Molecular and Cellular Biology, Apr. 1996, p. 1550–1556 Vol. 16, No. 4
0270-7306/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Analysis of the ERK-Stimulated ETS Transcription Factor ER81

RALF JANKNECHT*

Institute for Molecular Biology, Hannover Medical School, D-30623 Hannover, Germany

Received 1 November 1995/Returned for modification 6 December 1995/Accepted 15 January 1996

A plethora of extracellular signals leads to the stimulation of Ras, which triggers intracellular protein kinase cascades, resulting in activation of transcription factors and thus in enhanced gene activity. In this report, it is demonstrated that the ETS transcription factor ER81, which appears to be localized within the cell nucleus by virtue of its DNA binding domain, is transcriptionally activated by oncogenic Ras. Since this activation was dependent on the presence of Raf-1 and ERK-1, ER81 is a target of the Ras/Raf/MEK/ERK signaling cascade. Consistently, activated ERK-1 is capable to phosphorylate ER81. However, the carboxy-terminal region of ER81, which contains no potential ERK phosphorylation sites, is also transcriptionally activated by ERK-1, suggesting that an ERK-stimulated protein kinase phosphorylates and thus stimulates ER81 activity. Two acidic stretches of amino acids, which are conserved in the related PEA3 and ERM proteins, are localized within the amino- and carboxy-terminal transactivation domains of ER81. In addition, an inhibitory domain may dampen the activation function of these two domains. In conclusion, ER81 is a target of Ras-dependent signaling cascades and may thus contribute to the nuclear response upon stimulation of cells and also to cellular transformation due to oncogenic Ras.

Cellular differentiation, growth, and proliferation in a complex organism are elicited by extracellular signaling molecules. One class of these molecules comprises growth factors such as epidermal growth factor or nerve growth factor which interact with receptors traversing the cell membrane, induce their dimerization, and thus activate their intrinsic tyrosine kinase activity (13). This in turn leads via adaptor molecules to the activation of Ras (28), thereby initiating an intracellular kinase cascade consisting of Raf, MEK, and ERK kinases (7, 24).

ERKs enter the cell nucleus upon activation and phosphorylate transcription factors or other protein kinases (2, 22). Among the targets of ERKs are some ETS transcription factors, e.g., the c-*fos*-regulating proteins Elk-1 and Sap-1a (15–17, 23, 35) and the prototypical ETS protein c-Ets-1 (8, 44). ETS proteins are characterized by a common ETS domain mediating DNA binding (19, 43). Some of the ETS proteins have not only highly similar ETS domains but also other homologous regions, suggestive of a common ancestor as a founder of a subfamily of ETS proteins. For instance, the ER81 (4, 31), PEA3 (14, 45), and ERM (30) proteins display 95% identity within the ETS domain, compared with only approximately 60% identity to the ETS domain of c-Ets-1, and are additionally similar outside the ETS domain.

Northern (RNA) blot analysis has revealed that ER81 is not uniformly expressed (4, 31). High levels of expression have been observed in the heart, brain, and lung, moderate levels have been seen in the spleen, pancreas, intestine, and colon, and low levels were detectable in liver or skeletal muscle. In addition, ER81 expression varies within the mammalia, since levels of ER81 mRNA in the kidney were high in mice (4) but very low in humans (31). Also, ER81 is specifically expressed in some tumor cells lines (teratocarcinoma and prostate adenocarcinoma), while no ER81 mRNA was detected in cervix or hepatocarcinoma cells (31). These results suggest that ER81 may contribute to the transformation of certain cell lines.

Furthermore, the t(7;22)(p22;q12) chromosomal translocation characteristic for a minority of Ewing's sarcoma and primitive neuroectodermal tumors leads to the fusion of the last 168 amino acids of ER81, including its ETS domain, to the *EWS* gene (21). Similarly, the carboxy-terminal regions of Fli-1 and ERG, also including their ETS domains, are fused to the EWS protein in 85 or 10% of all Ewing's sarcomas by the chromosomal translocation t(11;22)(q24;q12) or t(21;22)(q22;q12), respectively (11, 40, 46). EWS–Fli-1 fusions function as true transcription factors, and their activity is dependent both on the DNA binding function of the Fli-1 ETS domain and on the amino-terminal EWS portion, which appears to contain a transactivation domain. Importantly, chimeric EWS–Fli-1 proteins efficiently transform cells, which strongly suggests that fusions of EWS with Fli-1, ERG, and ER81 lead to the development of Ewing's sarcoma (1, 26, 27). This notion is supported by a recent study using antisense RNA directed against EWS–Fli-1 and EWS-Erg, which led to a loss of tumorigenicity of Ewing's sarcoma cells (33).

In this report, it is demonstrated that the nuclear protein ER81 is capable of binding to the ETS binding site E74 and activates transcription upon stimulation of the Ras/Raf/MEK/ERK signaling pathway. ERKs can phosphorylate ER81 within a region that is juxtaposed to an acidic domain near the amino terminus and activate indirectly a carboxy-terminal transactivation domain. Collectively, these data identify ER81 as a novel target for Ras-dependent signaling.

MATERIALS AND METHODS

DNA cloning. ER81 cDNA was cloned into the pSG-new expression vector, a derivative of pSG5 (Stratagene), thereby generating pSG-ER81. Eukaryotic expression vectors for truncations of ER81 or the full-length molecule were constructed by using a derivative of the pEV series of vectors (25), which provides an amino-terminal hemagglutinin (HA) tag (12). The pABGal-linker plasmid (38) was used for cloning GAL4 fusions. The $E74_3$-tk80-luc and tk80-luc luciferase reporter constructs have been described before (17). Expression plasmids for BXB (5), ERK-1 (29), MEK-EE (9) are described in the literature. pGEX2T-6His-PL2 (kindly provided by R. A. Hipskind) was used for cloning of GST-

* Present address: The Salk Institute, Molecular Biology and Virology Laboratory, 10010 North Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 453-4100, ext. 1387. Fax: (619) 457-4765. Electronic mail address: rjanknecht@aim.salk.edu.

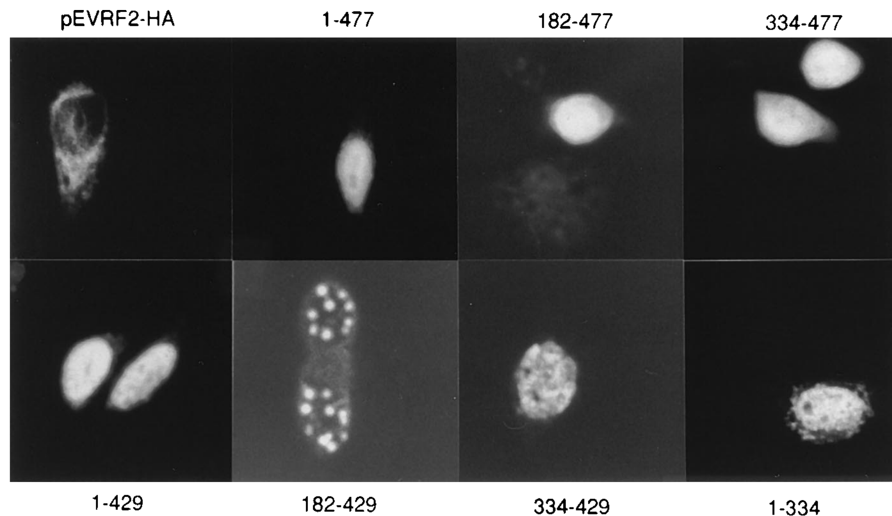

FIG. 1. Nuclear localization assayed by indirect immunofluorescence microscopy. Cells were transiently transfected with HA-tagged ER81 expression vectors or the empty expression vector pEVRF2-HA, leading to the generation of a small HA-tagged peptide. Staining of the cells was accomplished by virtue of anti-HA antibodies. Numbers refer to ER81 amino acids present in the respective HA-tagged proteins, with 1-477 representing the full-length ER81 molecule.

ER81 fusions, thus allowing purification of resulting proteins by virtue of a histidine tag.

Immunofluorescence studies. HeLa cells were grown on coverslips and transfected with expression plasmids for HA-tagged proteins by the calcium phosphate coprecipitation method. The precipitate was left on the cells for 24 h and then removed, and cells were further incubated for 18 h. Then cells were washed twice in phosphate-buffered saline (PBS) and subsequently fixed for 6 min in precooled methanol at −20°C. After three washes with PBS, the coverslips were incubated for 20 min in PBS–0.1% bovine serum albumin and then incubated for 60 min with a murine monoclonal antibody directed against the HA epitope (12). This was followed by a second incubation with a goat anti-mouse antibody coupled to fluorescein isothiocyanate. Finally, the coverslips were washed twice in PBS, once in PBS plus 1 μg of Hoechst dye 33258 per ml, twice again in PBS, and once with water and mounted with Entellan (Merck). Indirect immunofluorescence microscopy was performed under oil with a 100× objective and a 10× ocular, using Kodak Ektachrome ISO-400/27° film for photography.

Gel retardation assay. A 1.5-μg aliquot of the empty expression vector pSG-new or of pSG-ER81 was used for a combined in vitro transcription-translation reaction performed with the TNT coupled reticulocyte lysate system (Promega). Two microliters of the reaction mix was then used for a gel retardation reaction with ^{32}P-labeled E74 oligonucleotide (18). Complexes were resolved on a 4.5% native acrylamide gel at room temperature.

Luciferase reporter gene assays. Rabbit kidney epithelial-like cells (ATCC CCL37) were transiently transfected by the calcium phosphate coprecipitation method essentially as described previously (17). Cells were harvested 36 h after transfection and lysed, and the cleared lysate was used to measure luciferase activity and also β-galactosidase activity from a cotransfected, constitutively active β-galactosidase expression vector. The latter was used to correct luciferase activity for transfection efficiency.

Production of fusion proteins in *Escherichia coli*. *E. coli* BL21 cells transformed with GST-ER81 expression plasmids were grown to an optical density at 600 nm of 0.8 and then induced for 3 h with 0.5 mM isopropyl-β-D-thiogalactoside (AppliChem). Cells were washed once in PBS and then lysed in 6 M guanidine HCl–0.1 M NaP_i (pH 7.9) and frozen for 10 min at −80°C, and the lysate was shaken for 1 h at room temperature. After removal of debris, the lysate was incubated for 3 h with Ni^{2+}-nitrilotriacetic acid agarose (Qiagen), and bound proteins were eluted with 6 M guanidine HCl–0.1 M NaP_i (pH 4). Proteins were then renatured by extensive dialysis against 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6)–100 mM NaCl–10% glycerol–10 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride.

In vitro phosphorylation. Glutathione *S*-transferase (GST) fusion proteins were phosphorylated for 30 min at 30°C in a volume of 20 μl with 1 μCi [γ-^{32}P]ATP. For phosphorylation by purified activated ERK-1, the reaction buffer was composed of 10 mM morpholinepropanesulfonic acid (MOPS; pH 7.2), 10 mM $MgCl_2$, 15 mM β-glycerophosphate, 10 mM *p*-nitrophenylphosphate, 1 mM dithiothreitol, 2.5 mM EGTA, 0.25 mM Na_3VO_4, and 10 μM ATP. Alternatively, GST fusion proteins were phosphorylated by the catalytic subunit of protein kinase A from bovine heart (Sigma) in 20 mM Tris HCl (pH 7.5)–100 mM NaCl, 12 mM $MgCl_2$–1 mM dithiothreitol–10 mM *p*-nitrophenylphosphate–10 μM ATP.

RESULTS

Nuclear localization of ER81. Transcription factors exert their function within the cell nucleus. However, while many of them are constitutively nuclear, some are both cytoplasmic and nuclear or move from the cytoplasm to the nucleus upon phosphorylation as reported for the STAT transcription factors (10). Therefore, intracellular localization of murine ER81 that has been tagged with an HA epitope was analyzed in transiently transfected cells by using anti-HA antibodies (Fig. 1). While the empty expression vector directing the production of a short peptide encompassing the HA epitope resulted in predominantly cytoplasmic staining, the full-length ER81 molecule (amino acids 1 to 477) was localized exclusively within the nucleus. Truncating ER81 from the amino terminus did not alter this situation (Fig. 1, 182-477 and 334-477). Note that both of these truncations still contain an ETS domain (amino acids 333 to 415).

Deletion of carboxy-terminal amino acids without affecting the ETS domain in the 1-429 clone as well as jointly deleting amino- and carboxy-terminal sequences in $ER81_{182\text{-}429}$ and $ER81_{334\text{-}429}$ did not affect nuclear localization (Fig. 1). $ER81_{334\text{-}429}$ nearly matches the ETS domain, which suggests that the ETS domain determines nuclear localization of ER81. Interestingly, $ER81_{182\text{-}429}$ displayed a spot-like nuclear staining which seems to coincide with the presence of nucleoli. In addition, $ER81_{1\text{-}334}$, which has no ETS domain, displayed nuclear staining, albeit some speckles of staining were reproducibly observable outside the nucleus. Taken together, these data indicate that ER81 is a nuclear protein and that nuclear localization is due to the ETS domain, although amino acids 1 to 334 may also contain a region leading to (partial) nuclear localization of this protein.

Interaction of ER81 with the E74 binding site. The E74 binding site has been identified as a target for the *Drosophila* E74 ETS protein (42) and subsequently shown to be a high-affinity site for various vertebrate ETS proteins (16, 18, 36). Therefore, binding of ER81 to the E74 binding site was tested. To this end, full-length ER81 protein was generated by in vitro transcription-translation and tested in a gel retardation assay for an interaction with a ^{32}P-labeled E74 oligonucleotide. As

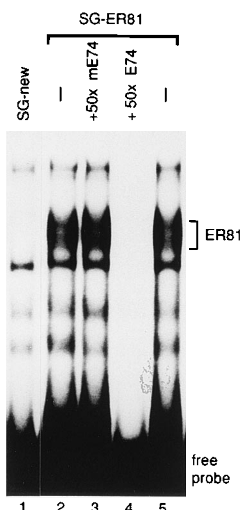

FIG. 2. DNA binding of ER81. In vitro-translated ER81 protein was used in gel retardation assays with ^{32}P-labeled E74 oligonucleotide (lanes 2 to 5). Lane 4 contains a 50-fold molar excess of unlabeled E74 oligonucleotide over the ^{32}P-labeled E74 oligonucleotide, while lane 3 contains a 50-fold excess of a mutated E74 oligonucleotide (mE74) in which the ETS binding site has been destroyed. In lane 1, the empty expression vector pSG-new was used for in vitro transcription-translation instead of pSG-ER81.

shown in Fig. 2, ER81 bound to the E74 oligonucleotide. Various other bands were visible on the autoradiogram; however, they are due to proteins within the reticulocyte lysate used for in vitro translation, since they were also generated with a control reaction mix containing the respective empty expression vector (compare lanes 1 and 2).

The specificity of the binding of ER81 to the E74 oligonucleotide was assessed by competition experiments with unlabeled oligonucleotides (Fig. 2). An excess of unlabeled E74 oligonucleotide abrogated complex formation. On the contrary, a 50-fold surplus of a mutated E74 oligonucleotide, in which the GGAA core sequence typical for ETS protein binding sites was mutated to CCAA (18, 36), did not affect DNA binding of ER81. Thus, ER81 can specifically interact with the E74 binding site.

Activation of ER81 by Ras. To study the transcriptional properties of ER81, an E74 binding-site-driven luciferase reporter construct was used in transient transfection experiments. As shown in Fig. 3A, expression of ER81 led to a repression of transcription to 40% of the basal level. This repression was specific, since a reporter construct devoid of E74 binding sites was unaffected (Fig. 3B). Cotransfection of an oncogenic Ras (Ras-onc) expression vector slightly alleviated this repression, while additional expression of both Raf-1 and ERK-1 led to a 7.2-fold activation of transcription relative to ER81 alone (Fig. 3A). Raf-1 or ERK-1 on their own were unable to do so, and also the combination of Ras-onc and Raf-1 or Ras-onc and ERK-1 showed little effect. These data suggest that a reconstituted Ras/Raf/MEK/ERK signaling cascade leads to the activation of ER81.

To obtain a more efficient degree of activation, constitutively active Raf-1 (BXB) or constitutively active MEK (MEK-EE) was used (Fig. 3A). These two kinases, in conjunction with ERK-1, were able to elevate ER81-mediated transcription drastically, while this degree of activation was relatively small in the absence of ER81. Again, these effects were binding-site specific (Fig. 3B). Taken together, ER81 is targeted by the Ras/Raf/MEK/ERK signaling cascade and can thereby elevate E74 binding-site-dependent transcription.

Mapping of transactivation domains. Transcription factors often display a modular structure with domains being responsible for DNA binding, transactivation, or protein-protein interaction. To identify potential transactivation domains within ER81, several truncations of ER81 were tested in transient transfection assays (Fig. 4). Deletion of the first 62 amino acids led to a severe reduction of ER81-mediated transcription upon stimulation of ERK-1 by the constitutively active Raf-1 kinase

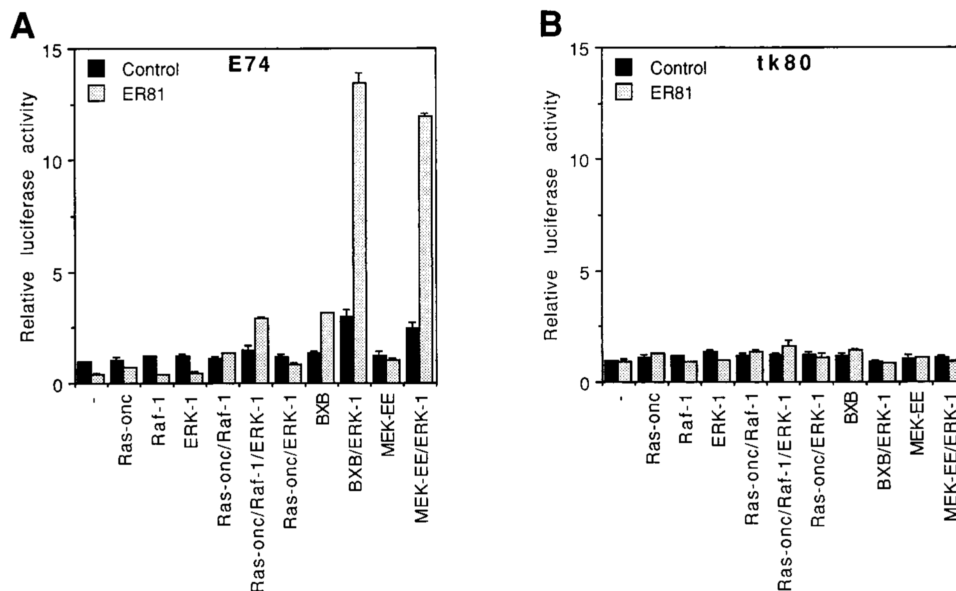

FIG. 3. Stimulation of ER81-mediated transcription by Ras/Raf/MEK/ERK. Cells were transiently transfected with the $E74_3$-tk80-luc (A) or tk80-luc (B) reporter construct. Where indicated, an expression plasmid for oncogenic Ras (Ras-onc), Raf-1, constitutively active Raf-1 (BXB), constitutively active MEK (MEK-EE), or ERK-1 was cotransfected. Black bars indicate transfections with an empty expression vector; stippled bars represent experiments with cotransfected full-length ER81 expression vector.

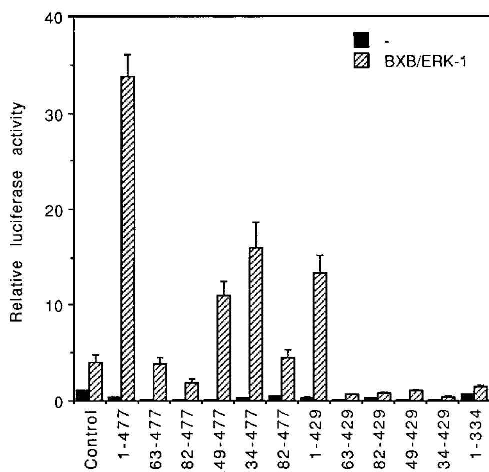

FIG. 4. Analysis of ER81 truncations. Cells were transiently transfected with the $E74_3$-tk80-luc reporter construct in either the absence or presence of BXB/ERK-1. Control denotes the empty expression vector; numbers indicate the ER81 amino acids expressed.

BXB (compare $ER81_{1-477}$ with $ER81_{63-477}$), implicating the existence of an amino-terminal transactivation domain. Surprisingly, further deleting amino-terminal residues to position 249 resurrected some of the transcriptional activity of ER81, implying that amino acids 182 to 249 exert an inhibitory effect on transactivation. More extensive truncation into the ETS domain ($ER81_{382-477}$) abolishes DNA binding and resulted in transcriptional levels comparable to the control levels.

Truncation of the last 48 residues in $ER81_{1-429}$ caused a 2.5-fold decrease of ERK-induced transcriptional activity relative to full-length ER81, indicating that the carboxy terminus contains another transactivation domain. Notably, the 249-429 and 334-429 clones were transcriptionally inactive compared with the respective 249-477 and 334-477 constructs, again hinting at a carboxy-terminal transactivation domain. In addition, the DNA-binding-incompetent 1-334 mutant resulted in a reduction of transcription below the level of the control. This finding may suggest that these amino acids can still serve the function of a target for ERK-1 and thereby competitively inhibit the activation of other endogenous proteins that would normally stimulate E74 binding-site-driven transcription.

As shown by indirect immunofluorescence microscopy with a subset of the ER81 truncations used for Fig. 1, the truncated molecules were expressed at levels comparable to that of the full-length ER81 molecule. In addition, fivefold variations in the amount of expression vector used for transient transfection did not significantly affect the transactivation data obtained with ER81 (data not shown). Thus, the different transactivation levels observed with the truncations relative to the full-length ER81 protein (Fig. 4) were not due to different intracellular protein concentrations.

To further demonstrate the existence of transactivation domains in ER81, fusions of ER81 amino acids to the DNA binding domain of the yeast protein GAL4 were constructed, and their transcriptional activities were tested with a GAL4 binding-site-driven luciferase reporter construct (Fig. 5A). Comparable expression of the GAL4-ER81 fusion proteins was investigated by Western blot (immunoblot) analyses using anti-GAL4 antibodies (data not shown). Significant increases of basal transcription were observed with the GAL4/1-182 and GAL4/1-63 constructs, corroborating the notion that the amino terminus of ER81 contains a transactivation domain. This transactivation domain (amino acids 1 to 182) could be stimulated with BXB/ERK-1 (Fig. 5B), suggesting that residues within this domain are phosphorylated by ERK-1 and thus activate it. Note that also GAL4/1-63 was approximately threefold activated by BXB/ERK-1, while GAL4/63-182 was not (Fig. 5B).

Furthermore, basal levels of transcription were slightly elevated with the GAL4/333-477 fusion protein but were repressed with the GAL4/333-429 protein (Fig. 5A). In addition,

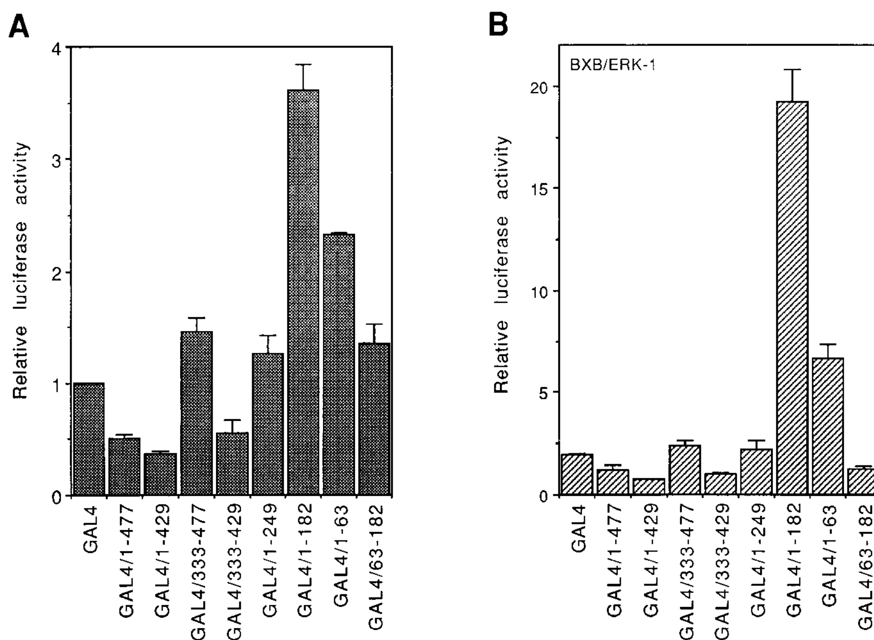

FIG. 5. Transcriptional activity of GAL4-ER81 fusion proteins. Activation of transcription from a GAL4 binding-site-driven luciferase reporter construct ($GAL4_2$-tk80-luc) by various GAL4-ER81 fusions was measured in the absence (A) or presence (B) of cotransfected BXB/ERK-1. Note the different scales in panels A and B.

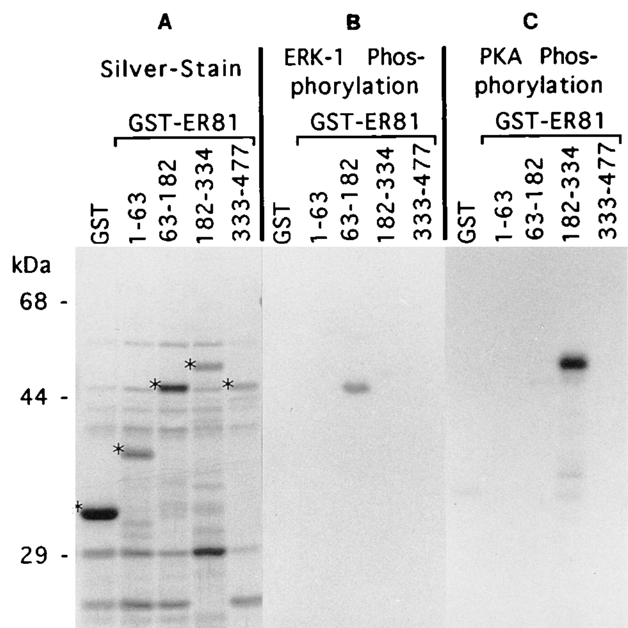

FIG. 6. ERK-1 phosphorylates ER81. Partially purified GST-ER81 fusion proteins were incubated in vitro with ERK-1 or protein kinase A (PKA) in the presence of [γ-^{32}P]ATP and then subjected to SDS-polyacrylamide gel electrophoresis. (A) Silver-stained protein gel (asterisks mark the fusion proteins); (B and C) corresponding autoradiograms of equivalent amounts of proteins after incubation with ERK-1 and protein kinase A, respectively.

GAL4/1-477 was slightly more active than GAL4/1-429. These data are in agreement with the identification of a transactivation domain at the carboxy terminus of ER81. However, in contrast to ER81$_{334-477}$, the GAL4/333-477 protein was nearly unresponsive to stimulation by BXB/ERK-1 (Fig. 5). The same holds true for GAL4/1-477 and GAL4/1-429. Since all of these three proteins possess the ETS domain, one may envisage that its presence prohibits stimulation by BXB/ERK-1 in the context of GAL4 fusion proteins.

In addition, the GAL4/1-249 protein was less active than GAL4/1-182 (Fig. 5). This finding suggests that amino acids 182 to 249 dampen the transcriptional activity of the amino-terminal transactivation domain.

Phosphorylation of ER81 by ERK-1. The transactivation data demonstrated that ER81 is a target of the Ras/Raf/MEK/ERK signaling cascade. However, this does not necessarily mean that ERK phosphorylates ER81, since ERKs can also phosphorylate and thus activate other protein kinases (2) which may utilize ER81 as a substrate. To investigate whether ER81 is a substrate for ERK-1, different portions of the ER81 protein were produced as GST fusions in *E. coli* and partially purified (Fig. 6). These fusion proteins were then incubated with purified, activated ERK-1 and [γ-^{32}P]ATP in vitro. As shown in Fig. 6, only ER81 amino acids 63 to 182 were phosphorylated by ERK-1. As a control, GST fusions were also incubated with protein kinase A, leading to the phosphorylation of GST-ER81$_{182-334}$, which is most likely due to phosphorylation of serine 334 (Fig. 7). These results strongly suggest that the amino-terminal transactivation domain of ER81 is directly targeted by ERK-1. Since the carboxy terminus of ER81 was not phosphorylated by ERK-1 despite being transcriptionally activated by ERK-1, an ERK-1-stimulated protein kinase may phosphorylate and thereby stimulate the carboxy-terminal transactivation domain of ER81.

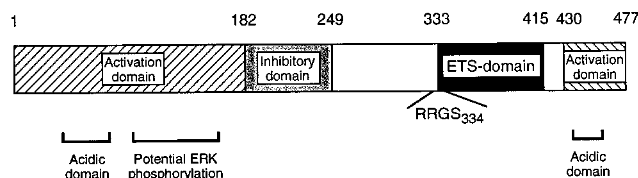

FIG. 7. Functional domains within ER81. Potential ERK phosphorylation motifs ([S/T]P) are located between amino acids 94 and 159. Acidic domains (amino acids 42 to 73 and 437 to 455) conserved in the ER81/PEA3/ERM subfamily of ETS proteins are also indicated. Serine 334 is part of a consensus site for protein kinase A.

DISCUSSION

The nuclear DNA-binding ER81 protein. In this study, murine ER81 was demonstrated to be localized within the nucleus. This result is in accordance with a recent report showing that the human homolog is also localized within the nucleus (31). Beyond this, murine ER81 requires neither amino acids amino terminal of the ETS domain nor those at the carboxy terminus, since the ER81$_{334-429}$ molecule was still localized within the nucleus. Thus, the ETS domain alone appears to be sufficient for nuclear localization. Although the ETS domain is also sufficient for nuclear localization of other transcription factors, e.g. Sap-1a (16), the ETS domain of Elk-1 alone is unable to direct nuclear localization (20). However, the second half of the ETS domain, which displays a basic character (19, 43), appears to be necessary for nuclear localization of Elk-1 and Ets-1 (3, 20).

Interestingly, the ER81$_{1-334}$ protein, which is devoid of the ETS domain, was still predominantly localized in the nucleus but also visible in the cytoplasm. Thus, amino acids 1 to 334 may contain a second nuclear localization signal, albeit not as strong as the one within the ETS domain.

DNA binding sites for ETS proteins reveal a strong conservation of a GGA(A/T) core sequence. Flanking nucleotides as well as neighboring binding sites for interaction partners may establish a certain degree of specificity for one of the multitude of ETS proteins (19, 43). However, a great variety of reported binding sites for ETS proteins are of comparable affinity for different ETS proteins; e.g., the E74 binding site can be targeted by the E74A (42), Elk-1 (36), Sap-1a (16), and also the ER81 protein. Thus, transcriptional regulation via one ETS binding site in vivo may be the result of a combinatorial operation of different ETS proteins, including ER81.

ER81 is a target for ERKs. ERK-1 is able to phosphorylate amino acids 63 to 182 of ER81 in vitro. Analysis of the protein sequence revealed the presence of six (S/T)P motifs that are potential targets for ERKs within the region 63–182. No further (S/T)P motifs are present in murine ER81, indicating that exclusively this region can be phosphorylated by ERKs. This phosphorylation may activate ER81, since in vivo activation of ERK-1 by overexpression of oncogenic Ras, constitutively activated Raf-1, or constitutively activated MEK resulted in an increase of ER81-mediated transcription. Thus, ER81 has been identified as a novel target of the Ras/Raf/MEK/ERK signaling cascade and may therefore represent an important player in the nuclear response to a multitude of extracellular signals activating Ras.

Previously, ETS proteins other than ER81 have been shown to activate transcription upon stimulation of Ras. These include c-Ets-1 and c-Ets-2 (8), the c-*fos*-regulating ternary complex factors (15), and the *Drosophila* Pointed-P2 protein, which is involved in eye development (32). It thus appears that many

ETS proteins are activated by Ras. Future studies should be directed to determining whether all of them, or only a subset, are required for Ras-mediated mitogenesis and transformation.

$ER81_{334-477}$ is also activated upon stimulation of ERK-1. However, these amino acids of ER81, as expected, are not phosphorylated by ERK-1 in vitro since they contain no potential ERK phosphorylation sites. This observation implies that ERK-1 indirectly stimulates the carboxy terminus of ER81. Since ERKs are known to activate $pp90^{rsk}$ (2), this protein kinase is a likely candidate for modulating ER81 activity by direct phosphorylation within the carboxy terminus. Thus, ER81 seems to be targeted both directly and indirectly by ERKs.

Transactivation domains within ER81. The region 1–182 of ER81 contains a transactivation domain (Fig. 7). Splitting this domain into two parts, 1–63 and 63–182, revealed that both parts coordinately transactivate, with 1–63 being the more potent part. Consistent with the notion that the amino terminus of ER81 is phosphorylated by ERK-1, transactivation mediated by GAL4/1-182 was enhanced by more than fivefold in the presence of BXB/ERK-1. Surprisingly, GAL4/63-182, which contains all potential ERK phosphorylation sites of ER81, was unresponsive to stimulation with BXB/ERK-1, while GAL4/1-63 was activated, possibly indirectly via another kinase activated by ERK-1. These data strongly suggest that ERK phosphorylation within the region 63–182 can activate transcription only in concert with the region 1–63. Interestingly, amino acids 42 to 73 represent an acidic domain characteristic for many transactivation domains, and this region is conserved in the ER81/PEA3/ERM subfamily of ETS proteins (31).

The carboxy-terminal amino acids 430 to 477 also contribute to transcription, since their deletion reduced ER81-mediated transcription as well as that of the GAL4/333-477 fusion. Thus, amino acids 430 to 477 may represent a second transactivation domain within ER81 (Fig. 7), whose activity may be indirectly modulated by ERK-1. Note that this carboxy-terminal transactivation domain also encompasses an acidic domain (amino acids 437 to 455) conserved among ER81, PEA3, and ERM.

Surprisingly, while the full-length ER81 protein was transcriptionally activated by ERK-1 when assayed with the E74 binding-site-driven reporter construct, it was inert when fused to the GAL4 moiety and tested with a GAL4 binding-site-driven reporter. A similar phenomenon has been observed with Sap-1a (16) and is likely due to the fact that the transactivation domains are shielded as long as the ETS domain is not contacting DNA (41). Indeed, a recent report has shown that binding of Ets-1 to DNA induces the unfolding of an α-helix outside the ETS domain without affecting the conformation of the ETS domain itself (34). It seems unlikely that the inhibitory effect of the ETS domain on GAL4/1-477-mediated transcription is due to its DNA-binding activity. If so, one would have to assume that GAL4/1-477, which can be regarded as a GAL4-tagged $ER81_{1-477}$ protein, binds to target sites within the genome by virtue of its ETS domain, thereby causing an unspecific inhibition of transcription. However, the same argument should apply for $ER81_{1-477}$, but huge degrees of transactivation were observable with this protein upon ERK-1 stimulation (Fig. 3A).

Furthermore, amino acids 182 to 249 (Fig. 7) may exert an inhibitory effect on the function of the carboxy-terminal transactivation domain, since $ER81_{249-477}$ was significantly induced by ERK-1 in comparison with $ER81_{182-477}$. Similarly, amino acids 182 to 249 appear to decrease the activity of the amino-terminal transactivation domain, because GAL4/1-249 was less active than GAL4/1-182.

Impact of ER81 on cell transformation. Ewing's sarcoma with a t(7;22)(p22;q12) chromosomal translocation contain a fusion of parts of the EWS protein, including a potential transactivation domain, to the last 168 amino acids of ER81 encompassing its DNA binding domain and also its carboxy-terminal acidic domain (21). This fusion protein is, in analogy to the respective EWS–Fli-1 and EWS-ERG fusion proteins, thought to trigger the transformation of cells. Thus, ER81 target genes, when regulated in an abnormal manner, may be responsible for the development of cancer. Consistent with such a notion, ER81 mRNA is found at high levels in certain tumor cell lines (31). These results also indicate how oncogenic forms of Ras or Raf may lead to cell transformation, namely, by constitutive activation of ER81.

Since different ETS proteins can occupy the same target site, gene regulation via an ETS binding site can be the result of an interplay between two (or more) ETS transcription factors. For instance, the *Drosophila* Pointed-P2 and Yan ETS proteins are required for eye development, and both are targeted by ERK (6, 32, 37). However, while Pointed-P2 is stimulated in its activity by the Ras-ERK pathway, Yan represents an inhibitor of transcription whose repressing activity is down-regulated by ERK phosphorylation. Thus, while transcription is shut off as a result of the repressor function of Yan in unstimulated cells, activation of ERK results in enhanced transcription levels by reducing Yan activity and simultaneously stimulating Pointed-P2. Such an opposing interplay of two ETS proteins may also occur in mammals. Here, ER81 could be the activator being stimulated by ERK phosphorylation, while the antagonist could be the recently identified ERF protein. This ETS protein strongly represses transcription and is hyperphosphorylated in the presence of upstream activators of ERK, and the hyperphosphorylated ERF protein is inhibited in its repressing function (39). Thus, ERF may be employed to counteract the ER81-associated tumorigenesis in Ewing's sarcoma.

ACKNOWLEDGMENTS

I thank K. Rollwage, U. Wiedemann, and U. zur Stadt for technical help, T. Brown for sending murine ER81 cDNA, and B. Lüscher for providing an expression vector for oncogenic Ras. I am also grateful to A. Nordheim for providing research facilities.

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Bailly, R.-A., R. Bosselut, J. Zucman, F. Cormier, O. Delattre, M. Roussel, G. Thomas, and J. Ghysdael.** 1994. DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. Mol. Cell. Biol. **14:**3230–3241.
2. **Blenis, J.** 1993. Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA **90:**5889–5892.
3. **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.
4. **Brown, T. A., and S. L. McKnight.** 1992. Specificities of protein-protein and protein-DNA interaction of GABPα and two newly defined *ets*-related proteins. Genes Dev. **6:**2502–2512.
5. **Bruder, J. T., G. Heidecker, and U. R. Rapp.** 1992. Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev. **6:**545–556.
6. **Brunner, D., K. Dücker, N. Oellers, E. Hafen, H. Scholz, and C. Klämbt.** 1994. The ETS domain protein Pointed-P2 is a target of MAP kinase in the Sevenless signal transduction pathway. Nature (London) **370:**386–389.
7. **Cano, E., and L. C. Mahadevan.** 1995. Parallel signal processing among mammalian MAPKs. Trends Biochem. Sci. **20:**117–122.
8. **Coffer, P., M. de Jonge, A. Mettouchi, B. Binetruy, J. Ghysdael, and W. Kruijer.** 1994. *junB* promoter regulation: Ras mediated transactivation by c-Ets-1 and c-Ets-2. Oncogene **9:**911–921.
9. **Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall.** 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77:**841–852.

10. **Darnell, J. E., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415–1421.
11. **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.
12. **Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler.** 1988. Purification of a *Ras*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8:**2159–2165.
13. **Heldin, C.-H.** 1995. Dimerization of cell surface receptors in signal transduction. Cell **80:**213–223.
14. **Higashino, F., K. Yoshida, Y. Fujinaga, K. Kamio, and K. Fujinaga.** 1993. Isolation of a cDNA encoding the adenovirus E1A enhancer binding protein: a new human member of the *ets* oncogene family. Nucleic Acids Res. **21:** 547–553.
15. **Janknecht, R., M. A. Cahill, and A. Nordheim.** 1995. Signal integration at the c-*fos* promoter. Carcinogenesis **16:**443–450.
16. **Janknecht, R., W. H. Ernst, and A. Nordheim.** 1995. SAP1a is a nuclear target of signaling cascades involving ERKs. Oncogene **10:**1209–1216.
17. **Janknecht, R., W. H. Ernst, V. Pingoud, and A. Nordheim.** 1993. Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J. **12:**5097–5104.
18. **Janknecht, R., and A. Nordheim.** 1992. Elk-1 protein domains required for direct and SRF-assisted DNA-binding. Nucleic Acids Res. **20:**3317–3324.
19. **Janknecht, R., and A. Nordheim.** 1993. Gene regulation by Ets proteins. Biochim. Biophys. Acta **1155:**346–356.
20. **Janknecht, R., R. Zinck, W. H. Ernst, and A. Nordheim.** 1994. Functional dissection of the transcription factor Elk-1. Oncogene **9:**1273–1280.
21. **Jeon, I.-S., J. N. Davis, B. S. Braun, J. E. Sublett, M. F. Roussel, C. T. Denny, and D. N. Shapiro.** 1995. A variant Ewing's sarcoma translocation (7;22) fuses the *EWS* gene to the ETS gene *ETV1*. Oncogene **10:**1229–1234.
22. **Karin, M., and T. Hunter.** 1995. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr. Biol. **5:**747–757.
23. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell **73:**381–393.
24. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. **4:**82–89.
25. **Matthias, P., M. M. Müller, E. Schreiber, S. Rusconi, and W. Schaffner.** 1989. Eukaryotic expression vectors for the analysis of mutant proteins. Nucleic Acids Res. **17:**6418.
26. **May, W. A., M. L. Gishizsky, 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 *FLI1* for transformation. Proc. Natl. Acad. Sci. USA **90:**5752–5756.
27. **May, W. A., S. L. Lessnick, B. S. Braun, M. Klemsz, B. C. Lewis, L. B. Lunsford, R. Hromas, and C. T. Denny.** 1993. 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*. Mol. Cell. Biol. **13:**7393–7398.
28. **McCormick, F.** 1993. How receptors turn Ras on. Nature (London) **363:**15–16.
29. **Meloche, S., G. Pagès, and J. Pouysségur.** 1992. Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, $p44^{mapk}$. Mol. Biol. Cell **3:**63–71.
30. **Monté, D., J.-L. Baert, P.-A. Defossez, Y. de Launoit, and D. Stéhelin.** 1994. Molecular cloning and characterization of human ERM, a new member of the Ets family closely related to mouse PEA3 and ER81 transcription factors. Oncogene **9:**1397–1406.
31. **Monté, D., L. Coutte, J.-L. Baert, I. Angeli, D. Stéhelin, and Y. de Launoit.** 1995. Molecular characterization of the ets-related human transcription factor ER81. Oncogene **11:**771–779.
32. **O'Neill, E. M., I. Rebay, R. Tjian, and G. M. Rubin.** 1994. The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. Cell **78:**137–147.
33. **Ouchida, M., T. Ohno, Y. Fujimura, V. N. Rao, and E. S. P. Reddy.** 1995. Loss of tumorigenicity of Ewing's sarcoma cells expressing antisense RNA to EWS-fusion transcripts. Oncogene **11:**1049–1054.
34. **Petersen, J. M., J. J. Skalicky, L. W. Donaldson, L. P. McIntosh, T. Alber, and B. J. Graves.** 1995. Modulation of transcription factor Ets-1 DNA binding: DNA-induced unfolding of an α helix. Science **269:**1866–1869.
35. **Price, M. A., A. Rogers, and R. Treisman.** 1995. Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). EMBO J. **14:**2589–2601.
36. **Rao, V. N., and E. S. P. Reddy.** 1992. A divergent *ets*-related protein, Elk-1, recognizes similar c-*ets*-1 proto-oncogene target sequences and acts as a transcriptional activator. Oncogene **7:**65–70.
37. **Rebay, I., and G. M. Rubin.** 1995. Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell **81:**857–866.
38. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
39. **Sgouras, D. N., M. A. Athanasiou, G. J. Beal, R. J. Fisher, D. G. Blair, and G. J. Mavrothalassitis.** 1995. ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress *ets*-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. EMBO J. **14:**4781–4793.
40. **Sorensen, P. H. B., S. L. Lessnick, D. Lopez-Terrada, X. F. Liu, T. J. Triche, and C. T. Denny.** 1994. A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat. Genet. **6:**146–151.
41. **Treisman, R., R. Marais, and J. Wynne.** 1992. Spatial flexibility in ternary complexes between SRF and its accessory proteins. EMBO J. **11:**4631–4640.
42. **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.
43. **Wasylyk, B., S. L. Hahn, and A. Giovane.** 1993. The Ets family of transcription factors. Eur. J. Biochem. **211:**7–18.
44. **Watson, D. K., M. J. McWilliams, P. Lapis, J. A. Lautenberger, C. W. Schweinfest, and T. S. Papas.** 1988. Mammalian *ets-1* and *ets-2* genes encode highly conserved proteins. Proc. Natl. Acad. Sci. USA **85:**7862–7866.
45. **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.
46. **Zucman, J., T. Melot, C. Desmaze, J. Ghysdael, B. Plougastel, M. Peter, J. M. Zucker, T. J. Triche, D. Sheer, C. Turc-Carel, P. Ambros, V. Combaret, G. Lenoir, A. Aurias, G. Thomas, and O. Delattre.** 1993. Combinatorial generation of variable fusion proteins in the Ewing family of tumours. EMBO J. **12:**4481–4487.