### Abstract

**Fli-1**, an *ets* related gene, was found to be rearranged in 75% of erythroleukemias induced by Friend murine leukemia virus. We have shown previously that the *Fli-1* gene codes for a sequence specific transcriptional activator which contains two autonomous transcriptional activation domains, one at the amino terminal region and the other at the carboxy terminal region. Recently human *Fli-1* gene was shown to be involved in Ewing's sarcoma and related subtypes of primitive neuroectodermal tumors which share t(11;22) (q24;q12) chromosome translocation. In these tumors the carboxy terminal region of *Fli-1* was found to be fused with the amino terminal region of a putative RNA binding protein, EWS. Because part of the amino terminal transcriptional activation domain of *Fli-1* was replaced with the amino terminal domain of the EWS (NTD-EWS) which shares homology with RNA polymerase II, it was speculated that NTD-EWS may interfere with RNA pol II function. Alternatively, NTD-EWS could also contribute to the transcriptional activation function of EWS/Fli-1 chimeric protein by providing either a modulatory/regulatory domain or a novel transcriptional activation domain. Here we show that EWS/Fli-1 chimeric protein functions as a transcriptional activator. Deletion analysis reveals that the EWS domain functions as a modulatory/regulatory domain for the transcriptional activation properties of the carboxy terminal transcriptional activation domain of EWS/Fli-1. We therefore propose that replacement of the amino terminal transcriptional activation domain of the *Fli-1* protein with the regulatory domain of NTD-EWS results in the activation of the carboxy terminal transcriptional activation domain of *Fli-1* which may be the molecular mechanism involved in these human tumors.

### Introduction

Analysis of erythroleukemic clones induced by F-MuLV reveals the presence of preferred integration site for F-MuLV, defined as *Fli-1* (Friend leukemia integration 1) (1). The *Fli-1* locus has been shown to have rearranged in 75% of erythroleukemias induced in either BALB/c or NIH/Swiss mice given injections of F-MuLV at birth. However, no rearrangements of *Fli-1* locus were seen in myeloid and lymphoid tumors induced by F-MuLV. Interestingly analysis of mouse erythroleukemias induced by spleen focus-forming virus showed rearrangement of cellular gene *Spi-1/PU.1* in 95% of these leukemias but showed no rearrangements of *Fli-1* gene. Similarly erythroleukemias induced by F-MuLV showed no rearrangements of *Spi-1/PU.1*, thus indicating a strict specificity for these viruses to integrate and activate the two genes, namely, *Fli-1* and *Spi-1/PU.1* in these erythroleukemias (5). In addition to the activation of *Fli-1* and *Spi-1* genes, inactivation of p53 was seen in these erythroleukemias suggesting that these steps are important for leukemic transformation (5). Characterization of both *Fli-1* (6–8) and *Spi-1* (3, 4) genes shows that they both belong to the *ets* oncogene family. The other members of *ets* oncogene superfamily consist of c-ets-1 (a cellular homologue of v-ets gene) (9–12), ets-2 (11, 13), erg (14, 15), elk-1, and elk-2 (16); E74 (17); GA binding protein α (18), Sap-1 and Sap-2 (19), elf-1 (20), PE3A (21), yan/pok (22, 23), ER 81 and ER 71 (24), Spi-1b (25) and pnt (26). *Ets* proteins were shown to be sequence specific transcriptional activators (3, 27–33). Characterization of the *Fli-1* gene by sequence analysis (6, 7) reveals that this gene is highly related (~80%) to the previously described *ets* related gene, *erg* (14, 15). Both these genes share two domains of *ets* homology at the 5' and 3' regions. These domains are referred as to 5'-*ets* domain and 3'-*ets* domain (7), respectively. The 5'-*ets* domain is shared by seven *ets* family members (Fli-1, erg, c-ets-1, ets-2, GA binding protein α, yan/pok, and pnt). Functional analysis of some of these members reveals that this 5'-*ets* domain contributes to the transcriptional activation function in the cases of *erg* (33), Fli-1 (34), ets-2, and other *ets* proteins. The 3'-*ets* domain is shared by all the *ets* family members and has been shown to contribute to the DNA binding properties of *ets* proteins. As a result of alternative splicing, *Fli-1* codes for at least two proteins, namely, Fli-1a and Fli-1b (7). Some of the *ets* proteins bind to DNA both autonomously and through association with other cellular factors (35–39). In addition, *ets* proteins have been shown to cooperate with other nuclear oncogenes in transcriptional activation (27, 40) and some act as transcriptional activators and/or repressors (22, 41).

We have shown previously that *Fli-1* proteins bind to DNA in a sequence specific manner and also function as a transcriptional activator (34). Deletion analysis of *Fli-1* revealed the presence of two autonomous transcriptional activation domains, one at the amino terminal region (ATA domain) and the other at the carboxy terminal region (CTA domain) (34). Similar transcriptional activation domains have also been observed in the case of *erg* proteins (33). It has been shown recently that human *Fli-1* gene is rearranged in the majority of cases of Ewing's sarcoma, neuroectodermal tumors that share t(11;22)(q24;q12) chromosome translocations (42), suggesting the involvement of human *Fli-1* in these solid tumors. Rearrangement of *Fli-1* gene (EWS/Fli-1) results in the fusion of the carboxy terminal region of *Fli-1* with amino terminal region of a putative RNA binding protein, EWS (42). Because the EWS domain replaces part of the *Fli-1* amino terminal transcriptional activation domain (34), it could be predicted that EWS/Fli-1 may show aberrant/alternated transcriptional activation property (34, 42). In order to understand functional differences in transcriptional activation between normal *Fli-1* and EWS/Fli-1, we have molecularly cloned EWS/Fli-1 cDNA and compared its transcriptional activation properties with those of *Fli-1* and found that EWS/Fli-1 functions as a transcriptional activator. Deletion analysis of EWS/Fli-1 revealed that EWS domain functions as a regulatory/modulatory domain. Implications of these findings in human diseases are discussed.

---

1. H. Siddique et al., unpublished results.
Materials and Methods

Molecular Cloning of EWS/Fli-1. Reverse transcription-PCR analysis was carried out by reverse transcription of the total RNA from Ewing's sarcoma cell lines using the Boehringer Mannheim kit, and the resulting cDNA was subjected to polymerase chain reaction using 5' and 3' primers. Restriction mapping, PCR analysis, and nucleotide sequence analysis were carried out as previously described (7).

In Vitro Transcription and Translation. Expression plasmids (pSG5) containing full length Fli-1 and EWS/Fli-1 (type 1) were linearized with XhoI restriction enzyme and transcribed in vitro with T7 RNA polymerase (Promega), according to the manufacturer's protocol. The resulting capped RNAs were purified by extraction with phenol and chloroform and translated in vitro with rabbit reticulocyte in the presence of [35S]methionine according to the manufacturer's protocol (Promega).

Transcriptional Activation Studies of EWS/Fli-1 and Fli-1 Proteins. Full length EWS/Fli-1 and Fli-1 cDNAs were cloned into pSG5 vector (Stratagene) for the expression of full length Fli-1 and EWS/Fli-1 proteins in cultured NIH 3T3 cells. Constructs containing a series of deletion mutants of EWS/Fli-1 (ΔE1-E4, Δ291/Fli-1) were also cloned into pSG5 vector. Appropriate initiation codons were introduced where needed. These expression plasmids were co-transfected with a reporter CAT plasmid carrying three copies of Fli-1 target sequences upstream of herpes simplex virus thymidine kinase promoter (E74-TK-CAT) (31), and a reference plasmid pCh110 carrying β-galactosidase gene under the SV40 early promoter. This reference plasmid was used for normalization of transfection efficiency. A mixture of 5 µg of reporter plasmid DNA, 5 µg of reference plasmid pCh110, and 1 µg of expression plasmid were transfected into NIH 3T3 cells as described (43). CAT and β-galactosidase activities were carried out as described (43). Transfection experiments were repeated at least five to seven times.

Results and Discussion

Isolation of EWS/Fli-1 cDNA Clone from Ewing's Sarcoma Cells. In order to understand the mechanism of activation of human Fli-1 gene in human tumors such as Ewing's sarcoma and neuroectodermal tumors which share t(11;22) chromosome translocation, we isolated RNA from a Ewing sarcoma cell line (Te-135D kindly provided by Dr. Triche). We isolated the EWS/Fli-1 cDNA by reverse transcription-PCR and subcloned the cDNA into SV40 expression vector (pSG5). The cDNA clone was subjected to nucleotide sequence analysis and was found to be identical with that of EWS/Fli-1 (type 1) reported previously (42). In order to verify the deduced open reading frame of EWS/Fli-1 and to compare it with that of normal Fli-1, both aberrant (EWS/Fli-1) and normal Fli-1 cDNA clones (in pSG5) (Fig. 1) were linearized, transcribed, and translated in vitro and expressed proteins were characterized on sodium dodecyl sulfate-gel electrophoresis (Fig. 1). EWS/Fli-1 and Fli-1 were expressed as Mr, 62,000 and Mr, 54,000 polypeptides close to the predicted size. The top two bands seen in the case of Fli-1 (Fig. 1, Lane 2) may result from internal initiation at the downstream methionine residue. Small differences observed between the deduced and actual sizes of these normal and chimeric Fli-1 proteins may reflect the high proline content found in these proteins. Comparison of the sequence of EWS/Fli-1 with Fli-1 revealed that the amino terminal region of Fli-1 was replaced with the proline, serine, threonine, and glutamine rich EWS coding region.

Comparison of Transcriptional Activation Properties between Normal and Aberrant Fli-1 Proteins. We have previously shown that human Fli-1 binds to DNA in a sequence specific manner and also acts as a sequence specific transcriptional activator (34). Unlike c-ets-1, ets-2, and elk-1, Fli-1 and erg proteins recognize limited ets target sequences (31, 34) suggesting that Fli-1 and erg proteins fall into a separate class of ets proteins. Both Fli-1 and erg proteins have been shown to bind to E74 target sequence and activate the transcription of the reporter plasmid (E74-TK-CAT) carrying three copies of E74 target sequence (31, 34). Because the 3'-coding region retained in the chimeric EWS/Fli-1 includes the 3'-ets domain (Fig. 2) which contributes to the DNA binding activity of the majority of ets proteins, it can be predicted that the DNA binding properties of EWS/Fli-1 and Fli-1 may not differ significantly. We have previously shown that the 5'-ets domain and the downstream FLS domain function as an amino terminal transcriptional activation domain of Fli-1 (Fig. 2) (34). Because the 5'-ets domain was replaced with a novel coding region from a putative RNA binding protein in EWS/Fli-1, it is speculated that it may have altered transcriptional activation function either positively or negatively (42). Because the EWS domain of the fusion product EWS/Fli-1 has moderate homology with the carboxy terminal domain of the large subunit of eukaryotic RNA polymerase II, it has also been predicted that EWS domain may interfere with the normal function of the large subunit of eukaryotic RNA polymerase II in gene expression by distorting or interfering in the transcription initiation complex which results in suppression of transcription (42). Alternatively because of high glutamine and proline content in EWS domain, it has also been suggested that these sequences may act as new transcriptional activation domains in EWS/Fli-1 protein resulting in aberrant transcriptional activation (42, 34). It is also possible that the EWS domain may not contribute to any function and mere truncation of the amino terminal region of the Fli-1 protein may be sufficient to activate the carboxy terminal transcriptional activation domain of the Fli-1 gene (33, 34) in Ewing's sarcoma and other solid tumors. In order to determine which of the above possibilities is correct, we compared the transcriptional activation properties of Fli-1, EWS/Fli-1, and truncated Fli-1 (at the breakpoint, Δ291/Fli-1) proteins (Fig. 2). Expression of these proteins was obtained by cloning the full length Fli-1, EWS/Fli-1, and the truncated Fli-1 (Δ291/Fli-1) cDNAs into pSG5 vector.
EWS/FLI-1 CODES FOR A TRANSCRIPTIONAL ACTIVATOR

Expression plasmids pSG-Fli-1, pSG-EWS/Fli-1, pSG-t219/Fli-1, and pSG5 (empty vector) were cotransfected with a β-galactosidase expression plasmid pCH110 (reference plasmid) into NIH 3T3 cells. Cells were incubated for 60–64 h and the cell lysates were assayed for β-galactosidase and CAT activities. Transfections were repeated at least 5 times in duplicate. (B) The chromatogram represents a typical transfection. Lane 1, TK-CAT; Lane 2, TK-CAT + pSG-EWS/Fli-1; Lane 3, E74-TK-CAT + pSG5 vector; Lane 4, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 5, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 6, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 7, E74-TK-CAT + pSG5-EWS/Fli-1 (opposite orientation).

Fig. 2. (A) Schematic representation of constructs used for comparison of transcriptional activation properties of Fli-1 and EWS/Fli-1. t219/Fli-1 represents the construct where the amino terminal region of EWS is deleted from EWS/Fli-1. Fold activation of transcription (compared to vector control) for various proteins is shown. Values represent the mean ± SD of 5 independent experiments. NIH 3T3 cells were transfected with E74-TK-CAT (reporter plasmid), pCH110 (reference plasmid), along with each of the various expression plasmids as described in “Materials and Methods.” Cells were incubated for 64 h and cell lysates were assayed for β-galactosidase and CAT activities. Transfections were repeated at least 5 times in duplicate. (B) The chromatogram represents a typical transfection. Lane 1, TK-CAT; Lane 2, TK-CAT + pSG5 vector; Lane 3, E74-TK-CAT + pSG5 vector; Lane 4, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 5, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 6, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 7, E74-TK-CAT + pSG5-EWS/Fli-1 (opposite orientation).

Fig. 3. (A) Functional role of EWS subdomain in transcriptional activation function of EWS/Fli-1. Reporter plasmid and reference plasmid were cotransfected with various deletion mutants of EWS/Fli-1 into NIH 3T3 cells as described in the legend to Fig. 2. Transcriptional activation of EWS/Fli-1 protein is taken as 100% which corresponds to a 9.5-fold increase in CAT activity compared to vector control. Each bar represents the normalized values for the mean ± SD of 5 independent experiments. (B) The chromatogram represents a typical transfection. Lane 1, E74-TK-CAT + pSG5 vector; Lane 2, E74-TK-CAT + pSG5-EWS/Fli-1; Lane 3, E74-TK-CAT + pSG5-AE1 (amino acids 65–499); Lane 4, E74-TK-CAT + pSG5-AE2 (amino acids 109–499); Lane 5, E74-TK-CAT + pSG5-AE3 (amino acids 210–414); Lane 6, E74-TK-CAT + pSG5-AE4 (amino acids 210–499); Lane 7, E74-TK-CAT + pSG5-AE5 (amino acids 265–499); Lane 8, E74-TK-CAT + pSG5-AE6 (amino acids 210–414); Lane 9, E74-TK-CAT + pSG5-AE6 (amino acids 265–414).
Analysis of target genes of Fli-1 and EWS/Fli-1 therefore may provide a clue to the activation of Fli-1 in these human solid tumors. It is also possible that the EWS domain of EWS/Fli-1 may interact with certain factors (unlike Fli-1), and these EWS/Fli-1 associated proteins may modulate sequence specificity and affinity to target sequences as observed in the case of elk-1 and SRF (35, 37). Perhaps T-L-T/S-T-S structures of EWS may be responsible for protein-protein interactions. We have previously observed such T-S-T-S structure in the elk-1 domain that interacts with SRF (37). Analysis of EWS/Fli-1 associated proteins therefore may provide insight into the mechanism of activation of Fli-1 in these human solid tumors. Because EWS/Fli-1 showed consistently higher level of trans activation when compared to t219/Fli-1 (devoid of EWS domain), our results appear to support the results that EWS domain is needed for transformation of NIH 3T3 cells by EWS/Fli-1 (45). Recently, EWS has been found to be fused with ATF-1, a transcription factor controlled by cyclic AMP in malignant melanoma of soft parts involving t(12;22) chromosome translocation (46). TLS, a gene related to EWS, has been shown to be fused to CHOP, a member of C/EBP family transcriptional factors in human myxoid liposarcomas involving chromosomal translocation (12;16)-(q13:p11) (47). EWS and TLS may provide regulatory/modulatory domain to the transcriptional activation/repression function of ATF-1 and CHOP similar to that observed with EWS/Fli-1. It remains to be seen whether replacement of transcriptional activation domain of transcriptional factors by regulatory domains of other factors (EWS, etc.) is a common mechanism of activation in these cancers.

Acknowledgments

We thank Dr. T. Triche for Ewing’s sarcoma cell lines. We thank Dr. C. M. Croce for support and encouragement. We also thank other colleagues of Dr. Reddy and Dr. Rao’s laboratories for their cooperation.

References

    sequence-specific DNA binding of the Moloney murine sarcoma virus. Genes Dev., 4:
29. Bosselut, R., Duvall, J. F., Geggone, A., Bailly, M., Hemar, A., Brady, J., and
EWS/Fli-1 Chimeric Protein Is a Transcriptional Activator


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/24/5859

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.