Cnr2*, were identified in the *Evi11* locus. The data suggest that *Cnr2* is the candidate target gene, since provirus is integrated in the *Cnr2* intron in NFS78 and NFS107, and although the mechanism needs further study, virus integrations in this locus lead to aberrant *Cnr2* mRNA expression in NFS78 and NFS107. In addition, all proviral integrations identified in the CasBrM-MuLV-induced primary

tumors reside within the 3′ UTR of *Cnr2*. These integrations resemble classical proviral integrations which generally enhance expression of the target gene (3, 30, 50). However, the possibility remains that another gene within the *Evi11* locus is affected due to retroviral insertion.

Cnr2 encodes a hematopoietic receptor that belongs to the class of seven-transmembrane G-protein-coupled receptors. *Cnr2* consists of a small 5′ noncoding exon 1 and a large exon 2 that contains the complete protein-coding region. Interestingly, small 5′ noncoding exons have been identified in a series of genes that encode G-protein-coupled receptors, e.g., the Burkitt's lymphoma receptor 1 gene (*BLR*1) (17), the *mas* oncogene-related rat thoracic aorta receptor gene (*RTA*) (43), and recently also *CNR1* (47). This could suggest that the small 5′ exons are important in regulation of gene expression. In NFS78 and NFS107 cells, proviruses are integrated in the intron that separates exon 1 from exon 2. Our data indicate abnormal expression of *Cnr2* exon 1 in NFS107 and NFS78. The RNase protection data are indicative of an mRNA in NFS107 that contains exon 1 only. This deleted transcript is probably the result of the polyadenylation signal introduced by the LTR of the provirus that is integrated in the proper orientation downstream of exon 1 (Fig. 7A). In NFS78, exon 1 is absent and an alternative exon is fused to the protein-coding exon 2 (Fig. 6A and 7A). If aberrant mRNA expression of exon 1 as the result of proviral integration leads to abnormal levels of *Cnr2* receptors and contributes to leukemic transformation, this would suggest that exon 1 contains important regulatory sequences. We are currently raising antibodies to examine the levels of *Cnr2* receptors in NFS78, NFS107, and control cell lines.

Interestingly, RNase protection experiments have demonstrated that *Cnr2* may be expressed in most hematopoietic lineages and that the ligand for cannabinoid receptors, anandamide, synergistically stimulates proliferation of hematopoietic progenitor cell lines with cytokines, e.g. IL-3, GM-CSF, erythropoietin, and G-CSF (52). Thus, *Cnr2* appears to encode an important hematopoietic receptor which following activation enhances the response to hematopoietic growth factors. This finding adds further support to the hypothesis that this receptor, when aberrantly expressed, may alter the proliferative response of hematopoietic cells and contribute to the development of leukemia. Several other genes that encode G-protein-coupled receptors have been implicated in oncogenic transformation (1, 49, 53, 54).

FISH analysis and interspecific backcrossing demonstrated that *Evi11* is located on the distal end of murine chromosome 4. This locus is distinct from known proto-oncogenes and common viral integration loci previously identified in the mouse. This particular region on murine chromosome 4 is homologous to a region on human chromosome 1p, i.e., 1p36. *Evi11* was found to localize between two genes, *Fgr* and *Nppa*. These comparative mapping results suggest that *FUCA1* and *CNR2* map to human 1p36 as well. In fact, it had already been shown that human *FUCA1* resides on chromosome 1p36 (20). FISH analysis using a human *CNR2* cDNA probe indeed demonstrated that this gene is also located on 1p36 in humans (data not shown). 1p36 is involved in breakpoints in certain cases of acute myeloid leukemia and myelodysplastic syndrome (7, 51) but also in other malignancies, e.g., neuroblastomas (6, 12, 33). This finding raises the question as to whether the human *Evi11* locus and possibly *CNR2* are mutated and aberrantly expressed in certain human diseases with 1p36 abnormalities.

ACKNOWLEDGMENTS

We thank E. M. E. Smit (Department of Genetics, Erasmus University Rotterdam) for carrying out FISH analysis, S. E. Verbakel for the assistance in cell culture experiments, M. Boschman and K. van Rooyen for preparation of the figures, S. Munro (MRC Laboratory of Molecular Biology, Cambridge, England) for providing the human *CNR2* cDNA, I. D. Hickson (Imperial Cancer Research Fund Laboratories, University of Oxford, Oxford, England) for the *GAPDH* RNase protection probe, and H. Morse III (NCI, Frederick, Md.) for the donation of the CasBrM-MuLV-producing NIH 3T3 cells. D. Meyer (Department of Genetics, Erasmus University Rotterdam) is acknowledged for his advice on the construction of the exon trap vector.

This work was supported by the Dutch Cancer Society Koningin Wilhelmina Fonds, the Netherlands Organisation for Scientific Research, and the Netherlands Academy of Sciences. This research was sponsored in part by the National Cancer Institute under contract with ABL.

REFERENCES

1. **Allen, L. F., R. J. Lefkowitz, M. G. Caron, and S. Cotecchia.** 1991. G-protein-coupled receptor genes as protooncogenes: constitutively activating mutation of the alpha1B-adrenergic receptor enhances mitogenesis and tumorigenicity. Proc. Natl. Acad. Sci. USA **88:**11354–11358.
2. **Arata, S., C. Newton, T. Klein, and H. Friedman.** 1992. Enhanced growth of *Legionella pneumophila* in tetrahydrocannabinol-treated macrophages. Proc. Soc. Exp. Biol. Med. **199:**65–67.
3. **Askew, D. S., C. Bartholomew, and J. N. Ihle.** 1993. Insertional mutagenesis and the transformation of hematopoietic stem cells. Hematol. Pathol. **7:**1–22.
4. **Askew, D. S., C. Bartholomew, A. M. Buchberg, M. B. Valentine, N. A. Jenkins, N. G. Copeland, and J. N. Ihle.** 1991. *His1* and *His2*: identification and chromosomal mapping of two commonly rearranged sites of viral integration in myeloid leukemia. Oncogene **6:**2041–2047.
5. **Auch, D., and M. Reth.** 1990. Exon trap cloning: using PCR to rapidly detect and clone exons from genomic DNA. Nucleic Acids Res. **18:**6743–6744.
6. **Biegel, J. A., P. S. White, H. N. Marshall, M. Fujimori, E. H. Zackai, C. D. Scher, G. M. Brodeur, and B. S. Emanuel.** 1993. Constitutional 1p36 deletion in a child with neuroblastoma. Am. J. Hum. Genet. **52:**176–182.
7. **Bloomfield, C. D., O. M. Garson, L. Volin, S. Knuntila, and A. de la Chapelle.** 1985. t(1;3)(p36,q21) in acute non-lymphoblastic leukemia: a new cytogenetic-clinicopathologic association. Blood **66:**1409–1413.
8. **Bouaboula, M., M. Rinaldi, P. Carayon, C. Carillon, B. Delpech, D. Shire, G. Le Fur, and P. Casellas.** 1993. Cannabinoid-receptor expression in human leukocytes. Eur. J. Biochem. **214:**173–180.
9. **Buchberg, A. M., H. G. Bedigian, N. A. Jenkins, and N. C. Copeland.** 1990. *Evi2*, a common integration site involved in murine myeloid leukemogenesis. Mol. Cell. Biol. **10:**4658–4666.
10. **Buckler, A. J., D. D. Chang, S. L. Graw, J. D. Brook, D. A. Haber, P. A. Sharp, and D. E. Housman.** 1991. Exon amplification: a strategy to isolate mammalian genes based on RNA splicing. Proc. Natl. Acad. Sci. USA **88:**4005–4009.
11. **Ceci, J. D., L. D. Siracusa, N. A. Jenkins, and N. G. Copeland.** 1989. A molecular genetic linkage map of mouse chromosome 4 including the localisation of several proto-oncogenes. Genomics **5:**699–709.
12. **Cheng, N. C., N. van Roy, A. Chan, M. Beitsma, A. Westerveld, F. Speleman, and R. Versteeg.** 1995. Deletion mapping in neuroblastoma cell lines suggests two distinct tumor suppressor genes in the 1p35-36 region, only one of which is associated with N-myc amplification. Oncogene **10:**291–297.
13. **Church, D. M., L. T. Banks, A. C. Rogers, S. L. Graw, D. E. Housman, J. F. Gusella, and A. J. Buckler.** 1993. Identification of human chromosome 9 specific genes using exon amplification. Hum. Mol. Genet. **2:**1915.
14. **Copeland, N. G., and N. A. Jenkins.** 1991. Development and applications of a molecular genetic linkage map of the mouse genome. Trends Genet. **7:**113–118.

14a. **Copeland, N. G., and N. A. Jenkins.** Unpublished results.

15. **Cuypers, H. T., G. Selten, W. Quint, M. Zijlstra, E. R. Maandag, W. Boelens, P. van Wezenbeek, C. Melief, and A. Berns.** 1984. Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. Cell **37:**141–150.
16. **Davis, R. W., M. Thomas, J. Cameron, T. P. St. John, S. Scherer, and R. A. Padgett.** 1980. Rapid DNA isolation for enzymatic and hybridization analysis. Methods Enzymol. **65:**404–411.
17. **Dobner, T., I. Wolf, T. Emrich, and M. Lipp.** 1992. Differentiation-specific expression of a novel G protein-coupled receptor from Burkitt's lymphoma. Eur. J. Immunol. **22:**2795–2799.
18. **Duyk, G. M., S. Kim, R. M. Myers, and D. R. Cox.** 1990. Exon trapping: a genetic screen to identify candidate transcribed sequences in cloned mammalian genomic DNA. Proc. Natl. Acad. Sci. USA **87:**8995–8999.
19. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabelling DNA

restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
20. **Fowler, M. L., H. Nakai, M. G. Byers, H. Fukushima, R. L. Eddy, W. M. Henry, L. L. Haley, J. S. O'Brien, and T. B. Shows.** 1986. Chromosome 1 localization of the human α-L-fucosidase structural gene with a homologous site on chromosome 2. Cytogenet. Cell. Genet. **43:**103–108.
21. **Gerard, C. M., C. Mollereau, G. Vassart, and M. Parmentier.** 1991. Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochem. J. **279:**129–134.
22. **Green, E. L.** 1981. Linkage, recombination and mapping, p. 77–113. *In* Genetics and probability in animal breeding experiments. Oxford University Press, New York, N.Y.
23. **Hamaguchi, M., H. Sakamoto, H. Tsuruta, H. Sasaki, T. Muto, T. Sugimura, and M. Terada.** 1992. Establishment of a highly sensitive and specific exon-trapping system. Proc. Natl. Acad. Sci. USA **89:**9779–9783.
24. **Holmes, K. L., E. Palaszynski, T. N. Frederickson, H. C. Morse III, and J. N. Ihle.** 1985. Correlation of cell-surface phenotype with the establishment of IL-3 dependent cell lines from wild-mouse murine leukemia virus induced neoplasms. Proc. Natl. Acad. Sci. USA **82:**6687–6691.
25. **Ihle, J. N., J. Keller, S. Oroszlan, L. E. Henderson, T. D. Copeland, F. Fitch, M. B. Prystowsky, E. Goldwasser, J. W. Schrader, E. Palaszynski, M. Dy, and B. Lebel.** 1983. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P-cell stimulating factor activity, colony stimulating factor activity and histamine producing cell stimulating factor activity. J. Immunol. **131:**282–287.
26. **Ihle, J. N., A. Rein, and R. Mural.** 1984. Immunological and virological mechanisms in retrovirus-induced murine leukemogenesis. Viral Oncol. **4:** 95–137.
27. **Ihle, J. N., and D. Askew.** 1989. Origins and properties of hematopoietic growth factor-dependent cell lines. Int. J. Cell Cloning **7:**68–91.
28. **Jenkins, J. R., P. Ayton, T. Jones, S. L. Davies, D. L. Simmons, A. L. Harris, D. Sheer, and I. D. Hickson.** 1992. Isolation of cDNA clones encoding the isoenzyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24. Nucleic Acids Res. **20:**5587–5592.
29. **Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee.** 1982. Organization, distribution and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. J. Virol. **43:**26–36.
30. **Jonkers, J., and A. Berns.** 1996. Retroviral insertional mutagenesis as a strategy to identify cancer genes. Biochim. Biophys. Acta **1287:**29–57.
31. **Justice, M. J., H. C. Morse III, N. A. Jenkins, and N. G. Copeland.** 1994. Identification of *Evi3*, a novel common site of retroviral integration in mouse AKXD B-cell lymphomas. J. Virol. **68:**1293–1300.
32. **Kretz, K. A., D. Cripe, G. S. Carson, H. Fukushima, and J. S. O'Brien.** 1992. Structure and sequence of the human α-L-fucosidase gene and pseudogene. Genomics **12:**276–280.
33. **Laureys, G., F. Speleman, G. Opdenakker, Y. Benoit, and J. Leroy.** 1990. Constitutional translocation t(1;17)(p36;q12-21) in a patient with neuroblastoma. Genes Chromosomes Cancer **2:**252–254.
34. **Liao, X., A. M. Buchberg, N. A. Jenkins, and N. G. Copeland.** 1995. *Evi5*, a common site of retroviral integration in AKXD T-cell lymphomas, maps near *Gfi1* on mouse chromosome 5. J. Virol. **69:**7132–7137.
35. **Matsuda, L. A., and T. I. Bonner.** 1995. Molecular biology of the cannabinoid receptor, p. 117–143. *In* R. Pertwee (ed.), Cannabinoid receptors. Academic Press, San Diego, Calif.
36. **Morishita, K., D. S. Parker, M. L. Muscenski, N. A. Jenkins, N. G. Copeland, and J. N. Ihle.** 1988. Retroviral activation of a novel gene encoding a zincfinger protein in IL-3-dependent myeloid leukemia cell lines. Cell **54:**831–840.
37. **Mucenski, M. L., B. A. Taylor, J. N. Ihle, J. W. Hartley, H. C. Morse III, N. A. Jenkins, and N. G. Copeland.** 1988. Identification of a common ecotropic viral integration site, *Evi1*, in the DNA of AKXD murine myeloid tumors. Mol. Cell. Biol. **8:**301–308.
38. **Müller, M. M., S. Ruppert, W. Schaffner, and P. Matthias.** 1988. A cloned octamer transcription factor stimulates transcription from lymphoid-specific promoters. Nature **336:**544–551.
39. **Munro, S., K. L. Thomas, and M. Abu-Shaar.** 1993. Molecular characterisation of a peripheral receptor for cannabinoids. Nature **365:**61–65.
40. **Murison, G., C. B. H. Chubb, S. Maeda, M. A. Gemmell, and E. Huberman.** 1987. Cannabinoids induce incomplete maturation of cultured human leukemia cells. Proc. Natl. Acad. Sci. USA **84:**5414–5418.
41. **Nakamura, T., D. A. Largaepada, J. D. Shaughnessy, Jr., J. A. Jenkins, and N. G. Copeland.** 1996. Cooperative activation of *Hoxa* and *Pbx1* related genes in murine myloid leukemias. Nat. Genet. **12:**149–153.
42. **North, M. A., P. Sanseau, A. J. Buckler, D. Church, A. Jackson, K. Patel, J. Trowsdale, and H. Lehrach.** 1993. Efficiency and specificity of gene isolation by exon amplification. Mamm. Genome **4:**466–474.
43. **Ross, P. C., R. A. Figler, M. H. Corjay, C. M. Barber, N. Adam, D. R. Harcus, and K. R. Lynch.** 1990. RTA, a candidate G protein-coupled receptor: cloning, sequencing and tissue distribution. Proc. Natl. Acad. Sci. USA **87:**3052–3056.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Sanger, F., and A. R. Coulsen.** 1975. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. **13:**441.
46. **Severne, Y., S. Wieland, W. Schaffner, and S. Rusconi.** 1988. Metal binding 'finger' structures in the glucocorticoid receptor defined by site-directed mutagenesis. EMBO J. **7:**2503–2508.
47. **Shire, D., C. Carillon, M. Kaghad, B. Calandra, M. Rinaldi-Carmona, G. Le Fur, D. Caput, and P. Ferrara.** 1995. An aminoterminal variant of the central cannabinoid receptor resulting from alternative splicing. J. Biol. Chem. **270:** 3726–3731.
48. **Shire, D., B. Calandra, M. Rinaldi-Carmona, D. Oustric, B. Pessègue, O. Bonnin-Cabanne, G. Le Fur, D. Caput, and P. Ferrara.** 1996. Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim. Biophys. Acta **1307:**132–136.
49. **Suarez, H. G., D. Russo, R. Wickler, J. A. Du Villard, S. Filetti, B. Caillou, and M. Schlumberger.** 1995. Role of somatic genetic alterations of the cAMP pathway in thyroid hyperfunctioning tumorigenesis. J. Cell. Biochem. Suppl. **19A:**44.
50. **Van Lohuizen, M., and A. Berns.** 1990. Tumorigenesis by slow-transforming retroviruses—an update. Biochim. Biophys. Acta **1032:**213–235.
51. **Viguie, F., J. P. Marie, F. Poler, and A. Bernadeau.** 1986. Three cases of preleukemic myelodysplastic disorders with the same translocation t(1;3). Cancer Genet. Cytogenet. **19:**213–218.
52. **Valk, P. J. M., S. Verbakel, Y. Vankan, S. Hol, B. Löwenberg, and R. Delwel.** 1996. Anandamide, a natural ligand for the peripheral cannabinoid receptor is a novel synergistic growth factor for hematopoietic cells. Blood **88:**339a.
53. **Whitehead, I., H. Kirk, and R. Kay.** 1995. Expression cloning of oncogenes by retroviral transfer of cDNA libraries. Mol. Cell. Biol. **15:**704–710.
54. **Young, D., G. Waitches, C. Birchmeier, O. Fasano, and M. Wigler.** 1986. Isolation and characterization of a new cellular oncogene encoding a protein with multiple potential transmembrane domains. Cell **45:**711–719.