

Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the *ets* gene family, *Fli-1*, closely linked to *c-ets-1*

Yaacov Ben-David, Elizabeth B. Giddens, Kenneth Letwin, and Alan Bernstein¹

Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5 Canada; ¹Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada

The retroviral integration site *Fli-1* is rearranged in 75% of the erythroleukemia cell clones induced by Friend murine leukemia virus (F-MuLV), whereas *Spi-1/PU.1*, a member of the *ets* family of DNA-binding proteins, is rearranged in 95% of the erythroleukemias induced by Friend spleen focus-forming virus (SFFV). To determine the transcriptional domain defined by *Fli-1*, we have isolated a cDNA clone that is highly expressed only in erythroleukemia cell lines with *Fli-1* rearrangements. The protein sequence of this cDNA is very similar to *Erg2*, another member of the *ets* gene family. The hydrophilic carboxy-terminal end of the *Fli-1* cDNA shares significant sequence similarity to the DNA-binding ETS domain found in all members of the *ets* family. PFGE analysis localized *Fli-1* within 240 kb of the *ets-1* proto-oncogene on mouse chromosome 9 and human chromosome 11q23, suggesting that *ets-1* and *Fli-1* arose from a common ancestral gene by gene duplication. The involvement of the murine *Fli-1*, *Spi-1*, and avian *v-ets* genes in erythroleukemia induction suggests that activation of *ets* gene family members plays an important role in the progression of these multistage malignancies.

[Key Words: F-MuLV; erythroleukemia; *Fli-1*; *ets-1*]

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The hematopoietic system is composed of a hierarchy of cells, ranging from the pluripotent stem cell, committed myeloid and lymphoid progenitor cells, to terminally differentiated blood cells (Metcalfe 1988; Sachs 1987). The molecular mechanisms that control the developmental and proliferative decisions of the pluripotent hematopoietic stem cell are largely unknown. The extreme cellular heterogeneity of hematopoietic populations, combined with the low frequency of stem cells, has hampered experimental approaches to this problem. The generation of mutations that affect stem cell function and the molecular analysis of existing developmental mutations that control hematopoiesis (Pawson and Bernstein 1990) provide two powerful genetic strategies for identifying and characterizing the genes that control normal blood cell formation. In addition, because cellular genes involved in leukemic transformation may also have a role in regulating normal stem cell function, the identification of such genes may provide insights into both normal and leukemic hematopoiesis.

Transformation by certain retroviruses can involve the integration of proviral DNA near or within specific cellular genes. Such integration events most frequently lead to the elevated expression of these genes (for review, see in Peters 1990), although the tumor suppressor gene *p53* can also be inactivated by retroviral integration of the Friend leukemia virus (Ben-David et al. 1988, 1990b). The analysis of common retroviral integration events can, therefore, be used to identify and clone novel transforming genes involved in tumorigenesis. Using this approach, a large number of genes important in the leukemic transformation of hematopoietic cells, including *pim-1*, *c-myc*, *c-myb*, *Evi-1*, *Fim-3/c-fms*, *Spi-1*, and *Fli-1* have been isolated (Peters 1990).

The *Fli-1* and *Spi-1* genes have been shown by us and others (Moreau-Gachelin et al. 1988; Ben-David et al. 1990) to be involved in erythroleukemia induction by various strains of Friend leukemia virus. *Fli-1* (Friend leukemia integration-site 1) is rearranged in 75% of independently isolated erythroleukemic clones from mice inoculated at birth with the replication-competent Friend murine leukemia virus (F-MuLV). In contrast, *Spi-*

¹Corresponding author.

1 (SFFV–proviral integration-site 1) is rearranged in the majority of erythroleukemias induced by injection of the polycythemia (FV-P)- or anemia (FV-A)-inducing strains of Friend virus (Moureaux-Gachelin et al. 1988,1990). The FV-P and FV-A strains of Friend virus are complexes of a replication-defective spleen focus-forming virus (SFFV-P and SFFV-A) and a replication-competent F-MuLV (for review, see Kabat 1990). *Spi-1* is rearranged only in FV-P- and FV-A-induced erythroleukemias and not in the leukemias induced by F-MuLV (Moreau-Gachelin et al. 1988; Ben-David et al. 1990a). In contrast, the *Fli-1* locus is only rearranged in the erythroleukemias induced by F-MuLV and not in the erythroleukemias induced by the FV-P or FV-A viral complexes (Ben-David et al. 1990a). These observations have raised the possibility that two distinct events are involved in the induction of erythroleukemias by F-MuLV or FV-P–FV-A complexes. Recently, another common viral integration site, *Sic-1*, rearranged in the non-T-, non-B-cell lymphomas induced by Cas-Br-E Moloney leukemia virus, has been shown to be identical to *Fli-1* (Bergeron et al. 1990).

A cDNA clone corresponding to the *Spi-1* transcript has been isolated from an FV-P-induced erythroleukemia cell line (Moreau-Gachelin et al. 1989). This transcript is highly expressed in both FV-P- and FV-A-induced erythroleukemia cell lines that have undergone rearrangements of the *Spi-1* gene due to the insertion of SFFV (Moreau-Gachelin et al. 1989 1990). The sequence of the *Spi-1* transcript has been shown recently to be identical to that of PU.1 (Goebel et al. 1990), a gene that encodes a DNA-binding protein (Klemsz et al. 1990). The PU.1 protein is also a transcriptional activator with 40% sequence identity to the proteins encoded by members of the *ets* oncogene family.

In the experiments reported here, we have identified a transcript that originates from sequences adjacent to the proviral integration sites defined by *Fli-1*. We show that this transcript is specifically up-regulated in leukemic clones with F-MuLV insertions at the *Fli-1* locus and that *Fli-1* encodes a new member of the *ets* family of DNA-binding proteins. Furthermore, we show that *Fli-1* and *c-ets-1* are located within 240 kb of each other on mouse chromosome 9. These studies raise intriguing questions concerning the role of the *ets* family in normal and leukemic hematopoiesis and the specificity of integration events in retrovirally induced malignancies.

Results

Isolation of cDNA corresponding to the *Fli-1* locus

Previously, we have identified a common site for integration of F-MuLV (*Fli-1*) in 75% of independently isolated erythroleukemia cell lines derived from either BALB/c or NIH/Swiss mice inoculated at birth with F-MuLV (Ben-David et al. 1990a). To determine whether this common integration event results in the activation of a novel transcript, we set out to identify and isolate a cDNA corresponding to the *Fli-1* locus by using genomic probes that map around this integration site. We first

determined the sites of F-MuLV integration at *Fli-1* in seven erythroleukemia cell lines induced by F-MuLV. As shown in Figure 1, the provirus is integrated in the same 5' to 3' orientation in a 2-kb region of genomic DNA. A number of erythroid and nonerythroid tumors were then analyzed by Northern blotting using several probes located adjacent to the *Fli-1* integration sites. The probe B4 (Fig.1), which maps close to the region of the proviral integration sites, detected a 4-kb mRNA in erythroleukemia cell lines with a rearranged *Fli-1* locus. A smaller RNA species was also observed in the TP1 cell line when total cellular RNA was used [this smaller transcript is not seen in poly(A)⁺ select RNA (data not shown)]. No *Fli-1* transcript was observed in erythroleukemia cell lines induced with FV-P or FV-A with the B4 probe (data not shown).

To determine whether expression of this 4-kb mRNA was activated due to retroviral insertion and to determine the structure and protein-coding sequences of *Fli-1*, we constructed a λ gt11 cDNA library with the poly(A)⁺ RNA isolated from an erythroleukemia cell line (CB7) that has a *Fli-1* rearrangement. A total of 5×10^5 clones were screened with the B4 probe, and four clones were isolated. These clones all had a similar 1.7-kb cDNA insert as determined by restriction enzyme mapping. Therefore, the insert from only one of these phage was purified and used in Northern blot analysis to determine its expression in erythroleukemia cell lines that either had a rearranged *Fli-1* locus or still contained the intact allele. All three erythroleukemia cell lines, CB7, CB3, and TP1, induced by F-MuLV, expressed high levels of the 4.0-kb transcript, whereas very low or undetectable levels of *Fli-1* mRNA were observed in three other erythroleukemia cell lines (DP25-15, DP27-17, and DP28-9) induced by FV-P and only low levels of *Fli-1* mRNA were observed in the erythroleukemia cell line DA24-23 induced by FV-A (Fig. 2). These latter four cell lines have a rearranged *Spi-1* locus, but their *Fli-1* locus was appar-

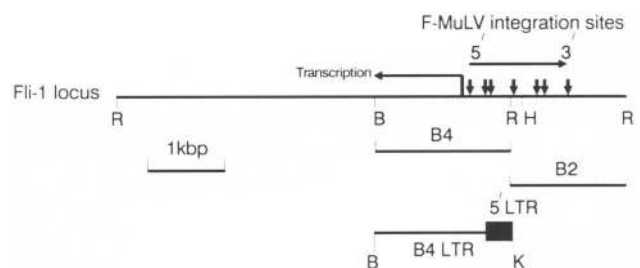


Figure 1. Identification of a transcription domain within the *Fli-1* locus. The arrows indicate the sites and orientation of F-MuLV integration within the *Fli-1* locus in seven erythroleukemia cell lines induced by F-MuLV. The 1.7-kb *Bam*HI–*Eco*RI fragment (B4 probe) detects a 4-kb transcript in erythroleukemias with a *Fli-1* rearrangement. This 4-kb transcript initiates within the B4 fragment (as indicated by arrow). The B4 LTR is a genomic fragment of *Fli-1* that includes a portion of the integrated F-MuLV LTR isolated from the phage clone λ C3, as described previously (Ben-David et al. 1990a). The B4 LTR fragment contains sequences that correspond to the 5' end of BB4 cDNA (see Fig. 3A).

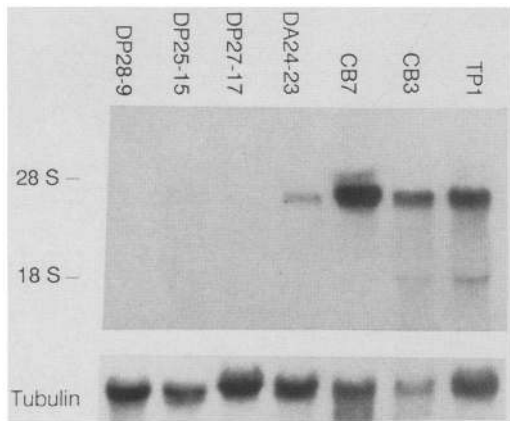


Figure 2. Expression of *Fli-1* in both FV-P/FV-A- and F-MuLV-induced erythroleukemias. Twenty micrograms of total RNA extracted from the FV-P-induced erythroleukemia cell lines (DP28-9, DP25-15, and DP27-17), the FV-A-induced erythroleukemia cell line DA24-23, and the F-MuLV-induced erythroleukemia cell lines (CB7, CB3, and TP1) were electrophoresed and transferred to nitrocellulose paper and hybridized with the *Fli-1* cDNA probe BB4 (see Fig. 3A). The same filter was rehybridized to the tubulin probe as a control and exposed for the same period of time (24 hr).

ently intact. The same filter was hybridized with a tubulin probe to control for RNA loading. The intensity of the bands detected with the *Fli-1* probe was similar to that detected with the highly expressed tubulin probe. These data strongly suggest that the *Fli-1* transcript detected with the B4 probe is highly expressed in F-MuLV-induced erythroleukemias and, therefore, might be a novel oncogene involved in the malignant progression of Friend leukemia.

Sequence analysis of the *Fli-1* transcript

The insert from the λ gt11 clone isolated with the B4 probe was subcloned into the PGEM-7Zf(+) vector (named BB4), and its complete nucleotide sequence was determined. As shown in Figure 3B, the sequence of the entire 1729-nucleotide insert contains a single long open reading frame of 1467 nucleotides. The 3'-untranslated region extends for an additional 129 bases and has no poly(A) tail. There are three in-frame stop codons within the untranslated 3' end. A putative translational initiation site is located 234 bp from the 5' end. The 5'-untranslated region contains two stop signals upstream of the first methionine that has no consensus sequence typically found in eukaryotic translation initiation sites (Kozak 1986). The distinct boundaries around the open reading frame of BB4 cDNA suggested that this clone is capable of encoding a primary translation product initiating at the 5'-most AUG codon of 452 amino acids in length with an expected molecular mass of 51 kD.

Fli-1 is a member of the *ets* gene family

A search of the GenBank and EMBL DNA sequence data bases with the BB4 sequence revealed that *Fli-1* is a new

member of the *ets* gene family. The greatest similarity (67% identity) of the BB4 sequence was to human *erg2* (*ets*-related gene; Fig. 4) isolated on the basis of its cross-hybridization with a *c-ets-1* probe (Rao et al. 1987). The strong similarity between *Fli-1* and *Erg2* was found over the entire coding sequence, except between amino acids 200–265 of *Fli-1*. The similarity with *erg2* extends from the fourth amino acid downstream of the putative initiation site of the *Fli-1* protein and both genes terminate with two conserved tyrosine residues at position 452 of *Fli-1*. The high degree of similarity immediately after the translational initiation site of *erg2* suggests that *Fli-1* translation also initiates at the same position. The overall protein identity between *Fli-1* and *Ets-1* is 30% (Table 1). However, greater similarity (68–100%) was found in an ~85 amino acid region located at the carboxy-terminal end of the three *ets*-related genes (Table 1). This region, termed the ETS domain (Karim et al. 1990), appears to be highly conserved among all of the *Ets* family members described to date. The *Spi-1/PU.1* gene, which is activated in FV-P/FV-A-induced erythroleukemias, has only 23% overall identity to the *Fli-1* protein sequence (Table 1), but 38% identity was found within the ETS domain of the *Fli-1* and *Spi-1/PU.1* proteins. The greatest similarity between *Spi-1/PU.1* with other *Ets* family members within the ETS domain is 38% (with *Ets-1*). In addition to the 85-amino-acid ETS domain, we have identified a conserved 18- to 20-amino-acid region of *Fli-1* (amino acids 117–207) that contains high levels of similarity with *Erg2* and *Ets-1* (Fig. 4). Similar related regions have been shown previously between the protein sequences of *Erg1* and *v-Ets* (Reddy et al. 1987).

The *Fli-1* protein shares with other members of the *Ets* family a hydrophilic sequence in the carboxy-terminal ETS domain. *Fli-1* does not have zinc-finger consensus sequences or the consensus sequences for α -helical turns found in other DNA-binding proteins with a helix-loop-helix structure (Murre et al. 1989; Struhl 1989). However, there are three highly conserved tryptophan residues in the ETS domain of *Fli-1*, *Erg2*, and *Ets-1* (Fig. 4, marked by asterisk). Similar patterns of tryptophan repeats are found in the *v-myb* DNA-binding region (Anton and Frampton 1988). Only the second and third tryptophan residues are conserved in the *Spi-1/PU.1* ETS domain. The tryptophan residues conserved in the *ets* gene family and *myb* are not present in other DNA-binding proteins and, hence, they may serve to define a new category of DNA-binding proteins. In addition, there are three other conserved tryptophan residues in the more amino-terminal conserved region of *Fli-1*, *Erg2*, and *Ets-1* (Fig. 4, shown by asterisk). The function and possible importance of these tryptophans are unknown at present.

Transcriptional orientation of the Friend provirus relative to *Fli-1*

Activation of a cellular oncogene by retroviral integration can occur by at least two known mechanisms, namely promoter insertion and enhancer activation. Pro-

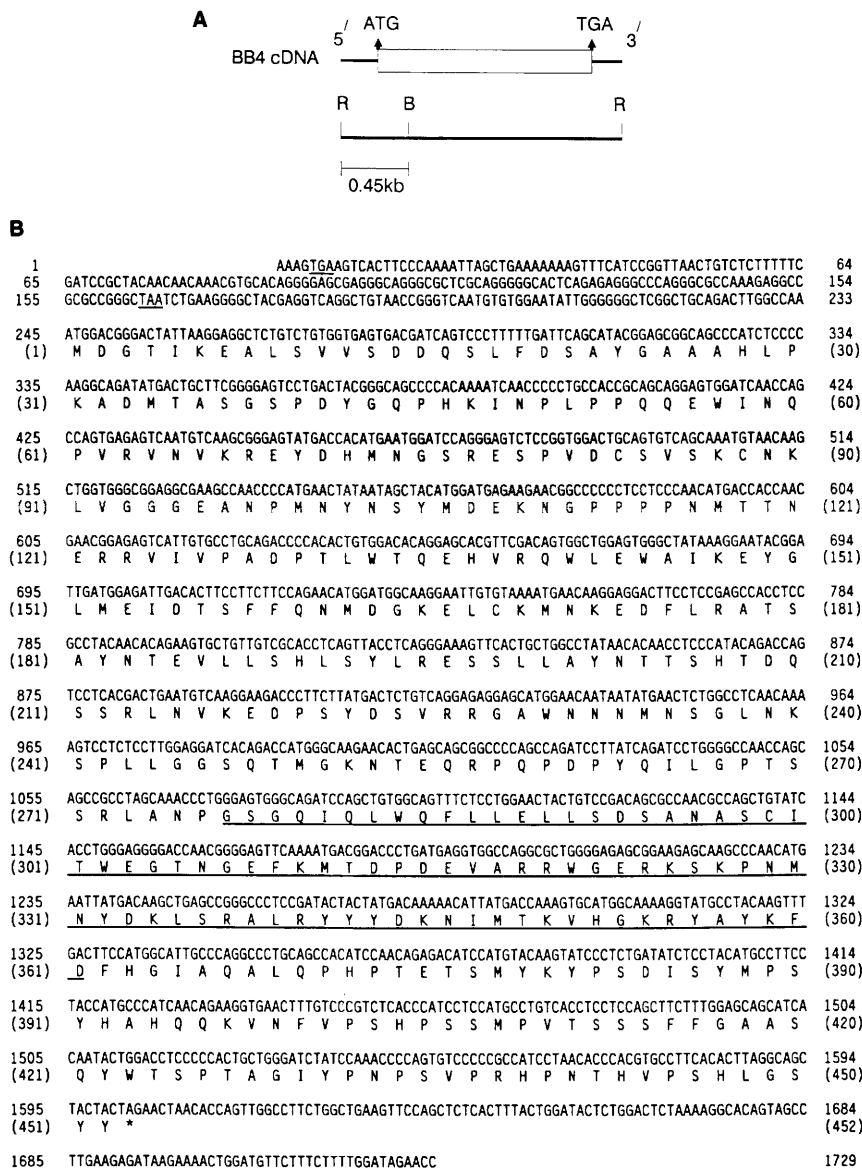


Figure 3. Sequence and structure of BB4 cDNA. (A) The 1.72-kb BB4 cDNA is shown with the presumed initiation and termination codons (open box). The restriction endonuclease sites are indicated. *EcoRI* (R); *BamHI* (B). (B) Sequence of BB4 cDNA and its deduced amino acid sequence. The ETS domain is underlined. In-frame stop codons located in the 5'-untranslated sequences region are underlined. The termination codon is marked with an asterisk (*).

moter insertion requires that the transcriptional orientation of the proto-oncogene and the provirus be parallel and that the provirus be located 5' of the start site of transcription, whereas enhancer activation can occur even if the provirus is in the transcriptionally opposite orientation and 3' of the proto-oncogene. To define the mode of gene activation of *Fli-1*, we determined the orientation of provirus integration relative to *Fli-1* in seven F-MuLV-induced erythroleukemia cell lines. As shown in Figure 1, the F-MuLV provirus integrated in the same 5' to 3' transcriptional orientation within 2 kb of *Fli-1* (Ben-David et al. 1990a). B4 LTR is a *BamHI*-*KpnI* fragment that includes a portion of the integrated F-MuLV LTR and its 5' *Fli-1*-flanking sequence derived from a genomic library constructed using DNA from the CB3 erythroleukemia cell line (Fig. 1). This 1.7-kb fragment hybridized with the 0.45-kb *EcoRI*-*BamHI* fragment from the 5' end of the BB4 cDNA (Fig. 3A). This result demonstrates that the 5' sequence of BB4 cDNA is in the

opposite transcriptional orientation relative to the orientation of the integrated provirus. Therefore, the *Fli-1* transcript appears to be activated through the enhancer element of the LTR and not by direct promoter activity of the LTR. We also isolated 17 kb of sequences 3' to the integration site and were not able to detect sequences that hybridized with BB4 cDNA or mRNA corresponding to the *Fli-1* transcript. Because the size of the activated transcript detected in Northern blot of F-MuLV-induced erythroleukemia cell lines is not altered, we suggest that the provirus integration sites are all located upstream of the *Fli-1* transcriptional initiation site.

Fli-1 lies within 240 kb of the *c-ets-1* proto-oncogene on mouse chromosome 9

Previously, we have mapped the *Fli-1* locus using recombinant inbred (RI) strains of mice and shown that it is located within 0–2 cM of the *c-ets-1* gene on mouse chro-

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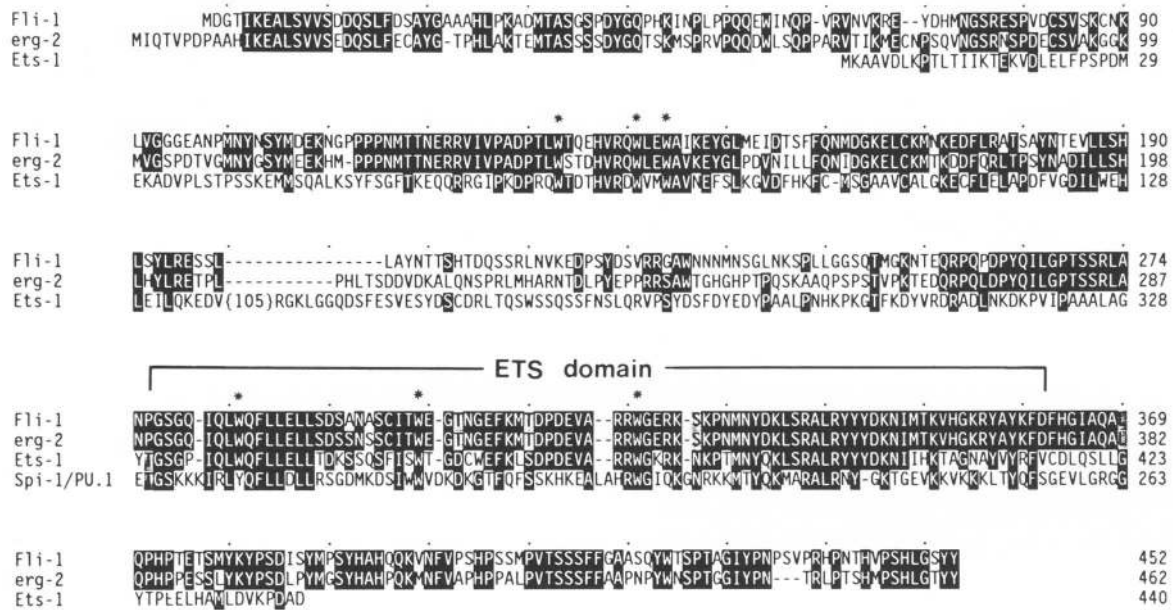


Figure 4. Comparative analysis of the Fli-1 coding sequence with the protein products of the Erg2, Ets-1, and Spi-1 genes. Amino acids shared by all four Ets proteins are marked in solid black. The amino acids shared by at least two of the four Ets proteins are either marked in solid black or are shaded. Gaps are indicated by hyphens. The [105] symbol on the Ets-1 coding sequence indicates an additional 105 amino acids with no similarity to Fli-1 and Erg2. The ~85-amino-acid ETS domain in the carboxyl terminus of Fli-1, Erg2, Ets-1, and Spi-1 is marked (Karim et al. 1990). Conserved tryptophan residues are indicated by asterisks (*).

mosome 9 (Ben-David et al. 1990a). To define the physical linkage between *Fli-1* and *ets-1*, we analyzed genomic fragments using pulsed field gel electrophoresis (PFGE). DNA from the erythroleukemia cell line DP27-17 that does not have a rearrangement of either the *Fli-1* or *ets-1* genes was digested with *Sall*, *SfiI*, *ClaI*, *NotI*, and *SacII* and analyzed by Southern blotting using the *c-ets-1* and B2 probes. The sizes of the DNA fragments that hybridized with the *ets-1* and B2 probes were compared to identify fragments common to both probes. As shown in Figure 5 and indicated by arrows, DNA fragments of identical sizes hybridized with both the B2 and *ets-1* probes when DNA was digested with *Sall*, *SfiI*, or *NotI* (indicated by arrows in lanes 1, 2, and 4, respectively).

However, digestion with *SacII* gave rise to a large fragment (>1000 kb) with the *ets-1* probe, whereas only a 200-kb *SacII* fragment was detected with the B2 probe (Fig. 5, lane 5; and bands are marked by asterisks). Similarly, digestion with *ClaI* gave rise to two hybridizing bands with both the B2 and *ets-1* probes, but these bands were of different sizes (Fig. 5, lane 3). Thus, the PFGE analysis suggests that *Fli-1* and *ets-1* are located on DNA fragments of the same size after digestion with *Sall*, *SfiI*, or *NotI*. The *NotI* digest was uninformative, as this fragment was >1000 kb and migrated in the compression zone. However, both *Fli-1* and *ets-1* are located on common 350-kb *Sall* and 240-kb *SfiI* bands, which are well separated by PFGE. Therefore, the common 240-kb *SfiI* fragment defines the upper limit for the distance between the *Fli-1* and *c-ets-1* genes.

Table 1. Comparative analysis between the protein sequences of Fli-1, Erg2, Ets-1, and Spi-1/PU.1

	Entire protein (%)		ETS domain (%)	
	identity	similarity	identity	similarity
Fli-1	100	100	100	100
Erg-2	67	76	98	98
Ets-1	30	41	68	80
Spi-1/PU.1	23	33	38	51

Percent identity scores were calculated by dividing the number of identical amino acids by the total number of aligned amino acids. Percent similarity scores were calculated in the same way except that similar amino acids were considered similar: G = A = L = I = V = M; S = T; D = E; H = K = R, Y = F = W.

Fli-1 expression in adult mouse tissues

As examined by Northern blot (Fig. 6), the *Fli-1* 4-kb mRNA is highly expressed in adult tissues, including thymus, heart, muscle, and spleen. Low levels of *Fli-1* expression were also detected in total RNA prepared from brain, kidney, and testes. In addition, small amounts of *Fli-1* RNA were observed in liver (data not shown). Although there were some differences in the amount of RNA loaded on the lanes in the blot shown in Figure 6, this Northern blot analysis clearly shows that *Fli-1* is expressed in both hematopoietic and nonhematopoietic tissues. The *c-ets-1* proto-oncogene has been shown previously to be expressed in adult mice

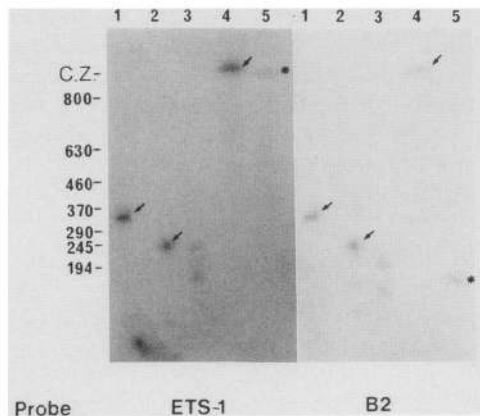


Figure 5. PFGE of the *Fli-1* and *ets-1* genes. High-molecular-weight DNA from the erythroleukemia cell lines DP27-17 was embedded in agarose and digested with the restriction enzymes *SalI* (lane 1), *SfiI* (lane 2), *ClaI* (lane 3), *NotI* (lane 4), and *SacII* (lane 5), run on a PFGE apparatus. DNA was transferred to nitrocellulose paper. The filter was first hybridized with an *ets-1* genomic probe and exposed to X-ray film. The probe was then stripped off and the same filter was rehybridized with an *Fli-1* probe (probe B2, Fig. 1). The arrows indicate the identical bands. The asterisks (*) show the two different bands generated with *SacII* digestion after hybridizing the same filter with either the *Fli-1* or *ets-1* probes. (C.Z.) Compression zone.

only in hematopoietic tissues, particularly in thymus (Bhat et al. 1987). Thus, despite the close structural homology and physical linkage between *Fli-1* and *c-ets-1*, these two genes appear to be under separate regulatory control.

Discussion

Insertional activation of cellular genes by replication-competent retroviruses is a common pathway to malignant transformation and tumor progression, and thus provides a unique strategy to identify and clone new transforming genes. The results presented in this study demonstrate that the induction of erythroleukemia by F-MuLV frequently involves the activation of a novel

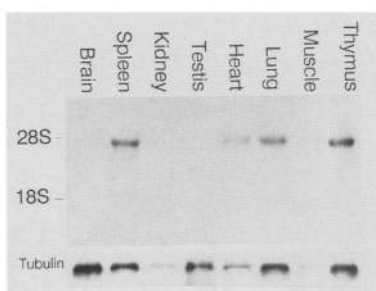


Figure 6. Expression of *Fli-1* in mouse adult tissues. Total RNA (20 μ g) from adult tissues was electrophoresed, blotted to nitrocellulose, and hybridized with 32 P-randomly primed BB4 cDNA. Tubulin is used to indicate the amount of RNA loaded on the gel.

member of the *ets* family of transcription factors as a result of the integration of F-MuLV upstream of the *Fli-1* locus. DNA and RNA analyses of *Fli-1* and the adjacent F-MuLV provirus suggest that F-MuLV integration activates *Fli-1* expression as the result of the strong enhancer activity of the Friend LTR in erythroid cells.

Fli-1 is a member of the ets family

A search of the translated GenBank and EMBL DNA sequence data bases for protein sequence similarity with the *Fli-1* gene product revealed an overall high similarity with members of the *ets* gene family, particularly with the human *erg2* gene. The *ets* gene family includes human *ets-1* (Watson et al. 1988), murine *ets-1* (Gunther et al. 1990), murine *ets-2* (Watson et al. 1988), human *erg* (Reddy et al. 1987), human *elk-1* and *elk-2* (Rao et al. 1989), *Drosophila* E74 (Burtis et al. 1990), *Drosophila ets-2* (Pribyl et al. 1988), and murine *PU.1* (Klemsz et al. 1990). The *Fli-1* protein is most similar to other members of the *ets* family in a region of ~85 amino acids close to the carboxyl terminus, designated the ETS domain (Karim et al. 1990). This domain appears to mediate the sequence-specific DNA binding of the Ets proteins to a purine-rich core DNA sequence. In addition, there is a region near the amino terminus that is conserved in the Ets-1 and Ets-2, Erg, v-Ets, and *Fli-1* proteins.

The *ets* family of proto-oncogenes was first identified as the cellular homologs of *v-ets*, one of the viral transforming genes of the avian retrovirus E26 (Leprince et al. 1983; Nunn et al. 1983). Both the *v-ets*-encoded protein and its cellular homologs (*c-Ets-1* and *c-Ets-2*) localize to the nucleus and have DNA-binding activity (Boulukos et al. 1988; Fujiwara et al. 1988a; Pognonec et al. 1989). The *c-ets-1* and *c-ets-2* genes are transiently activated and rapidly phosphorylated in response to mitogenic stimuli (Fujiwara et al. 1988b; Pognonec et al. 1988). These observations suggest that the *ets* gene family has regulatory roles, a view consistent with the oncogenic potential of *v-ets*. The products of the *c-ets-1* and *c-ets-2* proto-oncogenes can also *trans*-activate promoter/enhancer sequences contained within the HTLV-1 and Moloney murine sarcoma virus LTRs (Bosselut et al. 1990; Gunther et al. 1990). Furthermore, Ets proteins interact or cooperate with c-Fos and c-Jun to activate transcription (Waslyk et al. 1990). Taken together, these observations suggest that the mammalian *ets* family, including *Fli-1*, constitutes a novel and large group of transcriptional activators. Thus, the insertional activation of *Fli-1* may directly enhance the transcription of other genes involved in malignant transformation of erythroid cells during the progression of Friend leukemia.

The *ets*-binding consensus sequence that is present in the Moloney sarcoma virus LTR is also found in the promoter regions of the SFFV LTR (Clark and Mak 1983) and the F-MuLV LTR (Holland et al. 1987). Both SFFV and F-MuLV integrate upstream of the *Spi-1/PU.1* and *Fli-1* genes in Friend erythroleukemia cell lines, respectively. Thus, activation of *Fli-1* or *PU.1* may create a

positive feedback loop that enhances the expression of proviruses by binding to their LTR promoter sequences. Activation of the SFFV genome would also lead to increased synthesis of the SFFV-encoded gp55 glycoprotein, which has been shown to bind to the erythropoietin receptor and thereby eliminate dependency of erythroid cells for exogenous hormone (Li et al. 1990). In addition, an increase in the production of SFFV or F-MuLV could enhance the probability of insertional activation or inactivation of other genes involved in erythroleukemia induction. One example could be the p53 tumor suppressor gene that is inactivated in a significant number of erythroleukemia cell lines as the result of Friend virus integration (Ben-David et al. 1990b).

Amplification and dispersion of ets gene family members

The deduced amino acid sequence of the *Fli-1* protein is most similar to that of the human Erg2 protein and less so with Ets-1 and Spi-1/PU.1 (see Table 1). Overall, there is 67% identity between the mouse *Fli-1* and the human Erg2 protein, whereas within the 84-amino-acid ETS domain there is 98% sequence identity between these two proteins. Similarly, the Ets-1 and Ets-2 proteins share significant homology with each other and less so with Erg2 and *Fli-1*. The Spi-1/PU.1 protein appears to be an even more distant member of the Ets family.

Genetic and physical mapping has established that *Fli-1* and *c-ets-1* are very closely linked in both the mouse and human genomes on chromosome 9 and 11, respectively. Similarly, *c-ets-2* and *erg2* are closely linked to each other on human chromosome 21 band q22.3 (Watson et al. 1985; Rao et al. 1987); and in the mouse, *c-ets-2* maps to the syntenic region on mouse chromosome 16 (Cheng et al. 1988).

The chromosomal mapping and DNA sequence data discussed above suggest a model for the origin and dispersion of the various *ets* family members in which a primordial *ets* gene became duplicated and much later the pair of genes dispersed to other chromosomes. This model explains both the close sequence similarity between the unlinked *Fli-1/erg2* and *c-ets-1/c-ets-2* genes, as well as the more limited degree of similarity between the closely linked *ets* genes [*c-ets-1/Fli-1* and *c-ets-2/erg2*].

In addition to the considerable sequence divergence between *Fli-1* and *c-ets-1*, these genes display different patterns of expression in adult mouse tissues. These data suggest that despite their close physical linkage in mammalian genomes, *Fli-1* and *c-ets-1* are under distinct regulatory controls, reflecting perhaps sequence divergence extending into the transcriptional control regions.

Both *Fli-1* and *c-ets-1* are located in a region of the mouse genome that is syntenic with human chromosome 11q23. This region of the genome is a frequent site of breakpoints in a wide variety of human leukemias and lymphomas (Yunis and Brunning 1986). The 11q23 translocation can involve a number of other possible chromosomes, although the most common is chromosome 4q21

(Yunis et al. 1989). These rearrangements do not appear to be within the *c-ets-1* gene itself. The close physical linkage between *Fli-1* and *c-ets-1*, and the association of the *ets* family with experimental leukemias, raises the possibility that *Fli-1* may be involved in leukemic transformation in patients with the t(4;11) translocation. Experiments are currently in progress to test this possibility.

Multistage Friend erythroleukemia and sites for retrovirus integration

The erythroleukemias induced by the various strains of Friend virus are the culmination of a multistep process that has served as a model for our understanding of the molecular events that underlie tumor initiation and progression (Ben-David and Bernstein 1989). At least two distinct stages of Friend leukemia have been recognized. The first stage is characterized by the polyclonal expansion of infected but preleukemic erythroid progenitor cells. This is followed by a second stage characterized by the clonal emergence of malignant, immortalized cells (Mager et al. 1981; Wendling et al. 1981). The polyclonal proliferation that characterizes the early stages of the disease induced by the defective Friend SFFV appears to result from the binding of the SFFV-encoded membrane glycoprotein gp55 to the erythropoietin receptor (Li et al. 1990).

Studies on the leukemic clones that emerge in the late stages of the disease induced by either the Friend virus complexes (FV-P or FV-A) or by F-MuLV alone have shown that inactivation of the p53 gene is a common, and perhaps obligate, event. These inactivation events include deletions (Mowat et al. 1985; Rovinski et al. 1987; Ben-David et al. 1988; Munroe et al. 1988), retroviral insertions (Ben-David et al. 1988; Hicks and Mowat 1988; Ben-David et al. 1990b), and point mutations (Munroe et al. 1990), frequently accompanied by a reduction to homozygosity. These observations provided evidence that p53 might be a tumor suppressor gene, a conclusion substantiated by the subsequent analysis of the p53 gene in *in vitro* immortalization/transformation assays (Rovinski and Benchimol 1988; Eliyahu et al. 1989; Hinds et al. 1990), and alteration of the p53 gene in human tumors (Lane and Benchimol 1990; Levine 1990).

In addition to p53 inactivation, the cellular *Spi-1/PU.1* and *Fli-1* genes are almost always activated as the result of viral integration events. Although *Fli-1* and *Spi-1* are distinct genes, they are both members of the *ets* family of DNA-binding proteins. We have shown previously that *Fli-1* is only rearranged in erythroleukemias, not in myeloid or lymphoid tumors induced by F-MuLV (Ben-David et al. 1990a). Thus, *Fli-1* and *Spi-1* are both *ets* family members that, to date, only appear to be activated in erythroid neoplasms induced by different murine retroviruses. Given the biochemical similarities between *Fli-1* and *Spi-1/PU.1*, their specificity for erythroleukemias, and their similar mechanism of activation by retroviral integration, it is surprising that there is strict

specificity of oncogene activation; that is, *Fli-1* is only activated in erythroleukemias induced by F-MuLV, whereas *Spi-1* is only activated in leukemias induced by the Friend virus complexes. In 22 erythroleukemic clones, we have never observed *Fli-1* activation by FV-P or FV-A or *Spi-1* activation by F-MuLV (data not shown). There are two possible explanations for these observations. First, although *Spi-1* and *Fli-1* are both *ets* family members, they are clearly distinct, with limited identity in the highly conserved ETS domain (Table 1). Thus, *Fli-1* and *Spi-1* may *trans*-activate different genes because they recognize different DNA sequence elements and/or interact with distinct components of the transcriptional apparatus. However, the leukemic clones induced by infection with different strains of Friend virus are similar in their leukemic properties and in their erythroid characteristics, suggesting some overlap in the sets of genes *trans*-activated by *Fli-1* and *Spi-1*.

A second explanation for the strict specificity of *Fli-1* and *Spi-1* activation may be related to differences in their target cells for erythroid transformation. The FV-P and FV-A complexes can induce erythroleukemias in either newborn or adult animals, whereas F-MuLV only induces erythroleukemias in newborn mice (Silver and Kozak 1986). The susceptibility of adult mice to FV-P or FV-A may be due to the expression of the gp55 glycoprotein resulting, as discussed above, in the polyclonal proliferation of cells that might otherwise not be susceptible to infection. Thus, it is possible that the Friend virus complexes and F-MuLV have distinct target cells for transformation. If the chromatin structure around *Fli-1* and *Spi-1* undergoes changes during hematopoiesis, it is possible that the susceptibility of these genes to retroviral integration events also may change during cellular differentiation. Analysis of integration sites used after infection of turkey embryo fibroblasts by an avian retrovirus has suggested that there are a limited number of sites (~1000) available for integration events (Shih et al. 1988). The basis for this selectivity is unknown, but if the strict specificity for *Fli-1* and *Spi-1* integration events is related to stage of erythroid differentiation, the spectrum of sites available for retrovirus integration may vary with developmental stage. Thus, the total number of such sites within a species may be larger than estimated from examining a single cell type.

It should be possible to test these models by generating transgenic animals that either carry additional copies of *Fli-1* or *Spi-1*, or have sustained loss-of-function mutations in these genes by gene targeting. Such mutant mice should also be useful in determining whether *ets* genes are involved in the differentiation of normal hematopoietic stem cells, a possibility raised by the findings that three members of the *ets* family (*v-ets*, *Fli-1*, and *Spi-1*) are all involved in erythroleukemia induction in chickens and mice, respectively.

Materials and methods

Cells

The erythroleukemia cell lines DP25-15, DP27-27, DP28-9, and

DA24-33 were derived from methylcellulose colonies of spleen cells of DBA/2 adult mice that had been injected with either the polycythemia or anemia-inducing strains of Friend virus complex (FV-P or FV-A), as described previously (Ben-David et al. 1990b). The CB3 and CB7 cell lines were derived from the spleens of BALB/c mice injected at birth with F-MuLV helper virus (Shibuya and Mak 1983). The TP3 and TP1 cell lines were derived from the spleen of a NIH/Swiss mouse infected at birth with F-MuLV (Oliff et al. 1984). Cells were maintained in a minimal essential medium (α MEM) supplemented with 10% fetal calf serum.

Construction of cDNA library and isolation of cDNA clones

A λ gt11 cDNA expression library was constructed from 5 μ g of poly(A)⁺ RNA isolated from the erythroleukemia cell line CB7, using a Pharmacia cDNA synthesis kit as described previously (Ben-David et al. 1991). The library (10⁶ phages) was amplified once and stored at 4°C. To isolate cDNA clones, 5 \times 10⁵ phages were screened with the genomic probe B4; after three rounds of plaque purification, four colonies were recovered from the cDNA library.

Subcloning and nucleotide sequence determination

DNA was prepared from plaque-purified phage according to standard procedures using lambdaDASorb phage absorbent (Promega) and subcloned into pGEM-7Zf (+) or pGEM-7Zf (-). The plasmid DNA was isolated using cesium chloride gradients, as described previously (Chirgwin et al. 1979). For complete sequence determination, nested deletions spanning the cDNA insert of the desired clone were generated using the method of Henikoff (Henikoff 1987). These constructs were rescued as ssDNA after superinfection of bacteria with the coliphage M13K07 and sequenced by the dideoxynucleotide chain termination method using Sequenase enzyme, reagents, and protocols supplied by U.S. Biochemical. Sequence was obtained from both strands.

Nucleic acid probes

The B4 probe was a 1.7-kb *Bam*HI-*Eco*RI genomic fragment located within the site of provirus integration (Fig. 1) isolated from a λ phage genomic clone that contained the nonrearranged *Fli-1* locus (Ben-David et al. 1990a). The B4 LTR was a 1.7-kb *Bam*HI-*Kpn*I fragment that was isolated from a λ c3 phage clone (Ben-David et al. 1990a) and contained the sequences corresponding to the 5' F-MuLV LTR and sequences immediately adjacent to the provirus integration site (Fig. 1). The BB4 cDNA was a 1.7-kb *Eco*RI fragment subcloned from a λ gt11 phage clone that was isolated using the B4 probe (Fig. 3A). The B2 probe was the 1.5-kb *Eco*RI genomic fragment of the *Fli-1* locus (Fig. 1) and the *ets-1* probe was a genomic fragment of the mouse *c-ets-1* oncogene (kindly provided by R.H. Reeves, Frederick Cancer Research Center, Frederick, MD). The DNA probes were freed from plasmid sequences, gel-purified, and labeled by random priming (Feinberg and Vogelstein 1983).

RNA extraction and Northern blotting

Total cellular RNA from erythroleukemia cell lines was isolated essentially as described previously (Auffray and Rougeon 1980). In brief, 10⁸ cells were lysed in 200 μ l of 0.1 M NaCl, 0.1 M Tris (pH 7.9), and 0.65% NP-40. Cell extracts were centrifuged for 2 min in a microcentrifuge (Eppendorf). An equal volume of buffer containing 7 M urea, 0.35 M NaCl, 0.01 M Tris (pH

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7.4), 0.01 M EDTA, and 1% SDS was added to the lysate supernatant, and three extractions were performed with water-saturated phenol and chloroform (1 : 1) and one extraction was performed with chloroform. The final aqueous phase was precipitated with ethanol and stored in 100% ethanol at -70°C . Polyadenylated RNAs were selected by affinity chromatography on oligo(dT)-cellulose (Pharmacia) as described by the supplier.

Total RNA from mouse tissues was isolated according to the lithium chloride precipitation method described previously (Auffray and Rougeon 1980). In brief, tissues were removed from a number of adult BALB/c mice, homogenized in 10 ml of lithium chloride solution (3 LiCl, 6 M urea), and incubated for 20–24 hr at 4°C . The RNAs were precipitated by centrifugation for 20 min at 17,000g. The pellets were resuspended in 6 ml of lithium chloride/urea solution and centrifuged for 20 min at 17,000g. The RNA pellets were dried and sequentially extracted at room temperature twice with 2 ml of phenol/chloroform (1 : 1) and once with 2 ml of chloroform. The RNA was precipitated with ethanol and resuspended in TE.

Total RNA (20 μg) was dissolved in 2.2 M formaldehyde and 50% formamide denatured at 65°C for 5 min, and electrophoresed in a 1% agarose gel containing 0.66 M formaldehyde. After transfer to nitrocellulose, the filter was hybridized with 2×10^6 cpm of random primed probe per milliliter of hybridization mixture that contained 50% formamide, 10% dextran sulfate, $1.5 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M}$ sodium citrate at pH 7), $5 \times$ Denhardt's solution ($1 \times$ Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), and denatured salmon sperm DNA (100 mg/ml) at 42°C (Southern 1975; Sambrook et al. 1989). The filter was washed with $0.1 \times \text{SSC}$ and 0.1% SDS at 65°C .

PFGE

High-molecular-weight DNA for PFGE analysis was prepared from the erythroleukemia cell line DP27-17 as described previously (Sambrook et al. 1989). The DNA from $\sim 2 \times 10^5$ cells was embedded in agarose blocks and digested with the rare restriction enzyme cutters *SalI*, *SfiI*, *NotI*, *Clal*, and *SacII* by incubating for 16–20 hr at 37°C except for plugs digested with *SfiI*, which were incubated at 50°C . The digested DNA samples were run on gels (1% agarose in 45 M Tris-borate/1 M EDTA) for 24 hr with a switch time of 30 sec initial, 80 sec final, ramped over 24 hr and a field strength of 90 mA/170 V (Southern et al. 1987). Intact *Saccharomyces cerevisiae* YNN295 yeast chromosomal DNA and λ phage DNA were used as molecular weight markers. DNA was transferred to Gene Screen Plus membrane (Biotechnology Systems, Dupont), UV cross-linked, and hybridized to ^{32}P -labeled random primed probes as described above for RNA blotting.

Computer analysis

Sequences were compared with the GenBank (Bilofsky et al. 1986) and EMBL (Hamm and Cameron 1986) data bases using the Genetics Computer Group (GCC) programs (Devereux et al. 1984), and the automatic FASTA service provided by GenBank via electronic mail. The GCC programs were used for initial alignment while final alignment were done using the Maligned program (S. Clark, unpubl.).

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Note added in proof

Sequence data described in this paper have been submitted to EMBL/GenBank Data Libraries under accession number X59421 mouse, FLI-1 CDNA.

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