

# SRE elements are binding sites for the fusion protein EWS-FLI-1

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## ABSTRACT

**EWS-FLI-1 is a chimeric protein produced in most Ewing's sarcomas. It results from the fusion of the N-terminal-encoding region of the EWS gene to the C-terminal DNA-binding domain (the ETS domain) encoded by the FLI-1 ets family gene. Both EWS-FLI-1 and FLI-1 proteins function as transcription factors that bind specifically to ets sequences (the ets boxes) present in promoter elements. EWS-FLI-1 is a powerful transforming protein, whereas FLI-1 is not. In a search for potential DNA binding sites for these two proteins, we have tested their ability to recognize the serum responsive element (SRE) in the *c-fos* promoter. This *cis* element contains an ets box which can be occupied by members of the ETS protein family which do not bind DNA autonomously but form a ternary complex with a second protein, p67<sup>SRF</sup> (serum responsive factor). We demonstrate here that EWS-FLI-1, but not FLI-1, is able to form a ternary complex on the *c-fos* SRE. Using a GST pull-down assay, we show that both FLI-1 and EWS-FLI-1 interact *in vitro* with SRF in the absence of DNA. In electromobility shift assays, EWS-FLI-1 binding to the SRE is detectable in the absence of SRF whereas the binding of FLI-1 is not, suggesting that the interaction with DNA is the step which limits ternary complex formation by FLI-1. Deletion of the N-terminal portion of FLI-1 resulted in a protein which behaved as EWS-FLI-1, suggesting the existence of an N-terminal inhibitory domain in the normal protein. Taken together, our data indicate that there are intrinsic differences in the binding of EWS-FLI-1 and FLI-1 proteins to distinct ets sequences.**

## INTRODUCTION

A t(11;22) or t(21;22) chromosomal translocation is observed in the vast majority of Ewing's sarcoma and peripheral neuro-ectodermal tumors of childhood, suggesting a direct role for the corresponding fusion products in the formation of these tumors (1,2). The translocations juxtapose the 5' end of a gene encoding an RNA binding protein of undetermined function, EWS, and the 3' end of a proto-oncogene encoding a transcription factor: FLI-1 in the

t(11;22)(q24;q12) translocation (3) or ERG-1 in the t(21;22)(q22;q12) translocation (4). Both gene products belong to the ETS protein family which includes numerous members involved in developmental processes, cellular responses to various stimuli and cellular transformation (5). All proteins of the ETS family share an 85 amino-acid region, the ETS domain (6), usually located at their C-terminus, through which they specifically bind to, and transactivate through, promoter elements displaying a consensus GGAA core sequence called the ets box. The nucleotides flanking the core sequence define subclasses of ets boxes which are recognized by distinct ETS family members (7).

ETS family members have been shown to cooperate with other nuclear proteins in transcriptional activation, and often seem to function as parts of larger protein complexes. Two members of the ETS family, SAP-1 (8) and ELK-1 (9), are involved in the activation of promoters containing a serum responsive element or SRE (10). They barely bind the SRE autonomously, whereas they strongly bind this sequence in the presence of the serum response factor (SRF), a dimeric glyco-protein recognizing a CARG box adjacent to the ets element (11,12). This results in the formation of a ternary complex consisting of SAP-1 (or ELK-1), SRF and DNA (13,14). The SRE is a target for at least two signal transduction pathways, one which includes p21<sup>RAS</sup> and MAP kinases and results in the phosphorylation of SAP-1 and ELK-1 (12), and one which directly affects SRF through an unknown mechanism (10,15).

The Ewing's sarcoma associated fusion protein EWS-FLI-1 retains both the ETS domain and the transcriptional activity of FLI-1 (16,17). EWS-FLI-1, but not FLI-1, transforms NIH-3T3 cells (18) and induces in these cells the expression of transformation-associated genes including Stromelysin 1, cytokeratin 15 and CYP4F1 (17). Both the EWS domain and the FLI-1 portion of the fusion protein are required for transformation (16). The molecular basis for the difference between FLI-1 and EWS-FLI-1 is not fully understood. Both FLI-1 and the fusion protein localize to the nucleus (19). Both proteins display similar patterns of ets-box recognition (16). In co-transfection assays, both FLI-1 and EWS-FLI-1 display a transcriptional transactivating activity on promoters containing ets binding sites, such as the HTLV-1 and the glyco-protein IIb promoters (6,20). In these assays, EWS-FLI-1 is a more potent transactivator than FLI-1 (16,19), which might account, at least in part, for the difference between

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EWS-FLI-1 and FLI-1 with regard to cell transformation. However, another possibility is that EWS-FLI-1 and FLI-1 may recognize distinct panels of ets boxes. We show here that EWS-FLI-1, but not FLI-1, is able to form a ternary complex with the *c-fos* SRE, and thus displays a ternary complex factor (TCF) activity, as previously observed for ELK-1 and SAP-1. Both FLI-1 and EWS-FLI-1 are able to associate with the SRF protein *in vitro*, in the absence of DNA. However, EWS-FLI-1 binds to the ets-box of *c-fos* SRE in an autonomous manner in the absence of SRF, whereas binding of FLI-1 under the same conditions is barely detectable. We have analysed a deletion mutant of FLI-1, FLI-1(C) which is reduced to the C-terminal portion of the protein present in EWS-FLI-1. FLI-1(C) forms a ternary complex with SRF on the SRE and binds to the ets-box in an autonomous manner. This result suggests the existence of an inhibitory domain in the N-terminal part of the normal FLI-1 protein. Taken together, these data suggest that the loss of the N-terminal part of FLI-1 in EWS-FLI-1 modifies DNA binding-site selection by the fusion product.

## MATERIALS AND METHODS

### Plasmids

A 2993 bp EWS-FLI-1 cDNA (EF11), lacking the initiation codon but including the stop codon and the 3' untranslated sequence, was isolated from a Ewing's sarcoma cDNA library (21). A *SalI* site and a Kozak consensus translation initiation sequence were inserted at the 5'-end of the EF11 cDNA by PCR amplification, using as forward primer: 5'-TACAAAGTCGACCACCATGGCGTCCACGGATTACAGTACC-3'; the reverse primer, 5'-ATCTTAGAGCTCTAGTAGTAGCTGCCTA-3', included a *SacI* site and annealed to the 3'-end of the translated sequence. The amplification product (~1.6 kb) was subcloned between the *SalI* and *SacI* sites in the pSP64 poly(A) expression vector (Promega), giving the pSP64 EWS-FLI-1 plasmid.

The plasmid pCR3 FLI-1, which allows the *in vitro* translation of FLI-1 protein to high levels, was a gift of M. Duterque (Lille, France).

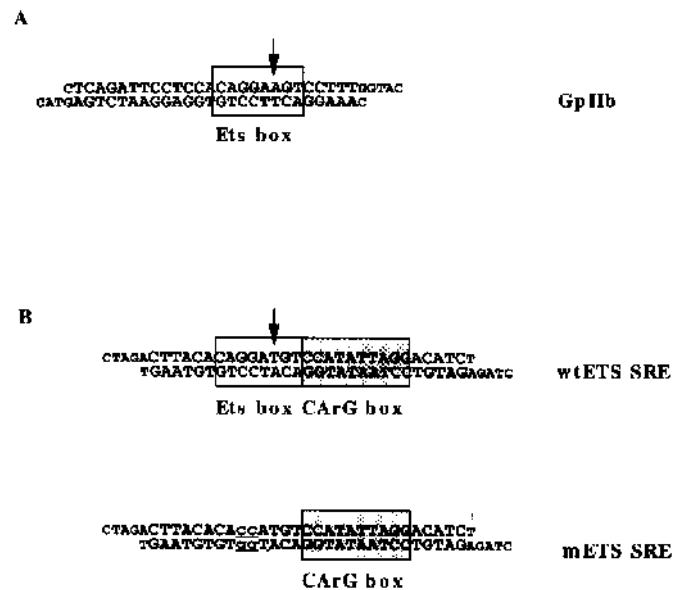
The ΔEB-78 FLI-1(C) vector, encoding for a truncated FLI-1 protein limited to C-terminal amino acid residues 225–452 of FLI-1 and conserving the ets binding domain, was described elsewhere and was a gift of J. Ghysdael (Orsay, France) (16). The FLI-1(C) cDNA was subcloned into the pSP64 vector.

pT7 ELK-1 (12) was a gift of R. Treisman (London, UK). pCDNA3 SRF was constructed by subcloning the SRF coding sequence from pG3.5 (also from R. Treisman) into pCDNA3 (Invitrogen). The SRFcore cDNA, encoding amino acid residues 131–266 of pG3.5 SRF and conserving the DNA binding domain and the ets protein interaction domain, was obtained by PCR amplification, using as forward primer 5'-CGGAAGCTTACCA-TGGTGAGCGGGGCCAA-3', and as reverse primer 5'-TCA-CAGGTTGGTACTGTGAACGCCGGC-3'. The amplification product was subcloned between the *HindIII* and *HincIII* sites in the HIV vector.

The ectodomain of the TM envelope glyco-protein of the HTLV-I retrovirus, subcloned in the pCDNA3 vector, and used as a negative control in this study, was a gift from A. Rosenberg (Villejuif, France).

### *In vitro* transcription and translation

Proteins were translated *in vitro* using a TnT kit, as recommended by the manufacturer (Promega). Experiments were run in parallel,



**Figure 1.** Oligonucleotides used in this study. (A) The GpIIb oligonucleotide covers a region of the human glycoprotein IIb promoter that contains an FLI-1 octanucleotide consensus sequence (boxed). (B) The wtETS SRE oligonucleotide includes the ets (open box) and the CArG (shaded box) boxes and covers a sequence between nucleotides –322 and –305 in the *c-fos* promoter; the mETS SRE oligonucleotide is a variant of this sequence which includes two mutations, underlined in the sequence, resulting in ets box inactivation. The arrow points to the position (6) in the ets box that differs between GpIIb and *c-fos*. Restriction sites at the ends of the oligonucleotides are indicated in small capitals.

in the presence or absence of <sup>35</sup>S-methionine. Radioactive products were analysed by SDS-PAGE using standard procedures, and non-radioactive products were used in EMSA.

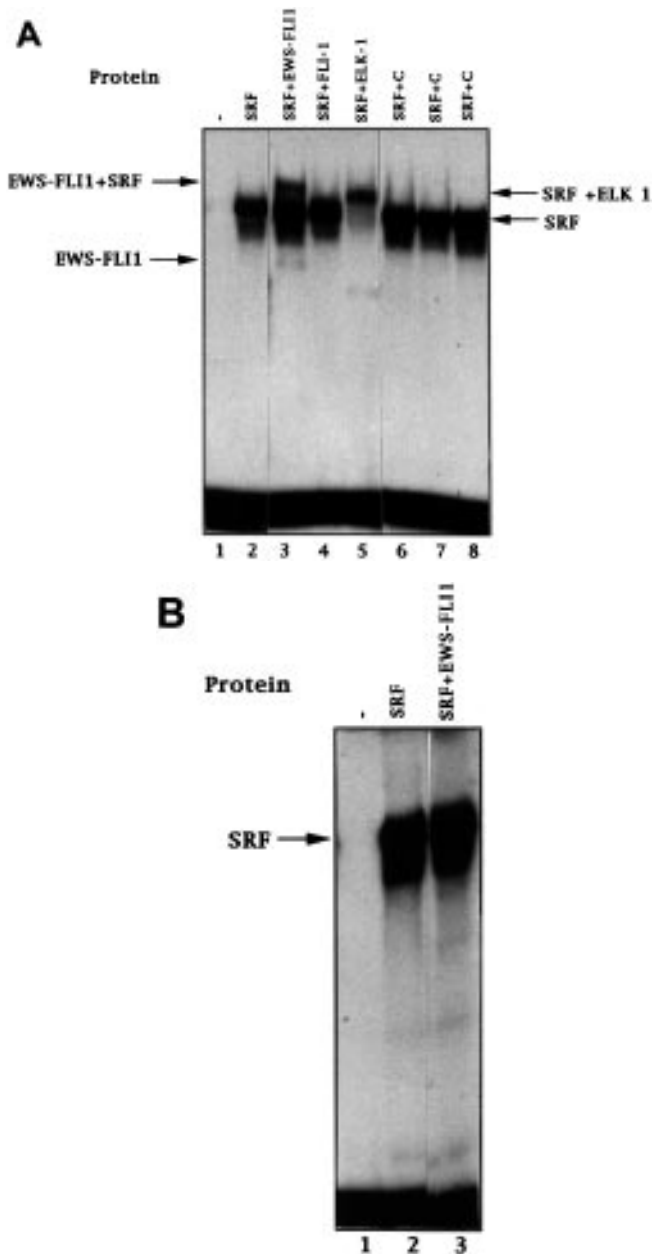
### Electrophoretic mobility shift assays (EMSA)

The GpIIb, wtETS SRE and mETS SRE oligonucleotides, described in Figure 1, were purified on denaturing acrylamide gels, annealed and end-labeled using T4 polynucleotide kinase (Bio-labs) and [ $\gamma$ -<sup>32</sup>P]ATP. *In vitro* translated proteins (2  $\mu$ l for SRF, 5  $\mu$ l for the others) were preincubated in 20  $\mu$ l of EMSA buffer (188 mM NaCl, 50 mM HEPES pH 7.9, 2.5 mM EDTA pH 8, 2.5 mM DTT, 12% glycerol) with 1–2  $\mu$ g salmon sperm DNA, together with an excess of unlabeled competitor oligonucleotide (where indicated). After 15 min on ice, <sup>32</sup>P-labeled oligonucleotide probes (2 ng, corresponding to  $\sim 9 \times 10^3$  c.p.m.) were added and the incubation was allowed to continue for an additional 15 min at room temperature. Samples were electrophoresed at room temperature on a 4% polyacrylamide gel in 0.25 $\times$  TBE buffer.

Gels were dried and analysed using a Phosphorimager (BAS-1000, FUJI), followed by conventional autoradiography.

### GST pull-down assay

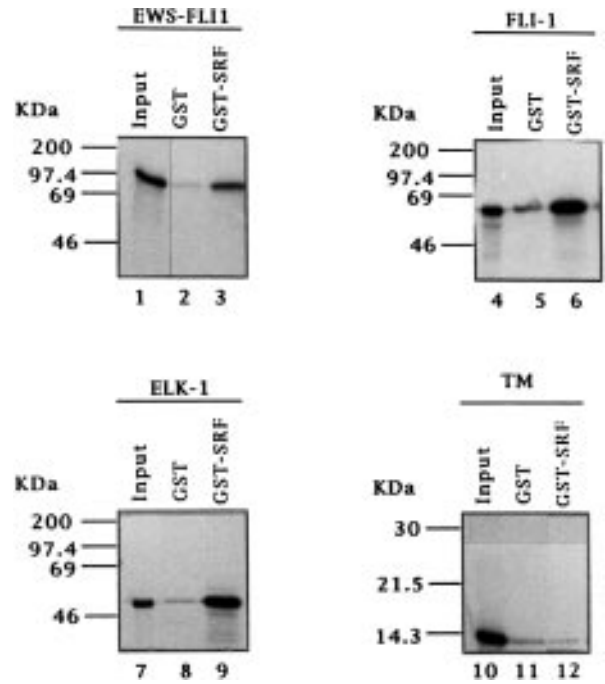
GST or GST-SRF coated beads were prepared according to Groisman *et al.* (22). GST or SRF-GST beads were incubated with equivalent amounts of *in vitro* translated <sup>35</sup>S-methionine-labeled EWS-FLI-1, FLI-1, FLI-1(C), ELK-1 or HTLV-1 TM proteins, in a final volume of 200  $\mu$ l of either NETN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) or ZBU buffer (25 mM HEPES pH 7.5, 12.5 mM MgCl<sub>2</sub>, 20% glycerol,



**Figure 2.** EWS-FLI-1 forms a ternary complex with SRF on *c-fos* SRE. FLI-1, EWS-FLI-1 and ELK-1 were transcribed and translated *in vitro*. Equivalent amounts of the translation products were analysed by EMSA in the presence of SRF, using the wtETS SRE (A) or mETS SRE (B) as a probe. (A) Lane 1, wtETS SRE (free probe); lane 2, SRF protein alone; lane 3, SRF and EWS-FLI-1; lane 4, SRF and FLI-1; lane 5, SRF and ELK-1; in lanes 6, 7 and 8, SRF protein was incubated with translation products of the corresponding vehicle vectors pSP64, pCR3 and pT7, respectively (C: control). (B) Lane 1, mETS SRE (free probe) (see Fig. 1B); lane 2, SRF protein; lane 3, SRF and EWS-FLI-1 proteins.

0.1% NP-40, 150 mM KCl, 0.5 M urea). After 1 h incubation at room temperature with gentle agitation, beads were washed five times in NETN buffer.

Adsorbed proteins were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and analysed using a Phosphorimager (Bas-1000, FUJI), followed by conventional autoradiography.

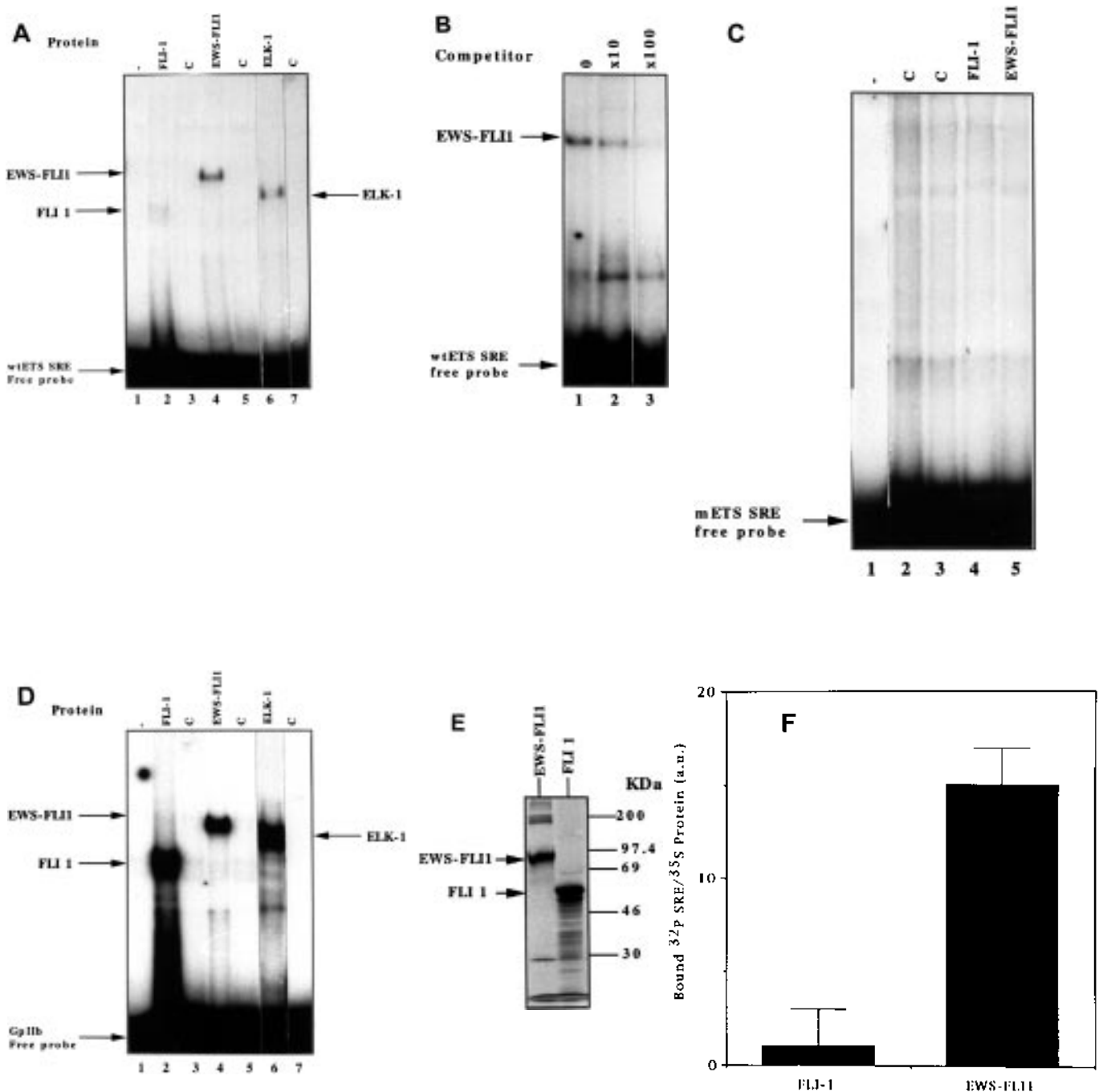


**Figure 3.** Analysis of interaction with SRF in the absence of DNA. EWS-FLI-1 and FLI-1 were transcribed and translated *in vitro* in the presence of <sup>35</sup>S-labeled methionine. Equivalent amounts of the translation products were then incubated with beads coated with GST or GST-SRF fusion protein. ELK-1 (positive control) and TM (negative control) were included in each experiment. Lanes 1, 4, 7 and 10 show the input (0.1 times the amount used in the assay) for EWS-FLI-1, FLI-1, ELK-1 and TM, respectively; lanes 2, 5, 8 and 11, EWS-FLI-1, FLI-1, ELK-1 and TM proteins incubated with GST beads; lanes 3, 6, 9 and 12, EWS-FLI-1, FLI-1, ELK-1 and TM proteins incubated with GST-SRF beads.

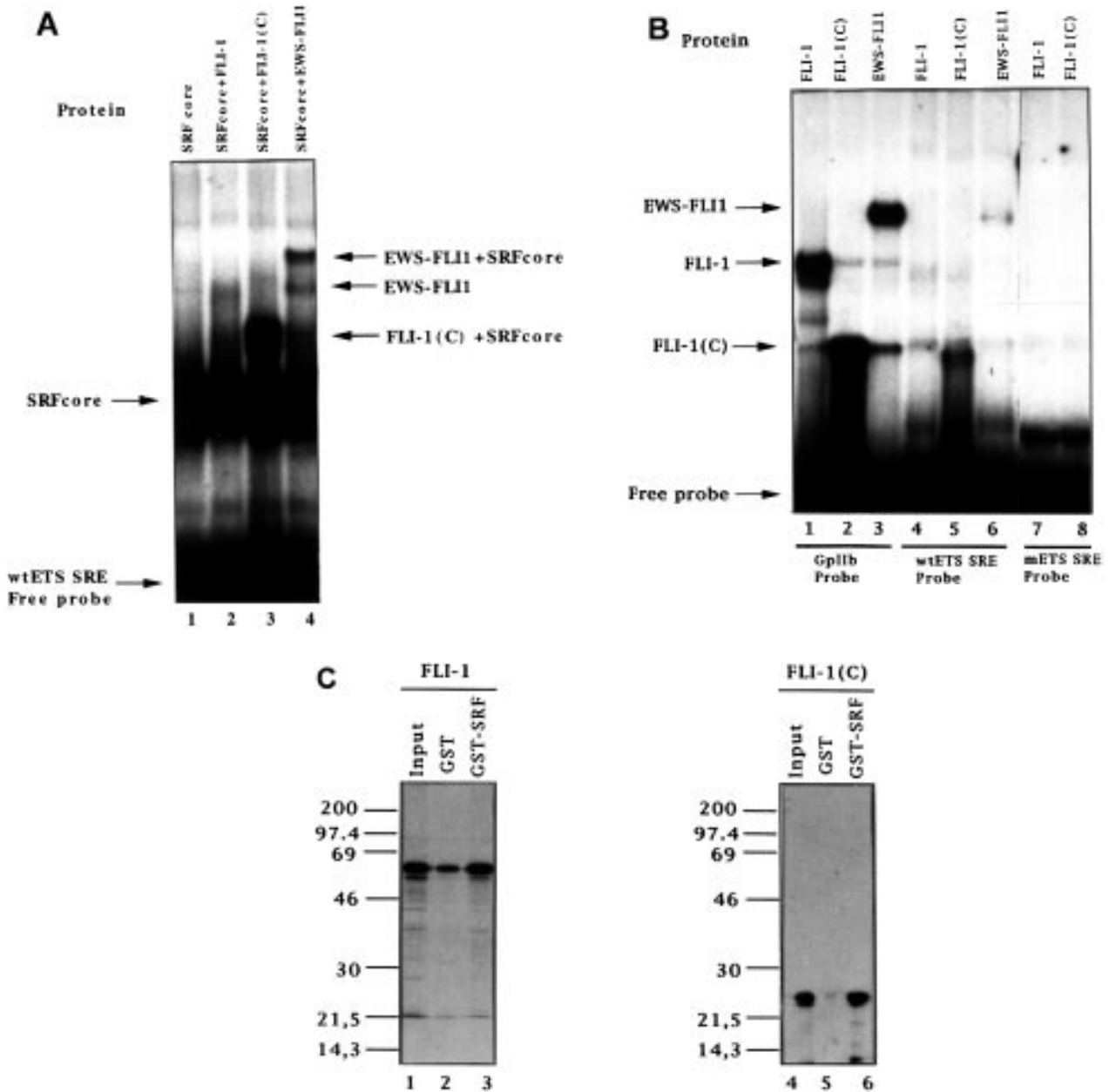
**RESULTS AND DISCUSSION**

**EWS-FLI-1 displays TCF activity on *c-fos* SRE**

The consensus element for FLI-1 or EWS-FLI-1 binding is an octanucleotide sequence, CC/AGGAAGT (Fig. 1A) (23). The *c-fos* SRE contains an ets box (Fig. 1B) which differs from this consensus sequence by a single nucleotide (T for A). SAP-1 and ELK-1, two ETS proteins involved in SRE modulation, barely bind the *c-fos* box in an autonomous manner. Rather, their binding requires the presence of SRF, which recognizes a CA<sub>n</sub>G box located adjacent to the ets box (Fig. 1), and with which they form a ternary complex (24). In order to test whether FLI-1 and EWS-FLI-1 were also able to form a ternary complex, the two proteins were translated *in vitro* and analysed by EMSA, in the presence of SRF. Results (Fig. 2A) suggest that the SRF complex (lane 2) was partially 'supershifted' in the presence of EWS-FLI-1 (lane 3), consistent with the formation of a ternary complex. No supershifted SRF could be detected with FLI-1 (lane 4), indicating that EWS-FLI-1 and FLI-1 do not behave similarly in this assay. Note, however, that in the presence of FLI-1 (compare lane 2 and lane 4), the SRF band was more diffuse, which could reveal highly unstable complexes between FLI-1, SRF and DNA. When a mutation was introduced into the ets binding site (mETS SRE) of the oligonucleotide used as a probe (Fig. 2B), no supershift could



**Figure 4.** Analysis of autonomous binding to *c-fos* ets box. FLI-1, EWS-FLI-1 and ELK-1 were transcribed and translated *in vitro*. Translation products were then incubated with  $^{32}\text{P}$ -labeled wtETS SRE (A and B), mETS SRE (C) or GpIIb (D) oligonucleotides, analysed on a 4% polyacrylamide gel by EMSA, and visualised by autoradiography. (A) Detection of EWS-FLI-1 binding to SRE. Lane 1, wtETS SRE (free probe); lane 2, FLI-1 protein; lane 4, EWS-FLI-1 protein; lane 6, ELK-1 protein; lanes 3, 5 and 7, *in vitro* translation product of the corresponding control vectors pCR3, pSP64 and pT7, respectively. (B) Analysis of EWS-FLI-1 binding specificity. *In vitro* translated EWS-FLI-1 protein was preincubated 15 min on ice with increasing amounts of unlabeled wtETS SRE oligonucleotide prior to incubation with the radiolabeled probe. Lane 1, no competitor; lane 2, 10-fold excess (20 ng); lane 3, 100-fold excess (200 ng). (C) EWS-FLI-1 does not bind to a mutated SRE probe. Lane 1, mETS SRE (free probe); lanes 2 and 3, *in vitro* translation products of the corresponding control vectors pCR3 and pSP64, respectively; lane 4, FLI-1 protein; lane 5, EWS-FLI-1 protein. (D) EWS-FLI-1 and FLI-1 bind to GpIIb in a similar manner. Lane 1, GpIIb (free probe); lane 2, FLI-1 protein; lane 4, EWS-FLI-1 protein; lane 6, ELK-1 protein; lanes 3, 5 and 7, *in vitro* translation products of the corresponding control vectors, pCR3, pSP64 and pT7, respectively. (E) EWS-FLI-1 and FLI-1 are *in vitro* translated with similar efficiencies. EWS-FLI-1 and FLI-1 were transcribed and translated *in vitro*, using  $^{35}\text{S}$  methionine. Products were analysed on an SDS-10% polyacrylamide gel. The major translation products (p80 for EWS-FLI-1 and p54 for FLI 1) are indicated. The amounts of the two proteins were calculated after quantification using a Bas 1000 Phosphorimager and based on their methionine contents. (F) Quantification of EWS-FLI-1 and FLI-1 binding to SRE. The amount of *c-fos* SRE probe retained in each complex was measured on a Bas 1000 Phosphorimager and standardised with respect to their EWS-FLI-1 or FLI-1 protein contents, as obtained in (E). The bars indicate standard deviations as calculated from four independent experiments.



**Figure 5.** The C-terminal region of FLI-1 is responsible for ternary complex formation with SRF on *c-fos* SRE and interacts physically with SRF. FLI-1, FLI-1(C) and EWS-FLI-1 were transcribed and translated *in vitro*. Equivalent amounts of the translation products were analysed by EMSA in the presence of SRFcore, using the wtETS SRE probe (A) or in the absence of SRF protein using GpIIB, wtETS SRE, or mETS SRE probe (B). (A) Lane 1, SRFcore protein alone; lane 2, SRFcore and FLI-1; lane 3, SRFcore and FLI-1(C); lane 4, SRFcore and EWS-FLI-1. (B) Lanes 1, 2 and 3, GpIIB probe; lanes 4, 5 and 6, wtETS SRE probe; lanes 7 and 8, mETS SRE probe. lanes 1, 4 and 7, FLI-1; lanes 2, 5 and 8, FLI-1(C); lanes 3 and 6, EWS-FLI-1. (C) FLI-1, FLI-1(C) and EWS-FLI-1 were transcribed and translated *in vitro* in the presence of <sup>35</sup>S-labeled methionine. Equivalent amounts of the translation products were then incubated with beads coated with GST or GST-SRF fusion protein, as in Figure 3. Lanes 1 and 4 show the input (0.1 times the amount used in the assay) for FLI-1 and FLI-1(C) respectively; lanes 2 and 5, FLI-1 and FLI-1(C) proteins incubated with GST beads; lanes 3 and 6, FLI-1 and FLI-1(C) proteins incubated with GST-SRF beads.

be observed with EWS-FLI-1 (Fig. 2B, lane 3), indicating that the integrity of the ets binding site is required.

Thus, like ELK-1, EWS-FLI-1 apparently displays a 'TCF' activity on *c-fos* SRE, a feature which is not observed for FLI-1. Formation of such a ternary complex by the ETS proteins SAP-1 and ELK-1 requires physical interaction with both SRF and DNA. We therefore used two different assays to investigate separately each of these interactions.

### Both FLI-1 and EWS-FLI-1 physically interact with SRF

Physical interaction between EWS-FLI-1 or FLI-1 and SRF was tested in the absence of DNA, using a GST pull-down assay. FLI-1 or EWS-FLI-1, as well as ELK-1 as a positive control, or the unrelated protein TM as a negative control, were incubated with beads coated with GST-SRF or GST alone. Results shown in Figure 3, indicate that significant amounts of EWS-FLI-1 and FLI-1 were

specifically retained on the SRF-coated beads (lanes 3 and 6), suggesting that both FLI-1 and EWS-FLI-1 interact with SRF. In control experiments, similar amounts of the TM protein did not show any detectable specific absorption on the SRF beads (lane 12). Furthermore, ethidium bromide did not inhibit the retention of EWS-FLI-1, ruling out the possibility of an artefactual result due to non-specific binding of the proteins to contaminating DNA (data not shown). Although this technique has some limitations (in particular a large excess of SRF on the beads allows the detection of low-affinity binding proteins), these results suggest that both EWS-FLI-1 and FLI-1 bind to SRF, similarly to what is observed with ELK-1 (lane 9). The interaction between SRF and other members of the ETS family requires a protein domain referred to as the B-box (8,25,26). Comparison of the amino-acid sequences of FLI-1 and the B-boxes of several known TCFs showed only a short stretch of homology (not shown). The involvement of these amino-acids is currently under investigation.

Thus EWS-FLI-1 and FLI-1 can both interact with SRF, whereas only the chimeric protein can form a ternary complex on the SRE ets box. This suggests that interaction with SRF is not sufficient for ternary complex formation.

### EWS-FLI-1 binds autonomously to the SRE ets box

We next investigated *c-fos* ets box recognition by FLI-1 and EWS-FLI-1. *In vitro* translated proteins were analysed by EMSA in the absence of SRF, using oligonucleotide probes containing the ets box from either the GpIIb promoter, as a reference, or the *c-fos* SRE. Significant amounts of wtETS SRE probe were retarded by EWS-FLI-1, comparable to that observed with ELK-1 (Fig. 4A, compare lanes 4 and 6), whereas binding of FLI-1 was hardly detectable (Fig. 4A, lane 2). The binding of EWS-FLI-1 was specific since it was inhibited by increasing amounts of the autologous oligonucleotide (Fig. 4B) but not by an oligonucleotide in which the ets box is mutated (mETS SRE; data not shown). Furthermore, the complexes were not observed when the mutant oligonucleotide was used as a probe (Fig. 4C). Such an autonomous binding could also be detected in the presence of SRF (see arrow 'EWS-FLI-1' in Fig. 2A), although most of the fusion protein was revealed in ternary complex under these conditions.

The quantitative difference in the amount of probe (wtETS SRE) retarded by EWS-FLI-1 and FLI-1 (Fig. 4A, lanes 2 and 4) was not due to a difference in the protein amounts used in these experiments. Indeed (Fig. 4D), both FLI-1 and EWS-FLI-1 retarded similar amounts of a GpIIb probe (described in Fig. 1A), which is expected since the two proteins display a similar affinity for this sequence (16). In addition, FLI-1 and EWS-FLI-1 proteins were repeatedly translated with similar efficiencies, as assessed by quantification of *in vitro* translated <sup>35</sup>S-labeled products (Fig. 4E) using a Bas 1000 Phosphoimager. Quantification of the amount of SRE probe (Fig. 4A) retained by EWS-FLI-1 and FLI-1 proteins showed that EWS-FLI-1 retained 10 times more probe than FLI-1 did.

### The C-terminal part of FLI-1 displays TCF activity on *c-fos* SRE

It therefore appears that a region outside the ETS domain (which is identical in the two proteins) may be critical for binding affinity to the *c-fos* ets sequence. For example, an inhibitory domain could be present in the FLI-1 protein, which could be masked or deleted in the fusion product EWS-FLI-1. In order to test this hypothesis,

we analysed next a deletion mutant of FLI-1, FLI-1(C) which is reduced to the portion of the protein present in the fusion product, EWS-FLI-1. FLI-1(C) was tested by EMSA in the presence or absence of SRF (in order to increase the resolution of potential ternary complexes with FLI-1(C), a short polypeptide, a short version of SRF, the SRF 'core' (11), was used instead of the full-length SRF). Results (Fig. 5) indicate that FLI-1(C) behaves like the fusion product EWS-FLI-1. Like EWS-FLI-1 (Fig. 5A, lane 4) and in contrast to FLI-1 (lane 2), FLI-1(C) induces a super shift of the core SRF-DNA complex (lane 3). Furthermore, FLI-1(C) binds to *c-fos* ets box in an autonomous manner, i.e. in the absence of SRF (Fig. 5, lane 5) as did EWS-FLI-1 (lane 6) but not FLI-1 (lane 4), and this autonomous binding of FLI-1(C) is completely abrogated when the SRE probe is modified by substitutions in the ets sequence (mETS SRE, Fig. 5B, lane 8). The difference between FLI-1 and FLI-1(C) was not observed when the GpIIb ets sequence was used as a probe (Fig. 5B, lanes 1-3). The amount of probe retained by FLI-1(C) in the presence of SRF was larger than that retained in the absence of SRF, suggesting a cooperative effect of SRF on probe recognition by FLI-1(C). This was also true for EWS-FLI-1. Finally, FLI-1 and FLI-1(C) display identical SRF-binding capacities in a GST-pull down assay (Fig. 5C), indicating that the region of the FLI-1 molecule responsible for the interaction with SRF is located in the C-terminal part of the molecule.

Taken together, the data show that, *in vitro*, EWS-FLI-1 recognizes target sequences distinct from those specifically bound by FLI-1 protein and that region outside the ETS-domain may be responsible for this difference. In conclusion, EWS-FLI-1 and FLI-1 can be distinguished not only on the basis of quantitative differences in their transactivation capability (16), but also qualitatively, by the target sequences which they are able to recognize. This suggests that the tumor-specific EWS-FLI-1 protein may have the potential to regulate the expression of various genes whose promoters cannot be bound by FLI-1. The functional relevance of this observation is currently under investigation.

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