Retroviral Insertions 90 Kilobases Proximal to the *Evi-1* Myeloid Transforming Gene Activate Transcription from the Normal Promoter

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Received 24 August 1990/Accepted 4 January 1991

The inappropriate production of the *Evi-1* zinc finger protein occurs in retrovirus-induced murine myeloid leukemias and human acute myelogenous leukemias. In murine leukemias, expression of the *Evi-1* gene is associated with retroviral insertions either in the *Evi-1* locus, which is immediately 5' of the coding region of the gene, or in the genetically linked *Cb-1/fim-3* locus. In these studies, we demonstrate by chromosomal walking and pulse field electrophoresis that the *Cb-1/fim-3* locus is located 90 kb 5' of the *Evi-1* locus. Primary structure analysis of *Evi-1* cDNA clones from a *Cb-1/fim-3* rearranged cell line (DA-3) demonstrates that transcription initiates 5' of the *Evi-1* locus and that the first noncoding exon of the gene is 681 bp larger than previously defined. S1 nuclease protection studies reveal multiple transcription initiation sites within this region. Comparable transcriptional initiation sites were identified in RNA from kidney and ovary, in which the gene is normally expressed, suggesting that retroviral insertions in the *Cb-1/fim-3* locus activate transcription from the normal promoter. In one myeloid cell line (DA-3), a single long terminal repeat (LTR) is present in the *Cb-1/fim-3* locus. No stable transcripts were detectable from this LTR. In cells with retroviral insertions in the *Cb-1/fim-3* locus, one allele of the *Evi-1* locus becomes hypermethylated in the 5' region of the gene. Together, these results are most consistent with an LTR-mediated, long-range *cis* activation of *Evi-1* gene expression.

The genes that transform myeloid cells in acute myelogenous leukemia (AML) by altering their ability to differentiate are largely unknown. To identify this class of transforming genes, we have used a series of interleukin-3 (IL-3)-dependent cell lines that were isolated from retrovirally induced myeloid leukemias (21). These cells are unaltered with regard to their growth factor requirements but are transformed as defined by their inability to terminally differentiate. Approximately 15% of these myeloid leukemia cell lines have been shown to have retroviruses inserted into a common site of viral integration termed the *Evi-1* locus (34, 35). Retroviral insertions in either of two 5' noncoding exons (34) activate transcription of the *Evi-1* gene, resulting in the production of a 145-kDa, nuclear zinc finger protein (28). Neither the *Evi-1* protein nor transcripts have been detected in normal hematopoietic cells. However, the gene has been shown to be normally expressed in kidney in the renal tubules in the corticomedullary junction and in developing oocytes in the ovary (33). On the basis of these observations, it has been hypothesized that the *Evi-1* gene may play a role in early oocyte development, yet when aberrantly expressed in myeloid cells can alter their ability to differentiate.

More recently, the human *Evi-1* gene has been molecularly cloned (32) and found to be 91 and 94% homologous to the murine gene in nucleotide and amino acid sequence, respectively. The human gene maps to chromosome 3q26 (31) near a region that is rearranged in cases of AML (2, 4, 37). Initial studies of one patient with AML with t(3;5)(q25;q34) failed to demonstrate either rearrangements of the 5' region or expression of the *Evi-1* gene (31). More recently, we have examined approximately 100 cases of AML for expression of

the *Evi-1* gene and have detected transcripts in six cases which had inversions or translocations involving chromosome 3q26 (34a). However, in none of these cases was the translocation breakpoint in the *Evi-1* gene or in the region defined by the *Evi-1* common site of integration, suggesting that activation of expression may occur by rearrangements that are quite distant from the gene.

The *Cb-1* and the *fim-3* common sites of integration were independently identified by two groups. We identified the *Cb-1* locus in IL-3-dependent myeloid leukemia cell lines derived from Moloney murine leukemia virus (Mo-MuLV)-induced leukemias (36). The *fim-3* locus was identified as a common site of integration in Friend virus-induced myeloblastic leukemias (5). It was subsequently demonstrated that the *Cb-1/fim-3* locus was genetically linked to the *Evi-1* locus because no recombination was observed among 159 interspecific backcrossed mice (36). The possible relationship between the *Cb-1/fim-3* and *Evi-1* loci was further indicated by the observation that myeloid cell lines containing retroviral insertions in the *Cb-1* locus expressed the *Evi-1* gene (36). More recently, it has been demonstrated that cell lines containing retroviral insertions in the *fim-3* locus also express the *Evi-1* gene (6). However, with the genomic clones that were available, no physical linkage could be established between these loci.

To investigate the possible mechanisms of viral activation of the *Evi-1* gene, we have determined the physical relationship of the *Cb-1/fim-3* and *Evi-1* loci. The results demonstrate that the loci are 90 kb apart and that *Evi-1* transcripts in cells containing viral insertions in the *Cb-1/fim-3* locus start in the same region as do normal *Evi-1* transcripts in kidney or ovary. The results further suggest that activation is due to an enhancer mechanism. These studies demonstrate that *Evi-1* gene expression can be activated by proviral

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sequences located a considerable distance away. This finding suggests that translocations involving chromosome 3q26 in human AMLs that are quite distant from the *Evi-1* gene may cause its aberrant expression.

MATERIALS AND METHODS

Cell lines. The DA-1, DA-3, and DA-34 cell lines were isolated from Mo-MuLV-induced tumors in BALB/c mice (23). The NFS-58, NFS-60, and NFS-78 cell lines were isolated from Casitas-brain-M isolate + murine leukemia virus-induced tumors (20). The FDC-P1 cell line was isolated from long-term bone marrow cultures (9). All of the cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum, glutamine, and 20 U of purified IL-3 per ml (22).

Isolation of bacteriophage genomic and cDNA clones. *Sau3A*I genomic libraries were prepared from DA-3 and DA-34 DNA as described previously (1). Phage were plated and screened by standard techniques (27), using random-primed (13) genomic DNA probes. Phage DNA was prepared and restriction maps were generated by standard techniques.

A random-primed cDNA library was prepared with poly(A)⁺-selected mRNA from DA-3 cells by published procedures (17), using the cDNA Synthesis Plus system (Amersham). Double-stranded cDNA was methylated with *Eco*RI methylase (New England BioLabs) ligated to *Eco*RI linkers (New England BioLabs) and cloned into *Eco*RI-digested λZAP DNA (Stratagene). After packaging, the recombinant phage were screened with the indicated probes as described above.

Northern (RNA) blot analysis. Total cellular RNA was extracted according to published techniques (12). Poly(A)⁺-containing RNA was isolated by retention on oligo(dT) columns (Collaborative Research). Total (20 µg) or poly(A)⁺ (2 µg) RNA was size fractionated by electrophoresis on 1% agarose gels (1× morpholine propanesulfonic acid [MOPS]) containing 0.6 M formaldehyde and was transferred to nitrocellulose, using 20× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), by Vacublot (LKB). Blots were hybridized with [α -³²P]dCTP-labeled random-primed DNA probes (13) as previously described (38, 41).

5' end amplification of cDNAs. Amplification of the 5' ends of cDNAs was performed according to published procedures (14), with the following modifications. One microgram of either FDC-P1 or DA-3 poly(A)⁺ RNA was reverse transcribed, using the Amersham cDNA Synthesis Plus system according to the manufacturer's instructions except for the use of a primer (5'-GTCACAGTCCTCACAGC-3') for reverse transcription which is complementary to nucleotides +558 to +542 of *Evi-1* cDNA clone 58.2 (34). After removal of excess primer on a Bio-Gel A-5M (Bio-Rad) column and poly(A)⁺ tailing with terminal deoxynucleotidyltransferase (Bethesda Research Laboratories), the reaction mixture was diluted and second-strand DNA synthesis was performed with *Taq* polymerase (Cetus) for 1 h at 50°C and 1 h at 72°C, using 25 pmol of the 5' adaptor primer A(dT)₁₇ (5'-GACTC GAGTCGACATCGAT-3'). DNA amplification was performed after the addition of 20 pmol of the primers A (5'-GACTCGAGTCGACATCG-3') and 5AMP (5'-CTTCGT GGATGTCAGGC-3'), which is complementary to nucleotides +529 to +513 of clone 58.2, using a Perkin Elmer DNA thermal cycler programmed for 40 cycles at 95°C for 1 min, 52°C for 2 min, and 72°C for 2 min, followed by a final cycle of 95°C for 1 min, 52°C for 2 min, and 72°C for 10 min. DNA amplification was also repeated as described above by

substituting the 5AMP primer for 5AMP3 (5'-GACCAGA ATTGAGCCGG-3'), which is located -3 to -19 nucleotides relative to clone 58.2. Specifically amplified DNA products were identified by Southern blot analysis using either of the 5'-end-labeled oligonucleotide probes 5H (5'-CCCAGG GAACGACAAAC-3') and 5H2 (5'-GTCTCACTACCTCCC TC-3'), which are located +66 to +82 and -78 to -94, respectively, relative to clone 58.2. cDNAs were subcloned into pKSII+ Bluescript (Stratagene) by using the *Sa*I site located in primer A and the *Ssr*I sites located at the 5' end of the *Evi-1* gene.

Southern blot analysis. DNAs (10 µg) were digested with appropriate restriction endonucleases according to the manufacturer's instructions and then size fractionated by electrophoresis on 0.8% agarose gels (0.5× TBE [1× TBE is 0.09 M Tris borate, 0.09 M boric acid, 0.002 M EDTA]). The DNAs were transferred (0.4 M NaOH-0.6 M NaCl) by Vacublot (LKB) to GeneScreen Plus (DuPont), and the blots were hybridized in 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaH₂PO₄·H₂O, 0.001 M EDTA)-2% sodium dodecyl sulfate (SDS)-10% dextran sulfate-100 mg of denatured salmon sperm DNA per ml at 55°C with α -³²P-labeled random-primed DNA probes (13).

Pulse field gel electrophoresis. DNA agarose blocks were prepared from FDC-P1 cells (0.5 × 10⁷ cells per ml) in 0.5% Incert agarose (FMC) as described previously (19). For enzyme digestion, 0.06-ml blocks were placed in 200 ml containing the appropriate enzyme (New England BioLabs) and buffer (recommended by manufacturer, supplemented with 2 mM spermidine) and incubated for 4 to 8 h with 20 to 40 U of enzyme. After digestion, blocks were incubated in 0.5 M EDTA-1% sarcosyl-1 mg of proteinase K per ml for 1 h at 50°C. Blocks were loaded into wells of a 1.5% agarose gel and sealed, and pulse field gel electrophoresis was performed with the CHEF-DR II Megabase DNA system (Bio-Rad) at 200 V, with a 40-s switch at 12°C for 24 h.

DNA sequencing. DNA fragments to be sequenced were subcloned into either M13mp18 or M13mp19, and single-stranded DNA was prepared (39) and sequenced, using Sequenase (U.S. Biochemical Corp.) according to the manufacturer's instructions.

Oligonucleotide hybridization. Poly(A)⁺ RNA samples on GeneScreen Plus membranes (DuPont) were hybridized with [γ -³²P]ATP-end-labeled oligonucleotide probes as described previously (16). Filters were prehybridized for 4 h in 5× SSC-20 mM sodium phosphate (pH 7.0)-10× Denhardt solution-7% SDS-100 µg of denatured salmon sperm DNA per ml and hybridized overnight in prehybridization solution with 10% dextran sulfate and 10⁶ dpm of probe per ml. Filters were washed twice at the hybridization temperature in 3× SSC-10 mM sodium phosphate (pH 7.0)-10× Denhardt solution-5% SDS for 1 min and then for 1 h in 1× SSC-1% SDS.

S1 nuclease analysis. S1 nuclease protection assays were performed according to published procedures (12, 43). An 800-bp *Rsa*I-*Ava*II fragment which flanks the 5' region of *Evi-1* exon 1 was end labeled at the *Ava*II site with [γ -³²P]ATP (6,000 Ci/mmol; NEN) by using T4 polynucleotide kinase (Bethesda Research Laboratories). Then 50 µg of total RNA was annealed overnight to the probe in 80% formamide (Fluka)-0.4 M NaCl-40 mM piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)-5 mM EDTA at 56°C. S1 nuclease digestion was performed in 0.25 M NaCl-30 mM sodium acetate (pH 4.6)-1 mM ZnSO₄-0.2 mg of denatured salmon sperm DNA per ml-400 U of S1 nuclease (Boehringer Mannheim) per ml at 30°C for 40 min.

FIG. 1. (A) Comparison of *Evi-1* transcripts in *Evi-1* and *Cb-1/fim-3* rearranged cell lines. Shown is a Northern blot analysis of nonrearranged FDCP1, *Evi-1* rearranged NFS-58, NFS-60, NFS-78, and DA-1, and *Cb-1/fim-3* rearranged DA-3 and DA-34 cell lines with a 2.2-kb *SstI*-digested *Evi-1* cDNA probe derived from clone 21.1. Positions of RNA size markers are indicated in kilobases. (B) Schematic diagram showing the locations of retroviral sequences (■) that have inserted into the *Evi-1* gene in NFS-58 cells. The first three exons, E1, E2, and E3, are indicated (□); below are shown the spliced transcripts derived from transcription initiating in the retroviral LTR promoter.

S1 nuclease-protected fragments were analyzed by electrophoresis through a 5% polyacrylamide–7 M urea gel and visualized by autoradiography.

RESULTS

Expression of the *Evi-1* gene in cells containing integrations in the *Cb-1/fim-3* locus. We have previously demonstrated that myeloid leukemia cells with retroviral insertions in either the *Evi-1* locus (34) or the genetically linked *Cb-1/fim-3*

locus (1) have *Evi-1* transcripts detectable on Northern blots. Examples (Fig. 1A) include RNA from IL-3-dependent myeloid leukemia cell lines that contain retroviral insertions in the *Evi-1* locus (NFS-58, NFS-60, NFS-78, and DA-1), in the *Cb-1/fim-3* locus (DA-3 and DA-34), or in neither locus (FDCP1). The predominant transcript seen in NFS-58 cells (Fig. 1B) has been previously shown (34) to originate in the viral long terminal repeat (LTR) and continue immediately into a proposed 5' noncoding exon (E1) of the *Evi-1* gene. Transcripts detected in DA-3 and DA-34 cells were larger than the variably sized transcripts seen in NFS-58, NFS-60, or DA-1 cells. In NFS-78 cells, a variety of transcripts are detected with *Evi-1* probes and are derived from variably spliced RNAs initiating in the integrated retrovirus. However, the transcripts in DA-3 and DA-34 cells were the same size as the normal 6-kb *Evi-1* transcript seen in kidney and ovary (33) (data not shown).

To confirm that the transcripts observed in myeloid cell lines containing retroviral insertions in the *Cb-1/fim-3* locus are derived from the *Evi-1* gene, cDNAs were isolated from DA-3 cells. In screening 500,000 phages of a cDNA library with an *Evi-1* cDNA probe, 13 independent cDNA clones were isolated. The majority of these clones contained inserts of 2.5 kb or larger and had restriction maps which were identical to portions of a previously characterized *Evi-1* cDNA clone termed 58.2 (34). A restriction map of the largest cDNA clone (21.1) is shown in Fig. 2, where it is compared with the map of the 58.2 cDNA clone. The cDNA clone spans the entire *Evi-1* gene coding region and has restriction sites identical to those of the 58.2 cDNA clone.

Identification of the 5' ends of *Evi-1* gene transcripts in DA-3 cells. Retroviral insertions in the *Evi-1* locus primarily activate *Evi-1* gene expression through a promoter insertion mechanism. To assess whether *Evi-1* activation by insertions in the *Cb-1/fim-3* locus were also due to promoter insertions, we characterized the 5' end of the transcripts detected in DA-3 and DA-34 cells. The major transcript observed in these cells was 6 kb, compared with the 5.4-kb transcript in NFS-58 cells (Fig. 1A). This finding suggested that DA-3 and DA-34 *Evi-1* gene transcripts contained additional 5' sequences relative to those observed in NFS-58 cells. Since none of the cDNA clones that we initially isolated were full length, cDNA clones of the 5' end of the transcripts were isolated from DA-3 cells by using a primer-extended cDNA library prepared by a polymerase chain reaction (PCR)-

FIG. 2. Restriction map of the *Evi-1* cDNA clone 21.1, which was derived from the *Cb-1/fim-3* rearranged cell line DA-3, compared with that of the previously described cDNA clone 58.2 isolated from NFS-58 cells (34). Sites for the restriction endonucleases *HindIII*, *SmaI*, *SphI*, and *SstI* are indicated. Above is shown a schematic diagram of the *Evi-1* protein, which has been aligned with the coding region of the cDNA clones. The zinc finger domains 1 and 2 and the acidic domain which have been described previously are indicated. Both cDNA clones span the coding region of the *Evi-1* gene.

FIG. 3. (A) Primer-extended cDNA clones derived from the 5' end of *Evi-1* transcripts in DA-3 cells. The cDNA clones DA-3 5AMP.42, DA-3 5AMP3.12, DA-3 5AMP3.24, and DA-3 5AMP3.37 were derived by PCR amplification using the oligonucleotide primer 5AMP or 5AMP3 (see Materials and Methods). (B) Southern blot analysis of PCR-amplified cDNA derived from the 5AMP3 oligonucleotide primer and hybridized with the 5H2 end-labeled oligonucleotide probe (see Materials and Methods). To determine specificity, the PCR amplification was performed on 5RT oligonucleotide-primed first-strand synthesis cDNA derived from either FDC-P1 mRNA or DA-3 mRNA (see Materials and Methods). The 123-bp size markers are indicated.

based procedure (14). Initially a primer 3' of an *SstI* site, located in exon 3, was used for reverse transcription. The amplified cDNA fragment from this reaction was cloned following digestion with *SstI* and *SalI*, which cleaves at a site in the reverse primer. The majority of the clones obtained were 511 bp and extended from the *SstI* site to another *SstI* site that was in exon 1, 5' of the site of the retroviral insertion in NFS-58 cells. One clone, however (DA-3 5AMP.42; Fig. 3A), extended 97 bp beyond the *SstI* site as a result of a nucleotide substitution that removed the site.

Since the additional 5' sequences represented in DA-3 5AMP.42 could not account for the size of the transcripts in DA-3, a new amplification primer was used that was complementary to sequences immediately 3' of the *SstI* site in exon 1. The products of the amplification reactions using this primer are shown in Fig. 3B. Amplification products of 300 to 700 bp were obtained from DA-3 cells, whereas no product was seen with RNA from a myeloid cell line (FDC-P1) that does not express the *Evi-1* gene. The amplified cDNAs were digested with *SalI* and *SstI*, and clones were obtained (Fig. 3A). The longest of the cDNA clones (DA-3 5AMP3.12 and DA-3 5AMP3.37) were sequenced. For comparison, a genomic clone containing the first exon region was also sequenced. The sequence of the genomic clone is shown in Fig. 4. The sequences of the 5' amplified cDNA clones were identical to the genomic sequence, and therefore only the 5' ends of the clones are shown. As indicated, the longest of the cDNA clones sequenced ended 681 bp 5' of the retroviral integration site in NFS-58 cells.

FIG. 4. Primary nucleotide sequence of genomic DNA of *Evi-1* exon 1 and 5' sequences. The boxed area indicates the region of complete homology between the *Evi-1* primer-extended cDNA clones DA-3 5AMP.42 and DA-3 5AMP3.12 with the genomic DNA sequences. The CCGG *MspI*-*HpaII* sites are boxed. The 5' ends of cDNA clones DA-3 5AMP 42, DA-3 5AMP3.12, 5AMP3.24, 5AMP3.37, and NFS-58 clone 58.2 are indicated (*). Arrows above the nucleotide sequences indicate the major sites of transcription initiation observed in DA-3 and DA-34 cells as determined by S1 nuclease protection studies. Arrows below the nucleotide sequence indicate the major sites of transcription initiation observed in DA-3 and DA-34 cells and also in murine kidneys and ovaries. The underlined sequence indicates a possible TATA box, located 26 nucleotides upstream of one of the two major initiation sites observed in DA-3, DA-34, kidney, and ovary cells.

These results suggested that in DA-3 cells transcripts were initiating from the exon 1 region and that this exon was larger than had been previously proposed (34).

Identification of transcriptional start sites. To further define the 5' end of the transcripts in DA-3 cells, S1 nuclease protection analysis was performed. For these experiments, an 800-bp *RsaI*-*AvaII* end-labeled genomic DNA fragment was used (Fig. 5B). Multiple protected fragments of between 425 and 72 nucleotides were seen with RNA from DA-3 or DA-34 cells but not with RNA from a myeloid cell line that does not express the *Evi-1* gene (FDC-P1) (Fig. 5A). These results indicate the presence of multiple transcription initiation sites within this region. The locations of the transcriptional start sites are depicted in Fig. 4. Importantly, the longest of the protected fragments (425 bp) corresponded precisely to the 5' end of the longest of the amplified cDNAs that were sequenced (DA-3 5AMP3.12 and DA-3 5AMP3.37; Fig. 4).

To determine whether the transcriptional start sites observed in DA-3 and DA-34 are similar to those normally used, S1 analysis was also performed with RNA from kidney and ovary. These tissues have previously been shown to be

FIG. 5. (A) S1 nuclease protection assay of total RNA derived from FDC-P1, DA-3, DA-34, kidney, and ovary cells. The two right-most lanes illustrate a longer exposure of the results with kidney and ovary RNA. Sizes of the major protected fragments are indicated in base pairs. The markers shown are derived from *MspI*-digested pBR322 DNA. (B) Partial restriction map of the exon 1 and 2 (E1 and E2; $\square$) region of *Evi-1* indicating the 800-bp *RsaI*-*AvaII* 5'-end-labeled probe used for S1 analysis.

the predominant sites of *Evi-1* expression in the mouse (33). A very similar profile of protected fragments was observed with these RNAs (Fig. 5A). Although the relative intensities varied somewhat, major S1 nuclease-protected fragments of 425 and 72 to 73 bp were found in all cases (Fig. 5A). These results suggest that transcripts in DA-3 and DA-34 cells are initiated at start sites that are utilized in normal tissues in which the *Evi-1* gene is expressed.

The *Cb-1/fim-3* locus is located 90 kb 5' of the *Evi-1* gene. The possible physical linkage of the *Cb-1/fim-3* and *Evi-1* retroviral common integration sites was indicated by their genetic linkage (36, 40). To initially establish the physical linkage, pulse field gel electrophoresis analysis was performed. Digestion of DNA with *SalI* and *SfiI* resulted in fragments of 120 and 150 kb, respectively, which were

FIG. 6. Pulse field gel analysis of FDC-P1 DNA (DBA/2) digested with the restriction endonuclease *SfiI* or *SalI* and hybridized to either the *Evi-1*-specific probe j (see Fig. 7) (A) or the *Cb-1/fim-3*-specific probe e (see Fig. 7) (B). Positions of *Saccharomyces cerevisiae* chromosome size markers (Bio-Rad) are indicated.

detected with probes from either the *Cb-1/fim-3* locus or the *Evi-1* locus (Fig. 6). These results demonstrated that the loci were maximally 120 kb apart. Since 3' probes from the *Evi-1* gene did not detect these fragments (data not shown), the *Cb-1/fim-3* locus was oriented 5' of the *Evi-1* locus.

To more precisely determine the physical linkage, chromosomal walking using *Sau3AI* partial genomic libraries in bacteriophage λ was undertaken. Genomic clones were isolated in both directions from the *Cb-1/fim-3* locus, since the relative orientation with the *Evi-1* locus was unknown. Genomic clones were also isolated 5' of the *Evi-1* locus. By using a number of overlapping genomic clones, it was possible to generate a restriction map for a region of approximately 130 kb 5' of the *Evi-1* gene (Fig. 7). The results established that the *Cb-1/fim-3* locus was 5' of the *Evi-1* common site of integration and that the sites were separated by 90 kb. The results also demonstrated that the orientations of the proviruses in DA-3 and DA-34 cells were opposite that of most of the retroviral insertions in the *Evi-1* locus and the *Evi-1* gene. For orientation, the first three exons of the *Evi-1* gene and the position of the first coding ATG are shown.

Consistent with the pulse field analysis, two restriction sites for *SalI* were identified that would generate a restriction fragment of 130 kb that would be detected with an *Evi-1* or a *Cb-1/fim-3* probe. Two *SfiI* sites were identified in the clones near the *Evi-1* locus. The most 5' site is polymorphic and is present in the BALB/c mouse DNA used for genomic cloning but is not present in DBA/2 mouse DNA that was used for the pulse field analysis. No other *SfiI* sites were identified within the cloned region, consistent with the pulse field data indicating that the next site would be beyond the region cloned.

Lack of stable transcripts from the LTR at the *Cb-1/fim-3* locus in DA-3 cells. Activation of transcription of the *Evi-1* gene in cells containing retroviral insertions in the *Cb-1/fim-3* locus could be due to *trans* activation by a gene that is activated by retroviral insertions at the locus. Alternatively, retroviral insertions might act as long-range enhancer elements to activate transcription from the normal promoter.

FIG. 7. Genomic organization of a 200-kb region surrounding the *Cb-1/fim-3* and *Evi-1* loci. The locations and orientations of retroviral insertions in the *Cb-1/fim-3* locus in DA-3 and DA-34 cells are indicated by arrows, as are the locations and orientations of retroviral insertions in the *Evi-1* locus in DA-1, NFS-48, NFS-58, NFS-60, and NFS-78. The first three *Evi-1* gene exons, including the first coding exon indicated by an ATG, are shown. The orientation of the *Evi-1* gene is indicated by the arrow. The DNA probes designated a through i were used to isolate overlapping bacteriophage clones upstream and downstream of the *Cb-1/fim-3* locus. The DNA probes designated I to IV were used to isolate overlapping bacteriophage clones upstream of the *Evi-1* locus. The j probe was used for both pulse field and methylation studies. Overlapping bacteriophage clones spanning the 200-kb region are labeled λCB1 to λCB40 and are depicted by horizontal lines beneath the restriction map. Clones λCB1 to λCB5 were isolated by using a full-length *Evi-1* cDNA probe (58.2).

To investigate these possibilities, we further characterized the retroviral integration in DA-3 cells. This cell line was particularly valuable since Southern blot analysis had indicated that the majority of the retrovirus had been deleted and that possibly only a single LTR remained in the locus (1). To confirm this, the regions flanking the LTR were subcloned and sequenced. The sequence confirmed the presence of a single LTR which has a structure consistent with a provirus that was deleted by homologous recombination between the LTRs (Fig. 8A). In particular, a comparison of the viral LTR 5' and 3' junction fragments demonstrated the expected four-base (TTCT) duplication of cellular sequences flanking the LTR and the loss of 2 bp from each end of the LTR (10, 42, 44).

If the LTR in DA-3 cells functioned as a promoter, stable transcripts that contained genomic sequences immediately 3' of the single LTR, but prior to potential splice donor sites, would be anticipated. We initially used 20 genomic probes from regions spanning 40 kb of the integration site; however, no transcripts were detected on Northern blots (data not shown). To further pursue the possibility of transcripts originating from the LTR, an oligonucleotide probe from immediately downstream of the LTR was used for Northern analysis. No transcripts were detected with this probe (Fig. 8B). As a control, a comparably sized oligonucleotide probe from the U5 region of the LTR was used. This probe readily detected full-length and spliced viral transcripts in the Mo-MuLV-infected cell line DA-1 but failed to detect any transcripts in DA-3 cells. As an additional control, we used a 17-mer from the 5' region of the *Evi-1* gene. This probe readily detected *Evi-1* transcripts in DA-1 and DA-3 cells (Fig. 8B). These results suggest that the isolated LTR in the *Cb-1/fim-3* locus of DA-3 cells either is inactive or does not give rise to stable transcripts.

Changes in methylation in the 5' region of one *Evi-1* allele in *Cb-1/fim-3* rearranged cells. A common modification of DNA is methylation of cytosines in CpG dinucleotides at their C5 position (3), and numerous studies have established a correlation between hypomethylation and gene activity (11). Moreover, the ability of proviruses to influence the methylation of flanking host sequences has been demonstrated (24).

Therefore, it was conceivable that proviral integrations in the *Cb-1/fim-3* locus might alter chromatin structure and affect the methylation state of the *Evi-1* gene, which in turn might influence expression. These changes would be expected to occur in the allele containing proviral integrations. We therefore looked for changes in methylation in the 5' C+G-rich region of the *Evi-1* gene. For these studies, we focused on a 5.5-kb *Eco*RI fragment that contains multiple potential *Msp*I-*Hpa*II sites and contains the entire exon 1

FIG. 8. (A) Comparison of partial sequence surrounding the solo viral LTR located in the *Cb-1/fim-3* locus in DA-3 cells with the Mo-MuLV sequence. The tetrameric repeat of genomic DNA sequences and the deleted Mo-MuLV sequences are highlighted. (B) Northern blot analysis of poly(A)⁺ RNAs derived from FDC-P1, DA-1, and DA-3 cells with the U5-specific oligonucleotide probe Mo-MuLVU5 (5'-CGCTGACGGGTAGTCAATCAC-3'), the 3'-end LTR-genomic DNA junction probe CB-1 (5'-CCAAGGCTATGTC TACACCAG-3'), or the *Evi-1*-specific 5AMP oligonucleotide probe described in Materials and Methods. Hybridization and washing were performed at 56°C with each probe. Positions of the RNA ladder (Bethesda Research Laboratories) size markers (in kilobases) and the 28S and 18S RNAs are indicated.

FIG. 9. Comparison of the methylation status of the *Evi-1* promoter region by Southern blot analysis of *EcoRI*, *EcoRI-MspI*, or *EcoRI/HpaII*-digested genomic DNAs derived from several myeloid cell lines, which either express (DA-3 and DA-34) or do not express (DA-13, DA-7, DA-22, DA-24, DA-28, and DA-31) the *Evi-1* gene, with probe j (see Fig. 7). Positions of specific fragments of 5.5, 4.7, and 3.2 kb and of λ *HindIII* size markers are shown.

region. In Southern blots, digestion of genomic DNAs with *EcoRI* and the methylation-insensitive enzyme *MspI* resulted in a 3.2-kb fragment that is detected with a 5' probe which identifies the first potential methylation site 3' from the *EcoRI* site (Fig. 9A). In a variety of myeloid cell lines that do not express the *Evi-1* gene, digestion with *EcoRI* and the methylation-sensitive enzyme *HpaII* generates a 4.7-kb fragment, indicating that on both alleles, the first site is methylated as well as all sites up to a site that is 0.8 kb from the end of the *EcoRI* fragment (Fig. 9). This corresponds to one of the three *MspI-HpaII* sites in exon 1 (Fig. 4). In contrast, *EcoRI-HpaII* digestion of DNA from either DA-3 or DA-34 cells generates two fragments of equal intensity at 4.7 and 5.5 kb, indicating that one allele was unchanged while all of the *MspI-HpaII* sites had become methylated on the other allele in both cases (Fig. 9A). These results demonstrate an increased, rather than decreased, methylation of one allele of the 5' region of the *Evi-1* gene in cell lines containing retroviral insertions in the *Cb-1/fim-3* locus.

DISCUSSION

Our studies have established a physical linkage between the *Cb-1/fim-3* and *Evi-1* common sites of retroviral insertion

which are associated with activation of *Evi-1* zinc finger gene expression. Activation of expression of the *Evi-1* gene is one of the most common potential transforming events identified in murine myeloid leukemias. Of our IL-3-dependent myeloid leukemia cell lines, 19% express the *Evi-1* gene and contain retroviral insertions in either the *Evi-1* or *Cb-1/fim-3* locus (1, 34). Among 68 Friend virus-induced myeloid leukemias, 23% were found to contain retroviral insertions in a 16-kb region that initially defined the *fim-3* common site of integration (5). More recently, cells with integrations in the *fim-3* locus have been shown to express the *Evi-1* gene (6). Conversely, no myeloid cell lines or tumors have yet been identified that express the *Evi-1* gene which do not have viral insertions in either the *Cb-1/fim-3* or *Evi-1* locus.

Although previous studies (1, 40) demonstrated that the *Evi-1* and *Cb-1/fim-3* loci were genetically linked, they failed to establish a physical linkage. The results presented here demonstrate, through chromosomal walking and pulse field gel electrophoresis, that the *Cb-1/fim-3* locus is 90 kb proximal to the *Evi-1* locus. The ability of retroviral insertions to affect gene expression over long distances has been demonstrated for the *myc* gene. In particular, the *Mlvi-4* locus maps 30 kb 3' of the *myc* gene (26), while the *Mlvi-1/Mis-1/pvt-1* locus maps 270 kb 3' of the *myc* gene (25, 26). All of the viral integrations in either locus are in the same transcriptional orientation as the *myc* gene and have been shown to affect *myc* expression in *cis* (26). Similarly, translocations in Burkitt's lymphomas have been shown to occur at sites 34, 140, and 260 kb 3' of the *myc* gene which also affect *myc* expression (18). The types of chromosomal changes that are brought about by either retroviral insertion or chromosomal translocations and that are associated with long-range alterations of gene expression are not known. Models that have been suggested include effects related to the introduction of nuclear matrix attachment sites (15), topoisomerase II binding sites (18), or alterations in chromatin structure (8).

The activation of expression of the *Evi-1* gene in cell lines containing retroviral insertions in the *Evi-1* common site of integration primarily occurs by a promoter insertion model (34). In these cases, proviruses are always oriented in the same transcriptional orientation as the *Evi-1* gene and transcripts initiate in the viral 5' or 3' LTR. The sole exception is the NFS-60 cell line, in which the provirus is in the opposite transcriptional orientation relative to the gene and a discrete *Evi-1* transcript is detected that does not contain proviral sequences (34b). Since the provirus is 3' of the normal transcriptional start sites, it is hypothesized that transcription initiates at a cryptic promoter 5' of the second, noncoding exon.

The mechanisms involved in the activation of *Evi-1* gene expression by retroviral integrations in the *Cb-1/fim-3* locus are less obvious. Several observations are relevant when considering possible mechanisms. (i) All of the proviruses are integrated in an orientation that is opposite the transcriptional orientation of the *Evi-1* gene. (ii) Proviral deletions can occur that leave a single LTR, although this is not essential (5). (iii) There appears to be specificity for the sites that can activate expression. In particular, no cell lines have been described that express the *Evi-1* gene that have proviral integrations in sites other than the *Cb-1/fim-3* or *Evi-1* loci. (iv) No stable transcripts have been detected from the *Cb-1/fim-3* locus. (v) Transcripts of the *Evi-1* gene start at sites that are observed in normal tissues in which the gene is expressed.

The *Evi-1* gene transcripts observed in the *Cb-1/fim-3* rearranged cell lines are slightly larger than those observed

in the *Evi-1* rearranged cells. The larger transcripts can be completely accounted for by additional sequences located at the 5' end of the gene. These sequences originate from a region immediately adjacent to the previously defined *Evi-1* exon 1 and extend the length of this noncoding exon by 681 nucleotides. By S1 nuclease protection assays, there is considerable heterogeneity in the transcriptional start sites that are observed at the 5' end of the *Evi-1* gene in DA-3 and DA-34 cells similar to that seen with a variety of genes.

The multiple transcription initiation sites observed in DA-3 and DA-34 cells were also observed in adult mouse kidneys and ovaries, in which the *Evi-1* gene is normally expressed (33). Although the patterns of transcription initiation sites are similar, the ratios differ significantly and indicate that minor sites are more frequently used in the myeloid cells. Importantly, the two major sites observed in kidney and ovary are also utilized in DA-3 and DA-34 cells. Thus, in cells with retroviral insertions in *Cb-1/fim-3*, *Evi-1* expression appears to be activated from the normal gene promoter.

In addition to the location of transcription initiation sites, there is other evidence to suggest that the promoter for the *Evi-1* gene is within this region. The exon 1 and 5'-flanking sequences are G+C rich, a feature frequently associated with eukaryotic gene promoters (3). Many genes which are transcribed by RNA polymerase II contain TATA box sequences (7), and transcripts are normally initiated approximately 25 nucleotides downstream from this motif. One of the major transcription initiation sites observed in kidney, ovary, DA-3, and DA-34 cells is located precisely 26 nucleotides downstream of a potential TATA box motif, TTATAA (Fig. 4). In addition, the consensus sequence 5'-CCCCAGGC-3', a motif specifically recognized by the enhancer-binding protein AP-2 (30, 45), is located between nucleotides 182 and 175 (Fig. 4). However the 5' region of the gene does not contain any other obvious sequences that are recognition sites for known transcription factors. Of potential interest is a long stretch of alternating TC then AC residues which are located immediately 5' of one of the major transcriptional start sites. Experiments are currently in progress to define a functional promoter region.

One possible mechanism of *Evi-1* gene expression in DA-3 and DA-34 cells is activation, *in trans*, via a putative transcription factor located at the *Cb-1/fim-3* site. Alternatively, proviral insertions may disrupt a transcriptional unit. Genomic probes spanning approximately 40 kb of the *Cb-1/fim-3* locus have failed to detect a transcriptional unit (data not shown). Additional evidence against activation of a gene at the *Cb-1/fim-3* locus comes from analysis of the DA-3 cell line. In this case, the activation of *Evi-1* gene expression is associated with the presence of a single LTR. Comparable deletions of proviruses have been described and are speculated to occur through homologous recombination between the LTRs (44). The presence of a single LTR considerably limits the types of transcripts of flanking cellular sequences that might be expected in the case of a promoter insertion mechanism. In DA-3 cells, no transcripts could be detected with probes immediately 3' to the LTR. Thus, either the LTR is transcriptionally inactive or no stable transcripts are synthesized.

To examine whether activation involves a mechanism occurring in *cis*, we looked for possible changes in the methylation state of the G+C-rich region in the 5' end of the *Evi-1* gene. Strikingly, one allele of the *Evi-1* gene was found to be hypermethylated in DA-3 and DA-34 cells but not in any of a variety of *Evi-1*-negative myeloid cell lines exam-

ined. Proviral sequences have been shown to induce *de novo* methylation of flanking DNA sequences (24), and therefore it is tempting to speculate that the hypermethylation of the allele is a consequence of viral sequences located at the *Cb-1/fim-3* locus. However, increased methylation is normally associated with lack of expression of genes (24). Methylation-specific DNA-binding proteins whose functions are not known have been described (29, 46); therefore, it is conceivable that methylation of DNA sequences around the *Evi-1* gene promoter may either enhance the binding of transcription factors required for expression or prevent binding of negative factors that normally suppress transcription.

Our results have particular importance for studies of the role of the *Evi-1* gene in human AML. We have previously shown (31) that the *Evi-1* gene maps to a region (3q26) that is often rearranged in cases of AML. More recently, we have identified six cases of AML in which the *Evi-1* gene is expressed (34a). All of these cases have inversions or translocations of the 3q26 region. In none of them, however, have we detected rearrangements within 100 kb 5' or 50 kb 3' of the gene. Taken together, the results indicate that activation of *Evi-1* gene expression by translocations may, like retroviral insertions in the *Cb-1/fim-3* locus, occur over considerable distances.

ACKNOWLEDGMENTS

This work was supported in part by National Cancer Institute Cancer Center Support (CORE) grant P30 CA21765, by grant RO1 CA51020 from the National Cancer Institute, and by the American Lebanese Syrian Associated Charities.

We thank S. Bartholomew for technical assistance and J. Cleveland, D. Askew, and S. Katzav for critical reading of the manuscript.

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