MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7393–7398
0270-7306/93/127393-06$02.00/0

Vol. 13, No. 12

The Ewing's Sarcoma *EWS/FLI-1* Fusion Gene Encodes a More Potent Transcriptional Activator and Is a More Powerful Transforming Gene than *FLI-1*

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Received 12 July 1993/Returned for modification 18 August 1993/Accepted 8 September 1993

EWS/FLI-1 is a chimeric protein formed by a tumor-specific 11;22 translocation found in both Ewing's sarcoma and primitive neuroectodermal tumor of childhood. *EWS/FLI-1* has been shown to be a potent transforming gene, suggesting that it plays an important role in the genesis of these human tumors. We now demonstrate that EWS/FLI-1 has the characteristics of an aberrant transcription factor. Subcellular fractionation experiments localized the EWS/FLI-1 protein to the nucleus of primitive neuroectodermal tumor cells. EWS/FLI-1 specifically bound in vitro an *ets-2* consensus sequence similarly to normal FLI-1. When coupled to a GAL4 DNA-binding domain, the amino-terminal EWS/FLI-1 region was a much more potent transcriptional activator than the corresponding amino-terminal domain of FLI-1. Finally, EWS/FLI-1 efficiently transformed NIH 3T3 cells, but FLI-1 did not. These data suggest that EWS/FLI-1, functioning as a transcription factor, leads to a phenotype dramatically different from that of cells expressing *FLI-1*. EWS/FLI-1 could disrupt normal growth and differentiation either by more efficiently activating *FLI-1* target genes or by inappropriately modulating genes normally not responsive to *FLI-1*.

Aberrant expression and structural alteration of transcription factors are frequent, primary molecular mechanisms in oncogenesis (11). In lower mammals and avian species, these alterations are often mediated by retroviral insertion. In humans, deregulation or structural alteration of transcription factors is frequently the result of somatic genomic rearrangement.

The 11;22 chromosomal translocation found in Ewing's sarcoma and primitive neuroectodermal tumor of childhood (PNET) juxtaposes the 5′ sequences from a newly described gene, termed *EWS*, with the 3′ sequences from *FLI-1*, which encodes a member of the Ets transcription factor family (6). Like most Ets family members (for a review, see reference 27), the carboxyl domain of FLI-1 mediates sequence-specific DNA binding. The FLI-1 amino terminus contains a putative transcription activation domain that may interact on a protein-protein level with other transcription factors. As with other Ets proteins, it is probably the combination of protein-DNA and protein-protein binding specificities that determines which genes are transcriptionally modulated by FLI-1. As a result of the 11;22 rearrangement, the amino-terminal domain of FLI-1 is replaced by a portion of EWS containing a series of degenerate, glutamine-rich repeats. The carboxyl terminus of EWS has amino acid similarity to proteins involved in RNA synthesis and processing (6). However, the function in tumor cells of the EWS amino-terminal domain that is fused to FLI-1 is unknown.

We have recently demonstrated that the *EWS/FLI-1* chimera is a potent transforming gene in NIH 3T3 cells (14). We have also shown that both *EWS* and *FLI* sequences are necessary for the transforming activity of this chimeric oncogene. These data suggest that EWS/FLI-1 may function as an aberrant transcription factor contributing to transformation in Ewing's sarcoma and PNET cells. The work presented here supports this hypothesis by demonstrating that (i) EWS/FLI-1 localizes to the cell nucleus, (ii) EWS/FLI-1 is able to bind DNA in a sequence-specific manner, and (iii) the EWS region can serve as a transcription activation domain that is more potent than the corresponding region of normal FLI-1. We also show that the EWS/FLI-1 fusion, coupling the activation domain of EWS to the DNA-binding domain of FLI-1, transforms rodent fibroblast cells much more effectively than wild-type FLI-1 does.

MATERIALS AND METHODS

Subcellular localization. Subcellular localization of EWS/FLI-1 protein was performed by modification of previously published protocols (29), using TC-32, a PNET-derived cell line containing the t(11;22) rearrangement (31), and HeLa cells. Briefly, cells grown in Dulbecco's minimal essential medium plus 10% calf serum were metabolically labeled with [^{35}S]methionine, washed with phosphate-buffered saline (PBS), subjected to hypotonic lysis in 10 mM Tris (pH 8)–1 mM $MgCl_2$–0.1 mM phenylmethylsulfonyl fluoride, and sheared through a 26-gauge needle. Nuclear and cytoplasmic fractions were separated as pellet and supernatant by low-speed centrifugation (2,000 rpm, 700 × *g*). Nuclei were washed and then resuspended in hypotonic lysis buffer.

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Staining with both hematoxylin-eosin and vimentin demonstrated less than 10% whole cell contamination in the nuclear fraction. Triton X-100 and sodium dodecyl sulfate (SDS) were added to both nuclear and cytoplasmic fractions to final concentrations of 1 and 0.1%, respectively. Two-cycle immunoprecipitations were performed on both cytoplasmic and nuclear fractions, using rabbit antisera raised to an EWS/FLI-1 fusion polypeptide, as previously described (14). Precipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiographed.

Gel shift assay. In vitro-translated proteins were made by using a rabbit reticulocyte lysate as recommended by the manufacturer (Promega). The constructs used for these in vitro translations have been described elsewhere (14). Protein production was confirmed by SDS-PAGE and autoradiography of ^{35}S-labeled proteins. The gel shift probes (see Fig. 2 for sequence) were excised from pBluescript II KS+ (Stratagene) with *Xba*I and *Hin*dIII and gel purified. The probe was labeled by Klenow filling in the presence of [^{32}P]dATP. Cold competitors were isolated in a similar fashion and left unlabeled.

DNA binding reactions were carried out for 15 min at room temperature in a final volume of 20 ml containing 10,000 cpm of labeled probe in a final buffer concentration of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–75 mM NaCl–0.5 mM EDTA–1 mM dithiothreitol–5% glycerol, using 2 μg of poly(dI-dC) (Pharmacia). Two microliters of in vitro-translated protein was used, and a 100-fold excess of cold competitor was added in indicated lanes. Samples were loaded onto a prerun (140 V for 30 min) 6% polyacrylamide gel (acrylamide/bisacrylamide ratio of 30:1) prepared with 0.25× Tris-borate-EDTA–5% glycerol and subjected to electrophoresis at 140 V at room temperature. The gels were dried, and autoradiography was performed by using two sheets of Kodak XAR film at −70°C with an intensifying screen.

Transformation assay. The cDNA for human *FLI-1* was modified by the addition of a nine-amino-acid epitope from influenza virus hemagglutinin to the amino-terminal end via polymerase chain reaction amplification as previously described (14). The modified cDNA was blunted with Klenow and cloned into the blunted *Eco*RI site of the retroviral vector SRαMSV tk neo(ΔHindIII) (19). The *EWS/FLI-1* cDNA constructed with the same epitope as previously described (14) and was also cloned into the *Eco*RI site of SRαMSV tk neo-(ΔHindIII). Replication-deficient retroviral stocks were created by transiently transfecting COS cells with *EWS/FLI-1* or *FLI-1* constructs together with a psi-minus packaging plasmid (19) by either calcium phosphate precipitation or electroporation. Conditioned medium containing virus was harvested and used to infect NIH 3T3 cells. These cells were then selected in G418 for 1 week. Selected populations were plated in soft agar at either 5,000 or 50,000 cells per 6-cm-diameter plate and at either 10 or 20% fetal calf serum in Iscove's medium as previously described (14). Agar plates were photographed approximately 3 weeks after plating. The levels of EWS/FLI and FLI proteins in these selected cell lines were determined by using two cycles of immunoprecipitation with polyclonal antisera as previously described (14).

GAL4 constructs. Constructs fusing amino acids 1 to 147 of the yeast transcription factor GAL4 to domains of interest were constructed as follows.

(i) **GAL4/EWS.** The 900-bp *Pst*I-*Pvu*II fragment from EWS/FLI-1 including nearly all of the EWS domain and a portion of the FLI-1 domain but excluding nearly all of the consensus DNA binding sequence was selected for fusion to GAL4 (see Fig. 4A). It was initially subcloned into pBluescript II KS+ (Stratagene). From this intermediate, two fragments were gel purified: a 5′ 450-bp *Bam*HI-*Avr*II fragment and a 3′ 430-bp *Avr*II-*Asp* 718 fragment. The EWS fragment was fused to GAL4 in a trimolecular ligation of these two fragments to the GAL4 fusion vector pSG424 (32) cut with *Bam*HI and *Asp* 718. The structure of the construct was confirmed with analytic digests.

(ii) **GAL4/FLI-1.** The 800-bp 5′ *Msc*I-*Pvu*II fragment from FLI-1 (see Fig. 4A) was cloned into the *Sma*I site of pUC19 in the proper orientation. The integrity of the 5′ junction was confirmed by sequencing. From this intermediate, the insert was excised with *Bam*HI and *Asp* 718 and cloned into pSG424 cut with the same enzymes.

Reporter gene assays. Approximately 10^6 HeLa cells were transfected by overlaying them with a mixture of 50 μl of Lipofectin (Gibco-BRL) plus 20 μg of total DNA (5 μg of GAL4 fusion plasmid plus 5 μg of reporter plasmid G_NE4tkCAT [courtesy of Mike Carey] with 2 μg of cytomegalovirus luciferase [courtesy of Tina Rhodes] and 8 μg of pBluescript II KS+) in serum-free Dulbecco's minimal essential medium. The cells were fed after 5 h to bring the serum concentration to 10% and harvested 48 h later. Cells were harvested with trypsin and washed in 1× PBS. Fifteen percent of each transfection was set aside and assayed for luciferase activity by using a commercially available kit and protocol (Promega).

The remaining cells were resuspended in 0.25 M Tris (pH 7.8) and lysed by three consecutive freeze-thaw cycles. The amount of lysate used was standardized by reference to the luciferase values. Chloramphenicol acetyltransferase (CAT) activity was assayed by incubation for 1 h at 37°C with 0.5 ml of [^{14}C]chloramphenicol (CAT assay grade; NEN) and 10 μl of acetyl coenzyme A in 0.25 M Tris (3.5 mg of lithium salt per ml; Sigma) in a final reaction volume of 70 ml of 0.25 M Tris (pH 7.8). Control reactions using 1 U of purified CAT enzyme (Pharmacia) were performed under the same conditions. Reactions were extracted with ethyl acetate, dried, and fractionated by thin-layer chromatography, using a 95:5 mixture of chloroform-methanol. Autoradiography was performed overnight, and bands were cut out and quantitated in 5 ml of scintillation fluid (Beckman).

RESULTS

EWS/FLI-1 localizes to the nucleus. Although FLI-1 is a known DNA-binding protein, the biologic function of EWS is unknown. The EWS/FLI-1 fusion protein could therefore be involved in a variety of cellular functions. To begin to determine the activity of EWS/FLI-1, subcellular localization analyses were performed. TC-32 cells, a PNET cell line containing the 11;22 translocation (31), and HeLa cells, which do not contain the 11;22 translocation and thus do not express the *EWS/FLI-1* fusion gene, were both metabolically labeled with [^{35}S]methionine. Nuclei were separated from cytoplasm and membranes by hypotonic lysis and low-speed centrifugation. Two-cycle immunoprecipitation experiments were then performed on both nuclear and cytoplasmic/membrane fractions, using an antiserum raised to a polypeptide from the TC-32 EWS/FLI-1 junction point (14). This polypeptide fragment included EWS amino acid residues 244 to 264 (6) fused to residues 241 to 284 of FLI-1 (30).

A 68-kDa band corresponding to the EWS/FLI-1 fusion protein was consistently present in the nuclear fraction of

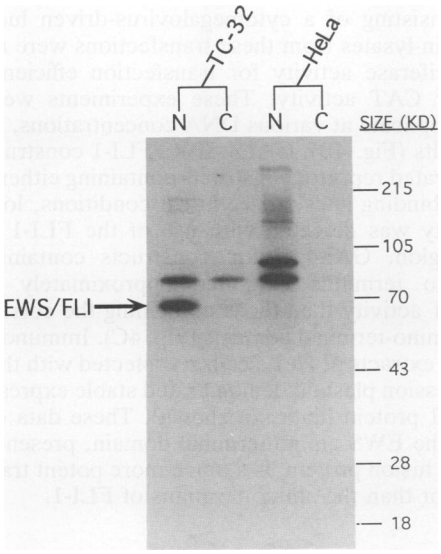

FIG. 1. Subcellular localization of EWS/FLI-1. Two-cycle immunoprecipitation of fractionated protein extracts with a polyclonal antiserum raised to an EWS/FLI-1 polypeptide fragment demonstrated that nearly all of the EWS/FLI-1 protein was present in the nuclear (N) fraction of TC-32 cells, a PNET cell line containing the 11;22 translocation. The additional 90- and 100 kDa bands present in TC-32 and also in t(11;22)-negative HeLa cells may represent unrearranged EWS proteins (see text). C, cytoplasmic fraction.

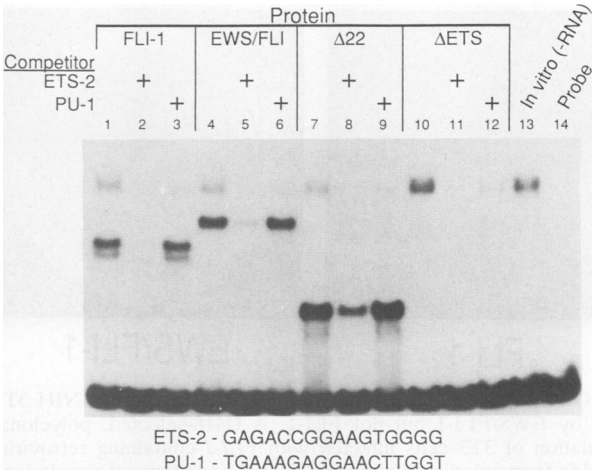

FIG. 2. Sequence-specific DNA binding by EWS/FLI-1 and FLI-1 proteins. In vitro-translated EWS/FLI-1 and FLI-1 proteins form stable complexes with ^{32}P-labeled *ets-2* oligonucleotide. Labeled DNA-protein complexes are attenuated by molar excess of unlabeled *ets-2* probe but not by PU-1/*spi-1* oligonucleotide. EWS/FLI-1 Δ22, a nontransforming mutant that lacks most of the EWS domain, also binds to the *ets-2* probe in a similar fashion. However, EWS/FLI-1 ΔETS, which lacks the FLI-1 DNA-binding domain, fails to bind labeled *ets-2* oligonucleotide. The background band visible in lanes 1, 4, 7, and 10 is probably due to endogenous Ets proteins present in the rabbit reticulocyte lysate since it is also seen in a mock in vitro translation mix lacking input RNA (lane 13).

TC-32 cells but absent in the cytoplasmic/membrane fraction (Fig. 1). This band comigrated with in vitro-translated *EWS/FLI-1* cDNA as well as immunoprecipitated protein from NIH 3T3 cells transformed by EWS/FLI-1 (14). Additional bands of 90 and 100 kDa were also detected in TC-32 and HeLa cells. It is likely that at least in part, these represent germ line EWS proteins. In vitro-translated *EWS* cDNA generated a protein that comigrated with the 90 kDa band and that immunoprecipitated with our EWS/FLI-1 antiserum (data not shown). Together, these data indicate that the EWS/FLI-1 fusion protein localizes to the cell nucleus.

EWS/FLI-1 binds specific DNA sequence. Many transcription factors bind DNA in a sequence-specific manner. Though there is considerable variation in EWS/FLI-1 fusion products from different translocation-positive tumors, in all instances the DNA-binding domain of FLI-1 remains intact (6). We previously showed that if the DNA-binding domain is disrupted, EWS/FLI no longer can transform NIH 3T3 cells (14). This result suggests that EWS/FLI-1 retains the ability to bind DNA and that its binding specificity might be similar to that of FLI-1.

The natural target genes of FLI-1 are unknown. FLI-1 is able to specifically bind to an *ets-2* consensus sequence but not a PU-1/*spi-1* sequence (10). To determine whether EWS/FLI-1 and FLI-1 share the same specificity for these DNA targets, gel shift analyses were performed (Fig. 2). In vitro-translated full-length FLI-1 (50 kDa) and EWS/FLI-1 (68 kDa) proteins were assayed for the ability to specifically bind a labeled *ets-2* consensus oligonucleotide. Approximately equal amounts of each protein, as measured by SDS-PAGE, were incubated with ^{32}P-labeled *ets-2* oligonucleotide and then fractionated on nondenaturing gels. Protein-DNA complexes were detected for both FLI-1 and EWS/FLI-1 proteins (Fig. 2, lanes 1 and 4). Binding was greatly attenuated by a molar excess of unlabeled *ets-2* oligonucleotide (lanes 2 and 5) but not by PU-1/*spi-1* oligonucleotide (lanes 3 and 6), demonstrating that for both EWS/FLI-1 and FLI-1 proteins, these protein-DNA interactions are sequence specific.

Two mutant EWS/FLI-1 constructs that were unable to induce transformation in 3T3 cells were also tested for the ability to bind *ets-2* sites. Mutant EWS/FLI-1 ΔETS contains a 54-amino-acid deletion within the FLI-1 DNA-binding domain (14) and was unable to bind *ets-2* oligonucleotide (lanes 10 to 12). Only a background band also present in the in vitro translation mix lacking input RNA (lane 13) was visible. This result directly confirms that sequence-specific DNA binding is essential to the transforming activity of EWS/FLI-1. In contrast, the mutant EWS/FLI-1 Δ22, which lacks nearly all of the EWS sequences, was able to specifically bind the *ets-2* probe (lanes 7 to 9). These data suggest that the inability of the Δ22 mutant to transform cells is due to the loss of biochemical function other than sequence-specific *ets* binding.

EWS/FLI-1 transforms NIH 3T3 cells, but FLI-1 does not. The *EWS/FLI-1* fusion gene is a potent single-step transforming gene in NIH 3T3 cells (14). Since FLI-1 and EWS/FLI-1 have similar DNA-binding characteristics, we wished to determine whether FLI-1 could also transform NIH 3T3 cells.

Low-passage NIH 3T3 cells were infected with recombinant retrovirus containing either *EWS/FLI-1* or *FLI-1* under the control of the retroviral long terminal repeat promoter. Pure polyclonal populations selected by growth in G418-containing medium were plated into soft agar at a variety of seeding densities and serum concentrations. In seven independent infections and under all plating conditions, *EWS/FLI-1*-infected 3T3 cells consistently formed numerous macroscopic colonies in agar after 8 to 10 days (Fig. 3). Three independent infections with *FLI-1* retroviruses produced no

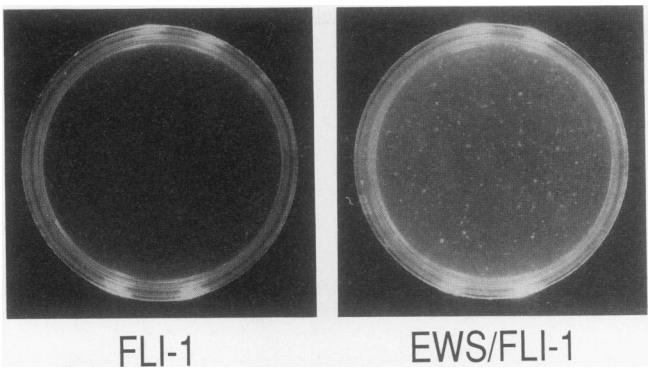

FIG. 3. Agar assays demonstrating transformation of NIH 3T3 cells by EWS/FLI-1 but not FLI-1. A G418-selected, polyclonal population of 3T3 cells infected with *FLI-1*-containing retrovirus failed to form colonies in soft agar. Clonal or polyclonal populations of NIH 3T3 cells expressing the EWS/FLI-1 fusion both formed macroscopic colonies in agar at equivalent efficiencies. For these plates, a polyclonal population 3T3 cells expressing *FLI-1* was compared with a clonal *EWS/FLI-1*-expressing 3T3 cell line. Cells were seeded into agar at a density of 50,000 cells per plate and at a serum concentration of 10%.

visible colonies in agar under any conditions, even after incubation for more than 4 weeks.

Expression of EWS/FLI-1 and FLI-1 proteins was documented by immunoprecipitation from [^{35}S]methionine metabolically labeled, infected 3T3 cell populations by using an EWS/FLI-1 antiserum (see above). EWS/FLI-1 and FLI-1 were present in 3T3 cells at approximately equal levels (data not shown). The difference in the transforming activity between EWS/FLI-1 and FLI-1 in NIH 3T3 cells is not due to disparate levels of intracellular protein but reflects differences in biologic function of these two proteins.

The EWS domain functions as a potent transcriptional activator. The amino-terminal domains of most Ets family members are thought to function as transcriptional activators. The principle difference between EWS/FLI-1 and FLI-1 is the substitution of the amino-terminal domain of EWS for the putative transcriptional activation domain of FLI-1. This substitution, which converts a nontransforming gene (*FLI-1*) into a transforming gene (*EWS/FLI-1*), might also alter the transcriptional activation properties of the amino-terminal domains of these two proteins.

To test this hypothesis, reporter gene experiments were performed in HeLa cells. FLI-1 has been shown to activate, at low levels, constructs containing concatemerized ets-binding sites upstream of an attenuated promoter and reporter gene (10, 33). We encountered high levels of nonspecific background with use of similar reporter constructs containing concatemerized *ets-2*-binding sites. To circumvent these problems, we used a reporter gene system based on the yeast *GAL4* gene. This system has been successfully applied to quantitating the transactivation properties of other mammalian transcription factors, including members of the Ets family (3, 22).

The DNA-binding domain of GAL4 was fused to the amino termini of either EWS/FLI-1 or FLI-1, truncated at the same point within the common FLI-1 DNA-binding domain (Fig. 4A). These constructs were transfected into HeLa cells, together with a reporter construct containing either one or five GAL4 binding sites upstream of a minimal promoter driving a CAT gene. Also included was a control plasmid consisting of a cytomegalovirus-driven luciferase gene. Protein lysates from these transfections were normalized by luciferase activity for transfection efficiency and assayed for CAT activity. These experiments were performed in triplicate at various DNA concentrations, all with similar results (Fig. 4B). GAL4–EWS/FLI-1 constructs efficiently activated reporter constructs containing either one or five GAL4 binding sites. At identical conditions, low-level CAT activity was detected with use of the FLI-1 amino-terminal region. GAL4 fusion constructs containing the EWS amino terminus exhibited approximately 30-fold greater CAT activity than those containing the corresponding FLI-1 amino-terminal domains (Fig. 4C). Immunoprecipitation from extracts of HeLa cells transfected with the same FLI-1 expression plasmid demonstrated stable expression of GAL4/FLI-1 protein (data not shown). These data demonstrate that the EWS amino-terminal domain, present in the EWS/FLI-1 fusion protein, is a much more potent transcription activator than the amino terminus of FLI-1.

DISCUSSION

Our data suggest that *EWS/FLI-1*, a transforming gene caused by a human tumor-specific chromosomal rearrangement, encodes a protein that acts as an aberrant transcription factor. We show that EWS/FLI-1 localizes to the nucleus of t(11;22)-positive PNET cells and that it can bind DNA in a sequence-specific manner. The fact that EWS/FLI-1 and FLI-1 can specifically bind the same nucleotide sequence is consistent with a modular nature for transcription factors, since both molecules have the same DNA-binding domain (for a review see reference 17). Finally, we demonstrate that the EWS sequences that displace the normal FLI-1 transactivation domain in the t(11;22) rearrangement can act as a potent transcriptional activation domain.

EWS/FLI-1 appears to act as a transcription factor that causes a phenotype that is distinct from that conferred by FLI-1. Although FLI-1 does not transform NIH 3T3 cells, EWS/FLI-1 does when expressed at comparable levels. These results suggest that the oncogenic effect of the 11;22 translocation is not simply deregulating an analog of FLI-1 by placing it under the transcriptional control of the ubiquitously expressed *EWS* gene. Rather, the structural fusion of EWS to FLI-1 and the creation of a chimeric protein is important for transformation activity.

There are several possible explanations for why *EWS/FLI-1* is a transforming gene but *FLI-1* is not. Both molecules may interact with the same target genes, but EWS/FLI-1 might be able to activate these genes much more effectively. We have shown that EWS/FLI-1 and FLI-1 can specifically bind the same *ets-2* consensus sequence. Part of the transforming activity of EWS/FLI-1 could be due to enhanced binding of FLI-1 target sites. EWS/FLI-1 differs from FLI-1 at its amino-terminal domain. The amino-terminal sequences of some Ets proteins can modulate the avidity of DNA binding (12, 28). Alternatively, EWS/FLI-1 and FLI-1 may bind target sites with similar affinities, but EWS/FLI-1 may be a more potent transcriptional activator. The fact that EWS acts as a more powerful transcription activation domain than FLI-1 in GAL4 reporter gene assays supports this concept. All of these models suggest quantitative differences in the ability of EWS/FLI-1 and FLI-1 to activate a common set of target genes.

Another possibility is that EWS/FLI-1 is able to transcriptionally activate target genes that FLI-1 cannot activate.

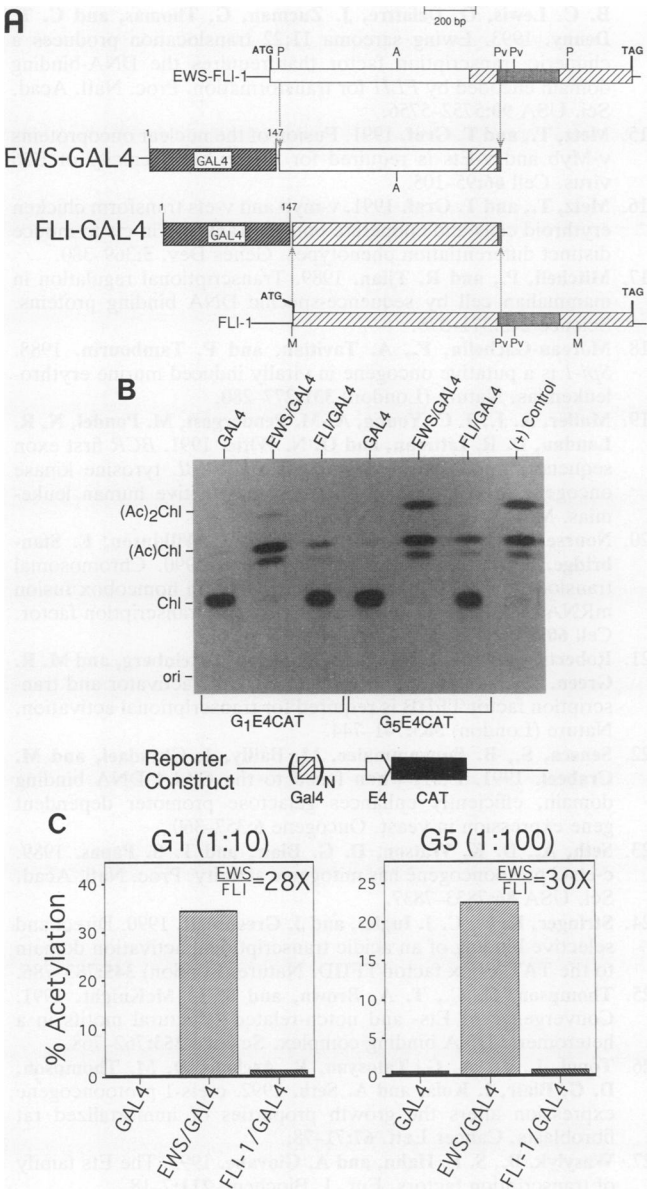

FIG. 4. *GAL4* reporter gene assays demonstrate that the EWS portion of EWS/FLI-1 is a potent transcriptional activation domain. (A) Schematic of EWS/GAL4 and FLI-1/GAL4 expression constructs. Expression constructs are flanked by simian virus 40 early promoters and poly(A) signal sequence. Restriction endonuclease sites shown: P, *Pst*I; A, *Avr*II; B, *Bam*HI; Pv, *Pvu*II; M, *Msc*I. Domains depicted are as labeled and as follows: EWS domain, white; FLI-1 domain, light cross-hatching; *ets* DNA-binding domain, grey. (B) Thin-layer chromatography autoradiogram demonstrating efficient activation of the CAT reporter gene by an EWS/GAL4 expression construct. An EWS/GAL4 or FLI-1/GAL4 plasmid was transiently transfected into HeLa cells together with a CAT reporter gene construct containing one (G_1E4CAT) or five (G_5E4CAT) GAL4 DNA-binding sites. EWS/GAL4 was a potent activator of both CAT reporter constructs. Under the same conditions, FLI-1/GAL4 induced a lower level of activation that was enhanced in the reporter construct containing five GAL4 binding sites. The control lane reflects the activity of 1 U of purified CAT enzyme at the same reaction conditions. $(Ac)_2$ Chl, diacetylated chloramphenicol; (Ac) Chl, acetylated chloramphenicol; Chl, chloramphenicol; ori, origin. (C) Histogram depicting quantitation of CAT activity. To compare EWS/GAL4 activation with FLI-1/GAL4 activation, lysates were diluted to place peak CAT activity within the linear range of the assay. As indicated, EWS/GAL4 constructs resulted in approximately 30-fold-greater CAT activity than did FLI-1/GAL4 constructs whether a reporter with one (G1) or five (G5) binding sites was used.

ETS proteins frequently work in conjunction with other proteins to transcriptionally activate genes. These protein-protein interactions often play a crucial role in defining the specificity of Ets proteins in transcriptional regulation. Such auxiliary proteins can be cofactors that do not bind DNA. For example, GABPβ is required together with GABPα, an Ets protein, for activation of herpes simplex virus early genes (25). Like GABPα, FLI-1 may require cofactors that are not expressed in NIH 3T3 cells, while EWS/FLI-1 can act independently of these factors. In other instances, Ets proteins work with other DNA-binding proteins to activate genes. The Ets protein SAP-1 forms a heteromeric complex with serum response factor for transcriptional induction of c-Fos (5). In this scenario, target genes would be limited to those that had binding sites for both a particular Ets protein and other requisite DNA-binding factors. EWS/FLI-1 may be able to function independently of DNA-binding factors needed by FLI-1 for transcriptional activation. In this way, EWS/FLI-1 could activate a broader repertoire of genes than FLI-1 can. The transcriptional activation domains of VP-16 and SP1 have been shown to interact directly with basal transcription complex proteins (8, 13, 21, 24). EWS/FLI-1 may be able to function similarly.

EWS/FLI-1 is the first example of an Ets protein that is directly involved in the genesis of a human malignancy. However, overexpression and structural alteration of Ets transcription factors have been implicated in cancers of lower vertebrates. Overexpression of either *FLI-1* or PU-1/*spi-1* by Friend virus promotes erythroleukemia in mice (1, 18). Fusion of Ets-1 to c-Myb in E26 virus causes leukemia in chickens (15, 16). In contrast to FLI-1, both Ets-1 and Ets-2 transform NIH 3T3 cells (23, 26).

The formation of chimeric transcription factors is a common theme in human oncogenesis and occurs through tumor-specific rearrangements in a variety of different cancers. Such rearrangements were first identified in hematopoietic malignancies. For example, the t(1;19) translocation associated with acute lymphoblastic leukemia fuses the transcriptional activation domain of the E2A gene to the DNA-binding region of the homeobox gene PBX (9, 20). The t(15;17) translocation found in acute promyelocytic leukemia juxtaposes the retinoic acid alpha receptor to the previously undescribed promyelocytic leukemia gene (2, 7).

Molecular characterization of chimeric transcription factors in nonhematopoietic human tumors has only recently been achieved. The t(12;16) translocation found in myxoid liposarcoma is of particular interest, because it generates a chimeric product similar to EWS/FLI-1 (4). This rearrangement fuses the amino terminus of a newly described gene, termed *TLS*, with the carboxyl portion of the *CHOP* gene, a member of the C/EBP family of transcription factors. Like *EWS*, the *TLS* gene is composed of a glutamine-rich amino-terminal domain coupled to a carboxyl region that is very similar to the *EWS* carboxyl terminus. This suggests that *EWS* and *TLS* belong to the same gene family. The *TLS* carboxyl-terminal domain will bind poly(A)$^+$ RNA in vitro, suggesting that this gene might normally be involved in RNA processing.

The Ewing's sarcoma 11;22 and myxoid liposarcoma 12;16

translocations are two of the first nonhematopoietic tumor-specific rearrangements to be molecularly characterized. It is striking that these two different tumors both form chimeric transcription factors, utilizing members of what appears to be the same gene family. No hematopoietic tumor-associated rearrangements that involve genes resembling *EWS* or *TLS* have yet been described. Whether this is due to rearrangement preference in the genome of the respective cells of origin or because *EWS/TLS* chimeric genes are most active in sarcomas is a point of future investigation.

ACKNOWLEDGMENTS

This work was supported by NCI grants CA12800 and CA50443. W.A.M. is supported by NIAID grant AI07126 and American Cancer Society grant PRTA-5. S.L.L. and B.S.B. are supported by NIH grant GM08042. C.T.D. is supported by a grant from the Leukemia Society of America.

We gratefully acknowledge the following individuals for their invaluable assistance: Michael Carey for help with *GAL4* reporter gene experiments, Owen Witte and Mikhail Gishizky for assistance in retroviral infection procedures and transformation experiments, Poul Sorensen and Timothy Triche for help with TC-32 histochemical staining, Kathleen Sakamoto and Neil Shah for assistance with reporter gene assays, and Harvey Herschman and Steven Smale for critical review of the manuscript.

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