DNA-Binding and Transcriptional Activation Properties of the EWS–FLI-1 Fusion Protein Resulting from the t(11;22) Translocation in Ewing Sarcoma

ROSE-AIMÉE BAILLY,¹ REMY BOSSELUT,¹ JESSICA ZUCMAN,² FRANÇOISE CORMIER,¹
OLIVIER DELATTRE,² MARTINE ROUSSEL,³ GILLES THOMAS,²
AND JACQUES GHYSDAEL^{1*}

Laboratoire d'Oncologie Virale et Cellulaire, Centre National de la Recherche Scientifique, URA 1443, Institut Curie, 91405 Orsay,¹ and Laboratoire de Génétique de Tumeurs, Institut National de la Santé et de la Recherche Médicale, C/JF 9201, Institut Curie, 75231 Paris,² France, and Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105³

Received 4 October 1993/Returned for modification 12 November 1993/Accepted 7 February 1994

The 5' half of the *EWS* gene has recently been described to be fused to the 3' regions of genes encoding the DNA-binding domain of several transcriptional regulators, including ATF1, FLI-1, and ERG, in several human tumors. The most frequent occurrence of this situation results from the t(11;22)(q24;q12) chromosome translocation specific for Ewing sarcoma (ES) and related tumors which joins *EWS* sequences to the 3' half of *FLI-1*, which encodes a member of the Ets family of transcriptional regulators. We show here that this chimeric gene encodes an EWS–FLI-1 nuclear protein which binds DNA with the same sequence specificity as the wild-type parental FLI-1 protein. We further show that EWS–FLI-1 is an efficient sequence-specific transcriptional activator of model promoters containing FLI-1 (Ets)-binding sites, a property which is strictly dependent on the presence of its EWS domain. Comparison of the properties of the N-terminal activation domain of FLI-1 to those of the EWS domain of the fusion protein indicates that EWS–FLI-1 has altered transcriptional activation properties compared with FLI-1. These results suggest that EWS–FLI-1 contributes to the transformed phenotype of ES tumor cells by inducing the deregulated and/or unscheduled activation of genes normally responsive to FLI-1 or to other close members of the Ets family. ES and related tumors are characterized by an elevated level of *c-myc* expression. We show that EWS–FLI-1 is a transactivator of the *c-myc* promoter, suggesting that upregulation of *c-myc* expression is under control of EWS–FLI-1.

Members of the Ets family form a novel class of sequence-specific DNA-binding proteins which are implicated in developmental processes, in the response of cells to extracellular signals, and in cellular transformation (for reviews, see references 23 and 70). They are characterized by an 85-amino-acid region of similarity, the Ets domain, which is essential for sequence-specific binding to DNA (20, 28, 30, 37, 50, 64, 69, 73). DNA sequences bound by several Ets family members have been analyzed in detail and found to include about 10 nucleotides centered over a central GGAA core sequence (11, 18, 50, 67, 78). These sequences when multimerized upstream of a minimal promoter or when present in the context of natural viral and cellular promoters/enhancers mediate transcriptional regulation by Ets proteins (8, 30, 55, 56, 71, 74, 79). Consistent with these properties, specific transcriptional activation domains have been mapped in Ets-1, Ets-2, and Elk-1 (20, 39, 59). Available evidence indicates, however, that Ets family members usually function as subunits of larger transcription complexes either in the form of heteromers in solution (GABP α /GABP β [33, 64]) or as ternary complexes with an unrelated factor on composite DNA elements (Elk and SAP1/SRF [15, 29], Pu1/NF-EM5 [53], and Ets-1/Sp1 [21]). These interactions as well as other, less well characterized forms of synergy between particular Ets family members and other transcription factors (63, 71) are likely to play a key role in target gene selectivity and/or fine regulation of their activity.

Three members of the Ets family have so far been implicated in oncogenic processes. *c-ets-1* is the cellular progenitor of the *v-ets* oncogene of avian leukemia virus E26 (35, 49). *v-ets*, together with a cotransduced *v-myb* oncogene, is expressed as a single Gag-Myb-Ets fusion protein in E26-transformed cells (6). Both *v-myb* and *v-ets* contribute in an interdependent and synergistic way to the transforming and leukemogenic properties of E26 (25, 43, 44, 48). *spi-1* is overexpressed in the leukemic cells of the vast majority of erythroleukemias induced in adult mice by the Friend virus complex as the result of a nearby proviral insertion of a replication-defective spleen focus-forming virus (45, 46). *fli-1* is rearranged and overexpressed by a similar mechanism in 75% of erythroleukemias induced in newborn mice by the replication-competent Friend murine leukemia virus (4). The precise contribution of the deregulated expression of Spi-1/Pu-1 and Fli-1 to the erythroleukemic process is unknown.

Ewing sarcoma (ES), a childhood bone tumor, and the less frequent peripheral neuroepitheliomas (PN) are tumors of neuroectodermal origin which are characterized by a highly specific and recurrent t(11;22)(q24;q12) balanced chromosomal translocation (2, 65, 66, 76, 77). This translocation results in the fusion, on the der(22) chromosome, of the 5' half of a resident gene of unknown function named *EWS* to the 3' portion of the chromosome 11-derived *FLI-1* gene (16, 82). This translocation results in the *EWS* promoter-driven expression of a chimeric transcript expected to encode an EWS–FLI-1 hybrid protein containing EWS-derived sequences at its amino terminus fused to the carboxy-terminal half of FLI-1. As a first step to understand how the EWS–FLI-1 protein contrib-

* Corresponding author. Mailing address: Institut Curie, Bâtiment 110, Centre Universitaire, 91405 Orsay Cedex, France. Phone: 33-1-69 86 31 52. Fax: 33-1-69 86 17 03.

FIG. 1. Schematic structure of the FLI-1 and EWS-FLI-1 proteins and of deletion and substitution mutants derived thereof. Proteins encoded by the expression plasmids used in this study are schematically depicted. Shown are *FLI-1*-encoded sequences (black boxes), *EWS*-encoded sequences (thinly hatched boxes), *ets-1*-encoded sequences (stippled boxes), and *GAL4*-encoded sequences (thick hatched boxes). The Ets domain within Ets-1 and FLI-1 is shown as a white box. A point mutation (W-321→R) in the FLI-1 Ets domain which abolishes FLI-1 DNA-binding activity is indicated. Details of the constructions are given in Materials and Methods.

utes to tumorigenesis, we analyzed its DNA-binding and transcriptional activation properties. We show that EWS-FLI-1 is a nuclear protein which binds DNA with a sequence specificity indistinguishable from that of the parental FLI-1 protein. EWS-FLI-1 is an efficient sequence-specific transcriptional activator, a property which is dependent on a transcriptional activation domain in the EWS sequence. Finally, in line with the elevated expression of the *c-myc* proto-oncogene in ES and PN tumor cells, EWS-FLI-1 is shown to upregulate the activity of a human *c-myc* promoter construct.

MATERIALS AND METHODS

Plasmid constructions. cDNAs encoding the normal human FLI-1 or a type 1 EWS-FLI-1 fusion protein (16) were inserted at the *EcoRI* and *HindIII* sites of the previously described simian virus 40 promoter-based expression vector Δ EB-78 (20). The DNA-binding-deficient mutant EWS-FLI-1(mut) protein was generated by oligonucleotide site-directed mutagenesis. The 764-bp *Bam*HI-to-*Hind*III fragment of EWS-FLI-1 was inserted in *Bam*HI-plus-*Hind*III-restricted M13 mp19. Mutation of the wild-type codon TGG (W-367 in EWS-FLI-1, corresponding to W-321 in FLI-1) into codon

CGG (R) was carried out on the corresponding single-stranded viral DNA, using an Amersham site-directed mutagenesis kit and the mutagenic oligonucleotide 5'-CCA GGCGCCGGGGCGAGTC-3'. The DNA-binding-deficient mutant EWS-FLI-1(R₂L₂) in which arginine residues 383 and 386 of EWS-FLI-1 (residues 337 and 340 in FLI-1) are converted into leucine was similarly obtained, using the mutagenic primer 5'-GCTGAGCCTGGCCCTCCTTTATTACT AT-3'. The FLI-1(C) deletion mutant was obtained by first creating a *de novo Hind*III site, at the junction between EWS and FLI-1 sequences of EWS-FLI-1 (position 669 in FLI-1), by oligonucleotide site-directed mutagenesis using the mutagenic primer 5'-AGCTCCTCTTCTGACAAGCTTATAAGAAGG GTTCG-3'. The *Hind*III insert fragment was then introduced at the *Hind*III site of the Δ EB-78 expression plasmid. This plasmid therefore encodes a truncated FLI-1 protein limited to amino acid residues 225 to 452 of FLI-1. The expression plasmid for Ets-1(C) was described previously as Ets-1 Δ 4 (20). Fusion of the FLI-1 amino-terminal sequences to Ets-1(C) to generate FLI-1(N)-Ets-1(C) was obtained by insertion of the 5' *Eco*RI fragment of FLI-1 (encoding amino acids 1 to 236) at the *Eco*RI site of Δ EB-78/Ets-1 Δ 4. Fusion of the EWS amino-terminal sequences to Ets-1(C) to generate EWS-Ets-1(C) was obtained by insertion of the 5' *Eco*RI fragment of the *EWS-FLI-1* cDNA, which encodes the entire 264 amino acids of the EWS domain of EWS-FLI-1, linked to 16 adjacent FLI-1-derived residues, at the *Eco*RI site of Δ EB-78/Ets-1 Δ 4.

The expression plasmid for the DNA-binding domain (residues 1 to 147) of Gal4 (pG4M polyII) was described previously (75). The Gal4-EWS fusion was obtained by inserting the *Bsa*HI-to-*Bam*HI fragment of the *EWS-FLI-1* cDNA, which encodes amino acid residues 2 to 246 of EWS, to *Cla*I- and *Bam*HI-restricted pG4M polyII. The Gal4-FLI-1(C) fusion was obtained by insertion of the 794-bp *Bam*HI fragment of Δ EB-78/EWS-FLI-1, which encodes the entire FLI-1-derived domain of EWS-FLI-1 preceded by 20 amino acid residues of adjacent EWS sequences, in frame at the *Bam*HI site of pG4M polyII. Gal4-FLI-1(C)(mut) was similarly constructed from Δ EB-78/EWS-FLI-1(mut). For construction of Gal4-FLI-1(N), the 5' portion of the *FLI-1* cDNA, encoding amino acid residues 1 to 234 of FLI-1, was amplified by using PCR and amplimers which included either a *Cla*I (5' amplimer) or an *Sst*I (3' amplimer) restriction enzyme cleavage site. The PCR-amplified fragment was digested by *Cla*I and *Sst*I and directionally inserted into *Cla*I- and *Sst*I-restricted pG4M polyII. To construct the baculovirus transfer vector for FLI-1 and EWS-FLI-1, the cDNA inserts were first bordered by *Bgl*II sites by subcloning in an appropriate Bluescript derivative; *Bgl*II-bordered inserts were next inserted at the *Bam*HI site of the pVL941 transfer vector (Pharmingen). To construct the human *c-myc* reporter construct, a 862-bp fragment corresponding to positions -511 to +351 of the human *c-myc* promoter obtained by *Pvu*II digestion of pMYC CAT-P (38) was subcloned into blunt-ended *Bgl*II-restricted plasmid pCAT-3M (32). The sequences of all DNA fragments subjected to oligonucleotide site-directed mutagenesis and PCR amplification were verified for the presence of the desired mutation and/or the absence of undesired alterations.

The *c-myc* probe used for Northern (RNA) blot analysis was derived by nick translation of a 400-bp *Pst*I fragment obtained from human *c-myc* exon II.

Immunoprecipitation, Western blotting (immunoblotting), and immunofluorescence analyses. Transfected HeLa cells (3×10^6 cells) and ES cell lines (10^7 cells) were rinsed once with methionine- and cysteine-free minimum Eagle's medium (Flow Laboratories) and incubated for 18 h in the same

FIG. 2. Expression studies and subcellular localization of FLI-1 and EWS-FLI-1. (A) An ES cell line (lanes 5 and 6) or HeLa cells transfected with either the control expression plasmid (lane 1) or expression plasmids for FLI-1(C) (lane 2), FLI-1 (lane 3), and EWS-FLI-1 (lane 4) were labeled for 18 h in the presence of an L-[³⁵S]methionine-L-[³⁵S]cysteine mixture and lysed. Immunoprecipitations were carried out on 10⁷ acid-insoluble counts of each cell lysates with a FLI-1-specific antiserum (lanes 1 to 5) or a control serum

medium supplemented with 1% dialyzed calf serum, 10% Dulbecco's modified Eagle's medium, and 250 μ Ci of a mixture of L-[³⁵S]methionine and L-[³⁵S]cysteine. Cells were washed twice in ice-cold phosphate-buffered saline and lysed in 3 ml of Tris-HCl (10 mM, pH 7.4)-NaCl (0.1 M)-EDTA (0.001 M)-Triton X-100 (1%)-sodium deoxycholate (0.5%)-sodium dodecyl sulfate (SDS; 0.1%)-aprotinin (1%)-phenylmethylsulfonyl fluoride (100 μ g/ml)-leupeptin (10 μ g/ml) and centrifuged at 100,000 $\times$ g for 30 min. Immunoprecipitation analyses were performed as described previously (22).

The FLI-1-specific antiserum used in this study was obtained from rabbits injected with a glutathione *S*-transferase fusion protein containing amino acid residues 359 to 447 of FLI-1. *Escherichia coli* cells transformed by pGEX1/FLI-1(362/452) (83) were induced to express the glutathione *S*-transferase fusion protein by treatment for 2 h in the presence of 0.4 mM isopropylthiogalactopyranoside (IPTG). The induced protein was purified by SDS-polyacrylamide gel electrophoresis and injected into rabbits as described previously (22). The rabbit antisera to the Ets domain of Ets-1 and to the carboxy-terminal domain (CTD) of ErbA have been described previously (22, 26). The polyclonal rabbit antiserum and the monoclonal antibody to the DNA-binding domain of Gal4 are generous gifts from S. Johnston (University of Texas, Medical School, Dallas) and Y. Lutz and P. Chambon (Laboratoire de Génétique Moléculaire des Eucaryotes, Strasbourg, France), respectively. Western blot analysis was performed after blotting of electrophoresed proteins on Immobilon membranes and incubation in the presence of a 1/1,000 dilution of the monoclonal antibody to the Gal4 DNA-binding domain. All procedures were performed by using the Amersham ECL kit as instructed by the manufacturer. Immunofluorescence analyses were performed on HeLa cells transfected with 5 μ g of the FLI-1 or EWS-FLI-1 expression vector or Δ EB-78 as a control. Two days after transfection, cells were fixed with 3.5% paraformaldehyde and permeabilized by incubation in 0.2% Triton X-100. After incubation for 1 h with a 1/300 dilution of the FLI-1-specific antiserum, cells were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins. After washing, immunofluorescence was analyzed with a Zeiss epifluorescence microscope.

Transfections and CAT assays. HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For transfection experiments, 5 $\times$ 10⁵ cells were seeded in 60-mm-diameter petri dishes and cotransfected 24 h later with 0.5 μ g of reporter plasmid and the indicated amounts of expression plasmids, using the calcium phosphate coprecipitation method. When the activities of different doses of expression plasmids are compared, the total amount of expression vector was kept constant by addition of the control expression plasmid. Conditions for cell lysis and chloramphen-

(lane 6). Immunoprecipitates were analyzed by electrophoresis on polyacrylamide gels in the presence of SDS, followed by fluorography. Light transmission scanning of the autoradiograms yielded relative band intensities of 1 for EWS-FLI-1 and FLI-1 and 2.1 for FLI-1(C). The methionine-plus-cysteine contents of EWS-FLI-1, FLI-1, and FLI-1(C) are 11, 20, and 9, respectively. (B) Subcellular localization of FLI-1 and EWS-FLI-1. HeLa cells transfected with the control expression plasmid (a) or an expression plasmid for FLI-1 (b) or EWS-FLI-1 (c) were seeded to collagen-treated coverslips, fixed in 3.5% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with the FLI-1-specific antiserum followed by a fluorescein isothiocyanate-conjugated goat serum to rabbit immunoglobulin G.

FIG. 3. DNA binding studies. (A) Electrophoretic mobility shift assays were carried out with 100 fmol of oligonucleotide A as a probe and extracts of HeLa cells transfected with either the control expression plasmid (lane 1) or an expression plasmid for FLI-1 (lane 2), EWS-FLI-1 (lane 3), or FLI-1 (C) (lane 4). Positions of the FLI-1 and EWS-FLI-1 complexes are indicated by a black arrowhead and an arrow, respectively; the position of the FLI-1(C) complex is indicated by a large open arrow. (B) Electrophoretic mobility shift assays were carried out with 100 fmol of oligonucleotide A as a probe and *S. frugiperda* Sf9 cells extracts as follows: extracts of Sf9 cells infected with an AcNPV-FLI-1 recombinant baculovirus (lanes 1 to 3), extracts of Sf9 cells infected with an AcNPV-EWS-FLI-1 recombinant baculovirus (lanes 4 to 6), extract of noninfected Sf9 cells (lane 7), or no extract (lanes 8 and 9). Two microliters of either the anti-FLI-1 antiserum (α -FLI-1) or, as a control, an anti-ErbA antiserum (α -ErbA) was included as indicated. Positions of the EWS-FLI-1 and FLI-1 complexes are indicated by an arrow and an arrowhead, respectively. (C) Electrophoretic mobility shift assays were carried out with 100 fmol of

oligonucleotide A as a probe and either control Sf9 cells extract (lane 1) or Sf9 cells extracts containing either FLI-1 (lanes 2 to 14) or EWS-FLI-1 (lanes 15 to 27). The binding reaction mixtures included either no competitor oligonucleotide (lanes 2 and 15), a 100-fold molar excess of oligonucleotide A (lanes 4 and 17) or oligonucleotide M (lanes 3 and 16), or a set of competitor oligonucleotides corresponding to specific EBSs (lanes 5 to 14 and 18 to 27). Position of the retarded complexes are indicated as in panel B. C, control.

ic acetyltransferase (CAT) assays were as described previously (8). The 6-2 and 6-2-57S+S as well as the tkD-2A, tkD-2B, and tkD-2D CAT reporter plasmids have been described previously (8, 21). The reporter construct for Gal4 fusion proteins contains five copies of a 17-mer Gal4 DNA-binding site (5'-CGGAGTACTGTCCTCCG-3'), inserted 5' of the adenovirus E1B TATA box/CAT reporter construct (36) (a generous gift of G. Gill, University of California, Berkeley).

Recombinant baculoviruses for FLI-1 and EWS-FLI-1 were obtained, using a Pharmingen kit, by cotransfection of *S. frugiperda* Sf9 cells with a linearized *Autographa californica* nuclear polyhedrosis virus (AcNPV) viral DNA lacking the polyhedrin gene with either the pVL941/FLI-1 or the pVL941/EWS-FLI-1 derivative. Recombinant viruses were plaque purified and further amplified by serial infection of Sf9 cells.

Electrophoretic mobility shift assays. Preparation of whole-cell extracts of transfected HeLa cells and of infected Sf9 cells as well as conditions for electrophoretic mobility shift assays were as described previously (8, 20). The Ets-binding site (EBS) used as a probe (oligonucleotide A) has the sequence (plus strand) 5'-ATAAACAGGAAGTGGT-3', flanked by *Ava*I restriction enzyme sites to allow for radioactive labeling by filling in with the Klenow fragment of DNA polymerase I in the presence of [α - 32 P]dCTP. The mutant EBS (oligonucleotide CAAGTGGT-3'), flanked by the same restriction site as the wild-type probe. Other EBS oligonucleotides used as competitors were as follows (plus strand, 5' to 3'): B, ATAAACAG GATGTGGT; C, ATAACCAGGAAGTGGG; D, AAATAT AGGAAGTGGT; E, TGAAACCGGAAGTGGG; F, TGA AAGAGGAAGTGG; G, CTTGGCGGGAAAAAGA; H, CAAAGGAGGAAAAACT; I, CAAAGGAGGAAATACT; J, ATGGGGAGGAAATGGG; and K, ATGGGGAGGATAT GGG. The Gal4-binding site used as a probe has the sequence (plus strand) 5'-CGGAGTACTGTCCTCC-3'.

RESULTS

To study the properties of the EWS-FLI-1 fusion protein, we made use of expression plasmids encoding either EWS-FLI-1, a deletion mutant restricted to the FLI-1-specific CTD of EWS-FLI-1 [FLI-1(C)], or a full-length human FLI-1 protein (Fig. 1). Expression of these proteins was analyzed by immunoprecipitation of extracts of HeLa cells transfected with the respective expression plasmids followed by metabolic labeling in the presence of a radioactive methionine and cysteine mixture. The antiserum used in these experiments is directed against 88 carboxy-terminal amino acid residues of FLI-1, a domain shared by all of these proteins. FLI-1 is detected as a doublet of 50 and 48 kDa, whereas FLI-1(C) and EWS-FLI-1 are expressed as polypeptides of 27 and 72 kDa (Fig. 2A). Analysis of ES cell lines expressing an *EWS-FLI-1* fusion transcript identified a single protein specifically immunoprecipitated by our antiserum which comigrates with the EWS-FLI-1 protein expressed in transfected HeLa cells (Fig. 2A and

data not shown). EWS-FLI-1 and FLI-1 were found to display half-lives of 240 and 200 min, respectively, in transfected HeLa cells, as analyzed by pulse-chase experiments (data not shown). This results in the accumulation of EWS-FLI-1 at a level approximately twofold higher (taking into account the content of methionine and cysteine residues of each protein; see the legend to Fig. 2) than that of *FLI-1*, as analyzed after an 18-h labeling period (Fig. 2A). In contrast, FLI-1(C) was found to be expressed at approximately fourfold-higher levels than FLI-1. Immunofluorescence analyses shows that both FLI-1 and EWS-FLI-1 localize to the nucleus of transfected cells (Fig. 2B).

The DNA-binding properties of these proteins were analyzed by electrophoretic mobility shift assay using an EBS oligonucleotide probe (oligonucleotide A; see Materials and Methods) and whole-cell extracts either of transfected HeLa cells or of Sf9 insect cells infected with recombinant baculoviruses. Extracts of nontransfected HeLa cells or of noninfected Sf9 cells were used as controls. Extracts of Sf9 cells infected with either the FLI-1 or EWS-FLI-1 recombinant baculovirus each generated a single retarded complex (Fig. 3B, lanes 1 and 4). These complexes are specific, since they are not observed in extracts of uninfected cells (Fig. 3B, lane 7) and since their formation is inhibited in the presence of the FLI-1-specific antiserum but not by a control serum (Fig. 3B, lanes 1 to 6). These complexes result from specific binding to DNA, since their formation is abolished by a 100-fold molar excess of unlabeled oligonucleotide A but not by the same excess of a mutant version of this oligonucleotide (oligonucleotide M; see Materials and Methods) carrying a GG-to-CC transversion in the EBS core sequence (Fig. 3C, lanes 2 to 4 and 15 to 17). The same specific complexes are observed in HeLa cells transfected with the FLI-1 and EWS-FLI-1 expression vectors, whereas expression of FLI-1(C) generated a faster-migrating complex (Fig. 3A). Taking into account their levels of expression in HeLa cells (Fig. 2), FLI-1, EWS-FLI-1, and FLI-1(C) appear to bind with the same efficiency to the oligonucleotide A probe. To study whether EWS-FLI-1 and FLI-1 recognize the same DNA sequences, competition electrophoretic mobility shift analyses were performed with oligonucleotide A as a probe and a series of EBS oligonucleotides as competitors (see Materials and Methods for sequences). The results show that the binding activities of FLI-1 and EWS-FLI-1 to the probe are similarly affected by all of the EBS competitors tested (Fig. 3C, lanes 4 to 14 and 17 to 27). We conclude from these experiments that EWS-FLI-1 and FLI-1 display similar DNA binding activities in vitro and bind DNA with similar specificities.

Transcription activation by EWS-FLI-1, FLI-1, and FLI-1(C) in HeLa cells was studied by cotransfection of the respective expression vectors with reporter plasmids containing the CAT gene under the control of distinct Ets-responsive promoters. We used two reporter plasmids which contain a duplicate copy of the Ets-responsive region (ERR1) of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat inserted upstream either of a -55 herpes simplex virus (HSV) thymidine kinase promoter (tkD-2A [21]) or of a minimal HTLV-I promoter (6-2-57S + S [8]). Previous analyses have shown that transcriptional activation of these reporters by Ets-1 depends not only on its binding to two specific EBS elements (ERE-A and ERE-B [8, 21, 24] but also on a tight synergy with factors like Sp1 which bind to a GC-rich element adjacent to ERE-A (21). FLI-1 and EWS-FLI-1 were found to activate CAT expression of both types of ERR1-based reporters in a dose-dependent manner (Fig. 4A and C). Mutation in ERE-A and ERE-B suppressed activation by FLI-1 and EWS-

FIG. 4. EWS-FLI-1 activates transcription from ERR1-containing recombinant promoters. HeLa cells were cotransfected with the indicated amounts of expression plasmids for FLI-1 (black bars), EWS-FLI-1 (hatched bars), FLI-1(C) (stippled bars), or the control expression plasmid (white bars) and 0.5 μg of the following reporter constructs: tkD-2A (A), either tkD-2A, tkD-2B, or tkD-2D, as indicated (B), or 6-2-57S+S (C). The total amount of expression plasmid was kept constant to 2 μg by addition of the control expression plasmid. Cells were lysed 48 h after transfection, and CAT assays were performed on cell lysates as described in Materials and Methods. For each reporter construct, fold stimulation is expressed with respect to CAT activity measured in cells transfected with the control (Co) expression plasmid. Results are means of three experiments, and standard error of the means are indicated by bars.

FIG. 5. Expression and transcriptional activation by Ets-1(C) chimeras. (A) HeLa cells transfected with either the control expression plasmid (lanes 1 and 2) or an expression plasmid for Ets-1 (C) (lane 3 and 4), EWS-Ets-1(C) (lane 5 and 6), or FLI-1(N)-Ets-1(C) (lane 7 and 8) were incubated for 18 h in the presence of L-[³⁵S]methionine-L-[³⁵S]cysteine and lysed. Immunoprecipitations were carried out on 10⁷ acid-insoluble counts of each lysates with an Ets-1 antiserum (α-Ets-1) (even-numbered lanes) or a control serum (odd-numbered lanes) and analyzed by polyacrylamide gel electrophoresis. Light transmission scanning of the autoradiogram yielded relative band intensities of 1 and 0.9 for EWS-Ets-1(C) and FLI-1(N)-Ets-1(C), respectively. The methionine-plus-cysteine contents of the proteins are 8 for EWS-Ets-1(C), 18 for FLI-1(N)-Ets-1(C), and 5 for Ets-1(C). (B) HeLa cells were cotransfected with the indicated amounts of expression plasmids for Ets-1(C) (stippled bars), FLI-1(N)-Ets-1(C) (black bars), and EWS-Ets-1(C) (hatched bars) or a control expression plasmid (white bar) and 0.5 µg of the tkD-2A reporter plasmid. The

FLI-1 (Fig. 4B, tkD-2B), and mutation of the Sp1-binding site inhibited activation by both proteins (Fig. 4B, tkD-2D). These results indicate that FLI-1 and EWS-FLI-1 are sequence-specific transcriptional activators which require the same determinants at the DNA level as those previously described for Ets-1 (21). The EWS-FLI-1 expression plasmid was found to maximally activate CAT expression from both types of ERR1-based reporters at concentrations approximately 10-fold lower than those required for the FLI-1 expression plasmid. The low level of induced CAT activity observed at a high concentration of the EWS-FLI-1 expression plasmid results from transcriptional squelching (Fig. 4A and C and data not shown). In contrast to FLI-1 and EWS-FLI-1, FLI-1(C) was found to only marginally activate CAT expression of these reporters (Fig. 4C), indicating that the amino-terminal domain of FLI-1 and the EWS region of EWS-FLI-1 play an essential role in the transcriptional activation properties of the respective proteins.

To investigate this point in further detail, we next analyzed the transcriptional activation properties of fusion proteins in which the EWS domain or the N-terminal region of FLI-1 was linked to the transcriptionally inactive DNA-binding domain of Ets-1 [Ets-1(C); Fig. 1]. The two fusion proteins were found to be expressed at similar levels in transfected HeLa cells (Fig. 5A) and to activate CAT expression from the tkD-2A reporter plasmid, with EWS-Ets-1(C) being more efficient than FLI-1(N)-Ets-1(C) (Fig. 5B). These results suggest that the FLI-1 amino-terminal half and the EWS region of EWS-FLI-1 each contain transcriptional activation domain(s).

To analyze whether the EWS and the FLI-1 amino-terminal domains can convey transcriptional activation independently of their association with an Ets domain, these regions were fused to the heterologous DNA-binding domain of the yeast transcriptional activator Gal4 [Gal4(1-147); Fig. 1] to generate Gal4-EWS and Gal4-FLI-1(N). We also analyzed the properties of a Gal4(1-147) fusion protein containing the carboxy-terminal domain of FLI-1, Gal4-FLI-1(C). To avoid the possible interference between the Gal4 and FLI-1 DNA-binding domains, a Gal4-FLI-1(C) fusion protein containing a point mutation (W-321→R) in the DNA-binding domain of FLI-1 [Gal4-FLI-1(C)(mut)] was also tested. Consistent with the effect of a similar mutation in Ets-1 (69), this substitution was found to abolish the specific DNA-binding activity of FLI-1 toward EBS (data not shown). Expression plasmids for each of these proteins were cotransfected in HeLa cells together with a CAT reporter plasmid containing five copies of a 17-mer Gal4-binding site inserted upstream of the adenovirus E1B TATA box (36). As shown in Fig. 6, both Gal4-FLI-1(N) and Gal4-EWS activate CAT expression from this reporter, the latter being more active by 2 orders of magnitude (Fig. 6A). In contrast, Gal4-FLI-1(C) and Gal4-FLI-1(C)(mut) were found to be inactive. The difference in activity between Gal4-FLI-1(N) and Gal4-EWS cannot be explained by a difference in expression levels, since Gal4-FLI-1(N) was found to be expressed at higher levels than Gal4-EWS in transfected cells, as analyzed by Western blotting with a monoclonal antibody specific for the DNA-binding domain of Gal4 (Fig. 6C; compare lanes 4 and 5). Extracts of transfected cells each generated distinct complexes by electrophoretic mobility shift assay using a Gal4 17-mer oligonucleotide probe

total amount of expression plasmid was kept constant to 2 µg by addition of the control (Co) expression plasmid. Cells were lysed 48 h after transfection, and CAT assays were performed as described in Materials and Methods. Fold stimulation is expressed with respect to the activity of the control expression plasmid.

(Fig. 6B, lanes 6, 8, 10, and 12). These complexes are specific, since they are not observed in extracts of nontransfected control cells (Fig. 6B, lane 1) and since they can be specifically supershifted by a Gal4-specific antiserum (Fig. 6B, lanes 7, 9, 11, and 13) but not by a control serum. The same relative binding activities of the different Gal4 fusion proteins were observed when the probe was used at subnanomolar concentration (0.3 nM; data not shown). Since the relative intensities of each of these complexes paralleled the level of expression as analyzed by Western blotting, the difference in transcriptional activity between the Gal4 fusion proteins cannot be ascribed to differences in DNA-binding activity. We conclude from these experiments that the FLI amino-terminal region and the EWS domain contain transcriptional activation domains and that the transcriptional activation properties of EWS-FLI-1 result from the substitution of the normal activation domain(s) of FLI-1 by the EWS sequences.

Proteins of the Ets family have recently been proposed to play a role in relaying the *c-myc* response to growth factor stimulation. Expression of a dominant negative form of Ets-2 in NIH 3T3 cells harboring an ectopic colony-stimulating factor 1 (CSF-1) receptor has been found to impair both induction of the *c-myc* gene in response to CSF-1 stimulation and the ability of CSF-1 to induce colony formation of these cells in semisolid medium (34). Furthermore, a mutant form of the CSF-1 receptor [CSF-1-R(Y809F) (57)] defective in its mitogenic response to CSF-1 and in its ability to induce *c-myc* expression can be rescued by overexpression of either *c-myc* or Ets-2 (34). Elevated expression of *c-myc* has been shown to be a hallmark of ES and PN tumor cells and cell lines, in contrast to the situation in other tumors of neuroectodermal origin such as neuroblastomas (41) (see Fig. 7A). We therefore examined whether EWS-FLI-1 could affect *c-myc* promoter activity. A 862-bp human *c-myc* promoter fragment (see Materials and Methods) was inserted 5' of the CAT gene of pCAT-3M (32). This fragment, which extends from positions -511 to +351 with respect to P2, includes the major P1 and P2 promoters but lacks the sequences in *c-myc* exon 1 which have been shown to be essential for transcriptional attenuation (5). Cotransfection of this reporter with an expression plasmid for EWS-FLI-1 in HeLa cells resulted in a dose-dependent acti-

FIG. 6. Expression and transcriptional activation by Gal4 chimeras. (A) HeLa cells were cotransfected with either 5 ng (white bars) or 20 ng (hatched bars) of the indicated expression plasmids and 0.5 μ g of Gal4/E1b TATA reporter construct. Cells were lysed 48 h after transfection, and CAT assays were carried out on amounts of cell lysates chosen to keep the percentage of conversion in the linear range of the reaction. (B) Expression and DNA binding of GAL4 derivatives used in cotransfection experiments. Electrophoretic mobility shift assays were carried out with 400 fmol of the 17-mer Gal4-binding site oligonucleotide as a probe and extracts of HeLa cells transfected with either the control expression plasmid (lanes 1 and 2) or the expression plasmid for Gal4(1-147) (lanes 3 to 5), Gal4-FLI-1(C) (lanes 6 and 7), Gal4-FLI-1(C)(mut) (lanes 8 and 9), Gal4-FLI-1(N) (lanes 10 and 11), or Gal4-EWS (lanes 12 to 14). To analyze the specificity of the each complex, antisera (1 μ l) directed against Gal4 (α -Gal4) and c-ErbA (α -ErbA) as a control (Co) were included in the binding reactions as indicated. (C) Western blot analysis of the expression of Gal4 derivatives. Proteins (0.5 μ g) of the whole-cell extracts used in panel B were separated by electrophoresis on polyacrylamide gels in the presence of SDS and electroblotted on a nylon membrane. The membrane was incubated in the presence of a 1/1,000 dilution of a monoclonal antibody to the DNA-binding domain of Gal4, washed, revealed by using an ECL (Amersham) chemiluminescence kit, and autoradiographed.

FIG. 7. Overexpression of *c-myc* in ES cell lines and transactivation of the human *c-myc* promoter by EWS-FLI-1. (A) Northern blot analysis showing *c-myc* overexpression in ES cell lines. Total cellular RNAs (15 μ g), prepared as described in Materials and Methods, were electrophoresed on a 1% agarose denaturing gel and transferred to a nylon membrane. The membrane was hybridized with an exon 2 *c-myc* probe. Lanes: 1 to 10, RNAs from cell lines derived from different ESs; 11, RNA from a neuroblastoma cell line. (B) HeLa cells were cotransfected with the indicated amounts of the expression plasmid for EWS-FLI-1 (hatched bars), EWS-FLI-1(R2L2) (black bars), or FLI-1(C) (stippled bars) or the control expression plasmid (white bar) and 0.5 μ g of either the MYCP-CAT-3M, pCAT-3M, or 6-2 reporter construct. The total amount of expression plasmid was kept constant by addition of the appropriate amount of control expression plasmid. Cells were lysed 48 h after transfection, and CAT assays were performed on cell lysates as described in Materials and Methods. Fold stimulation is expressed with respect to the activity of the control (Co) expression plasmid. The results shown are means of a triplicate experiment; standard errors of the means are indicated by bars. (C) EWS-FLI-1 binding to *c-myc* promoter oligonucleotides. Electropho-

retic mobility shift assays were carried out with 50 fmol of oligonucleotide A as a probe and extracts of *S. frugiperda* Sf9 cells infected with the AcNPV-EWS-FLI-1 baculovirus. The binding reaction mixtures included either no competitor (comp.) oligonucleotide (lane 1), a 25-fold molar excess of unlabeled oligonucleotide A (lane 4), or a 100-fold (lanes 2, 6, 8, 10, 12, 14, and 16) or 400-fold (lanes 3, 5, 7, 9, 11, 13, and 15) molar excess of the indicated nonlabeled competitor oligonucleotides. Oligonucleotides myc-1 to myc-7 correspond to the following *c-myc* promoter sequences (numbering is with respect to P2 and centered over the GGAA/T core): myc-1 (-62), 5'-CTCGAGCT TGGCGGGAAGAAAG-3'; myc-2 (-475), 5'-GACAAGGAT GCGGT-3'; myc-3 (-348), 5'-TTTACATCCTAGAG-3'; myc-4 (-222), 5'-AAAAGATCGTCTCT-3'; myc-5 (+191), 5'-GCCG CATCCACGAA-3'; myc-6 (+306), 5'-GACACTCCCCGCC-3'; and myc-7 (+111), 5'-GGGAGATCCGGAGC-3'.

retic mobility shift assays were carried out with 50 fmol of oligonucleotide A as a probe and extracts of the FLI-1(C) expression plasmid (Fig. 7B). Transactivation of the *c-myc* promoter construct by EWS-FLI-1 is specific, since no significant activation of CAT expression was observed when a pCAT-3M reporter either lacking any promoter fragment or containing a minimal (-52) HTLV-I promoter (6-2 [8]) was used (Fig. 7B). Activation of the *c-myc* promoter is also observed following cotransfection of expression vectors for FLI-1 or Ets-1 (data not shown). The DNA-binding activity of EWS-FLI-1 is required for activation of the *c-myc* promoter, since a mutant EWS-FLI-1 protein unable to bind DNA because of the mutation of two conserved arginine residues in its DNA-binding domain is inactive [EWS-FLI-1(R2L2); Fig. 7B]. The *c-myc* promoter fragment was found to be unable to bind EWS-FLI-1 in vitro, as analyzed by DNase I footprinting analysis (data not shown). Of note, although this fragment lacks a high-affinity EBS, it contains several motifs centered over a GGAA/T core sequence which may correspond to low-affinity binding sites (see the legend to Fig. 7C). Oligonucleotides spanning these sites were synthesized and used as competitors in electrophoretic mobility shift assays using oligonucleotide A as a probe. As shown in Fig. 7C, except for oligonucleotide myc-7, which was able to partially compete when tested at a 400-fold molar excess, none of these motifs were found to efficiently bind to EWS-FLI-1. The low-affinity in vitro binding site identified in this analysis does not appear to be instrumental in the *c-myc* promoter response in vivo, since its deletion failed to affect the activation of the *c-myc* promoter in response to FLI-1 or EWS-FLI-1 (data not shown). We conclude from these experiments that enforced expression of EWS-FLI-1 upregulates the activity of the human *c-myc* promoter by a mechanism which appears to be indirect.

DISCUSSION

This study establishes that the EWS-FLI-1 fusion transcript characteristic of ES and PN tumor cells encodes a nuclear protein in which the DNA-binding domain of FLI-1 is fused to the amino-terminal domain of EWS, a protein of unknown function. The fusion protein that we analyzed corresponds to the translation product of the previously defined type I fusion transcripts (16). Type I transcripts are found in about half of the ES and PN cases examined and result from the junction of exons 1 to 7 of the EWS gene to exons 6 to 9 of FLI-1 (83). The properties of the type I EWS-FLI-1 protein are very likely to be generalized to those of the products encoded by the variant fusion transcripts observed in other ES and PN cases (16, 40, 83). Indeed, the products of these variant fusion transcripts

retic mobility shift assays were carried out with 50 fmol of oligonucleotide A as a probe and extracts of *S. frugiperda* Sf9 cells infected with the AcNPV-EWS-FLI-1 baculovirus. The binding reaction mixtures included either no competitor (comp.) oligonucleotide (lane 1), a 25-fold molar excess of unlabeled oligonucleotide A (lane 4), or a 100-fold (lanes 2, 6, 8, 10, 12, 14, and 16) or 400-fold (lanes 3, 5, 7, 9, 11, 13, and 15) molar excess of the indicated nonlabeled competitor oligonucleotides. Oligonucleotides myc-1 to myc-7 correspond to the following *c-myc* promoter sequences (numbering is with respect to P2 and centered over the GGAA/T core): myc-1 (-62), 5'-CTCGAGCT TGGCGGGAAGAAAG-3'; myc-2 (-475), 5'-GACAAGGAT GCGGT-3'; myc-3 (-348), 5'-TTTACATCCTAGAG-3'; myc-4 (-222), 5'-AAAAGATCGTCTCT-3'; myc-5 (+191), 5'-GCCG CATCCACGAA-3'; myc-6 (+306), 5'-GACACTCCCCGCC-3'; and myc-7 (+111), 5'-GGGAGATCCGGAGC-3'.

differ from type I only by the presence of additional EWS sequences encoded by either exons 8 and 9 or 8, 9, and 10 of EWS and always include exons encoding the DNA-binding (Ets domain) of FLI-1 (16, 83).

EWS-FLI-1 contains a complete and nonmutated form of the Ets domain of FLI-1. Consistent with the fact that the Ets domain appears to be both necessary and sufficient for DNA binding (9, 20, 30, 37, 73), EWS-FLI-1 was found to bind DNA sequences indistinguishable from those bound by FLI-1.

In contrast, substitution of the FLI-1 amino-terminal domain by the chromosome 22-derived EWS sequence was found to confer upon EWS-FLI-1 distinct transcriptional activation properties. First, the FLI-1-encoded domain of EWS-FLI-1 does not efficiently activate transcription when acting either on its own on ERE-based reporter plasmids or as a fusion with the Gal4 DNA-binding domain on Gal4-responsive reporter constructs. Second, EWS-FLI-1 was found to activate transcription of ERE-based reporters at lower stoichiometric levels than FLI-1. Third, when fused to the heterologous DNA-binding domain of the yeast transcriptional activator Gal4, the EWS region was found to encode a stronger activation domain compared with the amino-terminal domain of FLI-1 analyzed in the same conditions.

Several lines of evidence implicate Ets family members in developmental processes and in the response of cells to extracellular signals. In *Drosophila melanogaster*, E74 is one of the early genes induced in response to ecdysone during the initiation of larva-to-adult metamorphosis, and Yan/Pokkuri appears to play a major role in the negative control of differentiation of the precursors of R7 photoreceptor cells in response to the *sevenless* signal transduction pathway (12, 31, 62). In higher eucaryotes, expression of distinct members of the Ets family has been shown to be rapidly and transiently induced in specific cell types in response to specific extracellular factors (7, 10). Furthermore, EBSs contribute to transcriptional activation of promoter/enhancer sequence in response to serum, phorbol esters, antigen, and nonnuclear oncogenes (27, 72, 80). Finally, Ets proteins are modified by specific phosphorylation events in response to extracellular stimuli, some of which regulate diverse aspects of their function (19, 39, 51, 52). The transcriptional activation domains of members of the Ets family are the direct targets of intracellular signaling pathways. For example, the Elk-1 activation domain is phosphorylated on several residues by mitogen-activated protein kinase, and these phosphorylation events are essential for the serum response element-mediated transcriptional activation of the *c-fos* proto-oncogene (39). Likewise, the transcriptional activation regions of Ets-1 and Ets-2 are modified by several protein kinases in response to extracellular signals, including mitogen-activated protein kinase (54a). If the amino-terminal domain of FLI-1 is also a target for intracellular signaling pathways, its substitution by EWS sequences would be expected to result in disruption of the regulated activity of FLI-1. On the other hand, since the DNA-binding specificity of FLI-1 is similar to that of ERG, ETS-1, and ETS-2 and since FLI-1 is undetectable in the majority of ES and PN cell lines analyzed (83), expression of EWS-FLI-1 could pervade regulatory circuits normally regulated by other members of the Ets family. Our results are consistent with a model in which abnormal expression of EWS-FLI-1 from the *EWS* promoter region would contribute to the transformed phenotype of ES and PN tumor cells by inducing the unscheduled and/or deregulated activation of genes normally controlled either by FLI-1 or by other select members of the Ets family. In line with this notion, although 85% of the cases of ES and PN are characterized by the expression of an EWS-FLI-1 protein as

the consequence of the t(11;22) translocation, most of the remaining cases express a novel protein in which EWS sequences are fused to the DNA-binding domain of ERG (83), a close relative of FLI-1. Furthermore, overexpression of either Ets-1 and Ets-2 (60, 61) or EWS-FLI-1 (40) in NIH 3T3 cells can induce morphological transformation of these cells.

Disruption of normal regulation of other families of transcription factors by fusion with EWS sequences appears to operate in other tumors as well. Recent evidence has shown that the amino-terminal domain of EWS is fused to the DNA-binding and dimerization motifs of ATF-1 in malignant melanoma of soft tissues as the result of a t(12;22) chromosomal translocation (81) to generate a hybrid transcriptional activator of cyclic AMP response element-based reporter plasmids (2a). Furthermore, the amino-terminal domain of the *TLS* gene, which is 55.6% identical to *EWS*, has recently been shown to be fused to the DNA-binding domain of CHOP, a member of the c/EBP family of transcriptional activators (14). Our results show that the EWS sequence found in EWS-FLI-1, EWS-ERG, and EWS-ATF-1 encodes a transcriptional activation domain. As noted previously (16), this sequence is characterized by a repetitive structure which resembles but is nevertheless markedly distinct from the heptapeptide repeats in the CTD of RNA polymerase II. Although the RNA polymerase II CTD is essential for cell viability in eucaryotes (1, 3, 47), its precise role in transcriptional initiation is still debated. Recent evidence has shown that the nonphosphorylated form of RNA polymerase II interacts directly with the TATA-binding protein (TBP) through the CTD (68). It is therefore conceivable that the EWS domain of these fusion proteins increases the probability of formation of a productive initiation complex by directly interacting with TBP. Alternatively, since the EWS domain of EWS-FLI-1 is rich in glutamine and proline residues, features which are reminiscent of the activation domains of other transcription factors (13, 42), it may act through specific TBP-associated factors (17, 54).

Our results suggest that upregulation of the *c-myc* proto-oncogene could be controlled by EWS-FLI-1 in ES and NP tumor cells, since elevated expression of *c-myc* is a hallmark of ES and NP tumor cells among other childhood tumors of neuroectodermal origin and since EWS-FLI-1 upregulates the activity of *c-myc* promoter constructs in transient transfection assays. In line with the model proposed above, this property is not restricted to EWS-FLI-1, since other closely related members of the Ets family such as FLI-1 or Ets-1 but not Pu-1/Spi-1 also activate *c-myc* promoter activity (data not shown). Available evidence suggests that *c-myc* promoter activation by EWS-FLI-1 occurs through an indirect mechanism, since EWS-FLI-1 was found unable to bind in vitro to the *c-myc* promoter fragment used in this study. Experiments are in progress to identify the EWS-FLI-1 response element(s) in the human *c-myc* promoter and to study how EWS-FLI-1 modifies the activity of these elements. Enforced expression of Ets-1 or Ets-2 has recently been shown to rescue both the G₁ block observed in NIH 3T3 cells expressing a mutant form of the CSF-1 receptor [CSF-1R(Y809F)] and the defective induction of *c-myc* expression of these cells in response to CSF-1 (34, 56a). Expression of EWS-FLI-1 as observed in ES and NP cells could therefore contribute, via its ability to constitutively deregulate *c-myc* expression, to release of these cells from normal growth and/or differentiation control.

ACKNOWLEDGMENTS

We thank Pierre Chambon (LGME, Strasbourg, France), Grace Gill (University of California, Berkeley), Stephen Johnston (University of Texas, Dallas), and Christian Larsen (Hôpital St. Louis, Paris, France)

for reagents; Martine Pironin for technical assistance; A. Sureau for help in scanning the autoradiograms; and Klara Beresnikoff and Sylvie Le Coidic for secretarial assistance.

This work was supported in part by NIH grant CA-56819 and St. Jude Children's Research Hospital (M.R.) and by funds from the Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Association pour la Recherche sur le Cancer, Ligue Nationale Française Contre le Cancer, and Institut Curie (G.T. and J. G.). R.-A.B. is a recipient of a predoctoral fellowship from the Ligue Nationale Française Contre le Cancer.

REFERENCES

- Allison, L. A., K.-C. Wong, V. D. Fitzpatrick, M. Moyle, and C. J. Ingles. 1988. The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammals: a conserved structure with an essential function. *Mol. Cell. Biol.* **8**:321-329.
- Aurias, A., C. Rimbaut, C. Buffe, J. Dubousset, and A. Mazabraud. 1983. Chromosomal translocation in Ewing's sarcoma. *N. Engl. J. Med.* **309**:496-497.
- Bailly, R.-A., et al. Unpublished data.
- Bartolomei, M. S., N. F. Halden, C. R. Cullen, and J. L. Corden. 1988. Genetic analysis of the repetitive carboxy-terminal domain of the largest subunit of mouse RNA polymerase II. *Mol. Cell. Biol.* **8**:330-339.
- Ben-David, Y., E. Gidden, K. Letwen, and A. Bernstein. 1991. Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the *ets* gene family, *FLI-1*, closely linked to *c-ets1*. *Genes Dev.* **5**:908-918.
- Bentley, D. L., and M. Groudine. 1988. Sequence requirements for premature termination of transcription in the human *c-myc* gene. *Cell* **53**:245-256.
- Beug, H., M. J. Hayman, and T. Graf. 1982. Myeloblasts transformed by the avian leukemia virus E26 are hormone-dependent for growth and for expression of a putative *myb*-continuing protein, p135 E26. *EMBO J.* **1**:1069-1075.
- Bhat, N. K., R. J. Fisher, S. Fujiwara, R. Ascione, and T. S. Papas. 1987. Temporal and tissue-specific expression of mouse *ets* genes. *Proc. Natl. Acad. Sci. USA* **84**:3161-3165.
- Bosselut, R., J. Duvall, A. Gégonne, M. Bailly, A. Hemar, J. Brady, and J. Ghysdael. 1990. The product of the *c-ets1* protooncogene and the related Ets2 protein act as transcriptional activators of the long terminal repeat of human T cell leukemia virus HTLV-1. *EMBO J.* **9**:3137-3144.
- Boulukos, K. E., P. Pognonec, B. Rabault, A. Begue, and J. Ghysdael. 1989. Definition of an Ets1 protein domain required for nuclear localization in cells and DNA-binding activity in vitro. *Mol. Cell. Biol.* **9**:5718-5721.
- Boulukos, K. E., P. Pognonec, E. Sariban, M. Bailly, C. Lagrou, and J. Ghysdael. 1990. Rapid and transient expression of *Ets2* in mature macrophages following stimulation with cMGF, LPS and PKC activators. *Genes Dev.* **4**:401-409.
- Brown, T. A., and S. L. McKnight. 1992. Specificities of protein-protein and protein-DNA interaction of GABP α and two newly defined *ets*-related proteins. *Genes Dev.* **6**:2502-2512.
- Burtis, K. C., C. S. Thummel, C. W. Jones, F. D. Karim, and D. S. Hogness. 1990. The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell* **61**:85-99.
- Courey, A. J., and R. Tjian. 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* **55**:887-898.
- Crozat, A., P. Aman, N. Mandahl, and D. Ron. 1993. Fusion of CHOP to a novel DNA-binding protein in human myxoid liposarcoma. *Nature (London)* **363**:640-644.
- Dalton, S., and R. Treisman. 1992. Characterization of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. *Cell* **68**:597-612.
- Delattre, O., J. Zucman, B. Plougastel, C. Desmaze, T. Melot, M. Peter, H. Kovar, I. Joubert, P. de Jong, G. Rouleau, A. Aurias, and G. Thomas. 1992. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature (London)* **359**:162-165.
- Dynlacht, B. D., T. Hoey, and R. Tjian. 1991. Isolation of coactivators associated with the TATA binding protein that mediate transcriptional activation. *Cell* **66**:563-576.
- Fisher, R. J., G. Mavrothalassitis, A. Konodoh, and T. S. Papas. 1991. High-affinity DNA-protein interactions of the cellular ETS1 protein; the determination of the ETS binding motif. *Oncogene* **6**:2249-2254.
- Fujiwara, S., R. J. Fisher, N. K. Bhat, S. M. Diaz de la Espina, and T. S. Papas. 1988. A short-lived nuclear phosphoprotein encoded by the human *ets-2* proto-oncogene is stabilized by activation of protein kinase C. *Mol. Cell. Biol.* **8**:4700-4706.
- Gégonne, A., B. Punyamalee, B. Rabault, R. Bosselut, S. Seneca, M. Crabeel, and J. Ghysdael. 1992. Analysis of the DNA binding and transcriptional activation properties of the *Ets1* oncoprotein. *New Biol.* **4**:512-519.
- Gégonne, A., R. Bosselut, R.-A. Bailly, and J. Ghysdael. 1993. Synergistic activation of the HTLV1 LTR Ets-responsive region by transcription factors Ets1 and Sp1. *EMBO J.* **12**:1169-1178.
- Ghysdael, J., A. Gégonne, P. Pognonec, D. Dernis, D. Leprince, and D. Stehelin. 1986. Identification and preferential expression in thymic and bursal lymphocytes of a *c-ets* oncogene-encoded M_R 54 000 cytoplasmic protein. *Proc. Natl. Acad. Sci. USA* **83**:1714-1718.
- Ghysdael, J., and M. Yaniv. 1991. Nuclear oncogenes. *Curr. Opin. Cell Biol.* **3**:484-492.
- Gitlin, S. D., R. Bosselut, A. Gégonne, J. Ghysdael, and J. N. Brady. 1991. Sequence-specific interaction of the Ets1 protein with the long terminal repeat of the human T-lymphotropic virus type I. *J. Virol.* **65**:5513-5523.
- Golay, J., M. Introna, and T. Graf. 1988. A single point mutation in the *v-ets* oncogene affects both erythroid and myelomonocytic cell differentiation. *Cell* **55**:1147-1158.
- Goldberg, Y., C. Glineur, J. C. Gesquiere, A. Ricouart, J. Sap, B. Vennström, and J. Ghysdael. 1988. Activation of protein kinase C or cAMP-dependent protein kinase increases phosphorylation of the *c-erbA*-encoded thyroid hormone receptor and of the *v-erbA*-encoded protein. *EMBO J.* **7**:2425-2433.
- Graham, R., and M. Gilman. 1991. Distinct protein targets for signals acting at the *c-fos* serum response element. *Science* **251**:189-192.
- Gunter, C. V., J. A. Nye, R. S. Bryner, and B. J. Graves. 1990. Sequence-specific DNA binding of the proto-oncoprotein *ets-1* defines a transcriptional activator sequence within the long terminal repeat of the Moloney murine sarcoma virus. *Genes Dev.* **4**:667-679.
- Hipskind, R. A., V. N. Rao, C. G. F. Mueller, E. S. P. Reddy, and A. Nordheim. 1991. Ets-related protein Elk-1 is homologous to the *c-fos* regulatory factor p62^{TCF}. *Nature (London)* **354**:531-534.
- Klemsz, M. J., S. R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki. 1990. The macrophage and B cell specific transcription factor PU-1 is related to the *ets* oncogene. *Cell* **61**:113-124.
- Lai, Z.-C., and G. M. Rubin. 1992. Negative control of photoreceptor development in *drosophila* by the product of the *yan* gene, an Ets domain protein. *Cell* **70**:609-620.
- Laimins, L. A., P. Gruss, R. Pozzatti, and G. Khoury. 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. *J. Virol.* **49**:183-189.
- La Marco, K., C. C. Thompson, B. P. Byers, E. M. Walton, and S. L. McKnight. 1991. Identification of Ets- and Notch-related subunits in GA binding proteins. *Science* **253**:789-792.
- Langer, S. L., D. M. Bortner, M. F. Roussel, C. J. Sherr, and M. C. Ostrowski. 1992. Mitogenic signaling by colony-stimulating factor 1 and *ras* is suppressed by the *ets-2* DNA-binding domain and restored by *myc* overexpression. *Mol. Cell. Biol.* **12**:5355-5362.
- Leprince, D., A. Gégonne, J. Coll, C. De Taisne, A. Schneeberger, C. Lagrou, and D. Stehelin. 1983. A putative second cell-derived oncogene of the avian leukemia retrovirus E26. *Nature (London)* **306**:395-397.
- Lillie, J. W., and M. R. Green. 1989. Transcription activation by the adenovirus E1a protein. *Nature (London)* **338**:39-44.
- Lim, F., N. Kraut, J. Frampton, and T. Graf. 1992. DNA binding by c-Ets-1, but not v-Ets, is repressed by an intramolecular mechanism. *EMBO J.* **11**:643-652.

38. Lipp, M., R. Schilling, S. Wiest, G. Laux, and G. W. Bornkamm. 1987. Target sequences for *cis*-acting regulation within the dual promoter of the human *c-myc* gene. *Mol. Cell. Biol.* 7:1393-1400.
39. Marais, R., J. Wynne, and R. Treisman. 1993. The SRF accessory protein Elk-1 contains a growth factor-regulator transcriptional activation domain. *Cell* 73:381-393.
40. May, W. A., M. L. Gishizky, S. L. Lessnick, L. B. Lunsford, B. C. Lewis, O. Delattre, J. Zucman, G. Thomas, and C. T. Denny. 1993. Ewing sarcoma 11,22 translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI-1 for transformation. *Proc. Natl. Acad. Sci. USA* 90:5752-5756.
41. McKeon, C., C. J. Thiele, R. A. Ross, M. Kwan, T. J. Triche, J. S. Miser, and M. A. Israel. 1988. Distinguishable patterns of protooncogene expression in two distinct but closely related tumors: Ewing's sarcoma and neuroepithelioma. *Cancer Res.* 48:4307-4311.
42. Mermod, N., O. A. O'Neil, T. J. Kelly, and R. Tjian. 1989. The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. *Cell* 58:741-753.
43. Metz, T., and T. Graf. 1991. *v-myb* and *v-ets* transform chicken erythroid cells and cooperate both in *trans* and in *cis* to induce distinct differentiation phenotypes. *Genes Dev.* 5:369-380.
44. Metz, T., and T. Graf. 1991. Fusion of the nuclear oncoproteins *v-Myb* and *v-Ets* is required for the leukemogenicity of E26 virus. *Cell* 66:95-105.
45. Moreau-Gachelin, F., D. Ray, M.-G. Mattei, P. Tambourin, and A. Tavittian. 1989. The putative oncogene Spi-1: murine chromosomal localization and transcription activation in murine acute erythroleukemias. *Oncogene* 4:1449-1456.
46. Moreau-Gachelin, F., A. Tavittian, and P. Tambourin. 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukemias. *Nature (London)* 331:277-280.
47. Nonet, M., D. Sweetser, and R. A. Young. 1987. Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. *Cell* 50:909-915.
48. Nunn, M., and T. Hunter. 1989. The *ets* sequence is required for induction of erythroblastosis in chickens by avian retrovirus E26. *J. Virol.* 63:398-402.
49. Nunn, M., P. H. Seeburg, C. Moscovici, and P. H. Duesberg. 1983. Tripartite structure of the avian erythroblastosis virus E26 transforming gene. *Nature (London)* 306:391-395.
50. Nye, J. A., J. M. Petersen, C. V. Gunther, M. D. Jonsen, and B. J. Graves. 1992. Interaction of murine Ets-1 with CGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes Dev.* 6:975-990.
51. Pognonec, P., K. E. Boulukos, J. C. Gesquiere, D. Stehelin, and J. Ghysdael. 1988. Mitogenic stimulation of thymocytes results in the calcium-dependent phosphorylation of *c-ets1* proteins. *EMBO J.* 7:977-983.
52. Pognonec, P., K. E. Boulukos, and J. Ghysdael. 1989. The *c-ets-1* protein is chromatin associated and binds to DNA *in vitro*. *Oncogene* 4:691-697.
53. Pongubala, J. M. R., S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison. 1992. PU.1 recruits a second nuclear factor to a site important for immunoglobulin K 3' enhancer activity. *Mol. Cell. Biol.* 12:368-378.
54. Pugh, B. F., and R. Tjian. 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev.* 5:1935-1945.
- 54a. Rabault, B., and J. Ghysdael. Unpublished data.
55. Rao, V. N., and E. S. P. Reddy. 1992. A divergent *ets*-related protein, Elk-1, recognized similar *c-ets-1* proto-oncogene target sequences and acts as a transcriptional activator. *Oncogene* 7:65-70.
56. Reddy, E. S. P., and V. N. Rao. 1991. Erg: an *ets*-related gene, codes for sequence-specific transcriptional activators. *Oncogene* 6:2285-2289.
- 56a. Roussel, M. F., J. N. Davis, J. L. Cleveland, J. Ghysdael, and S. W. Hiebert. 1994. Dual control of *myc* expression through a single DNA binding site targeted by *ets* family proteins and E2F1. *Oncogene* 9:405-415.
57. Roussel, M. F., J. L. Cleveland, S. A. Shurtleff, and C. J. Sherr. 1991. Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signaling. *Nature (London)* 353:361-363.
58. Roussel, M. F., S. A. Shurtleff, J. R. Downing, and C. J. Sherr. 1990. A point mutation at tyrosine 809 in the human colony stimulating factor 1 receptor impairs mitogenesis without abrogating tyrosine kinase activity, association with phosphatidylinositol-3-kinase, or induction of *fos* and *jun* genes. *Proc. Natl. Acad. Sci. USA* 87:6738-6742.
59. Schneikert, J., Y. Lutz, and B. Wasylyk. 1992. Two independent activation domains in *c-Ets-1* and *c-Ets-2* located in non-conserved sequences of the *ets* gene family. *Oncogene* 7:249-256.
60. Seth, A., and T. S. Papas. 1990. The *c-ets-1* proto-oncogene has oncogenic activity and is positively autoregulated. *Oncogene* 5:1761-1767.
61. Seth, A., K. Watson, D. G. Blair, and T. S. Papas. 1989. The *c-ets-2* protooncogene has mitogenic and oncogenic activity. *Proc. Natl. Acad. Sci. USA* 86:7833-7837.
62. Tei, H., I. Nihonmatsu, T. Yokokura, R. Ueda, Y. Sano, T. Okuda, K. Sato, K. Hirata, S. C. Fujita, and D. Yamamoto. 1992. pokkuri, a Drosophila gene encoding an E-26-specific (Ets) domain protein, prevents overproduction of the R7 photoreceptor. *Proc. Natl. Acad. Sci. USA* 89:6856-6860.
63. Thompson, C. B., C.-Y. Wang, I.-C. Ho, P. R. Bohjanen, B. Petryniak, C. H. June, S. Miesfeldt, L. Zhang, G. J. Nabel, B. Karpinski, and J. M. Leiden. 1992. *cis*-acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. *Mol. Cell. Biol.* 12:1043-1053.
64. Thompson, C. C., T. A. Brown, and S. L. McKnight. 1991. Convergence of Ets- and notch-related structural motifs in a heteromeric DNA binding complex. *Science* 253:762-768.
65. Turc-Carel, C., I. Philip, M. P. Berger, and G. Lenoir. 1983. Chromosomal translocation in Ewing's sarcoma. *N. Engl. J. Med.* 309:497-498.
66. Turc-Carel, C., I. Philip, M. P. Berger, T. Philip, and G. Lenoir. 1984. Chromosome study of Ewing's sarcoma cell-lines. Consistency of a reciprocal translocation t(11;22)(q24;q12). *Cancer Genet. Cytogenet.* 12:1-19.
67. Urness, L. D., and C. S. Thummel. 1990. Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the Drosophila ecdysone-inducible E74A protein. *Cell* 63:47-61.
68. Usheva, A., E. Maldonado, A. Goldring, H. Lu, C. Houbavi, D. Rainberg, and Y. Aloni. 1992. Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. *Cell* 69:871-881.
69. Wang, C.-Y., B. Petryniak, I.-C. Ho, C. B. Thompson, and J. M. Leiden. 1992. Evolutionarily conserved Ets family members display distinct DNA binding specificities. *J. Exp. Med.* 175:1391-1399.
70. Wasylyk, B., S. L. Hahn, and A. Giovane. 1993. The Ets family of transcription factors. *Eur. J. Biochem.* 211:7-18.
71. Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin. 1990. The *c-ets* proto-oncogene encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature (London)* 346:191-193.
72. Wasylyk, C., P. Flores, A. Gutman, and B. Wasylyk. 1989. PEA3 is a nuclear target for transcription activation by non nuclear oncogenes. *EMBO J.* 8:3371-3378.
73. Wasylyk, C., J. P. Kerckaert, and B. Wasylyk. 1992. A novel modulator domain of Ets transcription factors. *Genes Dev.* 6:965-974.
74. Watanabe, H., T. Wada, and H. Handa. 1990. Transcription factor E4TF1 contains two subunits with different functions. *EMBO J.* 9:841-847.
75. Webster, N., S. Green, J. Jin, and P. Chambon. 1988. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* 54:199-207.
76. Whang-Peng, J., T. J. Triche, T. Knutsen, J. Miser, E. C. Douglass, and M. A. Israel. 1984. Chromosome translocation in peripheral neuroepithelioma. *N. Engl. J. Med.* 311:584-585.
77. Whang-Peng, J., T. J. Triche, T. Knutsen, J. Miser, S. Kao-Shan, S. Tsai, and M. A. Israel. 1986. Cytogenetic characterization of

- selected small round cell tumors of childhood. *Cancer Genet. Cytogenet.* **4**:185–208.
78. **Woods, D. B., J. Ghysdael, and M. J. Owen.** 1992. Identification of nucleotide preferences in DNA sequences recognised specifically by c-Ets-1 protein. *Nucleic Acids Res.* **20**:699–704.
 79. **Xin, J.-H., A. Cowie, P. Lachance, and J. A. Hassell.** 1992. Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. *Genes Dev.* **6**:481–496.
 80. **Yamaguchi, Y., M. Satake, and Y. Ito.** 1989. Two overlapping sequence motifs within the polyomavirus enhancer are independently the target of stimulation by both the tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the Ha-*ras* oncogene. *J. Virol.* **63**:1040–1048.
 81. **Zucman, J., O. Delattre, C. Desmaze, A. L. Epstein, G. Stenman, F. Spelman, C. Fletcher, and G. Thomas.** 1993. EWS and ATF-1 gene fusion induced by the recurrent t(12;22) translocation in malignant melanoma of soft parts. *Nat. Genet.* **4**:341–345.
 82. **Zucman, J., O. Delattre, C. Desmaze, B. Plougastel, I. Joubert, T. Melot, M. Peter, P. de Jong, G. Rouleau, A. Aurias, and G. Thomas.** 1992. Cloning and characterization of the Ewing's sarcoma and peripheral neuroepithelioma t(11;22) translocation breakpoints. *Genes Chrom. Cancer* **5**:271–277.
 83. **Zucman, J., T. Melot, C. Desmaze, J. Ghysdael, B. Plougastel, M. Peter, J. M. Zucker, T. Triche, K. Patel, C. Turc-Carel, P. Ambros, V. Combaret, G. Lenoir, A. Aurias, G. Thomas, and O. Delattre.** 1993. Combinatorial generation of variable fusion proteins in peripheral primitive neuroectodermal tumors. *EMBO J.* **12**:4481–4487.